DISSERTATIONES
NEUROSCIENTIAE
UNIVERSITATIS
TARTUENSIS
31

HARDO LILLEVÄLI

Hyperphenylalaninaemias and neurophysiological disorders associated with the condition

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
31

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
31

HARDO LILLEVÄLI

Hyperphenylalaninaemias and neurophysiological disorders associated with the condition

UNIVERSITY OF TARTU
Press

Department of Clinical Genetics, Institute of Clinical Medicine, University of Tartu, Estonia

Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in Neurosciences on March, 6th, 2020 by the Joint Council for the Curriculum of Neurosciences.

Supervisors: Professor Katrin Õunap, MD, PhD, Department of Clinical Genetics, Institute of Clinical Medicine, University of Tartu, and Department of Clinical Genetics, United Laboratories, Tartu University Hospital, Tartu, Estonia.

Senior Research Fellow Kersti Lilleväli, PhD, Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia.

Reviewers: Associate Professor Eve Õiglane-Šlik, MD, PhD, Children's Clinic, Institute of Clinical Medicine, University of Tartu and Children's Clinic of Tartu University Hospital, Tartu, Estonia.

Senior Researcher Elin Org, PhD, Institute of Genomics and Estonian Genome Center, University of Tartu, Tartu, Estonia.

Opponent: Professor Johannes Zschocke MD, PhD, Chair of Human Genetics, Medical University Innsbruck, Austria.

Commencement: May, 12th 2020 at 15.00, at Biomedicum, 19 Ravila street, room 1006.

Publication of this dissertation is granted by the University of Tartu.

FUNDING: The current study has been supported by grants PUT0355 and PRG471 from the Estonian Research Council and partially by grants No. 592 and 1133 from the Estonian Science Foundation and grant No. E96-23.04-02 from the Open Estonian Foundation.

ISSN 1736-2792
ISBN 978-9949-03-320-1 (print)
ISBN 978-9949-03-321-8 (pdf)

University of Tartu Press
www.tyk.ee

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. Lilleväli H, Õunap K, Metspalu A. Phenylalanine hydroxylase gene mutation R408W is present on 84% of Estonian phenylketonuria chromosomes. Eur J Hum Genet. 1996;4(5):296–300.

II. Lilleväli H, Reinson K, Muru K, Simenson K, Murumets Ü, Möls T, Õunap K. Hyperphenylalaninaemias in Estonia: Genotype-Phenotype Correlation and Comparative Overview of the Patient Cohort Before and After Nation-Wide Neonatal Screening. JIMD Rep. 2018;40:39–45.

III. Lilleväli H, Reinson K, Muru K, Saarsalu S, Künnapas K, Kahre T, Murumets Ü, Õunap K. The evaluation of phenylalanine levels in Estonian phenylketonuria patients during eight years by electronic laboratory records. Molecular Genetics and Metabolism Reports, 2019; 19, 100467.10.

IV. Lilleväli H*, Pajusalu S*, Wojcik MH, Goodrich J, Collins RL, Murumets Ü, Tammur P, Blau N, Lilleväli K, Õunap K. Genome sequencing identifies a homozygous inversion disrupting *QDPR* as a cause for dihydropteridine reductase deficiency. Molecular Genetics & Genomic Medicine, 2020: e1154. doi:10.1002/mgg3.1154.

* Both authors contributed equally to this work

My contributions to the original articles:

Paper I: Collecting data; performing molecular tests, preparing figures and tables and writing the manuscript.

Paper II: Participation in the study design; analysing and interpreting data; preparing figures and tables and writing the manuscript.

Paper III: Participation in the study design; analysing and interpreting data; preparing figures and tables and writing the manuscript.

Paper IV: Collecting clinical data; participation in the study design and writing the manuscript.

ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptophan
5-MTHF	5-methyltetrahydrofolate
6-PTP	6-pyruvoyltetrahydropterin
AAAHs	aromatic amino acid hydroxylases
Arg	arginine
BBB	blood-brain barrier
BH_4	tetrahydrobiopterin (6R-dihydroxypro-2-pyl-L-erythro-5,6,7,8-tetrahydropterin)
bp	base pairs
BS	blood spots
cDNA	complementary DNA
CNV	copy number variation
CSF	cerebrospinal fluid
DHPR	dihydropteridine reductase
DNAJC12	DnaJ heat shock protein family (Hsp40) member C12
ES	exome sequencing
GABA	gamma amino butyric acid, 4-aminobutyric acid
GATK	genome analysis toolkit
GMP	glycomacropeptide
GS	genome sequencing
GTP	guanosine triphosphate
GTPCH	GTP-cyclohydrolase
HPA	hyperphenylalaninaemia
HSP	heat shock protein
HVA	homovanillic acid
IEM	inborn error of metabolism
IMD	inherited metabolic disease
kb	kilobases
LAT1	large amino acid transporter 1
LCL	lower confidence limit
LIMS	laboratory information management system
LNAAs	large neutral amino acids
Mb	megabases
MHP	mild hyperphenylalaninaemia
mmol/mol creat	millimoles per mole of creatinine
MS/MS	tandem mass spectrometry
$NADH^+$	reduced nicotinamide adenine dinucleotide
NGS	next generation sequencing
NO	nitric oxide
NOS	nitric oxide synthase
OMIM	Online Mendelian Inheritance in Man

PAH	phenylalanine hydroxylase
PAL	phenylalanine ammonia lyase
PCD	pterin-4-alpha-carbinolamine dehydratase
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
PKU	phenylketonuria
PTS	6-pyruvoyltetrahydropterin synthase
qBH_2	quinonoid-dihydropterin
QDPR	quinoid dihydropteridine reductase gene
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SR	sepiapterin reductase, 7,8-dihydrobiopterin:$NADP^+$ oxidoreductase
SSCP	single stranded conformational polymorphism
STR	short tandem repeat
SV	structural variant
TPH	tryptophan hydroxylase
Trp	tryptophan
TYH	tyrosine hydroxylase
Tyr	tyrosine
UCL	upper confidence limit
UL-TUH	United Laboratories of Tartu University Hospital
UTR	untranslated region
VNTR	variable number of tandem repeats
VQSR	variant quality score recalibration

1. INTRODUCTION

Phenylketonuria (PKU, OMIM #261600) is an autosomal recessive disorder of amino acid metabolism, wherein the level of phenylalanine (Phe) in body fluids is elevated due to the lack of the activity of the enzyme phenylalanine hydroxylase (PAH). This enzyme is normally responsible for converting dietary Phe into tyrosine (Tyr) of the resulting hyperphenylalaninaemia (HPA) can cause the build-up of cytotoxic compounds and block the transport of other amino acids across the blood brain barrier (BBB). These cause defects of brain development and severe intellectual disability if the condition is untreated. The treatment is primarily a special phenylalanine-free diet [Blau and Scriver 2004; Blau et al., 2010; Blau N 2014; Scriver 1995]. In the majority of Caucasoid populations PKU is one of the most frequent inherited metabolic diseases (IMDs) with the prevalence of approximately 1 in 10,000 [Williams et al., 2008].

The basis of PKU was discovered in 1934 by a Norwegian medical doctor Ivar Asbjørn Følling, who detected phenylpyruvic acid in the urine of some severely mentally retarded patients [Centerwall and Centerwall 2000; Folling 1994]. Furthermore, low-Phe diets were shown to improve the condition of the patients [Bickel et al., 1953]. These observations became the cornerstone for the treatment of PKU patients, who are now recommended to receive a protein-restricted diet supplemented with amino acids, except Phe.

PKU became the first IMD to be screened in newborns by analysing dried blood samples collected on filter paper, later known as Guthrie cards, named for the American microbiologist Robert Guthrie, who developed a bacterial inhibition assay for assessing elevated Phe in blood spots [Guthrie and Susi 1963]. The newborn screening programmes started in the beginning of the 1960s in the United States and later spread into most of the Western-European countries [Therrell et al., 2015]. Currently, newborn screening has been expanded to cover various treatable IMDs, taking into account technical developments and the specific genetic structure of particular populations [Bodamer et al., 2007; Burgard et al., 2012; Landau et al., 2014; Lindner et al., 2010].

Until the early 1990s, PKU in Estonia had been diagnosed only by the urinary Følling test [Folling 1994] and it had been assumed that the incidence of the disease was low, similar to that of the Finnish population, wherein its incidence had been established as about 1 in 200,000 births [Guldberg et al., 1995]. With the shift in paradigm taking place in the beginning of the 1990s, the concept of newborn screening for treatable metabolic disorders was re-evaluated, and in 1993, the national newborn screening programme was initiated [Ounap et al., 1998]. Retrospectively, data about available PKU patients were gathered in the Department of Clinical Genetics, United Laboratories of Tartu University Hospital, and is now permanently upgraded under the management of paediatricians and clinical geneticists in the same department. Since 2014, newborn screening in Estonia has been upgraded to a new level, as the

screening procedure using tandem mass spectrometry (MS/MS) was introduced to enable the detection of aberrations in metabolites to diagnose 19 treatable IMDs [Reinson et al., 2018].

The current study was initiated in the first half of the 1990s, when epidemiological study and mutation screening on Estonian PKU patients was performed in cooperation with Prof. K. Õunap. This is reflected in Publication I of the current thesis. Now, we saw an urgent need to update and refresh the information about PKU and to make it available to scientific and healthcare community. This emphasized the importance of analysing the efficiency of the dietary treatment and adherence of the patients and their families to the recommended dietary treatment, and also the need of finding the exact molecular lesion in the only Estonian patient with dihydropteridine reductase (DHPR) deficiency.

2. LITERATURE REVIEW

2.1. Phenlyalanine metabolism

Phenylalanine (Phe) is an essential amino acid regularly present in excess in nutritional proteins. In a normally functioning organism, the excess Phe is converted to tyrosine (Tyr) by phenylalanine hydroxylase (L-phenylalanine-4-monooxygenase, PAH, EC 1.14.16.1), which is the enzyme carrying out the obligatory and rate-limiting step in the catabolic pathway that leads finally to complete oxidation of Phe to CO_2 and water. The minimum requirements for a normal phenylalanine hydroxylation reaction by this enzyme are the presence of the cytosolic liver enzyme PAH, oxygen, L-phenylalanine, and the tetrahydrobiopterin (BH_4) cofactor [Scriver et al., 1994]. Alternatively, if the function of PAH is disturbed, transamination of excess Phe to phenylpyruvate following subsequent metabolism takes place, producing elevated levels of the neurotoxic compounds phenyllactate, phenylacetate, o-hydroxyphenylacetate and phenylacetylglutamate as well as decarboxylation to phenylethylamine (Figure 1) [Rausell et al., 2019; Williams et al., 2008].

Human PAH is a tetrameric enzyme composed of identical subunits [Fusetti et al., 1998], which are encoded by a single gene *PAH* (phenylalanine hydroxylase, OMIM *612349). The activity of this enzyme is regulated by phosphorylation/dephosphorylation. PAH activity is increased several times if phosphorylated by a cAMP-dependent protein kinase in rat, particularly by phosphorylation of serine at position 16 [Citron et al., 1994]. Human PAH is substantially maintained in an activated form and in vitro studies have demonstrated only moderate additional activation by phosphorylation [Kowlessur et al., 1996]. Each monomer (Figure 2) consists of three functional domains: an N-terminal regulatory domain (residues 1–142); a catalytic domain (residues 143–410) that includes binding sites for Fe^{3+} ion, which is reduced to the active Fe^{2+} form upon binding of the cofactor; and a C-terminal oligomerisation domain (residues 411–452) with dimerisation (residues 411–426) and tetramerisation motifs (residues 427–452) [Flatmark and Stevens 1999; Fusetti et al., 1998; Kobe et al., 1999]. The internal regions of the protein are very highly homologous to those of the other aromatic amino acid (tyrosine and tryptophan) hydroxylases (TYH, TPH). These common sequences are conserved around five cysteine residues [Grenett et al., 1987]. The full activation of PAH occurs only in the presence of the natural cofactor BH_4 and a non-heme iron atom. PAH is expressed mainly in the liver [Ayling et al., 1974], and additionally in the kidney [Lichter-Konecki et al., 1999; Rao and Kaufman 1986].

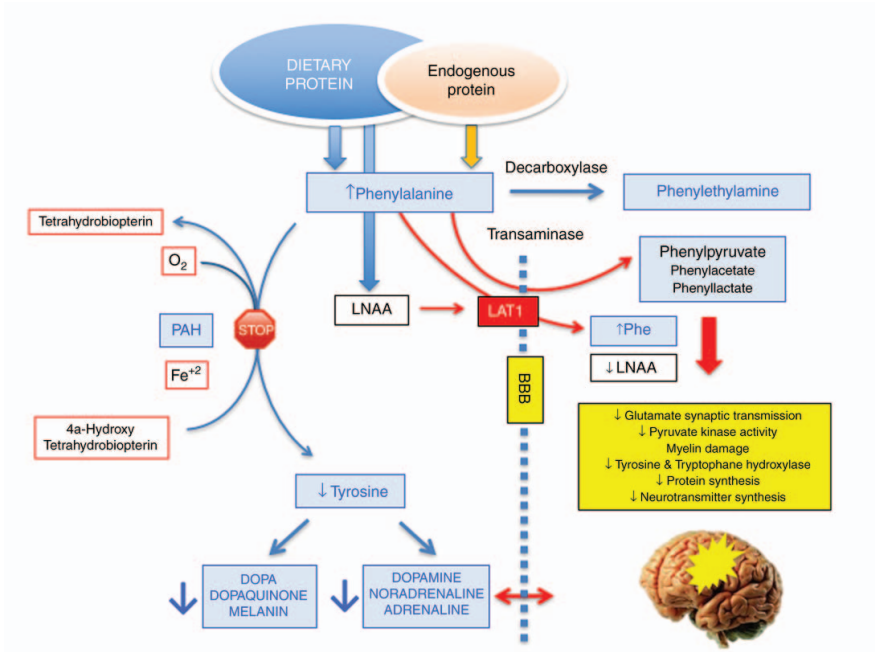

Figure 1. Under normal circumstances, phenylalanine (Phe) coming from the dietary contribution and endogenous protein is metabolized to tyrosine (Tyr) by phenylalanine hydroxylase (PAH) with the concourse of tetrahydrobiopterin, oxygen, and iron. In addition, Phe is converted by the action of Phe-decarboxylase to phenylethyamine. Patients with phenylketonuria (PKU) lack PAH, and as a consequence Phe plasma levels increase to toxic levels in the brain. Excess Phe is converted into phenylpyruvate, phenylacetate, and phenyllactate that are highly toxic for the brain. Phe competes with the other large neutral aminoacids (LNAA) for the same large amino acid transporter 1 (LAT-1) to cross the blood–brain barrier (BBB). In addition, circulating Tyr decreases and subsequently the synthesis of metabolites such as dopamine, noradrenaline, and adrenaline diminishes. The consequence of these metabolic alterations is a protracted brain damage [Rausell et al., 2019].

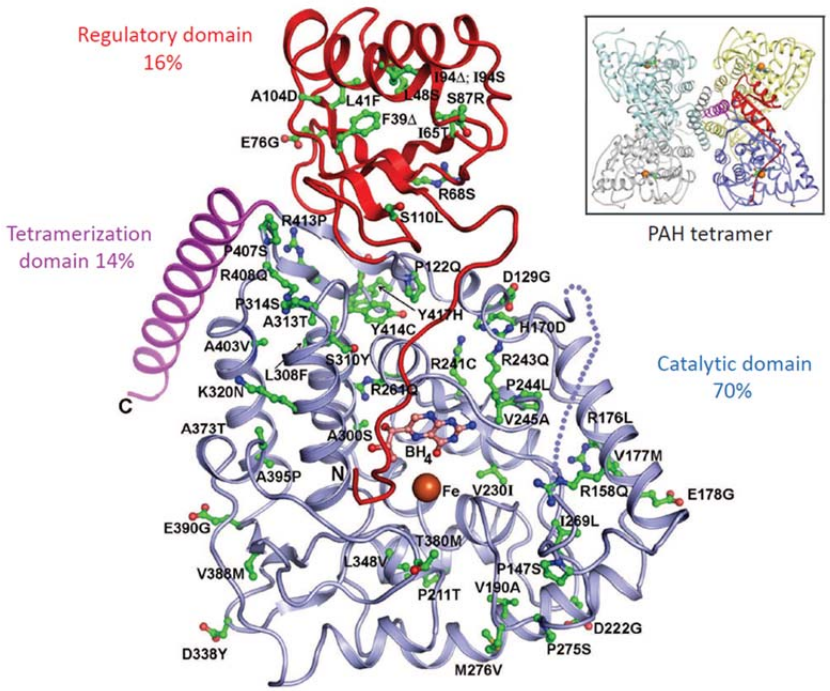

Figure 2. The 3D crystal structure of the PAH monomer. In the active site, the iron atom and BH_4 cofactor are shown in red. BH_4-responsive variations found in PKU patients are mapped in the structure. The N-terminus starting over the active site as well as the rest of the regulatory domain are highlighted in red; catalytic domain in blue; and tetramer domain is in purple. In the top right corner is the native tetramer form of the enzyme. From [Zurfluh et al., 2008].

PAH together with tyrosine hydroxylase (EC 1.14.16.2) and two isoforms of tryptophan hydroxylases (EC 1.14.16.4) make up the family of pterin-dependent aromatic amino acid hydroxylases (AAAHs) (Figure 3). The genes encoding these enzymes apparently evolved by duplication and divergence, beginning about 750 million years ago [Grenett et al., 1987; Xu et al., 2019]. The enzymes are 60% identical over their 330 C-terminal amino acids. All three enzymes are being phosphorylated at amino acid residues in the N-termini, although the effects of phosphorylation differ. The N-terminus of PAH contains an allosteric activation site dependent on the substrate – phenylalanine. Recombinant PAH with the deletion of 116 N-terminal amino acids remains active, but is not regulated by the substrate [Daubner et al., 1997].

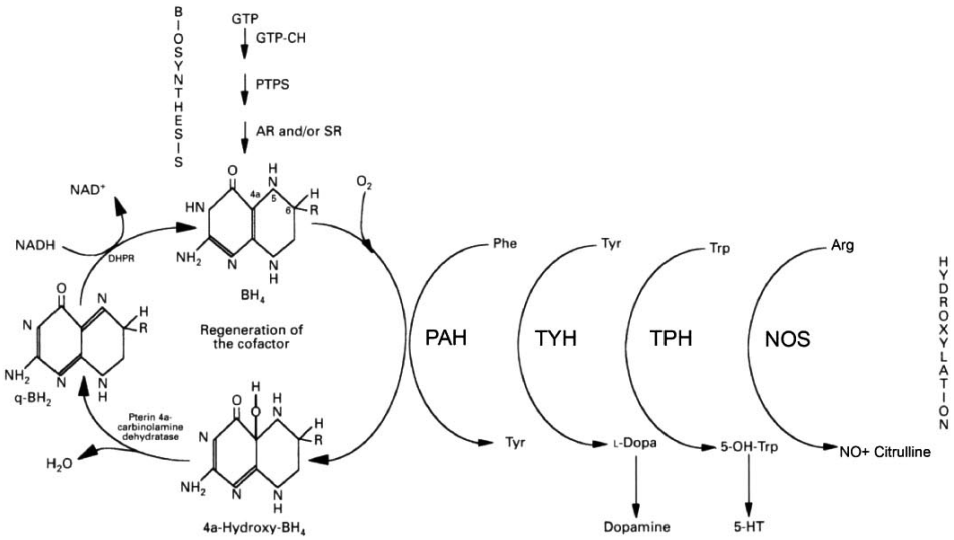

Figure 3. The metabolic pathways connected to the hydroxylation of aromatic amino acids are indicated. GTP-CH (GTP-cyclohydrolase), PTS (6-pyruvyltetrahydrobiopterin synthase), AR (aldose reductase) and/or SR (sepiapterin reductase) are all involved in the biosynthesis of BH_4 from GTP. Pterin 4α-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) are involved in the regeneration of BH_4 and act upon the substrates 4α-hydroxy-BH_4 (or 4α-carbinolamine) and q-BH_2 (quinonoid dihydrobiopterin) respectively. PAH, TYH and TPH catalyse the hydroxylation of phenylalanine, tyrosine and tryptophan to tyrosine, 3,4-dihydroxyphenylalanine (L-Dopa) and 5'-hydroxytryptophan (5-HT, 5-hydroxytryptamine, serotonin), respectively. Additionally, BH_4 catalyses NO synthesis from arginine to NO and citrulline by NOS (nitric oxide synthase). Adapted from [Hufton et al., 1995].

2.2. Biopterin metabolism

The BH_4 cofactor plays two independent roles in PAH: it acts both as a cofactor and as a negative regulator, at least in case of the tetrameric form of the enzyme [Davis et al., 1996]. Nomenclaturally, BH_4 is called 2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-tetrahydropteridine. It is synthesised in mammals via a pathway starting from the nucleotide guanosine triphosphate (GTP). GTP is converted to D-erythro-7,8-dihydroneopterin triphosphate by GTP-cyclo-hydrolase (GTPCH; EC 3.5.4.16). The subsequent pathway involves the re-action catalysed by 6-pyruvoyltetrahydropterin synthase (PTS; EC 4.6.1.10), historically called the “phosphate eliminating enzyme”. The product of PTS, 6-pyruvoyltetrahydropterin (6-PTP), is converted to BH_4 in the presence of NADPH and sepiapterin reductase (SR, EC 1.1.1.153) [Kim and Park 2010; Thony et al., 2000].

Similarly to the phenylalanine hydroxylation reaction, BH_4 is oxidised in the course of the hydroxylation reaction of the aromatic amino acids tyrosine and

tryptophan, catalysed by TYH and TPH, respectively (Figure 4). These amino acids are the essential precursors of the neurotransmitters dopamine (and subsequently other catecholamines) and serotonin. Tyr is hydroxylated to 3,4-dihydroxyphenylalanine (L-Dopa), which is the rate limiting step in the biosynthesis of catecholamines [Hufton et al., 1995]. Additionally, the importance of BH_4 for the full activity of nitric oxide synthases (NOS; EC 1.14.13.39) [Mayer et al., 1991] has been described as another possible origin of the pathogenic processes in BH_4 deficiencies [Werner et al., 2011]. In total, BH_4 is a cofactor for all AAAHs and three NOS isoforms, as well as the glyceryl-ether mono-oxygenase [Thony et al., 2000; Watschinger et al., 2009]

When aromatic amino acid hydroxylases perform the hydroxylation reaction, BH_4 is converted to pterin-4α-carbinolamine. The 4α-carbinolamine then undergoes a pterin-4α-carbinolamine dehydratase (PCD)-catalysed dehydration reaction and quinonoid dihydrobiopterin (qBH_2) is formed [Lei and Kaufman 1998]. This substance has no catalytic activity in the hydroxylation reaction performed by AAAHs and must be reduced back to BH_4 by the NADH-dependent dihydropteridine reductase (DHPR; EC 1.6.99.7). DHPR is a unique enzyme with no related gene sequences known in human genome and it is present in an active form in all tissues. In the absence of regeneration performed by DHPR, the hydroxylation reaction of Phe is stoichiometric for BH_4, i.e. one BH_4 added yields one tyrosine residue formed [Werner et al., 2011].

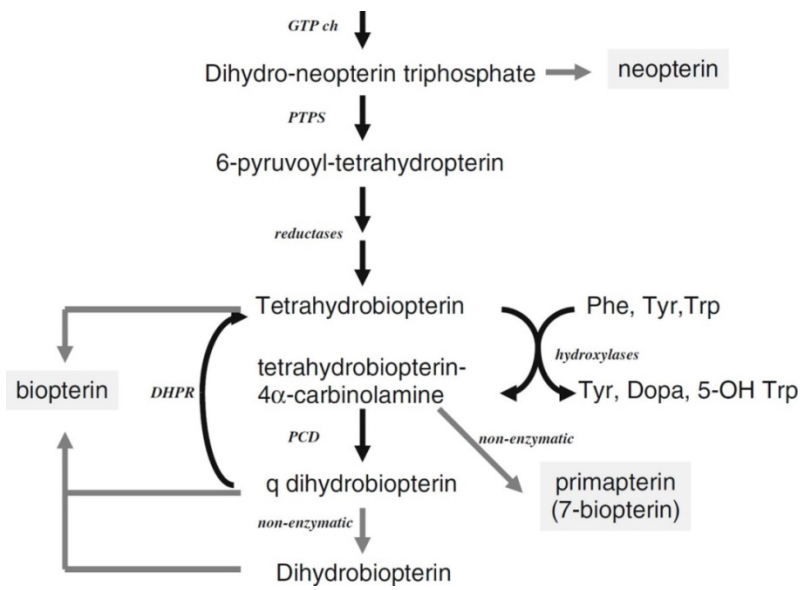

Figure 4. Simplified metabolic pathway of the synthesis and regeneration of tetrahydrobiopterin, and its implication in the synthesis of neurotransmitters [Dhondt 2010].

2.3. The phenylalanine hydroxylase gene

The human *PAH* locus has been mapped to chromosome 12q23.2 [Lidsky et al., 1985]. The first results in cloning human *PAH* were available in 1983 [Woo et al., 1983]. The cDNA of human *PAH* was cloned in 1985 [Kwok et al., 1985], and was shown to contain 2448 bases, with an open reading frame of 1356 bp capable of encoding a 452 amino acid protein monomer with a putative molecular weight of 51,672 daltons. The structure of the *PAH* gene was revealed in 1986, showing the whole peptide-coding region to span over 121.5 kb of genomic DNA and contain 13 coding exons, with intron sizes ranging from 1 to 23 kb [DiLella et al., 1986]. The schematic presentation of *PAH* is shown in Figure 5.

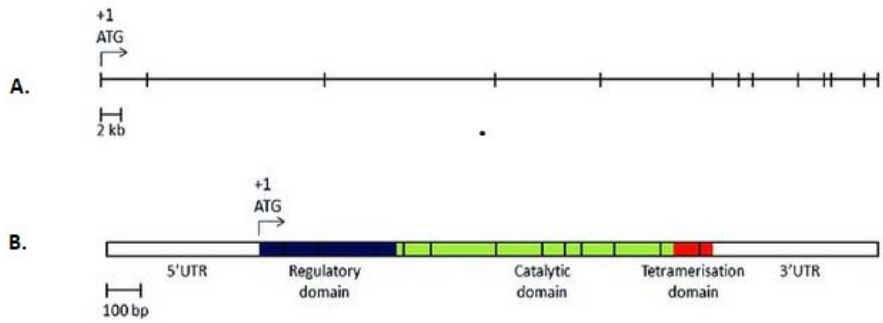

Figure 5. Structure of the human PAH gene. A) The horizontal line represents the full length of the PAH gene, spanning 79.3 kb. Each vertical bar represents an exon. The location of the start codon ATG in exon 1 is indicated (+1). B) Schematic representation of PAH mRNA. The vertical lines mark the boundaries between exons. The three functional domains of PAH are coloured purple for the regulatory domain, light green for the catalytic domain and orange for the tetramerisation domain [Ho and Christodoulou 2014].

Genetic alterations in *PAH* that cause the elevation of Phe in the human organism differ significantly between populations and geographical gradients of variations can be observed. In spite of the high number of described mutations, five molecular lesions are most prevalent among Caucasians [Eisensmith et al., 1992], which together usually cover more than half of the affected alleles in a population. Namely, these variations are p.Arg158Gln, p.Arg261Gln, c.1066-11G>A, p.Arg408Trp and c.1315+1G>A.

The number of reported variations in *PAH* is currently approaching one thousand (Table 1). Given the location of each particular variation in the gene and the character of amino acid substitutions, the phenotypes, together with the personal efficacy of modifications of treatment, can vary widely. These variations may involve complete lack of enzyme activity, responsiveness of the concentrations of the substrate (Phe) and cofactor (BH_4), modifications in protein

folding and tetramerisation, and response to the concentration of cofactor as a chaperon. Several more frequent variations have been assessed by *in vitro* assays [Danecka et al., 2015], providing clues to personalised approach to treatment.

2.4. Phenylketonuria (PKU), a metabolic condition occurring due to PAH deficiency

Phenylketonuria (PKU, OMIM #261600) is an autosomal recessive disorder of amino acid metabolism, wherein the lack of the activity of PAH converting Phe to Tyr leads to accumulation of Phe in the blood and brain. Untreated PKU is characterised by irreversible intellectual disability, microcephaly, motor deficits, eczematous rash, autism, seizures, developmental problems, aberrant behaviour and psychiatric symptoms. The precise pathogenesis of brain dysfunction is still unclear [van Wegberg et al., 2017].

Historically, accumulation of toxic metabolites has been considered one of the main factors in brain damage occurring in PKU; additionally lack of other large neutral amino acids (LNAAs) as well as oxidative stress together with disturbances in energy metabolism in brain is now taken into account as important causes that impact in the pathogenesis of PKU [Kyprianou et al., 2009].

A few pathogenic mechanisms have been proposed to be underlying the deleterious effects of elevated Phe in case of PKU. The excessive accumulation of Phe in plasma and tissues and its metabolites phenylpyruvate, phenyllactate, and phenylacetate, collectively known as phenylketones, provides a set of biochemically detectable compounds characteristic to PKU. These compounds exhibit toxicity to the developing brain, probably via several mechanisms, e.g. by inhibiting antioxidative enzymes like superoxide dismutase and glucose-6-phosphate dehydrogenase [Moraes et al., 2013; Rosa et al., 2012] and thereby inducing oxidative stress. Elevated level of Phe also affects neurotransmitter homeostasis in the developing brain, energy production, and protein synthesis. Transamination of Phe to form phenylpyruvate usually starts if Phe concentration exceeds 1.2 mmol/L. LNAAs need the same active transport channel as Phe for crossing BBB. Thus, the elevated Phe levels are considered to act as a competitive inhibitor of transport of other LNAAs across the BBB, reducing the entrance of Trp into cerebrospinal fluid (CSF) and thereby interfering with the production of serotonin [de Groot et al., 2010]. Moreover, phenylpyruvic, phenyllactic and phenylacetic acids are known to be inhibitors of 5-hydroxytryptophan decarboxylase, leading to a decrease in serotonin synthesis. Decreased amounts of adrenaline, noradrenaline and dopamine may be caused by the inhibition of dopamine decarboxylase. The toxic metabolites of Phe also inhibit glutamic acid decarboxylase, thereby decreasing the levels of 4-aminobutyric acid (GABA), an important neurotransmitter, which regulates neuronal excitability. Additionally, disturbed protein synthesis, lipid metabolism, bio-

energetics, and even calcium homeostasis may all contribute to the pathogenesis of untreated PKU [Schuck et al., 2015].

The mechanisms brought out above occur in persons with PKU. However, elevated levels of Phe and its metabolites can affect also a non-PKU foetus in a mother with PKU, if the treatment regime does not eliminate high Phe levels in maternal blood during gestation. This situation can cause a condition called maternal PKU with severe developmental consequences [Guttler et al., 1999; Levy and Ghavami 1996].

Table 1. Spectrum of the types of *PAH* variations according to Human Gene Mutation database as of August 2019 (http://www.hgmd.cf.ac.uk/) [Stenson et al., 2017].

Variation type	Total number of variations
Missense/nonsense	650
Splicing substitutions	127
Regulatory substitutions	2
Small deletions	106
Small insertions/duplications	24
Small indels	13
Gross deletions	58
Gross insertions/duplications	5
Complex rearrangements	2
Repeat variations	0
Total	987

2.5. Genotype and prevalence of PKU

Several geographically distinct populations show high predominance of some particular variations or at least a significantly increased ratio of one variant compared to the others. Thus, different founder populations of mutant alleles must have been existing; the effect of genetic drift is probable. For example, in Southern Europe and the Mediterranean area, the c.1066-11G>A variant, which introduces an alternative splice acceptor site in intron 9, counts for 10% to 40% of all PKU alleles. However in general in Southern European populations there is a greater heterogeneity in *PAH* variations than in Eastern Europe [Couce et al., 2013; Desviat et al., 1999; Ozguc et al., 1993; Rivera et al., 1998; Zschocke 2003], similar to the situation that exists in Latin America [Perez et al., 1993].

In Eastern Europe, p.Arg408Trp variation is highly predominant, forming a North-South gradient: up to 56% in Moscow region [Charikova et al., 1993], 62–65% in Poland [Bik-Multanowski et al., 2013; Kalaydjieva et al., 1991], 73.5% in Lithuania [Kasnauskiene et al., 2003], 51% in Russia [Gundorova et

al., 2019], 38% in Romania [Gemperle-Britschgi et al., 2016], 18% in Serbia and Montenegro [Stojiljkovic et al., 2006]. Historically, single nucleotide polymorphisms (SNPs) leading to detection of restriction fragment length polymorphisms (RFLPs), a variable number tandem repeat (VNTR) motif and a short tandem repeat (STR) polymorphism variations have been useful to create haplotypes in the *PAH* gene to trace the origins of variations and diagnostic properties [Goltsov et al., 1992; Goltsov et al., 1993; Woo 1988]. The previously mentioned variation p.Arg408Trp is widely present on a different VNTR/STR haplotype background in Ireland – 43% [Eisensmith et al., 1995], probably referring to independent recurrence in human history [Tighe et al., 2003]. This p.Arg408Trp variation is much less frequent in the Mediterranean area – about 4% [Berthelon et al., 1991]. East-European structure of *PAH* variations has been reported also from the Far-East of Russia (Pacific region), wherein p.Arg408Trp accounts for 63% of pathogenic variations [Sueoka et al., 1999]. The p.Arg408Trp variation fully abolishes PAH activity, resulting in severe PKU phenotype, if homozygous. The highly conserved Arg-408 is located on the hinge loop that connects the tetramerisation arm to the core of the PAH monomer (Figure 2) [Fusetti et al., 1998].

For Northern Europe, c.1315+1G>A is typical, especially for Denmark, which has been considered to be the founder population, with relative frequency of 45% among all PKU alleles [Eisensmith and Woo 1994]. Later studies have revealed a somewhat lower, but still outstanding, prevalence of c.1315+1G>A in Denmark (Table 2). In Switzerland and Turkey, p.Arg261Gln is relatively frequent: 32% and 10% of affected alleles, respectively [Eisensmith et al., 1992], as well as 9% in Sicily [Mirisola et al., 2001] and 10% in Portugal [Rivera et al., 1998]. Variation p.Arg158Gln is present in most populations, but no study has shown to contain it in a significantly higher percentage. Certain populations have specific mutations characteristic almost entirely to them, such as the Yemenite Jews, who carry a deletion of the whole exon 3 [Avigad et al., 1990]. Roma of Eastern Europe often carry the p.Arg252Trp allele [Kalanin et al., 1994]. Several less frequent mutations are distributed sporadically. The populations derived from various origins of migration (e.g. the United States) exhibit wide spectra of *PAH* variations where none of them clearly dominates over the others in frequency [Guldberg et al., 1996]. As expected, different and variable genotypic structure is present in China, wherein the p.Arg243Gln allele is the most prevalent mutation, with a relative frequency of 20% [Li et al., 2018; Wang et al., 2018]. The phenomenon of extreme PKU frequency among the Karachay people in North Caucasus exists with widespread prevalence of the p.Arg261* variant present in 68% of their PKU alleles [Gundorova et al., 2018]. In general, variations common in Caucasoid populations show very low frequency among Asian peoples, e.g. China and Japan, and vice versa.

Many populations with increased PKU incidence are present in Europe ranging from 1 in 3000 to 1 in 35,000 [Loeber 2007]. In Asians, the incidence has been shown to be lower – 1 in 20,000 in China [Chen et al., 2018], 1 in 53,000 in Japan [Yamaguchi-Kabata et al., 2019]; but 1 in 4370 live births in

Turkey [Ozguc et al., 1993]. Other frequencies include 1 in 5300 in Ireland, 1 in 7700 in Poland, 1 in 9000 in Czechia [Kozak et al., 1997], 1 in 12,000 in Portugal [Rivera et al., 1998], 1 in 16,700 in Italy, 1 in 13,000 in France, and 1 in 15,800 in Sweden [Ohlsson et al., 2017]. Our neighbours in Latvia have the incidence 1 in 8170 [Pronina et al., 2003]. Probably the highest frequency of PKU in the world can be found in the North-Caucasian Karachay-Cherkess Republic in the Russian Federation (1 in 850 newborns) and 1 in 332 among the titular nation of the Karachays, due to the tradition of monoethnic marriages, causing decreased genetic variability in those populations [Gundorova et al., 2018]. On the contrary, the prevalence among the population originating from Sub-Saharan Africa shows a difference of an order of magnitude lower frequency than in Caucasoid populations [Hardelid et al., 2008].

Estonian neighbourhood populations exhibit two different geographical gradients. Eastern-European countries with Baltic and Slavic background have a general incidence of PKU approximately between 1:6000 to 1:10,000, with a clear predominance of the variation p.Arg408Trp accounting for 50% to 80% of all PKU alleles in the population. In the North-West direction, over the Baltic sea, the same variation has much lower influence: 14% – 17%, while the "Nordic" variation c.1315+1G>A is responsible for 10% – 26% *PAH* deficient alleles. PKU incidence in the Nordic/Scandinavian countries is somewhat lower than in the East European populations, one in 12,000 to 14,000 newborns. As an outstanding exception, the ethnically close Finnish population has PKU incidence lower than 1 in 100,000 [Guldberg et al., 1995]. An overview of *PAH* variations in different populations with focus on variations present in Estonia is presented in Table 2.

It seems curious that a number of different *PAH* gene variations have become prevalent in various populations. The founder effect and genetic drift are obvious in case of common wide-spread mutations [Eisensmith and Woo 1994]. Selection in favour of the heterozygotes for the defective PAH gene can be proposed [Woo 1989], but no confirmed evidence about the mechanism that might bring profit to the carrier is known yet. At least in Ireland and Scotland women heterozygous for PKU have been shown to have had fewer pregnancies ended in spontaneous abortion, resulting in 7.4% more live-born offspring [Woolf 1994]. A hypothesis has been risen that slight HPA in the pregnant heterozygote can protect the foetus against abortifacient mycotoxin (ochratoxin A) found in stored grain infected by moulds from several species of *Aspergillus* and *Penicillum*. This might have been an advantage during lean years or hunger [Woolf 1986]. Possible over-dominant selection in PKU carriers may have occurred predominantly during periods of epidemics or famine [Krawczak and Zschocke 2003]. However, there have been numerous independent mutation events for PKU in Europe, and several variations have independently recurred in different founders and subsequently effectively spread over populations [Zschocke 2003].

The distribution of the phenotypic variations of PKU is in accordance with the incidences of the prevalent *PAH* gene variations with different influences on

the reduction of the enzymatic activity of PAH. A specific North-South gradient in Europe can be observed, while milder forms of HPA become more frequent towards Southern Europe, and severely disabled PAH activity is characteristic to Northern and Eastern Europe [Desviat et al., 1999; Zschocke 2003]. Variability in the severity of the disorder suggests that different mutations influence the phenotypic outcome in different ways. Evidence from in vitro expression analysis supports this opinion [Danecka et al., 2015; Okano et al., 1991].

2.6. Defects of biopterin metabolism

Deficiencies in the synthesis or regeneration system of BH_4, the cofactor in aromatic amino acid hydroxylation reactions, are rare in all populations. The estimated incidence of BH_4 deficiencies is 1–2 % of all patients with HPA detected by the newborn screening [Blau et al., 2011; Dhondt 2010; Opladen et al., 2012]. Current knowledge can be retrieved from the International Database of Tetrahydrobiopterin Deficiencies (http://www.biopku.org/biodef/) [Opladen et al., 2012]: 303 cases of DHPR deficiency, 37 with GTPCH deficiency, 735 with PTS deficiency, 55 with sepiapterin reductase (SR) deficiency, and 30 PCBD deficiency cases have been recorded. The severity of BH_4 disorders can vary widely, however, BH_4 disorders are considered among treatable IMDs. The therapies depend highly on the particular molecular lesion, from BH_4 monotherapy in the transient and benign case of HPA, the PCD deficiency, to strict low-Phe diet as in severe forms of PKU, together with the substitution of neurotransmitter precursors (L-DOPA/carbidopa, 5-hydroxytryptophan (5-HT), and folinic acid) in the case of severe DHPR deficiency [Opladen et al., 2012]. The therapies may be accompanied with symptomatic treatment such as anticonvulsive medication for reducing the deleterious effects caused by delay in diagnosis and treatment.

2.6.1. DHPR deficiency

DHPR deficiency (OMIM #261630, *612676) is the second most common cause of BH_4 deficiencies and accounts for about one-third of all forms of BH_4 deficiencies [Blau 2016]. The DHPR enzyme is encoded by the quinoid dihydropteridine reductase (*QDPR*) gene. The *QDPR* cDNA is 1.2 kb long and has been mapped to chromosome locus 4p15.3 [Dahl et al., 1987; Lockyer et al., 1987]. It encodes for a protein of 244 amino acids, active as a homodimer. The gene extends over more than 20 kb and the coding sequence consists of 732 bp. *QDPR* includes at least seven exons ranging within 84–564 bp and six introns within a range of approximately 1.7–10 kb. The intron–exon boundaries are flanked by canonic splice junctions [Dianzani et al., 1998]. Biallelic pathogenic variants in *QDPR* gene lead to BH_4-deficient HPA, accompanied with a severe biogenic amines deficiency. According to the online register of BH_4

deficiencies (http://www.biopku.org/biodef/BIODEF) [Opladen et al., 2012], 303 cases of DHPR deficiency have been recorded. The *QDPR* locus-specific database PNDdb (http://www.biopku.org/home/pnddb.asp) tabulates information of 85 disease-causing *QDPR* variants: 50 of them missense, 8 nonsense, 8 small deletions, 7 splice variants, 6 small insertions/duplication, 4 indels, 1 large deletion and 1 synonymous variant. One intronic variant resulting in cryptic splice site activation has been reported in a patient with DHPR deficiency [Ikeda et al., 1997].

The patients with DHPR deficiency exhibit the elevation of Phe as the first phenotypic feature, but in contrast to regular PKU children, the low-Phe dietary treatment is not sufficient for the reduction of the pathological processes induced by the lack of neurotransmitters due to the inactivation of AAAHs. The severity of the clinical picture can vary according to the molecular lesion and residual enzyme activity [de Sanctis et al., 2000]. The main symptoms characteristic to untreated or late-diagnosed DHPR deficiency include a cohort of severe neurologic symptoms, *e.g.* axial hypotonia and truncal hypertonia; abnormal thermogenesis, seizures, and microcephaly [Dianzani et al., 1998].

Individuals with severe DHPR deficiency require treatment with the hydroxylated precursors of the deficient neurotransmitters, phenylalanine-restricted diet, or substitutive therapy with BH_4, besides folinic acid supplementation. They display great clinical heterogeneity, similar to that observed in PKU, suggesting that DHPR deficiency may be the result of a wide range of mutations [Dianzani et al., 1998]. However, the treatment of DHPR deficiency differs from other BH_4 deficiencies. In the case of a block in the pathway of BH_4 biosynthesis, substitution with the synthetic coenzyme can provide a reservoir for several cycles of oxidation and subsequent regeneration to enable aromatic amino acid hydroxylases perform their function. In case of DHPR deficiency, however, the molecule cannot be regenerated and is used only once, and effective doses of BH_4 should be considerably higher. However, responsive cases of DHPR-deficient patients supplemented only with BH_4 have been reported [Coughlin et al., 2013; Kaufman et al., 1982].

2.6.2. GTPCH deficiency

GTPCH (EC 3.5.4.16) is the first and rate-limiting enzyme in BH_4 biosynthesis, catalysing the conversion of GTP into 7,8-DHNP-3'-TP. GTPCH deficiency (OMIM *600225, #233910) occurs in autosomal recessive and autosomal dominant forms. This enzyme is encoded by a single *GCH1* gene, and the corresponding locus has been mapped to chromosome 14q21–q22.2 [Ichinose et al., 1994]. The autosomal recessive inherited deficiency of GTPCH is clinically characterised by severe neurological symptoms unresponsive to the classic Phe-low diet [Thony and Blau 1997]. Early replacement therapy with BH_4 as well as L-dopa/carbidopa and 5-hydroxytryptophan (5-HT) can reduce the symptoms significantly [Sato et al., 2014].

The autosomal dominant version of GTPCH deficiency (OMIM #128230) is known also as Segawa syndrome or DOPA-responsive dystonia. During the first two decades of life, typically segmental or generalized dystonia occurs, and the disease may also present as parkinsonism that manifests as rigidity, bradykinesia and postural tremor. This condition is treatable with lifelong administration of L-DOPA/carbidopa and does not manifest with highly elevated Phe levels [Wijemanne and Jankovic 2015].

2.6.3. PTS deficiency

The most common deviation in BH_4 metabolism occurs due to abnormalities in PTS, 6-pyruvoyl-tetrahydropterin synthase (EC 4.6.1.10), which is encoded by a single *PTS* gene (OMIM *612719) and the corresponding locus has been mapped to chromosome 11q22.3–q23.3 [Thony et al., 1994]. Similarly to GTPCH deficiency, PTS deficiency is inherited autosomal-recessively and develops severe neurological symptoms that are unresponsive to the classic low-Phe diet. In contrast to GTPCH deficiency, PTPS deficiency is a more heterogeneous condition of HPA, occurring in mild, severe, or intermediate forms [Ponzone et al., 1990]. The heterozygotes for deficient PTS are clinically normal [Thony and Blau 1997].

2.6.4. PCBD deficiency

HPA due to PCBD deficiency (OMIM #264070) is caused by homozygous or compound heterozygous mutation in the *PCBD1* gene, which encodes the enzyme pterin-4-alpha-carbinolamine dehydratase (OMIM *126090; EC 4.2.1.96), and which is located onchromosome 10q22. This enzyme is involved in the salvage pathway for BH_4. PCBD deficiency is an autosomal recessive disorder characterized by mild transient HPA, often detected by newborn screening. Patients also show increased excretion of 7-biopterin. Affected individuals are asymptomatic and show normal psychomotor development, although transient neurologic deficits in infancy have been reported [Thony et al., 1998a]. This disorder is known also as primapterinuria, and is considered a transient and benign form of HPA [Thony et al., 1998b].

2.6.5. SR deficiency

Sepiapterin reductase (SR, 7,8-dihydrobiopterin:$NADP^+$ oxidoreductase; EC 1.1.1.153; OMIM *182125), belongs to a group of enzymes called aldo-keto reductases and is encoded by a gene at chromosome locus 2p13.2. SR catalyzes the NADPH-dependent reduction of various carbonyl substances, including derivatives of pteridines. Its deficiency (OMIM #612716) manifests in affected individuals as an L-DOPA-responsive, diurnally fluctuating movement disorder,

usually associated with cognitive delay and severe neurologic dysfunction [Bonafe et al., 2001]. Thus, this disorder has been diagnosed also as a DOPA-responsive dystonia. Patients with SR deficiency do not exhibit clear permanent HPA. Other signs of SR deficiency that are observed in some patients include parkinsonism, tremor, dysarthria, limb hypertonia, hyperreflexia, psychiatric disorders, autonomic dysfunction, and sleep disturbances [Wijemanne and Jankovic 2015].

2.6.6. DNAJC12 deficiency

Correct folding of aromatic amino acid hydroxylases, including PAH, depends on certain molecular chaperones, belonging to heat shock protein (HSP) families of 40 and 70 kD molecular weight. Variants in *DNAJC12* (OMIM #617384, DnaJ heat shock protein family (Hsp40) member C12) were recently described to lead to mild HPA, central biogenic amines deficiency, dystonia, intellectual disability and parkinsonism, thereby defining a new entity of HPA without PAH or BH_4 deficiency [Anikster et al., 2017]. DNAJC12 is a member of the HSP40 family that has been shown to interact with the aromatic amino acid hydroxylases PAH, TYH and TPHs. DNAJC12 binds to PAH through its peptide-binding domain and interacts with the HSP70-ATP complex through the HPD motif (a conserved His, Pro, Asp signature, crucial for stimulation of HSP70's ATPase activity) in the N-terminal J domain [Blau et al., 2018]. The cases with deficient DNAJC12 have been treated by substitution with BH_4 and/or neurotransmitter precursors L-DOPA/carbidopa and 5-HT, which have shown beneficial effects, resulting in the prevention of neurodevelopmental delay in individuals treated before the onset of symptoms [van Spronsen et al., 2017a].

2.7. Phenotypes of PAH deficiency

Normal blood phenylalanine concentration is considered to be 58 +/- 15 μmol/L in adults [Cleary and Walter 2001], 60 +/- 13 μmol/L in teenagers and 62 +/- 18 μmol/L in childhood. In healthy infants and children up to the age of 18 years, reference blood phenylalanine concentrations are between 21 and 137 μmol/L, and in adults, 35 to 85 μmol/L [Cleary et al., 2013].

The PAH deficiency trait is heterogeneous at the biochemical level with a continuum of metabolic phenotypes. It is usually classified as mild, moderate, or severe (also referred to as classic) PKU. This classification is commonly based on the highest untreated blood phenylalanine concentration following a clinical diagnosis or at newborn screening [van Spronsen et al., 2017b]. In 1980, for the first time, blood Phe levels were used to discriminate between three different phenotypes of PKU [Guttler 1980]. Classic PKU was defined by presenting with Phe pre-treatment levels >1200 μmol/L, variant PKU with Phe pre-treatment levels of 600–1200 μmol/L, and mild HPA with Phe pre-treat-

ment levels <600 μmol/L. More precisely, PAH deficiency has been classified into four different phenotypes: classic PKU presenting with Phe pre-treatment levels >1200 μmol/L, moderate PKU with Phe pre-treatment levels of 900–1200 μmol/L, mild PKU with Phe pre-treatment levels of 600–900 μmol/L, and mild HPA with Phe pre-treatment level <600 μmol/L [Blau et al., 2011; Guldberg et al., 1998].

In practice, classification of PKU is essential for choosing the optimal treatment. Therefore a simplified classification scheme recommended in the European guidelines on phenylketonuria [van Wegberg et al., 2017] is based on treatment requirements: a) patients who do need strict dietary treatment (PKU), b) patients who do not need any treatment (non-PKU HPA), and c) patients who may be treated with BH_4 (BH_4-responsive PKU) [Blau et al., 2011].

PKU always causes HPA, but not all HPA is PKU. In the 1970s, several children with HPA but unresponsive to dietary Phe restriction ("atypical" or "malignant" PKU), with developmental delay and neurological pathology, were described [Blau 2016; Cederbaum 1979; Danks et al., 1979]. These cases were further identified as BH_4 deficiencies, referring to disturbances in BH_4 synthesis and regeneration.

It is important not to confuse BH_4-responsive PKU and BH_4 deficiencies; the first ones are a subtype of PAH deficiency, which can be alleviated by supplementation with BH_4 and are caused by a number of variations in *PAH* gene. BH_4 works as a chaperon and supplementation in excess provides the PAH tetrameric protein better opportunities to regain its correct conformation and therefore also to restore its enzymatic activity. BH_4 deficiencies are caused by abnormalities in the synthesis or regeneration of the cofactor, and in these cases, elevated Phe is just one of the detrimental changes in the homeostasis of the organism. The even more serious changes include the malfunction of TYH and TPH as well as NOS, thereby disturbing much of the synthesis of basic components of neurotransmission like dopamine, noradrenaline, serotonin and nitric oxide and usually leading to more severe clinical phenotype than PKU, if untreated.

Table 2. Relative frequencies (%) of major disease-causing *PAH* gene variants among various PKU populations.

Population \ *PAH* variation (%)	p.Arg408Trp	p.Leu48Ser	c.1315+1G>A	c.1066-11G>A	p.Glu280Lys	p.Arg261 Gln	p.Arg158 Gln	p.Arg252Trp	p.Ala403Val	p.Pro281Leu	Total prevalence of PKU	Reference
Estonia	80.4	3.3	2.6	<1	<1	2	<1	<1	<1	<1	1 : 6700	[Lillevali et al., 2018]
Latvia	76		1	1	5.2	2.1	4.2	1		2.1	1 : 8200	[Pronina et al., 2003]
Lithuania	73.5		<1	<1	1	<1	7.1		2	1	1 : 9300	[Kasnauskiene et al., 2003]
Sweden	15.4	1.6	9.9	2.7	<1	5.5	<1	1.4	1.3	4.2	1 : 14 200	[Ohlsson et al., 2017]
Finland	50					12.5					< 1 : 100 000	[Guldberg et al., 1995]
Poland	54.9		2.7	4.9		2.2	6.6	1.1		<1	1 : 8000	[Zekanowski et al., 2001; Zekanowski et al., 1994]
Poland (West)	68		5.2	6				<1		2.2		[Dobrowolski et al., 2009]
Russia	50.9	1	3.1	2.6	1.2	5.3	2.4	1.6	<1	3.5	1 : 7000	[Gundorova et al., 2019]
Russia (St. Peters-burg area)	70.7		2.1	<1	<1	4.3	1.4	2.9				[Baranovskaya et al., 1996]
Russia (Far East	63		1.7			6.7	3.3	1.7			1 : 6100	[Sueoka et al., 1999]
Czech Republic	42.1	1.9	2.5	3.6		1.6	4.1	3.8	5.1	2	1 : 9000	[Reblova et al., 2013], [Kozak et al., 1997]
Hungary	48		6	2		2	8				1 : 9000	[Schuler et al., 1994; Schuler et al., 1996]
Slovakia	47		5.3	1.7		3.9	5.3	5.1	3.6	2.7	1 : 5900	[Polak et al., 2013]
Romania	37.7	9.3	3.1	6.8		5.6	1.2	1.2	1.2	5.6	1 : 8000	[Gemperle-Britschgi et al., 2016]
Germany (South)	23.2	1.2	10.4	2.7		3.1	5	<1	7.7	3.9		[Aulehla-Scholz and Heilbronner 2003]
Germany (North)	26.7	3.3	10	3.3		5	3.9	-	2.8	3.3		[Aulehla-Scholz and Heilbronner 2003]
Germany (East)	38.1	3.1	7.6	5.7	<1	4	3.3	<1	<1	2.9		[Hennermann et al., 2000]

Popu-lation / *PAH* variation (%)	p.Arg408Trp	p.Leu48Ser	c.1315+1G>A	c.1066-11G>A	p.Glu280Lys	p.Arg261 Gln	p.Arg158 Gln	p.Arg252Trp	p.Ala403Val	p.Pro281Leu	Total prevalence of PKU	Reference
Austria	22.8	1.7	11.6	3.7		6.8	4.4		1.7	3.4		[Sterl et al., 2013]
Denmark	16.9	<1	25.8	4.8	1.2	1.6	2.3	<1	1.7	1.5	1 : 12 000	[Bayat et al., 2016; Tighe et al., 2003]
Ireland	41		2.3	1.3	1.1	<1	<1	<1		<1	1 : 4500	[O'Donnell et al., 2002; Tighe et al., 2003]
Norway	14.4		14.7	<1	<1	8	<1	<1		1.7		[Eiken et al., 1996]
Serbia	16.4	31	2.6	1.7	-	3.4	3.4	-	1.7	6	1 : 12 300	[Djordjevic et al., 2013; Stojiljkovic et al., 2006]
Slovenia	29	2	3	2	<1	4	9	1	7	7		[Groselj et al., 2012]
Croatia	37	5	1.3	2.5		9	1.3	1.3		11	1 : 8275	[Zschocke et al., 2003]
Spain (Andalusia)	5		6.4	22.9	2.1	9.3	3.6		7.1			[Bueno et al., 2013]
Portugal	<1			10.8		10.4	3.2	4		4.5	1 : 12 000	[Rivera et al., 1998]
France	5.6	2.6	1.8	8.2	4.7	8.1	2.1	1.2	1.6	2.7	1 : 10 000	[Jeannesson-Thivisol et al., 2015; Tighe et al., 2003]
Italy (South)	6.1			12.1		9.1				7.6		[Trunzo et al., 2015]
Turkey	6.4	7		24.6		8.7	2.3	1.1		8.4	1 : 4370	[Dobrowolski et al., 2011; Ozguc et al., 1993]
Iran	2.1	1.4		26.1	<1	12.9	<1	2.1	1.8	19.3	1 : 6250 – 1 : 3700	[Esfahani and Vallian 2018]
Japan								4.4	<1	<1	1 : 52 700	[Okano et al., 2011; Yamaguchi-Kabata et al., 2019]
United States of America	18.7	1.7	7.8	4.4	2.7	2.4	1.7	1.7	1	2.7	1 : 15 000	[Guldberg et al., 1996; National Institutes of Health Consensus Development 2001]
China (Northwest)	1.4			<1		2.2	<1				1 : 11 200	[Yan et al., 2019]

2.8. Diagnosis of PKU

Ever since the fundamental discovery by Dr Asbjørn Følling in 1934 of phenyl-pyruvic acid in the urine of mentally handicapped patients (further developed into the so-called urinary Følling test with $FeCl_3$) [Centerwall et al., 1960], and the success of dietary low-Phe therapy to alleviate the symptoms of a PKU patient by German doctor Horst Bickel in 1953, the importance of diagnosis and early treatment of PKU has become the cornerstone of providing life quality for individuals with this inborn error of metabolism (IEM). Therefore, PKU became the first IEM for newborn screening programmes. The first successful screening method was introduced by Guthrie and Susi in 1963, who gathered blood samples on filter paper cards (nowadays, the well-known "Guthrie cards"). If a large amount of Phe were in the blood, it would overcome the metabolic block of a poison (β-2-thienylalanine) that would have otherwise inhibited the growth of a certain strain of *Bacillus subtilis.* The growth zones of the bacteria on agarose plates would indicate that the blood spot had an elevated Phe level [Guthrie and Susi 1963]. This enabled a technician to perform hundreds of screenings a day with very small amounts of blood, rather than the urine needed for the ferric chloride test [Zhu 2017].

Further, the fluorometric assay by McCaman and Robins, using quantitative fluorescence-based measuring of a ninhydrin-phenylalanine complex enhanced by L-leucyl-L-alanine dipeptide, later became available and with modifications, became widespread for newborn screening [McCaman and Robins 1962; Wu et al., 1979]. Together with the advancement of the availability of tandem MS/MS for public health care, multiple centres have now extended their newborn screening programmes, including multiple other metabolic disorders together with PKU [Bodamer et al., 2007]. This strategy has been used in Estonia since 2014 [Reinson et al., 2018].

In general, screening is performed mostly between the ages of 3 and 5 days, depending on the logistic capabilities employed in each newborn screening programme. A commonly used Phe cut-off level for diagnosis of PKU is 120–130 μmol/L (with a Phe/Tyr ratio > 2) with MS/MS employed [Blau et al., 2011]. In Estonia, according to the national PKU treatment guidelines, the cut-off level of Phe in newborn screening is 3 mg/l (180 μmol/L) [Uudelepp et al., 2012].

Currently, molecular diagnosis for finding the causative variants in the *PAH* gene has become an essential part of the diagnostic procedure of any person with the manifestation of HPA. This provides also early insight into the options and potential responses to treatment [Zschocke et al., 2008; Zschocke et al., 2012].

2.9. Treatment of PKU

The main and prevalent therapy for eliminating the hazardous effects of excess Phe on the nervous system has been the reduction of ingested natural proteins, substituting the lack of this essential nutritional compound with specifically designed amino acid mixtures devoid of Phe [MacDonald et al., 2011]. However, maintaining the recommended Phe levels (see Table 3) by diet may become a burden for an individual with PKU and his/her family. Therefore, efforts are being made to find solutions to alleviate the constant restrictions associated with the dietary therapy.

To date, three main different types of treatment are available and more are under development to lower the Phe level and keep it in the recommended range. The first and most important is the restriction of dietary Phe, which remains the mainstay of PKU management, and which usually begins immediately after confirmation of HPA in a neonate. Patients with PKU have to accept the Phe-free formula for covering the essential need for all other nutritional amino acids and to avoid foods rich in protein [Blau et al., 2010]. In contrast to earlier suggestions that dietary treatment may be terminated after the most vulnerable period of fast brain development and myelin stabilization, there has in recent decades been a strong urge toward a strict "diet for life" [Levy and Waisbren 1994]. Blood Phe levels in all patients are recommended to be maintained in the range of 120–360 μmol/l, with alleviation up to 600 μmol/l in adolescents and grown-ups, according to different national guidelines (see section 2.10).

Currently, monotherapy (low Phe diet) or, increasingly, a combination of two therapies – either low Phe diet and chaperone (BH_4) supplementation therapy or low Phe diet and enzyme substitution therapy – is used to control the blood Phe level of a given patient. Currently available drug therapy is infrequently used to allow for the discontinuation of the low Phe diet [Lichter-Konecki and Vockley 2019].

The second important treatment method is the dietary supplementation with BH_4, as a natural chaperon restoring PAH activity besides being an essential cofactor for PAH [Kure et al., 1999; Muntau et al., 2014]. BH_4 supplementation has become one preferred option since 2007, when sapropterin dihydrochloride (the pharmaceutically used version of BH_4) became commercially available and legally accepted under the name Kuvan ^{TM} [Levy et al., 2007]. However, this type of therapy is very dependent on the particular genotype of the patient and is effective only for a certain fraction of all patients [Burton et al., 2007], mainly those with milder phenotypes of PKU [Muntau et al., 2002]. *PAH* variation analysis together with BH_4 loading test enable one to predict the type of therapy to be recommended to each individual patient [Anjema et al., 2013]. Substantial research for getting the spectra of PAH enzyme activities in the presence of variable amounts of BH_4 cofactor and Phe substrate in 30 frequent homozygous and compound heterozygous genotypes has been performed to find possible personalised treatment regimens [Danecka et al., 2015]. Thus, the structure of

genotypic variability in each particular population may help to predict the most efficacious form of therapy to be recommended. For example, approximately only 8–10% of Polish patients with HPA may derive possibly benefit from the BH_4 treatment [Bik-Multanowski et al., 2013], while in Russia, genotype-based prediction proposes 20% of PKU patients as potential responders [Gundorova et al., 2019]. Responsiveness to BH_4 is predictably much higher (>75%) in Southern regions of Europe with a high frequency of BH_4-responsive alleles p.Arg261Gln, p.Val388Met, p.Ile65Thr, p.Arg158Gln, or p.Leu48Ser, than in Central Europe (50–70%), or in some Eastern European countries (<40%) with frequent severe alleles like p.Arg408Trp, p.Arg252Trp, or c.1315+1G>A [Zurfluh et al., 2008].

The third treatment option for alleviating the damage caused by excess Phe in CNS is the enrichment of the diet with LNAAs, which compete with Phe for the same transporter protein through BBB [Pietz et al., 1999] and thus reducing the concentration of Phe in the brain [Moats et al., 2003]. However, this method cannot be considered as monotherapy.

Combined therapy of low-Phe diet, LNAA and BH_4 supplementation has been considered in responding patients to relieve the possible burden of dietary restriction [Yano et al., 2016].

Currently, active development is being conducted on enzyme substitution therapy with phenylalanine ammonia lyase (PAL, pegvaliase, EC 4.3.1.5), an enzyme that degrades Phe via a different pathway than PAH. This therapy holds promise as a non-dietary way to control the Phe level in PKU. PAL is a non-mammalian enzyme that converts Phe to trans-cinnamic acid and ammonia. Clinical trials with injected pegvaliase have shown effect on lowering Phe levels in adult PKU patients [Harding et al., 2018]. Engineered versions of pegvaliase with attached polyethylene glycol (PEG) molecules to stabilize and protect the PAL protein in the intestine have now been approved in the United States under the name Palynziq™ as an enzyme substitution therapy for PKU [Levy et al., 2018] for adults with blood Phe levels ≥600 μM. In April, 2019, this treatment approach was approved for adolescent adults of >16 years of age by the European Medicines Agency [Grisch-Chan et al., 2019]. Variable methods of PAL administration both as cellular insertions and in cell-free units are in rapid progress.

A natural source of low-Phe protein called glycomacropeptide (GMP) is also available as a dietary supplement, which has an extraordinarily low level of Phe (2.5–5 mg Phe/g protein). GMP is a 63 amino acid C-terminal part of kappa-casein which is released in whey during cheese making by the action of chymosin [Neelima et al., 2013]. A variety of foods and beverages can be made with GMP to improve the taste, variety and convenience of the PKU diet [Ney et al., 2009].

For studying increasingly developing treatment variations for PKU, the creation of a mouse model *Pah*$^{enu2/2}$ was published by McDonald almost 30 years ago [McDonald et al., 1990]. This model is often used for viral vector based gene therapy experiments targeting mainly hepatocytes. More than a

dozen different attempts of such gene therapy on *Pah*$^{enu2/2}$ mouse model have been performed, with several promising outcomes [Grisch-Chan et al., 2019]. The most widespread genome editing tool, the CRISPR/Cas9 system, enables editing particular point mutations, but has raised safety concerns, especially due to the existence of other known therapies without these threats of unknown safety.

The U.S. PKU guidelines state (in the "Future Directions" section), that new therapies, including gene therapy and hepatocyte transplant, have shown some efficacy in animal or limited human trials but require further development and validation for routine clinical use [Vockley et al., 2014]. However, the recent European guidelines for PKU mention dietary and enzyme replacement therapy but not gene therapy as an option [van Spronsen et al., 2017b].

2.10. Dietary recommendations for PKU

Since the first observations made in 1953 [Bickel et al., 1953], dietary management of PKU has remained the main intervention to achieve proper development of the affected individuals. Much effort has been made to provide well-balanced nutritional formulae to substitute the restriction of proteins from diet [MacDonald et al., 2011]. Consensus for continuing the diet indefinitely has been agreed for 25 years already [Council 1993]. It has been shown, that the IQ levels in PKU patients are dependent on the strictness of the diet from birth to 12 years of age, and not as significantly afterwards [Pietz et al., 1998]. However, limiting the exposure to high levels of Phe after that age may help avoiding various neurological problems and may have additional benefits for the medical and cognitive status of these individuals [Koch et al., 2002]. Unified improved (international) guidelines for PKU, both for treatment of children, but also after childhood, should address additionally the nutritional, neuropsychological and psychosocial issues and focus not only on plasma Phe concentrations [van Spronsen and Burgard 2008]. The national guidelines vary significantly among nations for the recommended upper Phe values for follow-up of the adherence to low-Phe diet as well as for the constitution of the age groups for each recommended value [Demirkol et al., 2011].

2.10.1. European guidelines

In 2017, after substantial amount of work, a group of experts reached a consensus and published European guidelines for the diagnosis and management of patients with PKU [van Spronsen et al., 2017b; van Wegberg et al., 2017].

Low-Phe diet is the cornerstone of treatment, although some patients can benefit from tetrahydrobiopterin (BH_4). No intervention is required if the blood phenylalanine concentration is less than 360 μmol/L. Treatment is recommended up to the age of 12 years if the phenylalanine blood concentration is

between 360 μmol/L and 600 μmol/L, and lifelong treatment is recommended if the concentration is more than 600 μmol/L (Table 3). For women trying to conceive and during pregnancy (to avoid maternal PKU), untreated phenylalanine blood concentrations of more than 360 μmol/L need to be reduced. Treatment target concentrations are as follows: 120–360 μmol/L for individuals aged 0–12 years and for maternal PKU, and 120–600 μmol/L for non-pregnant individuals older than 12 years. Minimum requirements for the management and follow-up of patients with PKU are scheduled according to age, adherence to treatment, and clinical status. Nutritional, clinical, and biochemical follow-up is necessary for all patients, regardless of therapy [van Spronsen et al., 2017b].

2.10.2. Estonian guidelines

National guidelines for treatment, diagnostics and management of PKU were approved in Estonia already in 2012 [Uudelepp et al., 2012]. In comparison with the European guidelines, the Estonian recommendations differ mainly by being stricter during the first year of life, with a higher Phe value of 240 μmol/L allowed. The level of 240 μmol/L is desirable up to 12 years of age, but 360 μmol/L is considered maximum allowed level. After 12 years, 600 μmol/L is considered sufficient (Table 3). The recommendations urge the dietary treatment to be initiated before the 21st day of life to obtain the best outcome, but no later than eight weeks of life. Lifelong treatment is a goal, but minimal duration of the dietary treatment should be up to twelve years. Older patients with discontinued diet are encouraged to re-start with it, no matter if they have been treated since infancy or are late-diagnosed. Women of fertile age are recommended to maintain blood Phe levels below 240 μmol/L already 1 to 3 months before conception and keep it below 360 μmol/L during the whole pregnancy.

2.10.3. USA guidelines

In the USA, the recommendations of the NIH Consensus Development Conference (US Department of Health and Human Services, Public Health Service, NIH, NICHD, 2001) are to maintain blood Phe between 120 and 360 μmol/L up to age 12 and below 900 μmol/L for non-pregnant adults and adolescents. It is clear that excess Phe is highly detrimental to brain development prior to 10 years of age. Limiting exposure to high levels after that age may have additional benefits for the medical and cognitive status of these individuals. In light of findings that Phe levels are related to cognitive function in adolescents and adults, it is recommended that Phe levels be maintained between 120 and 900 μmol/L after 12 years of age. Considering the fact that brain development continues during adolescence, even lower Phe levels (120–600 μmol/L) are strongly encouraged during adolescence [National Institutes of Health Consensus Development 2001].

Table 3. Recommendations for follow-up of Phe values in PKU patients according to Estonian [Uudelepp et al., 2012], European [van Wegberg et al., 2017], US [Camp et al., 2014; Vockley et al., 2014] and Australian [Sharman et al., 2010] guidelines.

Age grup	Recommended highest Phe value according to Estonian (EST) guidelines (mg/dL; µmol/l)	Recommended frequency for dietary follow-up – EST	Recommended highest Phe value according to European (EU) guidelines (mg/dL; µmol/l)	Recommended frequency for dietary follow-up – EU	Recommended highest Phe value according to United States (US) guidelines (mg/dL; µmol/l)	Recommended frequency for dietary follow-up – US	Recommended highest Phe value according to Australian guidelines (µmol/l)
0 – 2 y	0–12 months – 4; 240 1–2 y – 4; 240 (max 6; 360)	0–12 months-weekly 1–2 y – twice per month	6; 360	0–12 months-weekly 1–2 y – twice per month	6; 360	0–12 months-weekly 1–2 y – twice per month	360
2 – 12 y	4; 240 (max 6; 360)	2–6 y – monthly 7–12y – 4 to 6 times yearly	6; 360	Twice per month	6; 360	Once to twice per month	360
12 – 18 y	6; 360 (max 10; 600)	4 to 6 times per year	10; 600	Monthly	6; 360	Monthly	500
Adult (18+ y)	6; 360 (max 10; 600)		10; 600	Monthly	6; 360	Monthly	500

Still, in the USA the recommendations have been varying about the need and adherence to therapy according to local policies of different treatment centres. The more up-to-date guidelines have come to the conclusion, that treatment for PAH deficiency should be lifelong for patients with untreated Phe levels above 360 μmol/l and maintaining a treated Phe level of 120–360 μmol/L is recommended for all patients of all ages [Vockley et al., 2014].

2.11. Summary of the literature

In summary, PKU together with other HPAs is one of the most well studied IMDs, and is still providing vast opportunities for research. These opportunities rise from the variability between phenotypes, the expanding number of causative genetic variations, the wide and variable distribution among populations, as well as intensive studies towards better management of dietary therapy and combinations with cofactor substitution therapy as well as other emerging treatment methods. This keeps PKU with other HPAs as a constant hotspot for diagnostic, epidemiological, molecular and therapeutic research. Understanding the molecular lesions of each particular individual as well as distribution of pathological variants among populations provides an important informational clue for finding the best possible options for the treatment of each individual as well as screening, diagnosis and treatment decisions for clinical practice and the whole healthcare system. While making decisions for choosing between existing and emerging therapies, the follow-up of blood Phe values in accordance with national guidelines remains the key indicator in the assessment of the efficacy of the therapy as well as the ability of each patient/family to adhere to recommendations in cooperation with clinical geneticists and nutritional therapists.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To establish the genotypes of Estonian patients with hyperphenylalaninaemia and correlate these with their phenotypes (Papers I and II);
2. To compare the spectrum of *PAH* gene variations among distinct ethnic groups in Estonia (Papers I and II);
3. To assess the geographical distribution of the most prevalent variant p.Arg408Trp in the *PAH* gene (Papers I and II);
4. To investigate the adherence of dietary therapy among Estonian PKU patients (Paper III);
5. To disclose the molecular lesion underlying the pathogenic status of an Estonian patient with DHPR deficiency (Paper IV).

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Patient group of genotype/phenotype study (Papers I and II)

Thirty-four Estonian PKU patients born since 1980 were included into the study performed in 1996 (Paper I), providing 68 independent affected chromosomes. This made up 87% of the known patients born during this period. Phenotypically, all patients expressed the 'classic' or severe PKU phenotype according to the pre-treatment Phe level or Phe tolerance in diet. In Estonia, the exact measurement of serum Phe concentration became available since 1992. Therefore, the previous patients' clinical subtype was determined by the physician only.

Out of the 34 analysed families, 20 were of Estonian origin not less than within two generations, six families were of Slavic (including Russian, Ukrainian, Polish) origin, eight families had at least one ancestor of non-Estonian origin, including Russian, Polish, Armenian, and German individuals.

For the retrospective study of 2018 (Paper II), we created the database of the 95 available Estonian HPA cases. The case histories of the patients either born or living in Estonia since 1974 up to 2016 (43 years) were selected and analysed. The following information was gathered: family and first name, the year of birth, personal ID-code, age at the diagnosis, Phe concentration level at the moment of diagnosis, method of diagnosis (either determination of Phe concentration fluorometrically, tandem MS/MS in blood or the Følling test from urine), highest known Phe concentration value of the particular individual, PAH mutation genotype, general remarks about medical condition, data about the start and continuation/discontinuation of the treatment, and genealogical data of the patient, including information about the ethnicity of the parents and grandparents of the proband. Additionally, data about the general medical/social status of the person was included into the database.

The whole cohort was divided into two subgroups according to the year of birth: 1974–1992 and 1993–2016, *i.e.* before and after the initiation of national newborn screening programme for PKU.

4.1.2. Patient group of phenylalanine measurement study (Paper III)

To analyse the adherence of Estonian PKU patients to the low-Phe diet, we created a sub-database of all available entries for the period 2010 to March 2018 from the general laboratory information management system (LIMS) database. LIMS is available for any diagnostic analysis performed in the United Laboratories of Tartu University Hospital (UL-TUH), concerning all Estonian patients

diagnosed with PKU or other forms of HPA. The created sub-database included the following fields: name, personal ID-code, date of sample collection, age at the moment of sample collection, assignment to age group at the moment of sample collection, Phe value in dried blood spot (either mg/dl or μmol/l), genotype, phenotype, time of diagnosis (either from the newborn screening or late diagnosis in case of persons born before 1993), and assessment of educational level. Initially, the database included 4290 entries from 69 patients. However, as the objective of the study was to acquire insight into the quality and proceeding of the ongoing therapy, nine of the subjects and entries concerning their blood Phe values were excluded from the database due to some of the following reasons: late diagnosis together with severe or profound intellectual disability and/or refusal of treatment. The final database for further analysis consisted of 4236 entries from 60 patients.

The following age groups were created: ≤1 year; 1 year 1 day to 2 years; 2 years 1 day to 6 years; 6 years 1 day to 12 years; 12 years 1 day to 18 years; > 18 years (for simplicity further referred to as: 0–1y, 1–2y, 2–6y, 6–12y, 12–18y, 18+y, respectively). The age group 0–1y contained 662 entries from 19 patients, 1–2y contained 548 entries from 19 patients, 2–6y contained 1140 entries from 20 patients, 6–12y contained 470 entries from 19 patients, 12–18y contained 477 entries from 16 patients and the adult group 18+y contained 933 entries from 27 patients. Median, maximum and minimum Phe values were counted for each group. All entries with Phe values above the maximum recommended value of 360 μmol/l (extrapolated to 6 mg/dl) in cases of patients up to 12 years of age and higher than 600 μmol/l (extrapolated to 10 mg/dl) in cases of patients older than 12 years of age were counted and the ratio of entries elevating the recommended level was calculated.

4.1.3. DHPR patient (Paper IV)

The proband with DHPR deficiency was born as the first child of unrelated Estonian parents following a normal pregnancy with birth weight 3365 g, length 48 cm and Apgar scores of 8 and 9 at 1 and 5 minutes, respectively. Since the first week of life, the proband's mother noticed muscular rigidity and further lack of eye contact. Later permanent constipation, and continuous periods of crying with only brief spontaneous laughter were noted. At the age of six months she was hospitalised for further investigations due to developmental delay and spasticity. On metabolic screening, HPA (plasma Phe = 1179 μmol/L) was detected, and a low-Phe diet was started assuming that the child had classical PKU. However, she did not improve clinically and seizures started one month later. Accordingly, a high suspicion of disturbance in BH_4 metabolism arose. At the age of 8 months, a Phe loading test (0.1 g Phe/kg body weight) was performed at Vilnius Children's Hospital, Lithuania. This test showed a sharp increase in Phe level: at 0 min after consumption – Phe 23.4 μmol/L, Tyr 79.9 μmol/L; at 65 min – Phe 629.3 μmol/L, Tyr 71.4 μmol/L; at 100 min – Phe 1083 μmol/L,

Tyr 91.4 μmol/L). Further, BH_4 loading test (7 mg BH_4/kg body mass) on the background of increased Phe level was performed at the age of 9 months. The second loading test showed variable changes in plasma Phe and Tyr: 0 h – Phe 1400 μmol/L, Tyr 55.8 μmol/L; 4 h – Phe 923.7 μmol/L Tyr 55.8 μmol/L; 8 h – Phe 1555.3 μmol/L Tyr 62.8 μmol/L; 24 h – Phe 892.3 μmol/L Tyr 48.8 μmol/L, indicating possible disturbance in BH_4 synthesis/regeneration.

At the age of 11 months, the child was reinvestigated for BH_4 cofactor disorders. BH_4 loading test, urinary biopterin analysis and CSF neurotransmitter analyses showed biochemical abnormalities indicating DHPR deficiency. Neopterin in urine was normal: 1.6 mmol/mol creat (normal: 1.1–4.0 mmol/mol creat) and biopterin was significantly increased: 12.3 mmol/mol creat (normal: 0.5–3.0 mmol/mol creat), resulting in elevated percentage of biopterin (88%). The BH_4 loading test (20 mg/kg body weight) resulted in blood Phe reduction from initial 496 μmol/L to 254 μmol/L and, Phe 169 μmol/L, 4 and 8 hours after BH_4 administration, respectively. Investigation of CSF neurotransmitters showed normal neopterin – 18 nmol/L (normal: 9–40 nmol/L) and elevated biopterin – 65 nmol/L (normal: 10–50 nmol/L), very low 5-HIAA – 19 nmol/L (normal: 114–336 nmol/L) and HVA – 170 nmol/L (normal: 295–932 nmol/L), 5-MTHF – 26 nmol/L (normal: 64–182 nmol/L), all compatible with DHPR deficiency. The diagnosis was confirmed by the absent DHPR activity in dried blood (<0.5 μU/g Hb; normal: 2.3–3.8).

4.2. Methods

4.2.1. Variation analysis in PAH deficient patients

In the work performed for Paper I, polymerase chain reaction (PCR) was used for amplifying *PAH* gene exons 5, 7, 11 and 12 as the regions of most common variations [Eisensmith et al., 1992]. Firstly, variations in these four exons were screened. If no results were obtained, other exons were considered. PCR primers were chosen using data from reported amplification systems. GoldStar Taq DNA polymerase (Eurogentec, Belgium) was used with an appropriate buffer system, 33 cycles (94°C for 45 s; 56°C for 1 min; 72 °C for 1 min 30 s) were performed.

Sequence Analysis: Solid-phase sequence analysis was performed by the Sanger dideoxychain termination method using the Sequenase™ Version 2.0 DNA Sequencing Kit (Amersham Life Science) and [^{35}S] α-ATP, according to the protocol provided by the manufacturer. One biotin- linked oligonucleotide PCR primer was used for preparing the single-stranded probes which were bound to streptavidine-coated magnetic beads (Dynabeads® M-280, Dynal A.S.) for strand separation. Exons 5, 7 and 12 were sequenced completely, if no p.Arg408Trp was found.

Restriction Analysis: Many of the proposed mutations in the DNA areas under research could be analysed by digesting the PCR product with restriction endonucleases [Eiken et al., 1991]. Special attention was paid to the

p.Arg408Trp variation which is distributed with high frequency in areas geographically close to Estonia and can be effectively identified by *Sty*I digestion [Ivaschenko and Baranov 1993], as it creates a new restriction site in exon 12. *Hinf*I, *Ava*I and *Bam*HI restriction enzymes were used for digestion exon 7 to check for probable p.Arg261Gln, p.Arg252Trp, Gly272ter variations accordingly and *Dde*I to detect c.1066-11G>A in exon 11 (flanking regions) [Eiken et al., 1991]. Restriction fragments were analysed by 2.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer. Single-Stranded Conformational Polymorphism (SSCP) Analysis: Denatured single-stranded PCR 185- to 295-bp products were separated by electrophoresis on homogeneous 12.5% polyacrylamide PhastGel® gels using two different temperatures: +4 and +15 °C and developed by silver-staining. SSCP was used if previously described methods did not reveal the variations.

Paper II: Variation analysis of the *PAH* gene of the probands as well as their parents, when available, was performed as described above or/and PCR and automated dideoxy sequencing with ABI 3130XL capillary sequencer (Applied Biosystems) of the *PAH* gene NM_000277.1 exons (1–13) and exon–intron boundaries. The presence of the prevalent p.Arg408Trp variation was checked first, if missing, all *PAH* gene exons were sequenced completely and MLPA analysis was performed using commercially available kit SALSA®MLPA® Probemix P055-PAH (MRC-Holland).

4.2.2. Measurement of Phe levels

Two different methods of Phe measurement from dried blood spots (BS) collected on filter paper (Schlieicher and Schuell filter paper No 2992) were used. The first used method was the modified McCaman and Robins quantitative fluorescence-based assay measuring ninhydrin-phenylalanine complex enhanced by L-leucyl-L-alanine dipeptide [Wu et al., 1979] using Labsystems neonatal phenylalanine kit (no. 6199 897) and FluoroScanTM (Labsystems Oy, Helsinki, Finland) device. Since 2014 we introduced LC-MS/MS tandem mass spectrometry on Waters AquityTM Ultra Performance LC device using ChromSystems MassChrom® Amino Acids and Acylcarnitines from Dried Blood kit (order nr 55000) applying neutral loss scan 120 detection, according to the methods provided by the manufacturer. In 2015, a switch in analytical methods from fluorescence measurement to LC-MS/MS analysis occurred. Thus, Phe values have been presented in different units. However, in order to unify the results, both types of values were converted (by either multiplying or dividing by a factor of 60.54, as one Phe unit measured in mg/dl equals to 60.54 µmol/l) to obtain comparable numeric values. As the values obtained by FluoroScan were not distinguished if lower than 1 mg/dl and higher than 25 mg/dL (shown as <1 and >25 mg/dl in the original database, respectively), aforementioned values were substituted with 0.9 and 25.1 mg/dl to enable analysis with numeric values. For simplicity, and considering the total number of measurements by

different methods, Phe values in mg/dl (instead of μmol/l) were used in calculations.

4.2.3. Assessment of educational level

Data about the educational level of the PKU patients were obtained during regular visits to outpatient clinic from the patients and/or their families. The patients younger than 18y of age were assigned as “normal school”, “assisted education” or “preschool age”. In the patients older than 18y of age, educational levels were evaluated according to ISCED 2011 scaling [Institute for Statistics and UNESCO. 2012].

4.2.4. Prevalence estimation

To estimate the prevalence of PAH-dependent HPA-s in Estonia, the period from 1993 to 01.09.2016 was taken under observation. The population data of all live births from 1993 to 2015 was obtained from national statistics agency Statistics Estonia (http://www.stat.ee). The number of screened newborns between 01.01.2016 and 01.09.2016 was added according to the data recorded in the screening laboratory of the Department of Clinical Genetics, United Laboratories of Tartu University Hospital. The data about all diagnosed HPA patients born during the whole period was collected at the Department of Clinical Genetics, United Laboratories of Tartu University Hospital.

4.2.5. Statistical analysis

Statistical analysis of the genealogical data was performed with SAS software (SAS® 9.2 Analytics, SAS Institute Inc.). Data collected about the birthplaces of the grandparents of PKU patients’ parental linage of Estonian ethnicity and carrying the p.Arg408Trp allele (all other mutation linages and ethnicities were removed) were determined with the fidelity of county. The analysed dataset consisted of 52 multivariate independent observations corresponding to 52 observed PKU patients. The pool of known localisations contained 162 birthplaces of grandparents. The number of possible carrier grandparents in each of the 15 counties of the Republic of Estonia and the pre-World War II Petseri County per county was normalised to the population number of each county (taking into account the percentage of inhabitants of Estonian ethnicity). Population data and administrative structure were selected as of the year 2009 with the predisposition that demographic tendencies in the window of two generations have been similar over the whole country; as an exception data from the year 1934 were used for Petseri County now remaining out of the administration of the Republic of Estonia. Confidence limits to the results were obtained by bootstrap method [Efron 1981].

Knowing the number of Estonians in a county, and the number of registered p.Arg408Trp alleles in this county, we calculated the expected number of p.Arg408Trp alleles per 10,000 Estonians in the county. To find confidence limits for the number of p.Arg408Trp per 10,000 Estonians, we used the B. Efron's estimation approach [Efron 1981]. For this, we replaced the real sample for a county with 1000 random samples of the same size as the real sample. With each of these 1000 bootstrap samples, we repeated the same calculations as with the real sample and calculated 1000 bootstrap estimates for the number of known p.Arg408Trp carriers per 10,000 Estonians. Ordering these 1000 estimates increasingly, we considered the 25^{th} lower estimate as the lower confidence limit (LCL) and the 976^{th} estimate as the upper confidence limit (UCL).

All data concerning the numbers and locations of Estonian population were obtained from official public database Statistics Estonia http://www.stat.ee.

4.2.6. Genealogical survey

Parents of the PKU patients were requested to fill a questionnaire for genealogical search. The questionnaire comprised fields about the names, maiden names, birth dates and birthplaces of the parents and grandparents of the PKU patients, who had at least one grandparent of Estonian ethnic origin. The birthplace data were preferably intended to contain information about the village, parish and county. Further an illustrative map was created, wherein each birthplace of a known grandparent carrying the p.Arg408Trp mutation in the PAH gene with 50% probability was determined with a spot. In case birthplaces were approximate, the spots were located into the centre of respective parish or county. Altogether 160 birthplaces of grandparents were available to be located to the map.

4.2.7. Variation analysis of the DHPR deficient patient

In an effort to identify the causative variants for DHPR deficiency, *QDPR* gene NM_000320.2 exons with their flanking regions were Sanger sequenced four times by different laboratories in the index patient and her parents.

4.2.8. Exome sequencing of the DHPR patient

Exome sequencing of index patient was carried out in the Estonian Genome Centre at the University of Tartu. DNA library was prepared using Nextera Rapid Capture Exome 37 Mb kit (Illumina Inc.) according to the manufacturer's protocols. The HiSeq 2500 (Illumina Inc.) platform was used for paired-end 2×100 bp sequencing. The bioinformatics data processing made use of BWA [Li and Durbin 2009], which mapped the reads to the hg19 reference genome, and different Picard (v2.2.2) and Genome Analysis Toolkit (GATK) (v3.5-0)

tools to mark duplicate reads, recalibrate base quality scores. GATK Haplotype Caller v3.5-0 was used to call variants.

An in-house variant annotation pipeline was used. Annotations included, but were not limited to reference databases from ExAC [Lek et al., 2016] and 1000 Genomes Project [Genomes Project et al., 2015], and ClinVar pathogenicity annotations [Landrum et al., 2016], as well as HPO terms [Kohler et al., 2014] and OMIM disorders as gene-based annotations. Additionally, allele counts from our in-house database of variants detected among all NGS analyses (panels and ES) performed in our department (654 samples) were annotated to every detected variant.

CNVs were called using CoNIFER software [Krumm et al., 2012]. First, reads per thousand bases per million reads sequenced (RPKM) values were calculated for each sample separately. Second, all available samples using the same library preparation kit were joined for CNV calling. CNV detection and plot generation for detected CNVs were carried out subsequently according to CoNIFER guidelines.

4.2.9. mRNA study

Blood for mRNA analysis was collected from the parents and a control, to obtain cDNA from *QDPR* and analyze the integrity of the cDNA by PCR. PCR was performed from the cDNA synthesized from blood extracted RNA (Tempus™ Spin RNA Isolation Kit, incl DNAse treatment) with SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol; the first strand cDNA was synthesized using Random Hexamers (Applied Biosystem). The primers (QDPR_Rev GTGACTTTTCTGGCAGGCCCCTCATA and QDPR_For GGAGCTGCGGGAGCCGGGCT) were designed from UTR regions of the transcript (NCBI Reference Sequence: NM_000320.3), thus an alternative (93 bp) exon was included into the PCR products with predicted 809 and 716 bp fragments depending on the presence/absence of alternative exon. Phusion Hot Start II DNA Polymerase (ThermoFisher) was used for the PCR reaction.

4.2.10. Genome sequencing

Genome sequencing (GS) and data processing were performed by the Genomics Platform at the Broad Institute of MIT and Harvard. PCR-free preparation of sample DNA (350 ng input at >2 ng/μl) was accomplished using Illumina HiSeq X Ten v2 chemistry. Libraries were sequenced to a mean target coverage of >30x. GS data was processed through a pipeline based on Picard, using base quality score recalibration and local realignment at known indels. The BWA aligner was used for mapping reads to the human genome build 38. Single Nucleotide Variants (SNVs) and insertions/deletions (indels) are jointly called across all samples using Genome Analysis Toolkit (GATK) HaplotypeCaller

package version 3.4. Default filters were applied to SNV and indel calls using the GATK Variant Quality Score Recalibration (VQSR) approach. Annotation was performed using Variant Effect Predictor (VEP). Lastly, the variant call set was uploaded to Seqr for collaborative analysis between the Center Mendelian Genomics and investigator.

4.2.11. Validation of possible breakpoint

To validate the possible inversion we designed oligonucleotide primers (Table 4) to perform PCR amplification, and subsequent Sanger validation around the breakpoints inside exon 2 of *QDPR* gene and intron 8 of *ACOX3* genes.

Table 4. PCR primers for validation studies of the breakpoint detected in *ACOX3* and *QDPR* genes of the DHPR deficient patient and her family.

ACOX3	
F1	5'-TGCATGAAGACAGTGGAATCA-3'
R1	5'-AGGAATCACAGTCTCGTTGT-3'
QDPR	
F2	5'-TCATGAAACTGGGGAAAGAGGT-3'
R2	5'-AGTTTCGCTTGTCTCCCAGG-3'

4.2.12. Chromosome analysis

Karyotype analysis from peripheral blood lymphocytes was performed by using conventional GTG-banding technique (G-bands by trypsin using Giemsa; band level 550). Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN2016), [Gonzalez Garcia and Meza-Espinoza 2006]. Both karyotypes were performed at the 550-band level.

4.3. Ethics

All activities performed during this study were approved by Research Ethics Committee of the University of Tartu (approval date 21.09.2015 number 251/T-6) and were strictly in accordance with the Declaration of Helsinki. Informed consent for carrying out research was obtained from the patients and/or their parents/caretakers. The raw data of the patients was maintained only in the servers of the Tartu University Hospital and any information made available for the public was coded in a manner not enabling linking delicate personal data with particular persons.

5. RESULTS AND DISCUSSION

5.1. The genotype of Estonian HPA patients

5.1.1. The genotype of Estonian HPA patients known by 1996 (Paper I)

In the study from 1996, variations in 63 alleles out of the 68 affected ones (34 individuals) were identified. In these alleles under study, 57 (84%) were detected as defective due to p.Arg408Trp. Twenty four patients were homozygotes for p.Arg408Trp, nine were compound heterozygotes for p.Arg408Trp, and only one patient was a compound heterozygote with p.Arg158Gln / p.(?)/IVS12+1G>A variations. These data correlate well with Hardy-Weinberg equilibrium, providing evidence that the Estonian PKU patient pool is balanced and that it is likely that other (milder) forms had not been missed. The other identified variations included p.Arg252Trp, p.Arg261Gln, and p.Ser349Pro [Lillevali et al., 1996].

Although the number of samples studied was not very large, it became clear that Estonia is one of the most homogeneous countries for the structure of mutant *PAH* alleles. According to data published by 1996, no other population displayed such a high frequency (84%) of the p.Arg408Trp variation [Eisensmith et al., 1995]. It has been suggested that p.Arg408Trp on the East-European haplotype is of Balto-Slavic origin [Eisensmith and Woo 1994; Kalaydjieva et al., 1991], or may even originate from more ancient pre-Indo-European people [Giannattasio et al., 1997]. So far, the highest frequency of this mutation had been described in Byelorussia, where it was present on 75% of mutant alleles [Zekanowski et al., 2001].

Linguistically and ethnoculturally, the Estonians belong to the Finno-Ugric peoples, and the results of the current study indicate that p.Arg408Trp, as one option, may originate from early Finno-Ugric tribes who inhabited large areas of Eastern Europe including northwest Russia and the Volga River region. They were largely assimilated by Slavic and Baltic tribes and may have donated the mutant allele to them, later spreading widely. Another possibility that can explain the very high frequency of one mutant allele is genetic drift. Periods with decreasing number of inhabitants clearly exist in the history of Estonia, most recently in the beginning of the 18th century. In this way, one allele can occasionally reach a higher frequency, or in case of proposed over-dominant selective advantage, contributed to the survival of the carriers [Krawczak and Zschocke 2003].

The rest of the identified variations were well known: p.(?)/IVS12+1G>A was detected twice (3%), providing evidence of contacts with Scandinavian peoples; alleles with p.Arg261Gln, p.Arg252Trp, and p.Arg158Gln have a random distribution in several Caucasoid populations [Eisensmith et al., 1992].

Since the Estonian population is rather homogeneous for the p.Arg408Trp variation, which is easily detectable by the PCR/ *Sty*I restriction test, this test

was introduced as quick and reliable first tier analysis for any newborn identified in the screening before performing other tests to eliminate BH_4 deficiencies. The presence of this affected allele (in homozygous or heterozygous state) confirms that the person is a PAH-deficient PKU patient without the need for further investigations of the BH_4 system. If absent, the BH_4 loading test was performed as recommended [Dhondt 1991].

5.1.2. The genotype of Estonian HPA patients known by 2016 (Paper II)

By 2016, genotype data for 92 PKU probands (out of 94) was available. Two patients from the elder cohort have emigrated, one had died and consequently data about only one of her alleles exists. In addition, variation analysis of one proband (with a mild PKU phenotype) has been successful for only one allele. Taken together, we were able to include 182 alleles into the study. Among these, sixteen were from siblings of a previously identified proband, while three more alleles had to be removed from the list of independent alleles, as the families could be genealogically traced back to the same great-grandparent of the probands. Accordingly, the known Estonian PAH allele pool contained 153 independent alleles [Lillevali et al., 2018]. Among these, 123 independent alleles were harbouring the p.Arg408Trp variation characteristic to East-European populations, constituting 80.4% of all PKU alleles and thus being one of the highest reported prevalence [Tighe et al., 2003]. In general, the distribution of this disease-causing allele highly resembles the total genetic structure of various European populations obtained by wide-screen analysis of neutral SNPs [Nelis et al., 2009]. The detailed results of the pathogenic alleles are presented in Table 5.

Table 5. PAH variations among PKU patients (N=92) in Estonia (independent alleles) [Lillevali et al., 2018].

Variation in the *PAH* gene	Number of independent alleles	% of all independent alleles	ethnic origin
p.Arg408Trp (c.1222 C > T)	123	80.4%	Therein Estonian – 89 Slavic – 31 Mixed – 3
p.Leu48Ser (c.143 T > C)	5	3.3%	
p.(?)/IVS12+1G>A (c.1315 + 1G > A) (IVS12+1 G > A)	4	2.6%	
p.Arg261Gln (c.782 G > A)	3	2%	
p.Arg252Trp (c.754 C > T)	2	<1%	
p.Glu280Lys (c.838 G >A)	2	<1%	
p.Pro281Leu (c.842 C > T)	2	<1%	
p.Ile306Val (c.916 A > G)	2	<1%	
p.Ile65Thr (c.194 T > C)	1	<1%	
p.Arg158Gln (c.473 G > A)	1	<1%	
p.Asp222* (c.663_664 del AG)	1	<1%	Armenian
p.Ala300Ser (c.898 G > T)	1	<1%	
p.Ser349Pro (c.1045 T > C)	1	<1%	
p.Glu390Gly (c.1169 A > G)	1	<1%	Georgian
c.Gln355_Tyr356insGlyLeuGln/ IVS10-11G>A c.1066-11G>A	1	<1%	Slavic
p.Ala403Val (c.1208 C > T)	1	<1%	Azerbaijan
Unknown (not p.Arg408Trp)	2	1.3%	
Total	**153**	**100%**	

The upgraded analysis of the genetic structure of the Estonian PAH-deficient HPA patients revealed sixteen different pathogenic variations in the *PAH* gene. Two more alleles remained unidentified in this gene pool, as the DNA of one patient with classical severe PKU phenotype (with p.Arg408Trp in one allele) was not available anymore, and in another patient with a mild phenotype, we were not able to identify the pathogenic variation (beside p.Arg408Trp) despite repeated Sanger sequencing and MLPA analysis of the *PAH* gene.

5.3. Comparison between the results of the early and updated patient groups

The patient group of Paper I was included in the cohort of Paper II. However, in 1996 the majority of the known 34 PKU patients in Estonia had been diagnosed late, after significant clinical manifestations had become obvious and accordingly different extent of damage to psychomotor development had already occurred. Thanks to developed diagnostic capabilities, all except one of the pathological variations of *PAH* gene alleles of the patients in Paper I were identified by the time of the publication of Paper II, the one that was not unveiled was due to lack of material and the patient had disappeared from the reach of clinical geneticists. All of these patients had classical type of PKU with low tolerance to Phe.

The analyses performed by the time of the publication of Paper II in 2018 added ten more pathological variations into the pool of HPA-causing *PAH* alleles in Estonia, while the whole cohort covered 153 independent alleles (from unrelated families). Here we could also identify alleles with mild or moderate effect on PAH activity (Ala300Ser, Ala403Val), including the ones responding to sapropterin hydrochloride administration (Leu48Ser, Arg261Gln), while the vast majority of all pathogenic variations remained among the ones with deleterious effect on PAH activity. The incidence of the predominant variation p.Arg408Trp did not exhibit much change between the two studies: 84% in 1996 and 80.4% in 2018. Obviously, the launch of newborn screening for PKU in 1993 [Ounap et al., 1998] became the cornerstone for constant diagnostic supervision for this disorder and enabled also the finding and treatment of the few milder versions.

5.4. Genotype phenotype correlation of Estonian patients with HPA

Clinically, all our patients known by 1996 exhibited the classical PKU phenotype with low phenylalanine tolerance. Variation p.Arg408Trp leaves no residual enzyme activity [Kayaalp et al., 1997] and the fact that our patient group is balanced explains its clinical homogeneity.

The same is characteristic for the patients presented in the later study (Paper II). The vast majority of Estonian PKU patients (87%) exhibit the classical PKU phenotype with high Phe levels, which if untreated, is due to minimal or zero PAH activity. This is in good correlation with the genotypic data, as the variations p.Arg408Trp, p.Arg158Gln, p.Pro281Leu, p.Arg252Trp, p.(?)/IVS12+1G>A [Danecka et al., 2015] retain the mutated PAH negligible residual enzymatic activity. Only nine patients exhibited mild HPA, harbouring p.Arg261Gln, p.Ala403Val, p.Ala300Ser, p.Glu390Gly beside the p.Arg408Trp in the second allele, whereas four patients exhibited good response to BH_4

supplementation and are constantly on Kuvan® treatment now. Genotype/ phenotype correlation of Estonian HPA patients is presented in Table 6.

We have now obtained a larger cohort of patients and thus we are able to present more adequate results about the genotypic and phenotypic distribution of Estonian PKU patients. Despite the fact that no PAH deficient patients exhibiting mild HPA phenotype have been identified in the pre-screening subgroup, the relative frequency (80%) of the major p.Arg408Trp variation has remained among the highest across diverse populations. The exceptionally high prevalence (84%) of this mutation reported two decades ago in Paper I [Lillevali et al., 1996] could have been considered as a result of insufficient diagnostic capabilities in the past, leaving mild HPA cases out of the reach of paediatricians and clinical geneticists. It may have occurred in single undetermined cases, but the presence of only six PKU patients exhibiting mild HPA phenotype in the whole cohort suggests that this kind of possible diagnostic shortcoming must not have been prevalent.

Our cohort included a relatively small number of BH_4-sensitive patients. Previous research on BH_4 responsiveness in other populations has provided evidence on the need to study each patient individually, as the response may differ significantly due to the particular molecular structure of the mutant PAH protein [Danecka et al., 2015]. However, based on the genotypic structure of the Estonian population with high prevalence of the p.Arg408Trp mutation, this number is not surprising at all. It has been clearly shown that this mutation is responsible for almost complete ablation of the PAH enzymatic activity with no change if substrate and cofactor are provided in excess [Danecka et al., 2015]. Since 2010, every HPA newborn has undergone a loading test with sapropterin hydrochloride, while the patients born earlier were not tested, and therefore in our data, three older patients with the p.Arg408Trp/p.Leu48Ser genotype have been considered as having classical PKU, and the more recent one as BH_4 responsive. This practice has helped to discover six BH_4-responsive patients, which enables to provide better quality of life to them and their families.

In conclusion, Estonia exhibits a notably homogenous mutation pool of disease-causing PKU alleles with high prevalence of the classical severe form of PKU. Contemporary clinical practices and newborn screening have helped in diagnosing a few additional mild HPA cases.

Table 6. Allelic combinations and phenotype distribution of Estonian PKU patients [Lillevali et al., 2018].

Genotype (variations in the *PAH* gene)	Number of patients	Frequency (%)	Phenotype
p.[Arg408Trp];[Arg408Trp] c.1222 [C>T];[C>T]	58	62%	Classical PKU
p.[Arg408Trp];[Leu48Ser] c.[1222C>T];[143T>C]	4	4.3%	3 Classical PKU / 1 BH_4-responsive PKU
p.[Arg408Trp];[(?) / IVS12+1G>A] c.[1222C>T];[1315+1G>A]	3	3.2%	Classical PKU
p.[Arg408Trp];[Pro281Leu] c.[1222C>T];[842C>T]	2	2.1%	Classical PKU
p.[Arg408Trp];[Arg261Gln] c.[1222C>T];[782G>A]	4	4.3%	2 Classical PKU / 2 BH_4-responsive PKU
p.[Arg408Trp];[Glu390Gly] c.[1222C>T];[1169A>G]	1	1.1%	BH_4-responsive PKU
p.[Arg158Gln];[(?) / IVS12+1G>A] c.[473>A];[1315+1G>A]	1	1.1%	Classical PKU
p.[Arg408Trp];[Gln355_Tyr356 insGlyLeuGln / IVS10-11G>A] c.[1222C>T];[1066-11G>A]	1	1.1%	Classical PKU
p.[Arg408Trp];[Arg252Trp] c.[1222C>T];[754C>T]	4	4.3%	Classical PKU
p.[Arg408Trp];[Asp222*] c.[1222C>T];[c.663_664delAG]	2	2.1%	Classical PKU
p.[Arg408Trp];[Ser349Pro] c.[1222C>T];[1045T>C]	1	1.1%	Classical PKU
p.[Arg408Trp];[Ile306Val] c.[1222C>T];[916A>G]	2	2.1%	Mild HPA
p.[Arg408Trp];[Glu280Lys] c.[1222C>T];[838G>A]	2	2.1%	Classical PKU
p.[Leu48Ser];[Glu280Lys] c.[143T>C];[838G>A]	1	1.1%	Classical PKU
p.[Arg408Trp];[Ile65Thr] c.[1222C>T];[194T>C]	1	1.1%	Classical PKU
p.[Arg408Trp];[Ala300Ser] c.[1222C>T];[898G>T]	2	2.1%	Mild HPA
p.[Arg408Trp];[Ala403Val] c.[1222C>T];[1208C>T]	1	1.1%	Mild PKU
p.[Arg408Trp];[NA] c.[1222C>T];[NA]	1	1.1%	Classical PKU
p.[Arg408Trp];[NA] c.[1222C>T];[NA]	1	1.1%	Mild PKU
c.[NA];[NA] (no DNA)	2	2.1%	Classical PKU
Total	**94**	100%	

5.5. The comparison of the spectrum of *PAH* gene variations among distinct ethnic groups in Estonia

We revealed, that among the patients of Estonian origin, the incidence of p.Arg408Trp is even higher than in Slavic and mixed groups and in the whole population (87.5, 75 and 81.3%), respectively [Lillevali et al., 1996]. The relative frequency of p.Arg408Trp in the Slavic group is close to other Slavic populations as described before [Charikova et al., 1993; Eisensmith et al., 1995]. Statistical χ^2 analysis did not reveal a significant difference in the distribution of the p.Arg408Trp allele among ethnically distinct groups of the Estonian population – the Estonians and Russians (approximately 30% of the Estonian population).

The ethnic structure of Estonian PKU patients resembles the general ethnical structure of the republic. Out of the 94 patients, 63 (67%) were Estonians, 24 (26%) Slavic (Russian or Ukrainian) and seven of mixed origin, including Estonian, Latvian, Armenian and Azerbaijan [Lillevali et al., 2018]. This structure is highly similar to general Estonian population, which comprises mostly of Estonians (68.8%) and people of East Slavic ethnicities (27.8%), according to Statistics Estonia (http://www.stat.ee) (01.01.2016).

Accordingly, no differentiation between distinct ethnic groups for the incidence and genotypic variability of PKU in Estonia can be drawn.

5.6. The geographical distribution of the variation p.Arg408Trp in the *PAH* gene inside Estonia

An interesting observation, derived from the genealogical data and the localisation of the birthplaces of the grandparents carrying the prominent p.Arg408Trp variation, is that a significant proportion of these are located in the relatively sparsely populated areas of Southern and South-Eastern Estonia. Statistical analysis of the birthplaces of the grandparents of PKU patients of Estonian ethnicity carrying the p.Arg408Trp variation revealed several counties of Estonia providing higher input of this allele into Estonian *PAH* variation pool. Considering the population density, the local 'hotspot' of p.Arg408Trp locates to three (and one former) counties of South-Eastern Estonia, especially to former Petseri County, as well as Võru, Põlva and Valga Counties (3.5, 2.63, 2.04 and 2.01 carrier origins per 10,000 Estonians, respectively), while the number of p.Arg408Trp carrier origins per 10,000 Estonians for the whole country was 0.88. Relatively high input came from Estonian bigger islands, Saaremaa and Hiiumaa, exhibiting similar disproportionally high ratio of birthplaces of putative p. Arg408Trp carrying grandparents. However, with wider confidence limits; a 'hotspot' was also found in North-Eastern Ida-Viru County (2.1 carrier origins per 10,000), while Northern, Western mainland and Central Estonia had relatively few carriers in comparison with their population density (Table 7). The birthplaces of 160 grandparents of PKU patients were marked on

a map with red dots and presented in Figure 6. Each dot shows the origin of p.Arg408Trp with 50% probability [Lillevali et al., 2018]. We speculate for a possible bottleneck effect or genetic drift, as the period of plagues in the 17th century as well as the tremendous population loss due to the Great Northern War in the beginning of the 18th century may have led to the observed distribution of this particular allele.

Table 7. Relative impact of geographically distinct regions of Estonia (counties) to historical formation of Estonian pool of *PAH* alleles carrying the p.Arg408Trp variation [Lillevali et al., 2018].

County	No of grand-parents	All residents	Estonians	p. Arg408Trp carrier count per 10,000 Estonians	Lower Confidence Limit	Upper Confidence Limit
Harjumaa	30	524,938	314,490	0.48	0.2702789	0.6677478
Hiiumaa	4	10,097	9,930	2.01	0	4.5317221
Ida-Virumaa	14	169,688	33,343	2.10	0.8997391	3.2990433
Järvamaa	2	36,130	33,803	0.30	0	0.7395793
Jõgevamaa	4	36,780	33,234	0.60	0.1504483	1.2035867
Lääne-Virumaa	8	67,151	57,239	0.70	0.2620591	1.2229424
Läänemaa	1	27,477	24,124	0.21	0	0.6217874
Pärnumaa	7	88,466	77,522	0.45	0.0644978	1.0319651
Petserimaa	11	48,536	15,706	3.50	0.955049	6.3669935
Põlvamaa	12	31,002	29,371	2.04	0.8511797	3.234483
Raplamaa	8	36,678	34,235	1.17	0.4381481	2.0446911
Saaremaa	11	34,723	34,139	1.61	0.2929201	3.0756613
Tartumaa	14	150,139	127,498	0.55	0.2352978	0.9411912
Valgamaa	10	30,176	24,934	2.01	0.8021176	3.4089998
Viljandimaa	7	55,657	52,501	0.67	0.1904726	1.2380717
Võrumaa	19	37,888	36,069	2.63	1.3862319	3.8814494

Figure 6. Geographical distribution of the origins of the p.Arg408Trp variation in the phenylalanine hydroxylase (*PAH*) gene in Estonia. Birthplaces of the grandparents of PKU patients with the p.Arg408Trp variation in the *PAH* gene are shown with red dots. Note that each dot presents 50% probability of the grandparent being a carrier of the variation [Lillevali et al., 2018].

5.7. The prevalence of PAH deficiency in Estonia

The number of live births in Estonia during 1993 to 01.09.2016 was 321,210. This number was divided by 48, the number of the second sub-cohort of HPA patients, and thus the prevalence of PAH-dependent HPA-s in Estonia was estimated as 1 in 6,700. Although some newborns miss from the screening programme due to lack of parental consent, the likelihood of missing a PKU patient from medical observation during last 24 years is negligible; therefore, total national statistics of live births was used. The two probands of Estonian origin born in Belgium and one prenatally terminated diagnosed pregnancy were taken into account when determining the prevalence of PAH-deficient HPA-s [Lillevali et al., 2018].

It has been a subject of discussions that heterozygosity for PAH deficiency might possess some selective advantage, as suggested by the prevalence of PKU in distinct populations and with a wide diversity in the mutational spectrum [Saugstad 2006; Zschocke 2003]. We speculate for a possible bottleneck effect or genetic drift, as the period of plagues in the 17th century as well as the tremendous population loss due to the Great Northern War in the beginning of the 18th century may have led to the observed distribution of this particular allele.

5.8. The adherence of dietary therapy among Estonian PKU patients (Paper III)

Among all of Estonian PKU patients, we found the median of medians of Phe values during the observed period as 394 µmol/l, reflecting that approximately half of the patients were able to sustain the recommended dietary treatment for half of the measurement instances. The summary of the average Phe levels for the whole cohort is presented in Figure 7. Only four of the 60 patients never exceeded the recommended Phe level during the entire evaluation period. Only one patient of these four had the benign PKU phenotype, with the other three having classical PKU. As this assessment took into account all entries (N = 4236, 60 individuals) without discrimination of the age of the patient, the regularity of observations, or concomitant health problems, next we therefore split the database into more distinct age groups.

We divided the whole cohort into six age groups to give insight into the actual values of blood spot Phe during various periods of growth and life, taking into account the proposed values from the Guidelines.

All values of blood spot Phe analyses presented in relation to the recommended cut-off values in Estonia are shown in a diagram (Figure 8). All data are presented in groups by age of the patients. Figure 8 shows the percentage of patients in an age group with median Phe values falling below the national recommendation levels of the respective age.

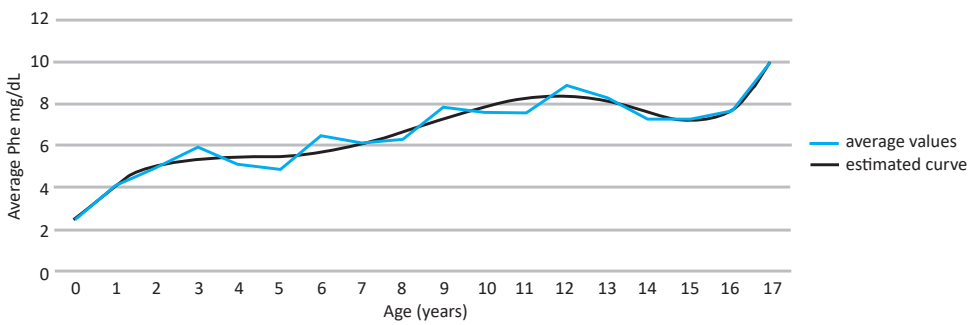

Figure 7. Average Phe values of all Estonian PKU patients of ages 0 to 18 years together during 2010 to 2018 (N = 4236) [Lillevali et al., 2019].

5.8.1. Age group 0 to 1 years

The results of the age group of 0-1year (19 patients, Estonian reference value 240 µmol/L; European reference value 360 µmol/l – results given in brackets) were remarkably good for this cohort: for 79% of all measurements and 95% of the patients, the median Phe values were within the range of the nationally-recommended levels (Figures 8 and 9). There were three (five) patients whose Phe value never exceeded the recommended level, while for six (nine) patients the elevated levels occurred in less than 10% of cases, likely referring to occasional fluctuations possibly due to infections or random uncontrolled ingestions of unchecked products. In three patients, the elevated levels occurred between 10 and 20%, while four patients exhibited elevated levels in 50, 44 and 35 % of measurements, which may indicate that the latter families were not able to consistently follow the advised dietary instructions. The median number of samples arriving to the laboratory was 35, which is lower than the recommended weekly frequency, but still reflects the desire of the families to adhere to the recommendations. During the first year of life, three of the patients, all with classical PKU, never exceeded the recommendation of 240 µmol/L Phe level [Lillevali et al., 2019].

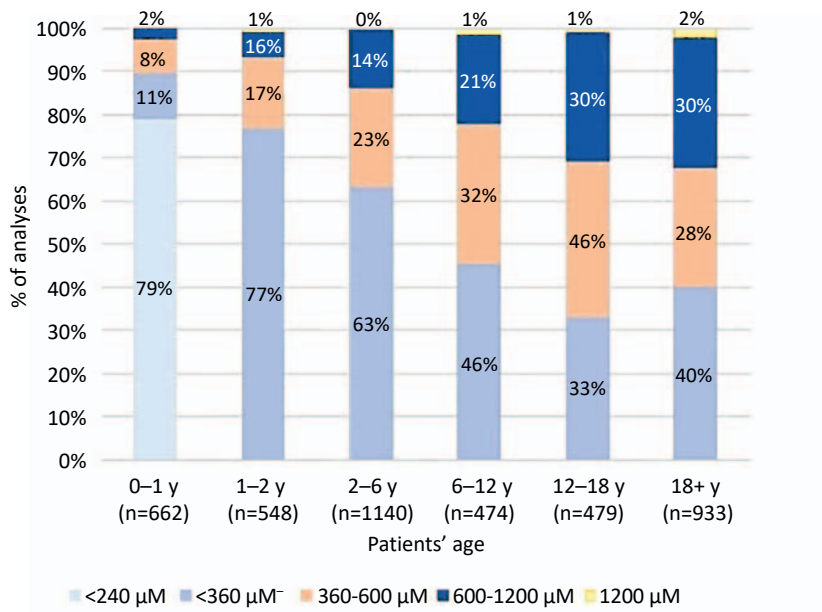

Figure 8. All entries ($N = 4236$) of blood spot Phe values of Estonian PKU/HPA patients in LIMS of UL-TUH are shown on a diagram regarding recommended limits as percentage along age groups. The diagram presents generalized overview of adherence to dietary recommendations in particular age groups and draws out the proportion of samples in well-managed patients and cases with lower dietary adherence [Lillevali et al., 2019].

5.8.2. Age group 1 to 2 years

The results of the age group of 1-2year (19 patients) exhibited some change compared to 0-1y: for 77% of all measurements and 84% of the patients, the median Phe values were in the range of the nationally-recommended levels (Figures 8 and 9). The median of medians of Phe values remained at 224 µmol/L, suggestive of generally acceptable adherence to diet. The group contained the same 19 patients as the 0-1y cohort, however in only three patients did the Phe value exceed suggested recommendations (360 µmol/L) in no more than 10% of measurements, while six patients exhibited elevated levels in more than 25% of cases, including two patients whose Phe values did not fit into the recommended level for a single time. Similarly to the first age group, the median count of samples per patient was 32, being in good accordance with the fortnightly recommendation [Lillevali et al., 2019].

5.8.3. Age group 2 to 6 years

In this group of 20 patients, for 63% of all measurements and 70% of the patients, the median Phe values were in the range of the nationally-recommended levels (Figures 8 and 9). The median of medians of Phe values was 285 µmol/L, but on the background of drastically diverging individual scores, the adherence to diet seems more questionable. In only four patients did elevations above the recommendation (360 µmol/L) occur in less than 10% of cases; three of them having classical PKU and one has BH_4-sensitive PKU. In three patients, the elevated levels occurred between 10 and 25% of samples, and all the remaining 13 patients exceeded the level more frequently, with four of them exceeding the level in 90-100% of entries, reflecting severe difficulties in keeping the diet. Additionally, the sampling frequency deviates substantially: while the families of some patients send the samples even more frequently than recommended, the others either are seemingly attached to the dietary routine and feel confident, or have lost interest in constant monitoring [Lillevali et al., 2019].

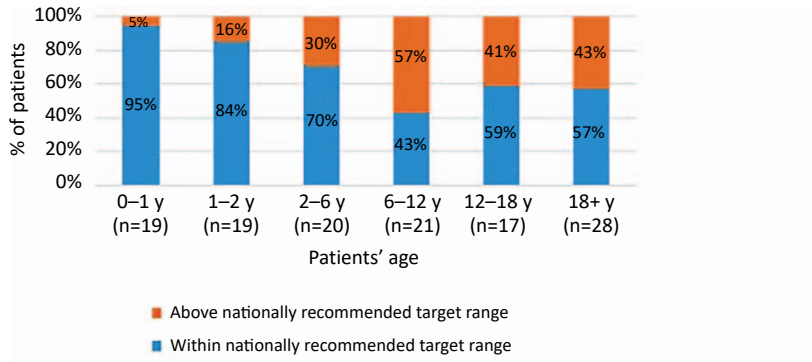

Figure 9. Median blood spot Phe values of Estonian PKU/HPA patients ($N = 60$) are shown in the diagram as percentage fitting under the recommended national values according to age groups. Here the medians of gathered blood spot values of each patient are assembled into the graph to present the proportion of well-managed individual diet among every age group [Lillevali et al., 2019].

5.8.4. Age group 6 to 12 years

In the age group of 6–12 year (19 patients), for 46% of all measurements and 43% of the patients, median Phe values were in the range of the recommended levels (Figures 8 and 9). The tendency for elevated Phe increased as shown earlier (recommended 360 µmol/L). For only three cases of 19 patients was an elevated Phe level observed in less than 10% of entries, including one patient with mild HPA. Simultaneously, 11 of the patients had Phe increase above 360 µmol/L in more than 50% of analysed samples. This high ratio of elevated results may refer to difficulties in maintaining the dietary regimen in rapidly changing social context of early school years. Blood spot sampling frequency also declined in this age group, as the median number of samples per patient was only 14 during six years. Only in single cases did sampling occur monthly [Lillevali et al., 2019].

5.8.5. Adolescent group of 12 to 18 years

Referring to the Guidelines, the recommended Phe levels of the adolescent group of 12–18y (16 patients) should not exceed 600 µmol/L. For 79% of all measurements and 59% of the patients, the median Phe values were in the range of the nationally recommended levels (Figure 9). The distribution of elevated values among patients was notably more variable than in the younger groups: while in half of the patients, the ratio of elevated Phe samples remained in the limits from 0 to 40% of measured samples, another half exceeded the recommended level in more than half of the measured cases. Here we also observed relatively low activity in sending blood samples, as the median count was 14.5 [Lillevali et al., 2019].

5.8.6. The adult group of 18+ years

In the adult group of 18+ years (27 patients), the results varied even more widely, and for 68% of all measurements and 57% of the patients, the median Phe values were in the range of the nationally-recommended levels (Figure 9). For three patients, the Phe value never exceeded the recommended level, two of them having late-diagnosed classical phenotype, and one with a mild PKU version. In five cases exceeding the value happened only once or twice, and each of these were individuals with classical phenotype, late diagnosis and p.Arg408Trp/p.Arg408Trp genotype; and one from screening, classical phenotype, not taking into account those who presented their samples very rarely. Still, in 11 patients the elevated values were present in more than half of the measurements. Wide differences occurred in the frequency of sending control samples: from a single sample during the whole period to constant monitoring with 100 – 150 samples presented, resulting in a frequency of sampling of up to 14 to 18 times per year [Lillevali et al., 2019].

5.8.7. Assessment of the tendencies of the adherence to diet

Maintaining acceptable blood Phe levels in PKU patients as suggested in national guidelines [Uudelepp et al., 2012; Vockley et al., 2014] or more widely agreed among international consortiums [Camp et al., 2014; van Spronsen et al., 2017b] can be burdensome for families [Walter et al., 2002], even though it is clear that adherence to these recommendations is important for avoiding undesirable neuropsychiatric symptoms as well as intellectual disability [Didycz and Bik-Multanowski 2017; Lindegren et al., 2012]. Our results from the Estonian cohort of PKU patients reflect the tendencies of gradually occurring deviation from the suggested recommendations over time (Figure 7).

Previously, similar observations have been presented by Walter et al., 2002, wherein about a quarter of all samples from ages 0 – 4 years and 5 – 9 years exceeded the recommended level. Similarly, our results show that blood Phe concentration was not always maintained below the recommend value [Walter et al., 2002].

The adherence to the recommendations in younger age groups remains remarkably higher, while with the increase of age and in adolescence the results became increasingly divergent. A report involving data from ten European PKU centres has shown similar results, reflecting that these same obstacles are to be faced in any country and population [Ahring et al., 2011]. Ahring et al. has demonstrated that blood Phe concentrations increase with age, and we observed a similar tendency (Figure 7), although we also observed a small decrease in average Phe concentrations at the age of 14 to 16 years that is not fully understood. One possible explanation is that dietary control in adolescents may be better than reported previously [Ahring et al., 2011]. In comparison with the work of Jurecki et al. [Jurecki et al., 2017], our data also show better com-

pliance with the recommendations in our adolescent patients than the pre-adolescence age group.

Our approach was to observe each of the patients during the entire available period, up to eight years. The median Phe value for either the whole period or selected age gives a better understanding of a particular patient's general adherence to dietary management if fluctuations in Phe over the recommended level remain sporadic. However, if the median value is observed to be higher than the recommendation, the family may warrant closer attention and observation.

The variability of dietary adherence was not connected to the severity of the genotype, as 20 of the patients with median Phe values under the recommended reference have the PAH genotype fully depleting PAH activity (14 of them are homozygotes for the p.Arg408Trp variant). A few patients exhibited exceedingly elevated Phe levels: nine patients had a median Phe level of 12 mg/dl or higher. Phenotypically, they all exhibit the classical PKU phenotype, which is in accordance with their genotype, harbouring the predominant p.Arg408Trp variation of the PAH gene in one or both of the alleles, and in compound heterozygotes the second allele (p.E221D222FSdelAG, p.Arg252Trp, c.1315+1G>A) has been shown to have a deleterious effect on PAH activity. Six of these patients had been diagnosed late, before the launch of national screening program, providing an explanation to their inability to adhere to the recommendations, as elevated Phe during their infancy had already caused cognitive damage. However, three remaining patients were not diagnosed late and exhibited normal progress in education. Surprisingly, five of the late diagnosed patients with low educational results manifest well-controlled Phe levels, probably referring to well established family support or institutional care.

PKU patients of the same family exhibit usually very similar Phe level patterns; in one pair the divergence was greater and, interestingly, in one pair the late-diagnosed sibling has excellent dietary adherence, while his sister with a more timely diagnosis demonstrated more fluctuations and higher median Phe value.

We observed that during the first two years of life, the families show good dietary adherence and follow the recommendations, with the exception of only a few families. However, the number of the cases of elevated Phe values subsequently increases with age, especially during early school age (6 to 12 y). In adolescence the picture slightly improves, but the relaxed dietary threshold for adults is still frequently crossed by most patients.

The ability to maintain the diet among the patients with classical PKU phenotype shows great variability, though, surprisingly, good results were seen among a few late-diagnosed patients, even those with poor cognitive functioning. There were only four patients in our cohort whose Phe values have been constantly under the reference value: one with benign HPA, other with the classical PKU version, while two of the latter belong to the group of late-diagnosed patients.

Our data reflect similar tendencies observed previously in studies from different PKU management centres, that an increase in cases of elevated Phe levels is seen in parallel with age.

5.9. The molecular lesion in the patient with DHPR deficiency (Paper IV)

The following part of the study comprises the final results of an Estonian patient with biochemically diagnosed DHPR deficiency, but whose molecular diagnosis remained unsolved during 27 years.

5.9.1. Sanger and exome sequencing

Despite repeated sequencing of the exonic regions with the flanking areas of the QDPR gene in the proband and both parents, no pathogenic variations were determined.

Singleton ES failed to reveal pathogenic variants in *QDPR* gene or in other genes involved in pterin metabolism. However, ES analysis indicated homozygosity around the *QDPR* gene locus, as only homozygous variants were detected in 10 Mb region chr4:7433652-17817262 (GRCh38).

5.9.2. Genome sequencing

Following the initial investigations, predominantly after obtaining the results from ES, the main hypothesis was that the patient may have a homozygous non-coding rearrangement which disrupts *QDPR* expression. To explore this hypothesis, trio genome sequencing (GS) was performed. Although there were no rare coding variants, concordant with previous studies, two rare homozygous intronic variants in the 3' region of *QDPR* were detected (c.*119+4759T>C and c.*119+12119G>A, ENST00000513615). Both variants were heterozygous in both parents. In the gnomAD database, both variants have an allele frequency below 0.1% with no homozygotes [Karczewski et al., 2019]. However, as the functional consequence is difficult to predict for intronic SNVs, we searched further for other classes of variants. By visual inspecting aligned sequencing reads, a possible structural variant (SV) breakpoint at Chr4(GRCh38): g.17505522 (Figure 10 C) was detected, which locates to intron 2 of *QDPR*. The other breakpoint was discovered by paired read mapping and split read analysis, revealing it localised to intron 8 of the *ACOX3* gene (Chr4(GRCh38): g.8398067) (Figure 10 C). Thus, a possible 9 megabase (Mb) inversion in 4p was suspected (Figure 10 A,B). We also performed post hoc analysis using three structural variant callers on GS data: Manta [Chen et al., 2016], DELLY [Rausch et al., 2012], and Smoove [Layer et al., 2014]. All three SV callers detected the inversion and genotyped it as homozygous in the proband and heterozygous in both parents. To validate the inversion we designed oligonucleotide primers to perform PCR amplification, and subsequent Sanger validation around the breakpoints (Figure 10 B). As expected, the proband did not have any PCR product using F1-R1, and F2-R2 primer pairs, whereas F1-F2 and R1-R2 primer pairs gave PCR product with predicted sequence over both

breakpoints (Figure 10 D). This 9-Mb inversion was not observed in 10,782 unrelated genomes with SV calls in gnomAD [Collins et al., 2019]; in fact, no inversions within 544 kb of *QDPR* were observed in gnomAD, further supporting the rareness and probable pathogenicity of this inversion. Importantly, gnomAD database has 2297 Estonians in its GS dataset, thus we can conclude that this inversion is very rare among Estonians as well. Homozygosity mapping from GS data was performed using PLINK 1.9 [Chang et al., 2015], and the homozygous stretch encompassing the inversion was confirmed for the region chr4:7452118-18823503 (GRCh38), and no other homozygous stretches larger than 5 Mbs were detected, thus excluding the parental consanguinity. The detected inversion was submitted to ClinVar database (accession number SCV000898485) and to the locus-specific PNDdb [Lillevali et al., 2020].

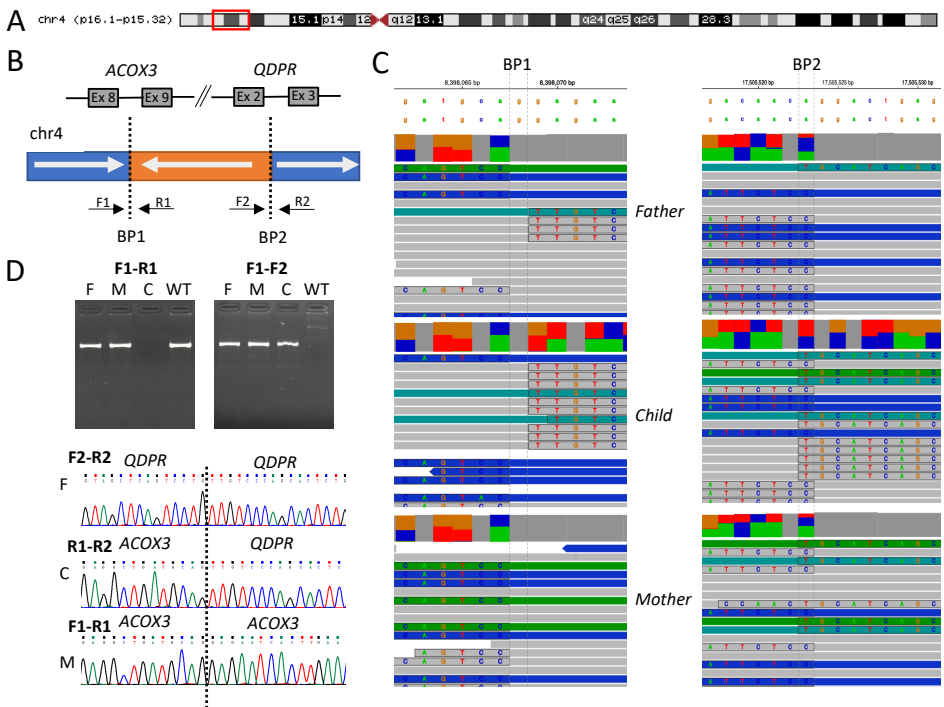

Figure 10. (A) Chromosome 4 ideogram. The described 9-Mb inversion is marked by the red box. (B) Scheme of the inversion in the context of *ACOX3* and *QDPR* genes. F1, R1, F2, R2 schematically represent the PCR primer design relative to the reference (WT) genome. (C) The aligned sequencing reads around detected breakpoints BP1 and BP2 visualised using The Integrative Genomics Viewer (IGV). Soft-clipped nucleotides are highlighted and nucleotides shown. (D) Validation studies confirming the variant by PCR using primers F1-R1 and F1-F2. C – child, M – mother, F – Father, WT – wild-type control [Lillevali et al., 2020].

5.9.3. Chromosome analysis

After finding the large inversion, we investigated whether the inversion could be detected with a regular chromosomal microscopy analysis (karyotyping with G bands) in both heterozygous parents. Our result showed that the inversion of 4p16.1-p15.32 is not detectable by GTG-banding techniques, as inversion points 4p16.1 and 4p15.32 form symmetrical pattern around the band 4p16.2 (Figure 11). Accordingly, in spite of the almost 10 Mb size of the inversion, the inversion is not detectable by G-banding.

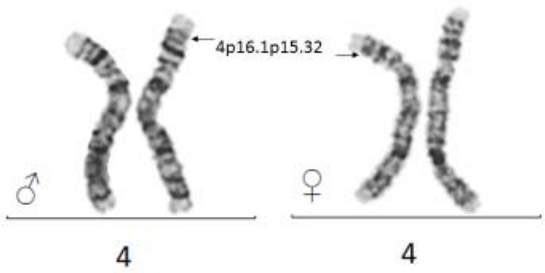

Figure 11. Karyotype analysis from peripheral blood lymphocytes of the parents of the DHPR deficient proband with 9 Mb inversion does not reveal observable pattern abnormality [Lillevali et al., 2020].

5.9.4. mRNA analysis

For the identification of variants in *QDPR*, we performed mRNA studies to analyse the integrity of the cDNA by PCR in heterozygous parental samples (no RNA samples were stored from the proband). This analysis did not reveal any different patterns between the parents of the proband and the control, thus indicating the lack of possible mRNA products with abnormal length (Figure 12). Despite the qualitative essence of the performed reactions, a hint for decreased quantity of obtained *QDPR* cDNA could be retrieved from the visually observed lower intensity of the PCR products of the parents compared to the ones from the control.

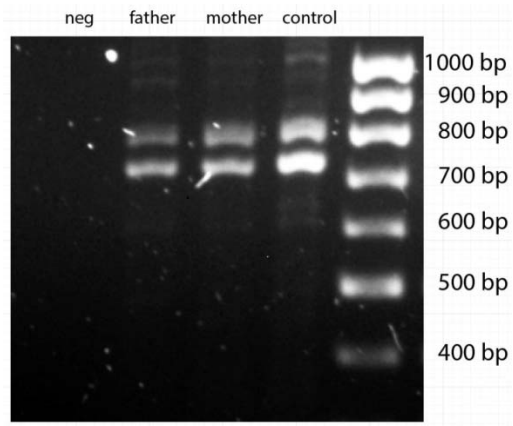

Figure 12. PCR analysis of *QDPR* from the cDNA obtained from peripheral blood mRNA of the parents of the proband and control. No products of abnormal length can be observed, predicted 809 and 716 bp fragments are present. neg: includes all ingredients and primers without cDNA; marker: SolisBiodyne 100 bp DNA ladder [Lillevali et al., 2020].

5.9.5. The importance of genome sequencing in solving the molecular diagnosis

The case illustrates the advantages of GS and the clinical importance of structural variants (SVs) in disease-associated genes. In this family, GS enabled the resolution of a 27-year-long diagnostic odyssey, and now provides the possibility to adequately counsel and test family members at risk for being carriers for this disorder. Since the parents are non-consanguineous, but carry the same inversion and haplotype, this variant may not be unique to this family.

SVs, including inversions, are well recognized as a disease mechanism [Collins et al., 2017], but balanced SVs in particular remain difficult to detect using common molecular DNA variant detection assays that typically focus only on the coding regions of the genome. Heterozygous and hemizygous inversions are known to cause many different disorders. However, karyotyping does not allow for the identification of exact breakpoints and thus the discovery of disrupted genes.

To our knowledge, this is the first report on a large, canonical homozygous pathogenic inversion detected by GS. In addition to simple inversions, GS can be used to resolve more complex SVs, where inversions may be accompanied by deletions or duplications [Sanchis-Juan et al., 2018].

The second breakpoint of our patient's inversion was mapped into the intronic area between exons 8 and 9 of the *ACOX3* gene encoding pristanoyl-CoA oxidase. This enzyme has been shown to be involved in the degradation of the branched-chain fatty acids [Ferdinandusse et al., 2018; Vanhooren et al.,

1997], but its deficiency is probably compensated by other peroxisomal acyl-CoA oxidases and is unlikely to be causing disease in our patient.

From a clinical perspective, the current report presents an additional strategy for making a molecular diagnosis, if biochemical features are suggestive of a disorder and conventional or even the most up- to-date methods fail to identify the causative variant. Although high-throughput targeted sequencing has been shown to be very effective in solving BH_4-deficient HPAs [Trujillano et al., 2014], our case reiterates the additional benefits associated with GS. This inversion maintained all of the exons of *QDPR* intact, as the breakpoint occurred deep inside of an intron, thus presented completely normal results from PCR and Sanger sequencing as well as ES. Obviously, the presence of a breakpoint inside a gene eliminates the possibility of synthesizing normal functional mRNA and respective protein. Standard cytogenetic methods were not capable of revealing this inversion. After mapping the breakpoints in the genome of the index patient, further diagnostic testing in the family can be performed by simple PCR reactions like those presented in Figure 10D, and/or Sanger sequencing, enabling significantly faster analysis and consultation.

6. CONCLUSIONS

1. The studies on the genotype and phenotype of Estonian HPA patients were published in 1996 and 2018, providing an overview of the cohort of our patients with an interval of two decades. Our cohort consisted of 34 patients in 1996 and 94 patients in 2018.
   1.1. Estonian patients with HPA have a relatively homogenous pool of phenylalanine hydroxylase variations. Most common of all affected alleles is p.Arg408Trp (80.4%), which is specific for Eastern Europe and exhibits the highest proportion known in all previously described populations. This predomination was even higher in the study from 1996 (84%).
   1.2. The spectrum of pathogenic variations has widened to seventeen variations in the *PAH* gene compared to the study performed in 1996, when six variations were determined.
   1.3. A vast majority of Estonian patients exhibit classical PKU (87%) with low tolerance to Phe in nutrition and high pre-treatment Phe levels.
   1.4. In the refreshed cohort of 94 patients, four patients receiving BH_4 treatment were found, two with mild PKU with moderate elevation of Phe, and four patients with mild manifestation of HPA, who do not receive specific treatment for lowering their Phe values.

2. The distribution of PKU among Estonian patients could not be distinguished ethnically; the proportions of ethnic Estonians and people of Slavic ethnic background (Russians, Ukrainians) were similar to the ethnic proportions in the population of the country.

3. The local domicile of the ancestors of the PKU patients of Estonian ethnicity harbouring the most widely distributed pathological version of *PAH*, the p.Arg408Trp variant, revealed that the hotspot of the origin of p.Arg408Trp in Estonia is located mostly to Southern and South-Eastern Estonia, to Põlva, Võru and Valga counties, and especially to former Petseri county.

4. In the adherence to dietary therapy, Estonian PKU patients follow similar behavioural pattern as the patients described elsewhere.
   4.1. During the early childhood, rather well-maintained Phe levels during the constant follow-up were observed (with a few deviations in single families).
   4.2. The situation worsens with the start of elementary school, when the median of analyses elevates the nationally recommended levels for 57% of the patients.
   4.3. The adherence to dietary therapy improves during adolescence (43%) and remains similar in adults.

4.4. As an interesting observation, some late-diagnosed adult patients with variable levels of disability, exhibit exceptionally good adherence to the dietary therapy.
4.5. The laboratory information management system of Tartu University Hospital enables personal tracking of each patient as well as obtaining generalised view of different age groups.

5. The patient with biochemically diagnosed DHPR deficiency was subjected to multiple molecular and cytological diagnostic methods, but the existing molecular lesion was discovered by genome sequencing.
5.1. Genome sequencing identified a homozygous 9-Mb inversion in chromosome 4, harbouring a structural variant breakpoint at Chr4(GRCh38): g.17505522 in intron 2 of *QDPR* gene, and another breakpoint in intron 8 of the *ACOX3* gene at Chr4(GRCh38):g.8398067.
5.2. The observed structural variant has never been described before and is obviously causative for the ablation of DHPR activity.

REFERENCES

Ahring K, Belanger-Quintana A, Dokoupil K, Gokmen-Ozel H, Lammardo AM, MacDonald A, Motzfeldt K, Nowacka M, Robert M, van Rijn M. 2011. Blood phenylalanine control in phenylketonuria: a survey of 10 European centres. Eur J Clin Nutr 65(2):275–278.

Anikster Y, Haack TB, Vilboux T, Pode-Shakked B, Thony B, Shen N, Guarani V, Meissner T, Mayatepek E, Trefz FK, Marek-Yagel D, Martinez A, Huttlin EL, Paulo JA, Berutti R, Benoist JF, Imbard A, Dorboz I, Heimer G, Landau Y, Ziv-Strasser L, Malicdan MCV, Gemperle-Britschgi C, Cremer K, Engels H, Meili D, Keller I, Bruggmann R, Strom TM, Meitinger T, Mullikin JC, Schwartz G, Ben-Zeev B, Gahl WA, Harper JW, Blau N, Hoffmann GF, Prokisch H, Opladen T, Schiff M. 2017. Biallelic Mutations in DNAJC12 Cause Hyperphenylalaninemia, Dystonia, and Intellectual Disability. Am J Hum Genet 100(2):257–266.

Anjema K, van Rijn M, Hofstede FC, Bosch AM, Hollak CE, Rubio-Gozalbo E, de Vries MC, Janssen MC, Boelen CC, Burgerhof JG, Blau N, Heiner-Fokkema MR, van Spronsen FJ. 2013. Tetrahydrobiopterin responsiveness in phenylketonuria: prediction with the 48-hour loading test and genotype. Orphanet J Rare Dis 8:103.

Aulehla-Scholz C, Heilbronner H. 2003. Mutational spectrum in German patients with phenylalanine hydroxylase deficiency. Hum Mutat 21(4):399–400.

Avigad S, Cohen BE, Bauer S, Schwartz G, Frydman M, Woo SL, Niny Y, Shiloh Y. 1990. A single origin of phenylketonuria in Yemenite Jews. Nature 344(6262):168–170.

Ayling JE, Pirson WD, al-Janabi JM, Helfand GD. 1974. Kidney phenylalanine hydroxylase from man and rat. Comparison with the liver enzyme. Biochemistry 13(1): 78–85.

Baranovskaya S, Shevtsov S, Maksimova S, Kuzmin A, Schwartz E. 1996. The mutations and VNTRs in the phenylalanine hydroxylase gene of phenylketonuria in St Petersburg. J Inherit Metab Dis 19(5):705.

Bayat A, Yasmeen S, Lund A, Nielsen JB, Moller LB. 2016. Mutational and phenotypical spectrum of phenylalanine hydroxylase deficiency in Denmark. Clin Genet 90(3):247–251.

Berthelon M, Caillaud C, Rey F, Labrune P, Melle D, Feingold J, Frezal J, Briard ML, Farriaux JP, Guibaud P, et al. 1991. Spectrum of phenylketonuria mutations in western Europe and north Africa, and their relation to polymorphic DNA haplotypes at the phenylalanine hydroxylase locus. Hum Genet 86(4):355–358.

Bickel H, Gerrard J, Hickmans EM. 1953. Influence of phenylalanine intake on phenylketonuria. Lancet 265(6790):812–813.

Bik-Multanowski M, Kaluzny L, Mozrzymas R, Oltarzewski M, Starostecka E, Lange A, Didycz B, Gizewska M, Ulewicz-Filipowicz J, Chrobot A, Mikoluc B, Szymczakiewicz-Multanowska A, Cichy W, Pietrzyk JJ. 2013. Molecular genetics of PKU in Poland and potential impact of mutations on BH4 responsiveness. Acta Biochim Pol 60(4):613–616.

Blau N. 2016. Genetics of Phenylketonuria: Then and Now. Hum Mutat 37(6):508–515.

Blau N, Hennermann JB, Langenbeck U, Lichter-Konecki U. 2011. Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies. Mol Genet Metab 104 Suppl:S2–9.

Blau N, Martinez A, Hoffmann GF, Thony B. 2018. DNAJC12 deficiency: A new strategy in the diagnosis of hyperphenylalaninemias. Mol Genet Metab 123(1):1–5.

Blau N, Scriver CR. 2004. New approaches to treat PKU: how far are we? Mol Genet Metab 81(1):1–2.

Blau N, van Spronsen FJ, Levy HL. 2010. Phenylketonuria. Lancet 376(9750):1417–1427.

Blau N vSF. 2014. Disorders of Phenylalanine and Tetrahydrobiopterin Metabolism. In: Blau N. DM, Gibson K., Dionisi Vici C. (eds), editor. Physician's Guide to the Diagnosis, Treatment, and Follow-Up of Inherited Metabolic Diseases Berlin, Heidelberg: Springer. p 3–21.

Bodamer OA, Hoffmann GF, Lindner M. 2007. Expanded newborn screening in Europe 2007. J Inherit Metab Dis 30(4):439–444.

Bonafe L, Thony B, Penzien JM, Czarnecki B, Blau N. 2001. Mutations in the sepiapterin reductase gene cause a novel tetrahydrobiopterin-dependent monoamine-neurotransmitter deficiency without hyperphenylalaninemia. Am J Hum Genet 69(2):269–277.

Bueno MA, Gonzalez-Lamuno D, Delgado-Pecellin C, Aldamiz-Echevarria L, Perez B, Desviat LR, Couce ML. 2013. Molecular epidemiology and genotype-phenotype correlation in phenylketonuria patients from South Spain. J Hum Genet 58(5):279–284.

Burgard P, Rupp K, Lindner M, Haege G, Rigter T, Weinreich SS, Loeber JG, Taruscio D, Vittozzi L, Cornel MC, Hoffmann GF. 2012. Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 2. From screening laboratory results to treatment, follow-up and quality assurance. J Inherit Metab Dis 35(4):613–625.

Burton BK, Grange DK, Milanowski A, Vockley G, Feillet F, Crombez EA, Abadie V, Harding CO, Cederbaum S, Dobbelaere D, Smith A, Dorenbaum A. 2007. The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicentre, open-label, screening study. J Inherit Metab Dis 30(5):700–707.

Camp KM, Parisi MA, Acosta PB, Berry GT, Bilder DA, Blau N, Bodamer OA, Brosco JP, Brown CS, Burlina AB, Burton BK, Chang CS, Coates PM, Cunningham AC, Dobrowolski SF, Ferguson JH, Franklin TD, Frazier DM, Grange DK, Greene CL, Groft SC, Harding CO, Howell RR, Huntington KL, Hyatt-Knorr HD, Jevaji IP, Levy HL, Lichter-Konecki U, Lindegren ML, Lloyd-Puryear MA, Matalon K, MacDonald A, McPheeters ML, Mitchell JJ, Mofidi S, Moseley KD, Mueller CM, Mulberg AE, Nerurkar LS, Ogata BN, Pariser AR, Prasad S, Pridjian G, Rasmussen SA, Reddy UM, Rohr FJ, Singh RH, Sirrs SM, Stremer SE, Tagle DA, Thompson SM, Urv TK, Utz JR, van Spronsen F, Vockley J, Waisbren SE, Weglicki LS, White DA, Whitley CB, Wilfond BS, Yannicelli S, Young JM. 2014. Phenylketonuria Scientific Review Conference: state of the science and future research needs. Mol Genet Metab 112(2):87–122.

Cederbaum SD. 1979. Diagnosis and management of "malignant hyperphenyl-alaninemia". N Engl J Med 301(8):441–442.

Centerwall SA, Centerwall WR. 2000. The discovery of phenylketonuria: the story of a young couple, two retarded children, and a scientist. Pediatrics 105(1 Pt 1):89–103.

Centerwall WR, Chinnock RF, Pusavat A. 1960. Phenylketonuria: screening programs and testing methods. Am J Public Health Nations Health 50:1667–1677.

Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience 4:7.

Charikova EV, Khalchitskii SE, Antoshechkin AG, Schwartz EI. 1993. Distribution of some point mutations in the phenylalanine hydroxylase gene of phenylketonuria patients from the Moscow region. Hum Hered 43(4):244–249.

Chen T, Xu W, Wu D, Han J, Zhu L, Tong F, Yang R, Zhao Z, Jiang P, Shu Q. 2018. Mutational and phenotypic spectrum of phenylalanine hydroxylase deficiency in Zhejiang Province, China. Sci Rep 8(1):17137.

Chen X, Schulz-Trieglaff O, Shaw R, Barnes B, Schlesinger F, Kallberg M, Cox AJ, Kruglyak S, Saunders CT. 2016. Manta: rapid detection of structural variants and indels for germline and cancer sequencing applications. Bioinformatics 32(8):1220–1222.

Citron BA, Davis MD, Kaufman S. 1994. Electrostatic activation of rat phenylalanine hydroxylase. Biochem Biophys Res Commun 198(1):174–180.

Cleary M, Trefz F, Muntau AC, Feillet F, van Spronsen FJ, Burlina A, Belanger-Quintana A, Gizewska M, Gasteyger C, Bettiol E, Blau N, MacDonald A. 2013. Fluctuations in phenylalanine concentrations in phenylketonuria: a review of possible relationships with outcomes. Mol Genet Metab 110(4):418–423.

Cleary M, Walter JH. 2001. Assessment of adult phenylketonuria. Ann Clin Biochem 38(Pt 5):450–458.

Collins RL, Brand H, Karczewski KJ, Zhao X, Alföldi J, Khera AV, Francioli LC, ., Talkowski ME. 2019. An open resource of structural variation for medical and population genetics. bioRxiv.

Collins RL, Brand H, Redin CE, Hanscom C, Antolik C, Stone MR, Glessner JT, Mason T, Pregno G, Dorrani N, Mandrile G, Giachino D, Perrin D, Walsh C, Cipicchio M, Costello M, Stortchevoi A, An JY, Currall BB, Seabra CM, Ragavendran A, Margolin L, Martinez-Agosto JA, Lucente D, Levy B, Sanders SJ, Wapner RJ, Quintero-Rivera F, Kloosterman W, Talkowski ME. 2017. Defining the diverse spectrum of inversions, complex structural variation, and chromothripsis in the morbid human genome. Genome Biol 18(1):36.

Couce ML, Boveda MD, Fernandez-Marmiesse A, Miras A, Perez B, Desviat LR, Fraga JM. 2013. Molecular epidemiology and BH4-responsiveness in patients with phenylalanine hydroxylase deficiency from Galicia region of Spain. Gene 521(1): 100–104.

Coughlin CR, 2nd, Hyland K, Randall R, Ficicioglu C. 2013. Dihydropteridine reductase deficiency and treatment with tetrahydrobiopterin: a case report. JIMD Rep 10:53–56.

Council MR. 1993. Recommendations on the dietary management of phenylketonuria. Report of Medical Research Council Working Party on Phenylketonuria. Arch Dis Child 68(3):426–427.

Dahl HH, Hutchison W, McAdam W, Wake S, Morgan FJ, Cotton RG. 1987. Human dihydropteridine reductase: characterisation of a cDNA clone and its use in analysis of patients with dihydropteridine reductase deficiency. Nucleic Acids Res 15(5):1921–1932.

Danecka MK, Woidy M, Zschocke J, Feillet F, Muntau AC, Gersting SW. 2015. Mapping the functional landscape of frequent phenylalanine hydroxylase (PAH) genotypes promotes personalised medicine in phenylketonuria. J Med Genet 52(3):175–185.

Danks DM, Schlesinger P, Firgaira F, Cotton RG, Watson BM, Rembold H, Hennings G. 1979. Malignant hyperphenylalaninemia--clinical features, biochemical findings, and experience with administration of biopterins. Pediatr Res 13(10):1150–1155.

Daubner SC, Hillas PJ, Fitzpatrick PF. 1997. Expression and characterization of the catalytic domain of human phenylalanine hydroxylase. Arch Biochem Biophys 348(2):295–302.

Davis MD, Parniak MA, Kaufman S, Kempner E. 1996. Structure-function relationships of phenylalanine hydroxylase revealed by radiation target analysis. Arch Biochem Biophys 325(2):235–241.

de Groot MJ, Hoeksma M, Blau N, Reijngoud DJ, van Spronsen FJ. 2010. Pathogenesis of cognitive dysfunction in phenylketonuria: review of hypotheses. Mol Genet Metab 99 Suppl 1:S86–89.

de Sanctis L, Alliaudi C, Spada M, Farrugia R, Cerone R, Biasucci G, Meli C, Zammarchi E, Coskun T, Blau N, Ponzone A, Dianzani I. 2000. Genotype-phenotype correlation in dihydropteridine reductase deficiency. J Inherit Metab Dis 23(4): 333–337.

Demirkol M, Gizewska M, Giovannini M, Walter J. 2011. Follow up of phenylketonuria patients. Mol Genet Metab 104 Suppl:S31–39.

Desviat LR, Perez B, Gamez A, Sanchez A, Garcia MJ, Martinez-Pardo M, Marchante C, Boveda D, Baldellou A, Arena J, Sanjurjo P, Fernandez A, Cabello ML, Ugarte M. 1999. Genetic and phenotypic aspects of phenylalanine hydroxylase deficiency in Spain: molecular survey by regions. Eur J Hum Genet 7(3):386–392.

Dhondt JL. 1991. Strategy for the screening of tetrahydrobiopterin deficiency among hyperphenylalaninaemic patients: 15-years experience. J Inherit Metab Dis 14(2): 117–127.

Dhondt JL. 2010. Lessons from 30 years of selective screening for tetrahydrobiopterin deficiency. J Inherit Metab Dis 33(Suppl 2):S219–223.

Dianzani I, de Sanctis L, Smooker PM, Gough TJ, Alliaudi C, Brusco A, Spada M, Blau N, Dobos M, Zhang HP, Yang N, Ponzone A, Armarego WL, Cotton RG. 1998. Dihydropteridine reductase deficiency: physical structure of the QDPR gene, identification of two new mutations and genotype-phenotype correlations. Hum Mutat 12(4):267–273.

Didycz B, Bik-Multanowski M. 2017. Dynamics of hyperphenylalaninemia and intellectual outcome in teenagers with phenylketonuria. Acta Biochim Pol 64(3):527–531.

DiLella AG, Kwok SC, Ledley FD, Marvit J, Woo SL. 1986. Molecular structure and polymorphic map of the human phenylalanine hydroxylase gene. Biochemistry 25(4):743–749.

Djordjevic M, Klaassen K, Sarajlija A, Tosic N, Zukic B, Kecman B, Ugrin M, Spasovski V, Pavlovic S, Stojiljkovic M. 2013. Molecular Genetics and Genotype-Based Estimation of BH4-Responsiveness in Serbian PKU Patients: Spotlight on Phenotypic Implications of p.L48S. JIMD Rep 9:49–58.

Dobrowolski SF, Borski K, Ellingson CC, Koch R, Levy HL, Naylor EW. 2009. A limited spectrum of phenylalanine hydroxylase mutations is observed in phenylketonuria patients in western Poland and implications for treatment with 6R tetrahydrobiopterin. J Hum Genet 54(6):335–339.

Dobrowolski SF, Heintz C, Miller T, Ellingson C, Ellingson C, Ozer I, Gokcay G, Baykal T, Thony B, Demirkol M, Blau N. 2011. Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish PKU population. Mol Genet Metab 102(2):116–121.

Efron. 1981. Nonparametric estimates of standard error: The jackknife, the bootstrap and other methods. Biometrika 68(3):589–599.

Eiken HG, Knappskog PM, Boman H, Thune KS, Kaada G, Motzfeldt K, Apold J. 1996. Relative frequency, heterogeneity and geographic clustering of PKU mutations in Norway. Eur J Hum Genet 4(4):205–213.

Eiken HG, Odland E, Boman H, Skjelkvale L, Engebretsen LF, Apold J. 1991. Application of natural and amplification created restriction sites for the diagnosis of PKU mutations. Nucleic Acids Res 19(7):1427–1430.

Eisensmith RC, Goltsov AA, O'Neill C, Tyfield LA, Schwartz EI, Kuzmin AI, Baranovskaya SS, Tsukerman GL, Treacy E, Scriver CR, et al. 1995. Recurrence of the R408W mutation in the phenylalanine hydroxylase locus in Europeans. Am J Hum Genet 56(1):278–286.

Eisensmith RC, Okano Y, Dasovich M, Wang T, Guttler F, Lou H, Guldberg P, Lichter-Konecki U, Konecki DS, Svensson E, et al. 1992. Multiple origins for phenylketonuria in Europe. Am J Hum Genet 51(6):1355–1365.

Eisensmith RC, Woo SL. 1994. Population genetics of phenylketonuria. Acta Paediatr Suppl 407:19–26.

Esfahani MS, Vallian S. 2018. A comprehensive study of phenylalanine hydroxylase gene mutations in the Iranian phenylketonuria patients. Eur J Med Genet.

Ferdinandusse S, Denis S, van Roermund CWT, Preece MA, Koster J, Ebberink MS, Waterham HR, Wanders RJA. 2018. A novel case of ACOX2 deficiency leads to recognition of a third human peroxisomal acyl-CoA oxidase. Biochim Biophys Acta Mol Basis Dis 1864(3):952–958.

Flatmark T, Stevens RC. 1999. Structural Insight into the Aromatic Amino Acid Hydroxylases and Their Disease-Related Mutant Forms. Chem Rev 99(8):2137–2160.

Folling I. 1994. The discovery of phenylketonuria. Acta Paediatr Suppl 407:4–10.

Fusetti F, Erlandsen H, Flatmark T, Stevens RC. 1998. Structure of tetrameric human phenylalanine hydroxylase and its implications for phenylketonuria. J Biol Chem 273(27):16962–16967.

Gemperle-Britschgi C, Iorgulescu D, Mager MA, Anton-Paduraru D, Vulturar R, Thony B. 2016. A novel common large genomic deletion and two new missense mutations identified in the Romanian phenylketonuria population. Gene 576(1 Pt 1):182–188.

Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. 2015. A global reference for human genetic variation. Nature 526(7571):68–74.

Giannattasio S, Jurgelevicius V, Lattanzio P, Cimbalistiene L, Marra E, Kucinskas V. 1997. Phenylketonuria mutations and linked haplotypes in the Lithuanian population: origin of the most common R408W mutation. Hum Hered 47(3):155–160.

Goltsov AA, Eisensmith RC, Konecki DS, Lichter-Konecki U, Woo SL. 1992. Associations between mutations and a VNTR in the human phenylalanine hydroxylase gene. Am J Hum Genet 51(3):627–636.

Goltsov AA, Eisensmith RC, Naughton ER, Jin L, Chakraborty R, Woo SL. 1993. A single polymorphic STR system in the human phenylalanine hydroxylase gene permits rapid prenatal diagnosis and carrier screening for phenylketonuria. Hum Mol Genet 2(5):577–581.

Gonzalez Garcia JR, Meza-Espinoza JP. 2006. Use of the International System for Human Cytogenetic Nomenclature (ISCN). Blood 108(12):3952–3953; author reply 3953.

Grenett HE, Ledley FD, Reed LL, Woo SL. 1987. Full-length cDNA for rabbit tryptophan hydroxylase: functional domains and evolution of aromatic amino acid hydroxylases. Proc Natl Acad Sci U S A 84(16):5530–5534.

Grisch-Chan HM, Schwank G, Harding CO, Thony B. 2019. State-of-the-Art 2019 on Gene Therapy for Phenylketonuria. Hum Gene Ther 30(10):1274–1283.
Groselj U, Tansek MZ, Kovac J, Hovnik T, Podkrajsek KT, Battelino T. 2012. Five novel mutations and two large deletions in a population analysis of the phenylalanine hydroxylase gene. Mol Genet Metab 106(2):142–148.
Guldberg P, Henriksen KF, Sipila I, Guttler F, de la Chapelle A. 1995. Phenylketonuria in a low incidence population: molecular characterisation of mutations in Finland. J Med Genet 32(12):976–978.
Guldberg P, Levy HL, Hanley WB, Koch R, Matalon R, Rouse BM, Trefz F, de la Cruz F, Henriksen KF, Guttler F. 1996. Phenylalanine hydroxylase gene mutations in the United States: report from the Maternal PKU Collaborative Study. Am J Hum Genet 59(1):84–94.
Guldberg P, Rey F, Zschocke J, Romano V, Francois B, Michiels L, Ullrich K, Hoffmann GF, Burgard P, Schmidt H, Meli C, Riva E, Dianzani I, Ponzone A, Rey J, Guttler F. 1998. A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. Am J Hum Genet 63(1):71–79.
Gundorova P, Stepanova AA, Kuznetsova IA, Kutsev SI, Polyakov AV. 2019. Genotypes of 2579 patients with phenylketonuria reveal a high rate of BH4 non-responders in Russia. PLoS One 14(1):e0211048.
Gundorova P, Zinchenko RA, Kuznetsova IA, Bliznetz EA, Stepanova AA, Polyakov AV. 2018. Molecular-genetic causes for the high frequency of phenylketonuria in the population from the North Caucasus. PLoS One 13(8):e0201489.
Guthrie R, Susi A. 1963. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. Pediatrics 32:338–343.
Guttler F. 1980. Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. Acta Paediatr Scand Suppl 280:1–80.
Guttler F, Azen C, Guldberg P, Romstad A, Hanley WB, Levy HL, Matalon R, Rouse BM, Trefz F, de la Cruz F, Koch R. 1999. Relationship among genotype, biochemical phenotype, and cognitive performance in females with phenylalanine hydroxylase deficiency: report from the Maternal Phenylketonuria Collaborative Study. Pediatrics 104(2 Pt 1):258–262.
Hardelid P, Cortina-Borja M, Munro A, Jones H, Cleary M, Champion MP, Foo Y, Scriver CR, Dezateux C. 2008. The birth prevalence of PKU in populations of European, South Asian and sub-Saharan African ancestry living in South East England. Ann Hum Genet 72(Pt 1):65–71.
Harding CO, Amato RS, Stuy M, Longo N, Burton BK, Posner J, Weng HH, Merilainen M, Gu Z, Jiang J, Vockley J, Investigators P-. 2018. Pegvaliase for the treatment of phenylketonuria: A pivotal, double-blind randomized discontinuation Phase 3 clinical trial. Mol Genet Metab 124(1):20–26.
Hennermann JB, Vetter B, Wolf C, Windt E, Buhrdel P, Seidel J, Monch E, Kulozik AE. 2000. Phenylketonuria and hyperphenylalaninemia in eastern Germany: a characteristic molecular profile and 15 novel mutations. Hum Mutat 15(3):254–260.
Ho G, Christodoulou J. 2014. Phenylketonuria: translating research into novel therapies. Transl Pediatr 3(2):49–62.
Hufton SE, Jennings IG, Cotton RG. 1995. Structure and function of the aromatic amino acid hydroxylases. Biochem J 311 (Pt 2):353–366.

Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, Nomura Y, Endo K, Tanaka H, Tsuji S, et al. 1994. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat Genet 8(3):236–242.

Ikeda H, Matsubara Y, Mikami H, Kure S, Owada M, Gough T, Smooker PM, Dobbs M, Dahl HH, Cotton RG, Narisawa K. 1997. Molecular analysis of dihydropteridine reductase deficiency: identification of two novel mutations in Japanese patients. Hum Genet 100(5–6):637–642.

Institute for Statistics, UNESCO. 2012. International Standard Classification of Education ISCED 2011. Available from: http://uis.unesco.org/sites/default/files/documents/international-standard-classification-of-education-isced-2011-en.pdf.

Ivaschenko T, Baranov VS. 1993. Rapid and efficient PCR/StyI test for identification of common mutation R408W in phenylketonuria patients. J Med Genet 30(2):153–154.

Jeannesson-Thivisol E, Feillet F, Chery C, Perrin P, Battaglia-Hsu SF, Herbeth B, Cano A, Barth M, Fouilhoux A, Mention K, Labarthe F, Arnoux JB, Maillot F, Lenaerts C, Dumesnil C, Wagner K, Terral D, Broue P, de Parscau L, Gay C, Kuster A, Bedu A, Besson G, Lamireau D, Odent S, Masurel A, Gueant JL, Namour F. 2015. Genotype-phenotype associations in French patients with phenylketonuria and importance of genotype for full assessment of tetrahydrobiopterin responsiveness. Orphanet J Rare Dis 10:158.

Jurecki ER, Cederbaum S, Kopesky J, Perry K, Rohr F, Sanchez-Valle A, Viau KS, Sheinin MY, Cohen-Pfeffer JL. 2017. Adherence to clinic recommendations among patients with phenylketonuria in the United States. Mol Genet Metab 120(3):190–197.

Kalanin J, Takarada Y, Kagawa S, Yamashita K, Ohtsuka N, Matsuoka A. 1994. Gypsy phenylketonuria: a point mutation of the phenylalanine hydroxylase gene in Gypsy families from Slovakia. Am J Med Genet 49(2):235–239.

Kalaydjieva L, Dworniczak B, Kucinskas V, Yurgeliavicius V, Kunert E, Horst J. 1991. Geographical distribution gradients of the major PKU mutations and the linked haplotypes. Hum Genet 86(4):411–413.

Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, Laricchia KM, ., MacArthur DG. 2019. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. bioRxiv.

Kasnauskiene J, Giannattasio S, Lattanzio P, Cimbalistiene L, Kucinskas V. 2003. The molecular basis of phenylketonuria in Lithuania. Hum Mutat 21(4):398.

Kaufman S, Kapatos G, McInnes RR, Schulman JD, Rizzo WB. 1982. Use of tetrahydropterins in the treatment of hyperphenylalaninemia due to defective synthesis of tetrahydrobiopterin: evidence that peripherally administered tetrahydropterins enter the brain. Pediatrics 70(3):376–380.

Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR. 1997. Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a meta-analysis of genotype-phenotype correlations. Am J Hum Genet 61(6):1309–1317.

Kim HL, Park YS. 2010. Maintenance of cellular tetrahydrobiopterin homeostasis. BMB Rep 43(9):584–592.

Kobe B, Jennings IG, House CM, Michell BJ, Goodwill KE, Santarsiero BD, Stevens RC, Cotton RG, Kemp BE. 1999. Structural basis of autoregulation of phenylalanine hydroxylase. Nat Struct Biol 6(5):442–448.

Koch R, Burton B, Hoganson G, Peterson R, Rhead W, Rouse B, Scott R, Wolff J, Stern AM, Guttler F, Nelson M, de la Cruz F, Coldwell J, Erbe R, Geraghty MT, Shear C, Thomas J, Azen C. 2002. Phenylketonuria in adulthood: a collaborative study. J Inherit Metab Dis 25(5):333–346.

Kohler S, Doelken SC, Mungall CJ, Bauer S, Firth HV, Bailleul-Forestier I, Black GC, Brown DL, Brudno M, Campbell J, FitzPatrick DR, Eppig JT, Jackson AP, Freson K, Girdea M, Helbig I, Hurst JA, Jahn J, Jackson LG, Kelly AM, Ledbetter DH, Mansour S, Martin CL, Moss C, Mumford A, Ouwehand WH, Park SM, Riggs ER, Scott RH, Sisodiya S, Van Vooren S, Wapner RJ, Wilkie AO, Wright CF, Vulto-van Silfhout AT, de Leeuw N, de Vries BB, Washingthon NL, Smith CL, Westerfield M, Schofield P, Ruef BJ, Gkoutos GV, Haendel M, Smedley D, Lewis SE, Robinson PN. 2014. The Human Phenotype Ontology project: linking molecular biology and disease through phenotype data. Nucleic Acids Res 42(Database issue):D966–974.

Kozak L, Blazkova M, Kuhrova V, Pijackova A, Ruzickova S, St'astna S. 1997. Mutation and haplotype analysis of phenylalanine hydroxylase alleles in classical PKU patients from the Czech Republic: identification of four novel mutations. J Med Genet 34(11):893–898.

Kowlessur D, Citron BA, Kaufman S. 1996. Recombinant human phenylalanine hydroxylase: novel regulatory and structural properties. Arch Biochem Biophys 333(1):85–95.

Krawczak M, Zschocke J. 2003. A role for overdominant selection in phenylketonuria? Evidence from molecular data. Hum Mutat 21(4):394–397.

Krumm N, Sudmant PH, Ko A, O'Roak BJ, Malig M, Coe BP, Project NES, Quinlan AR, Nickerson DA, Eichler EE. 2012. Copy number variation detection and genotyping from exome sequence data. Genome Res 22(8):1525–1532.

Kure S, Hou DC, Ohura T, Iwamoto H, Suzuki S, Sugiyama N, Sakamoto O, Fujii K, Matsubara Y, Narisawa K. 1999. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 135(3):375–378.

Kwok SC, Ledley FD, DiLella AG, Robson KJ, Woo SL. 1985. Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. Biochemistry 24(3):556–561.

Kyprianou N, Murphy E, Lee P, Hargreaves I. 2009. Assessment of mitochondrial respiratory chain function in hyperphenylalaninaemia. J Inherit Metab Dis 32(2):289–296.

Landau YE, Lichter-Konecki U, Levy HL. 2014. Genomics in newborn screening. J Pediatr 164(1):14–19.

Landrum MJ, Lee JM, Benson M, Brown G, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Hoover J, Jang W, Katz K, Ovetsky M, Riley G, Sethi A, Tully R, Villamarin-Salomon R, Rubinstein W, Maglott DR. 2016. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res 44(D1):D862–868.

Layer RM, Chiang C, Quinlan AR, Hall IM. 2014. LUMPY: a probabilistic framework for structural variant discovery. Genome Biol 15(6):R84.

Lei XD, Kaufman S. 1998. Identification of hepatic nuclear factor 1 binding sites in the 5' flanking region of the human phenylalanine hydroxylase gene: implication of a dual function of phenylalanine hydroxylase stimulator in the phenylalanine hydroxylation system. Proc Natl Acad Sci U S A 95(4):1500–1504.

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper

DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG, Exome Aggregation C. 2016. Analysis of protein-coding genetic variation in 60,706 humans. Nature 536(7616):285–291.

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

Levy HL, Ghavami M. 1996. Maternal phenylketonuria: a metabolic teratogen. Teratology 53(3):176–184.

Levy HL, Sarkissian CN, Scriver CR. 2018. Phenylalanine ammonia lyase (PAL): From discovery to enzyme substitution therapy for phenylketonuria. Mol Genet Metab 124(4):223–229.

Levy HL, Waisbren SE. 1994. PKU in adolescents: rationale and psychosocial factors in diet continuation. Acta Paediatr Suppl 407:92–97.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25(14):1754–1760.

Li N, He C, Li J, Tao J, Liu Z, Zhang C, Yuan Y, Jiang H, Zhu J, Deng Y, Guo Y, Li Q, Yu P, Wang Y. 2018. Analysis of the genotype-phenotype correlation in patients with phenylketonuria in mainland China. Sci Rep 8(1):11251.

Lichter-Konecki U, Hipke CM, Konecki DS. 1999. Human phenylalanine hydroxylase gene expression in kidney and other nonhepatic tissues. Mol Genet Metab 67(4):308–316.

Lichter-Konecki U, Vockley J. 2019. Phenylketonuria: Current Treatments and Future Developments. Drugs 79(5):495–500.

Lidsky AS, Law ML, Morse HG, Kao FT, Rabin M, Ruddle FH, Woo SL. 1985. Regional mapping of the phenylalanine hydroxylase gene and the phenylketonuria locus in the human genome. Proc Natl Acad Sci U S A 82(18):6221–6225.

Lillevali H, Ounap K, Metspalu A. 1996. Phenylalanine hydroxylase gene mutation R408W is present on 84% of Estonian phenylketonuria chromosomes. Eur J Hum Genet 4(5):296–300.

Lillevali H, Pajusalu S, Wojcik MH, Goodrich J, Collins RL, Murumets U, Tammur P, Blau N, Lillevali K, Ounap K. 2020. Genome sequencing identifies a homozygous inversion disrupting QDPR as a cause for dihydropteridine reductase deficiency. Mol Genet Genomic Med:e1154.

Lillevali H, Reinson K, Muru K, Saarsalu S, Kunnapas K, Kahre T, Murumets U, Ounap K. 2019. The evaluation of phenylalanine levels in Estonian phenylketonuria patients during eight years by electronic laboratory records. Mol Genet Metab Rep 19:100467.

Lillevali H, Reinson K, Muru K, Simenson K, Murumets U, Mols T, Ounap K. 2018. Hyperphenylalaninaemias in Estonia: Genotype-Phenotype Correlation and Comparative Overview of the Patient Cohort Before and After Nation-Wide Neonatal Screening. JIMD Rep 40:39–45.

Lindegren ML, Krishnaswami S, Fonnesbeck C, Reimschisel T, Fisher J, Jackson K, Shields T, Sathe NA, McPheeters ML. 2012. Adjuvant Treatment for Phenylketonuria (PKU). Rockville (MD).
Lindner M, Hoffmann GF, Matern D. 2010. Newborn screening for disorders of fatty-acid oxidation: experience and recommendations from an expert meeting. J Inherit Metab Dis 33(5):521–526.
Lockyer J, Cook RG, Milstien S, Kaufman S, Woo SL, Ledley FD. 1987. Structure and expression of human dihydropteridine reductase. Proc Natl Acad Sci U S A 84(10):3329–3333.
Loeber JG. 2007. Neonatal screening in Europe; the situation in 2004. J Inherit Metab Dis 30(4):430–438.
MacDonald A, Rocha JC, van Rijn M, Feillet F. 2011. Nutrition in phenylketonuria. Mol Genet Metab 104 Suppl:S10–18.
Mayer B, John M, Heinzel B, Werner ER, Wachter H, Schultz G, Bohme E. 1991. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. FEBS Lett 288(1–2):187–191.
McCaman MW, Robins E. 1962. Fluorimetric method for the determination of phenylalanine in serum. The Journal of Laboratory and Clinical Medicine 59(5):885–890.
McDonald JD, Bode VC, Dove WF, Shedlovsky A. 1990. Pahhph-5: a mouse mutant deficient in phenylalanine hydroxylase. Proc Natl Acad Sci U S A 87(5):1965–1967.
Mirisola MG, Cali F, Gloria A, Schinocca P, D'Amato M, Cassara G, Leo GD, Palillo L, Meli C, Romano V. 2001. PAH gene mutations in the Sicilian population: association with minihaplotypes and expression analysis. Mol Genet Metab 74(3):353–361.
Moats RA, Moseley KD, Koch R, Nelson M, Jr. 2003. Brain phenylalanine concentrations in phenylketonuria: research and treatment of adults. Pediatrics 112(6 Pt 2):1575–1579.
Moraes TB, Jacques CE, Rosa AP, Dalazen GR, Terra M, Coelho JG, Dutra-Filho CS. 2013. Role of catalase and superoxide dismutase activities on oxidative stress in the brain of a phenylketonuria animal model and the effect of lipoic acid. Cell Mol Neurobiol 33(2):253–260.
Muntau AC, Leandro J, Staudigl M, Mayer F, Gersting SW. 2014. Innovative strategies to treat protein misfolding in inborn errors of metabolism: pharmacological chaperones and proteostasis regulators. J Inherit Metab Dis 37(4):505–523.
Muntau AC, Roschinger W, Habich M, Demmelmair H, Hoffmann B, Sommerhoff CP, Roscher AA. 2002. Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria. N Engl J Med 347(26):2122–2132.
National Institutes of Health Consensus Development P. 2001. National Institutes of Health Consensus Development Conference Statement: phenylketonuria: screening and management, October 16–18, 2000. Pediatrics 108(4):972–982.
Neelima, Sharma R, Rajput YS, Mann B. 2013. Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: a review. Dairy Sci Technol 93(1):21–43.
Nelis M, Esko T, Magi R, Zimprich F, Zimprich A, Toncheva D, Karachanak S, Piskackova T, Balascak I, Peltonen L, Jakkula E, Rehnstrom K, Lathrop M, Heath S, Galan P, Schreiber S, Meitinger T, Pfeufer A, Wichmann HE, Melegh B, Polgar N, Toniolo D, Gasparini P, D'Adamo P, Klovins J, Nikitina-Zake L, Kucinskas V, Kasnauskiene J, Lubinski J, Debniak T, Limborska S, Khrunin A, Estivill X, Rabionet R, Marsal S, Julia A, Antonarakis SE, Deutsch S, Borel C, Attar H,

Gagnebin M, Macek M, Krawczak M, Remm M, Metspalu A. 2009. Genetic structure of Europeans: a view from the North-East. PLoS One 4(5):e5472.
Ney DM, Gleason ST, van Calcar SC, MacLeod EL, Nelson KL, Etzel MR, Rice GM, Wolff JA. 2009. Nutritional management of PKU with glycomacropeptide from cheese whey. J Inherit Metab Dis 32(1):32–39.
O'Donnell KA, O'Neill C, Tighe O, Bertorelle G, Naughten E, Mayne PD, Croke DT. 2002. The mutation spectrum of hyperphenylalaninaemia in the Republic of Ireland: the population history of the Irish revisited. Eur J Hum Genet 10(9):530–538.
Ohlsson A, Bruhn H, Nordenstrom A, Zetterstrom RH, Wedell A, von Dobeln U. 2017. The Spectrum of PAH Mutations and Increase of Milder Forms of Phenylketonuria in Sweden During 1965–2014. JIMD Rep 34:19–26.
Okano Y, Eisensmith RC, Guttler F, Lichter-Konecki U, Konecki DS, Trefz FK, Dasovich M, Wang T, Henriksen K, Lou H, et al. 1991. Molecular basis of phenotypic heterogeneity in phenylketonuria. N Engl J Med 324(18):1232–1238.
Okano Y, Kudo S, Nishi Y, Sakaguchi T, Aso K. 2011. Molecular characterization of phenylketonuria and tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Japan. J Hum Genet 56(4):306–312.
Opladen T, Hoffmann GF, Blau N. 2012. An international survey of patients with tetrahydrobiopterin deficiencies presenting with hyperphenylalaninaemia. J Inherit Metab Dis 35(6):963–973.
Ozguc M, Ozalp I, Coskun T, Yilmaz E, Erdem H, Ayter S. 1993. Mutation analysis in Turkish phenylketonuria patients. J Med Genet 30(2):129–130.
Ounap K, Lillevali H, Metspalu A, Lipping-Sitska M. 1998. Development of the phenylketonuria screening programme in Estonia. J Med Screen 5(1):22–23.
Perez B, Desviat LR, Die M, Cornejo V, Chamoles NA, Nicolini H, Ugarte M. 1993. Presence of the Mediterranean PKU mutation IVS10 in Latin America. Hum Mol Genet 2(8):1289–1290.
Pietz J, Dunckelmann R, Rupp A, Rating D, Meinck HM, Schmidt H, Bremer HJ. 1998. Neurological outcome in adult patients with early-treated phenylketonuria. Eur J Pediatr 157(10):824–830.
Pietz J, Kreis R, Rupp A, Mayatepek E, Rating D, Boesch C, Bremer HJ. 1999. Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria. J Clin Invest 103(8):1169–1178.
Polak E, Ficek A, Radvanszky J, Soltysova A, Urge O, Cmelova E, Kantarska D, Kadasi L. 2013. Phenylalanine hydroxylase deficiency in the Slovak population: genotype-phenotype correlations and genotype-based predictions of BH4-responsiveness. Gene 526(2):347–355.
Ponzone A, Blau N, Guardamagna O, Ferrero GB, Dianzani I, Endres W. 1990. Progression of 6-pyruvoyl-tetrahydropterin synthase deficiency from a peripheral into a central phenotype. J Inherit Metab Dis 13(3):298–300.
Pronina N, Giannattasio S, Lattanzio P, Lugovska R, Vevere P, Kornejeva A. 2003. The molecular basis of phenylketonuria in Latvia. Hum Mutat 21(4):398–399.
Rao DN, Kaufman S. 1986. Purification and state of activation of rat kidney phenylalanine hydroxylase. J Biol Chem 261(19):8866–8876.
Rausch T, Zichner T, Schlattl A, Stutz AM, Benes V, Korbel JO. 2012. DELLY: structural variant discovery by integrated paired-end and split-read analysis. Bioinformatics 28(18):i333-i339.
Rausell D, Garcia-Blanco A, Correcher P, Vitoria I, Vento M, Chafer-Pericas C. 2019. Newly validated biomarkers of brain damage may shed light into the role of

oxidative stress in the pathophysiology of neurocognitive impairment in dietary restricted phenylketonuria patients. Pediatr Res 85(2):242–250.
Reblova K, Hruba Z, Prochazkova D, Pazdirkova R, Pouchla S, Zeman J, Fajkusova L. 2013. Hyperphenylalaninemia in the Czech Republic: genotype-phenotype correlations and in silico analysis of novel missense mutations. Clin Chim Acta 419:1–10.
Reinson K, Kunnapas K, Kriisa A, Vals MA, Muru K, Ounap K. 2018. High incidence of low vitamin B12 levels in Estonian newborns. Mol Genet Metab Rep 15:1–5.
Rivera I, Leandro P, Lichter-Konecki U, Tavares de Almeida I, Lechner MC. 1998. Population genetics of hyperphenylalaninaemia resulting from phenylalanine hydroxylase deficiency in Portugal. J Med Genet 35(4):301–304.
Rosa AP, Jacques CE, Moraes TB, Wannmacher CM, Dutra Ade M, Dutra-Filho CS. 2012. Phenylpyruvic acid decreases glucose-6-phosphate dehydrogenase activity in rat brain. Cell Mol Neurobiol 32(7):1113–1118.
Sanchis-Juan A, Stephens J, French CE, Gleadall N, Megy K, Penkett C, Shamardina O, Stirrups K, Delon I, Dewhurst E, Dolling H, Erwood M, Grozeva D, Stefanucci L, Arno G, Webster AR, Cole T, Austin T, Branco RG, Ouwehand WH, Raymond FL, Carss KJ. 2018. Complex structural variants in Mendelian disorders: identification and breakpoint resolution using short- and long-read genome sequencing. Genome Med 10(1):95.
Sato H, Uematsu M, Endo W, Nakayama T, Kobayashi T, Hino-Fukuyo N, Sakamoto O, Shintaku H, Kure S. 2014. Early replacement therapy in a first Japanese case with autosomal recessive guanosine triphosphate cyclohydrolase I deficiency with a novel point mutation. Brain Dev 36(3):268–271.
Saugstad LF. 2006. From genetics to epigenetics. Nutr Health 18(3):285–300.
Schuck PF, Malgarin F, Cararo JH, Cardoso F, Streck EL, Ferreira GC. 2015. Phenylketonuria Pathophysiology: on the Role of Metabolic Alterations. Aging Dis 6(5):390–399.
Schuler A, Somogyi C, Mate M, Pataki L, Toros I, Woo SL, Eisensmith RC, Fekete G. 1994. Cognitive development related to metabolic phenotype and mutation genotype in 25 Hungarian patients with phenylketonuria. J Inherit Metab Dis 17(3):372.
Schuler A, Somogyi C, Toros I, Pataki L, Mete M, Kiss E, Nagy A. 1996. A longitudinal study of phenylketonuria based on the data of the Budapest Screening Center. Eur J Pediatr 155 Suppl 1:S50–52.
Scriver CR. 1995. Whatever happened to PKU? Clin Biochem 28(2):137–144.
Scriver CR, Eisensmith RC, Woo SL, Kaufman S. 1994. The hyperphenylalaninemias of man and mouse. Annu Rev Genet 28:141–165.
Sharman R, Sullivan K, Young R, McGill J. 2010. A preliminary investigation of the role of the phenylalanine:tyrosine ratio in children with early and continuously treated phenylketonuria: toward identification of "safe" levels. Dev Neuropsychol 35(1):57–65.
Sterl E, Paul K, Paschke E, Zschocke J, Brunner-Krainz M, Windisch E, Konstantopoulou V, Moslinger D, Karall D, Scholl-Burgi S, Sperl W, Lagler F, Plecko B. 2013. Prevalence of tetrahydrobiopterine (BH4)-responsive alleles among Austrian patients with PAH deficiency: comprehensive results from molecular analysis in 147 patients. J Inherit Metab Dis 36(1):7–13.
Stojiljkovic M, Jovanovic J, Djordjevic M, Grkovic S, Cvorkov Drazic M, Petrucev B, Tosic N, Karan Djurasevic T, Stojanov L, Pavlovic S. 2006. Molecular and phenotypic characteristics of patients with phenylketonuria in Serbia and Montenegro. Clin Genet 70(2):151–155.

Sueoka H, Moshinetsky A, Nagao M, Chiba S. 1999. Mutation screening of phenylketonuria in the Far East of Russia. J Hum Genet 44(6):368–371.
Zekanowski C, Jurkowska M, Bal J. 2001. Association between minihaplotypes and mutations at the PAH locus in Polish hyperphenylalaninemic patients. Hum Hered 51(1–2):117–120.
Zekanowski C, Nowacka M, Zgulska M, Horst J, Cabalska B, Mazurczak T. 1994. Frequencies of the most common mutations responsible for phenylketonuria in Poland. Mol Cell Probes 8(4):323–324.
Zhu M. 2017. The Guthrie Test for Early Diagnosis of Phenylketonuria. http://embryo.asu.edu/handle/10776/11460. Embryo Project Encyclopedia. p ISSN: 1940–5030
Zschocke J. 2003. Phenylketonuria mutations in Europe. Hum Mutat 21(4):345–356.
Zschocke J, Aulehla-Scholz C, Patton S. 2008. Quality of diagnostic mutation analyses for phenylketonuria. J Inherit Metab Dis 31(6):697–702.
Zschocke J, Haverkamp T, Moller LB. 2012. Clinical utility gene card for: Phenylketonuria. Eur J Hum Genet 20(2).
Zschocke J, Preusse A, Sarnavka V, Fumic K, Mardesic D, Hoffmann GF, Baric I. 2003. The molecular basis of phenylalanine hydroxylase deficiency in Croatia. Hum Mutat 21(4):399.
Zurfluh MR, Zschocke J, Lindner M, Feillet F, Chery C, Burlina A, Stevens RC, Thony B, Blau N. 2008. Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. Hum Mutat 29(1):167–175.
Therrell BL, Padilla CD, Loeber JG, Kneisser I, Saadallah A, Borrajo GJ, Adams J. 2015. Current status of newborn screening worldwide: 2015. Semin Perinatol 39(3):171–187.
Thony B, Auerbach G, Blau N. 2000. Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem J 347 Pt 1:1–16.
Thony B, Blau N. 1997. Mutations in the GTP cyclohydrolase I and 6-pyruvoyl-tetrahydropterin synthase genes. Hum Mutat 10(1):11–20.
Thony B, Heizmann CW, Mattei MG. 1994. Chromosomal location of two human genes encoding tetrahydrobiopterin-metabolizing enzymes: 6-pyruvoyl-tetrahydropterin synthase maps to 11q22.3-q23.3, and pterin-4 alpha-carbinolamine dehydratase maps to 10q22. Genomics 19(2):365–368.
Thony B, Neuheiser F, Kierat L, Blaskovics M, Arn PH, Ferreira P, Rebrin I, Ayling J, Blau N. 1998a. Hyperphenylalaninemia with high levels of 7-biopterin is associated with mutations in the PCBD gene encoding the bifunctional protein pterin-4a-carbinolamine dehydratase and transcriptional coactivator (DCoH). Am J Hum Genet 62(6):1302–1311.
Thony B, Neuheiser F, Kierat L, Rolland MO, Guibaud P, Schluter T, Germann R, Heidenreich RA, Duran M, de Klerk JB, Ayling JE, Blau N. 1998b. Mutations in the pterin-4alpha-carbinolamine dehydratase (PCBD) gene cause a benign form of hyperphenylalaninemia. Hum Genet 103(2):162–167.
Tighe O, Dunican D, O'Neill C, Bertorelle G, Beattie D, Graham C, Zschocke J, Cali F, Romano V, Hrabincova E, Kozak L, Nechyporenko M, Livshits L, Guldberg P, Jurkowska M, Zekanowski C, Perez B, Desviat LR, Ugarte M, Kucinskas V, Knappskog P, Treacy E, Naughten E, Tyfield L, Byck S, Scriver CR, Mayne PD, Croke DT. 2003. Genetic diversity within the R408W phenylketonuria mutation lineages in Europe. Hum Mutat 21(4):387–393.

Trujillano D, Perez B, Gonzalez J, Tornador C, Navarrete R, Escaramis G, Ossowski S, Armengol L, Cornejo V, Desviat LR, Ugarte M, Estivill X. 2014. Accurate molecular diagnosis of phenylketonuria and tetrahydrobiopterin-deficient hyperphenylalaninemias using high-throughput targeted sequencing. Eur J Hum Genet 22(4):528–534.

Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C, Leccese A, Bafunno V, Lillo V, Papadia F, Margaglione M. 2015. Phenylalanine hydroxylase deficiency in south Italy: Genotype-phenotype correlations, identification of a novel mutant PAH allele and prediction of BH4 responsiveness. Clin Chim Acta 450:51–55.

Uudelepp M-L, Joost K, Žordania R, Õunap K. 2012. Fenüülketonuuria Eesti ravijuhend (Treatment guidelines of phenylketonuria in Estonia; in Estonian) Eesti Arst 96:46–51.

Walter JH, White FJ, Hall SK, MacDonald A, Rylance G, Boneh A, Francis DE, Shortland GJ, Schmidt M, Vail A. 2002. How practical are recommendations for dietary control in phenylketonuria? Lancet 360(9326):55–57.

van Spronsen FJ, Burgard P. 2008. The truth of treating patients with phenylketonuria after childhood: the need for a new guideline. J Inherit Metab Dis 31(6):673–679.

van Spronsen FJ, Himmelreich N, Rufenacht V, Shen N, Vliet DV, Al-Owain M, Ramzan K, Alkhalifi SM, Lunsing RJ, Heiner-Fokkema RM, Rassi A, Gemperle-Britschgi C, Hoffmann GF, Blau N, Thony B. 2017a. Heterogeneous clinical spectrum of DNAJC12-deficient hyperphenylalaninemia: from attention deficit to severe dystonia and intellectual disability. J Med Genet.

van Spronsen FJ, van Wegberg AM, Ahring K, Belanger-Quintana A, Blau N, Bosch AM, Burlina A, Campistol J, Feillet F, Gizewska M, Huijbregts SC, Kearney S, Leuzzi V, Maillot F, Muntau AC, Trefz FK, van Rijn M, Walter JH, MacDonald A. 2017b. Key European guidelines for the diagnosis and management of patients with phenylketonuria. Lancet Diabetes Endocrinol 5(9):743–756.

van Wegberg AMJ, MacDonald A, Ahring K, Belanger-Quintana A, Blau N, Bosch AM, Burlina A, Campistol J, Feillet F, Gizewska M, Huijbregts SC, Kearney S, Leuzzi V, Maillot F, Muntau AC, van Rijn M, Trefz F, Walter JH, van Spronsen FJ. 2017. The complete European guidelines on phenylketonuria: diagnosis and treatment. Orphanet J Rare Dis 12(1):162.

Wang R, Shen N, Ye J, Han L, Qiu W, Zhang H, Liang L, Sun Y, Fan Y, Wang L, Wang Y, Gong Z, Liu H, Wang J, Yan H, Blau N, Gu X, Yu Y. 2018. Mutation spectrum of hyperphenylalaninemia candidate genes and the genotype-phenotype correlation in the Chinese population. Clin Chim Acta 481:132–138.

Vanhooren JC, Marynen P, Mannaerts GP, Van Veldhoven PP. 1997. Evidence for the existence of a pristanoyl-CoA oxidase gene in man. Biochem J 325 (Pt 3):593–599.

Watschinger K, Keller MA, Hermetter A, Golderer G, Werner-Felmayer G, Werner ER. 2009. Glyceryl ether monooxygenase resembles aromatic amino acid hydroxylases in metal ion and tetrahydrobiopterin dependence. Biol Chem 390(1):3–10.

Werner ER, Blau N, Thony B. 2011. Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem J 438(3):397–414.

Wijemanne S, Jankovic J. 2015. Dopa-responsive dystonia--clinical and genetic heterogeneity. Nat Rev Neurol 11(7):414–424.

Williams RA, Mamotte CD, Burnett JR. 2008. Phenylketonuria: an inborn error of phenylalanine metabolism. Clin Biochem Rev 29(1):31–41.

Vockley J, Andersson HC, Antshel KM, Braverman NE, Burton BK, Frazier DM, Mitchell J, Smith WE, Thompson BH, Berry SA, American College of Medical G, Genomics Therapeutics C. 2014. Phenylalanine hydroxylase deficiency: diagnosis and management guideline. Genet Med 16(2):188–200.

Woo SL. 1988. Collation of RFLP haplotypes at the human phenylalanine hydroxylase (PAH) locus. Am J Hum Genet 43(5):781–783.

Woo SL. 1989. Molecular basis and population genetics of phenylketonuria. Biochemistry 28(1):1–7.

Woo SL, Lidsky AS, Guttler F, Chandra T, Robson KJ. 1983. Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria. Nature 306(5939):151–155.

Woolf LI. 1986. The heterozygote advantage in phenylketonuria. Am J Hum Genet 38(5):773–775.

Woolf LI. 1994. Phenylketonuria in Turkey, Ireland and west Scotland. J Inherit Metab Dis 17(2):246–247.

Wu JT, Wu LH, Ziter FA, Ash KO. 1979. Manual fluorometry of phenylalanine from blood specimens collected on filter paper: a modified procedure. Clin Chem 25(3):470–472.

Xu J, Li Y, Lv Y, Bian C, You X, Endoh D, Teraoka H, Shi Q. 2019. Molecular Evolution of Tryptophan Hydroxylases in Vertebrates: A Comparative Genomic Survey. Genes (Basel) 10(3).

Yamaguchi-Kabata Y, Yasuda J, Uruno A, Shimokawa K, Koshiba S, Suzuki Y, Fuse N, Kawame H, Tadaka S, Nagasaki M, Kojima K, Katsuoka F, Kumada K, Tanabe O, Tamiya G, Yaegashi N, Kinoshita K, Yamamoto M, Kure S, Tohoku Medical Megabank Project Study G. 2019. Estimating carrier frequencies of newborn screening disorders using a whole-genome reference panel of 3552 Japanese individuals. Hum Genet.

Yan Y, Zhang C, Jin X, Zhang Q, Zheng L, Feng X, Hao S, Gao H, Ma X. 2019. Mutation spectrum of PAH gene in phenylketonuria patients in Northwest China: identification of twenty novel variants. Metab Brain Dis 34(3):733–745.

Yano S, Moseley K, Fu X, Azen C. 2016. Evaluation of Tetrahydrobiopterin Therapy with Large Neutral Amino Acid Supplementation in Phenylketonuria: Effects on Potential Peripheral Biomarkers, Melatonin and Dopamine, for Brain Monoamine Neurotransmitters. PLoS One 11(8):e0160892.

SUMMARY IN ESTONIAN

Hüperfenüülalanineemiad ja seotud neurofüsioloogilised häired

Fenüülalaniin (Phe) on üks 20-st tavatoidu valkudes esindatud aminohappest, mis normaalse ainevahetuse puhul hüdrolüüsitakse türosiiniks. Kui selle protsessi eest vastutav ensüüm fenüülalaniini hüdroksülaas (PAH) ei toimi korrektselt, tekib organismis hüperfenüülalanineemia (HPA) – seisund, mille püsimisel võib kujuneda vaimne ja motoorne mahajäämus. Peamine HPA sümptomaatikaga ainevahetushaigus on fenüülketonuuria (PKU, OMIM #261600), autosomaalse retsessiivse päritavusega häire, mis kuulub tuntuimate ja levinuimate ainevahetushaiguste sekka, sagedusega ca 1:10 000 vastsündinu kohta valge rassi seas [Williams et al., 2008]. PKU on ravitav, kui eemaldada igapäevasest toidust võimalikult suurel määral tavaline valk ja asendada see aminohapete seguga, millest puudub Phe [Blau and Scriver 2004; Blau et al., 2010; Blau N 2014; Scriver 1995].

PKU olemusest hakati aru saama 1930ndatel aastatel, mil Norra arst Asbjørn Følling avastas grupi vaimse mahajäämusega patsientide uriinist Phe alternatiivse metaboliidi fenüülpüruvaadi, mille kaudu haigust hakati kutsuma PKU-ks [Centerwall and Centerwall 2000; Folling 1994]. 1950. aastatel avastati fenüülalaniinivaese dieedi oluline kasulikkus PKU patsientidele ja selle tulemuslikkus seostati ravi varajase alustamisega. [Bickel et al., 1953].

USA mikrobioloog Dr Robert Guthrie töötas välja meetodi Phe taseme tuvastamiseks filterpaberile kogutud vereplekist ning tema algatusel alustati Phe taseme rutiinset määramist vastsündinutel. Test seisnes kindla bakteritüve võimes kasvada ainult kõrgenenud Phe taseme juuresolekul. [Guthrie and Susi 1963]. Dr Guthrie järgi tuntakse vastsündinute sõeluuringuks kasutatavaid filterpabereid Guthrie kaartidena. Arenenud lääneriikides loodi PKU varajaseks avastamiseks riiklikud vastsündinute sõeluuringu-programmid, millele lisandus järk-järgult ka programme teiste ravitavate ainevahetushaiguste varajaseks tuvastamiseks ja rutiinseks testimiseks. [Therrell et al., 2015]. Tänapäevaks katab vastsündinute sõeluuring hulka ravitavaid ainevahetushaigusi ja riiklikud programmid on üles ehitatud, võttes arvesse iga populatsiooni geneetilist struktuuri ja tehnilist võimekust [Bodamer et al., 2007; Burgard et al., 2012; Landau et al., 2014; Lindner et al., 2010].

Eestis diagnoositi kuni 1990. aastate alguseni PKU-d ainult Føllingi testi abil, mis määrab uriinist fenüülpüruvaati [Folling 1994] ja eeldati, et tegu on väga haruldase haigusega nagu Soome populatsioonis, kus oli PKU sageduseks hinnatud umbes 1 patsient 200 000 sünni kohta [Guldberg et al., 1995]. Alates 1990. aastate algusest asuti Eestis suurt tähelepanu pöörama ravitavate ainevahetushaiguste leidmisele ning 1993. aastal käivitati riiklik vastsündinute sõeluuring PKU suhtes [Ounap et al., 1998]. Tänaseks toimub SA TÜ Kliinikumi ühendlabori kliinilise geneetika keskuses riikliku sõeluuringu programmi raames otsing 20 ravitava ainevahetushaiguse suhtes, neist 19, sealhulgas PKU

leidmiseks kasutatakse alates 2014. aastast tandem mass-spektromeetrilist (MS/MS) analüüsi [Reinson et al., 2018].

Käesolev töö sai alguse 1990 aastate esimesel poolel, kui Eesti PKU patsientide kohta said tehtud koos prof. K. Õunapiga esimesed epidemioloogilised ja molekulaarsed uuringud, mis kajastuvad käesolevas doktoritöös hõlmatud publikatsioonis I [Lillevali et al., 1996]. Praeguseks ajaks on tekkinud vajadus ajakohastada meie andmeid ja teha need kättesaadavaks meedikutele ja teadlaskonnale, uurida dieetravi tõhusust koos meie patsientide ja nende perekondade võimekusega pidada kinni ettenähtud raviskeemidest. Kuna madala Phe hoidmine dieedis on peredele omaette lisakoormus, vajasime ülevaadet meie patsientide (ja perede) võimekuse kohta pidada kinni ravijuhistes ettenähtud soovitustest lubatava Phe taseme kohta patsiendi veres. Tänu alates 2010. aastast TÜ Kliinikumis toimivale e-labori süsteemile saime koostada andmebaasi kõikidest kirjetest, mis on seotud Eesti PKU patsientide vereanalüüsidega Phe taseme suhtes, hinnata iga patsiendi ravi dünaamikat individuaalselt ja samuti üldistada dünaamikat ajas ja vanusegruppide vahel.

PAH ensüümi toimimiseks on vaja piisava hulga koensüümi tetrahüdrobiopteriini (BH_4) olemasolu. Seetõttu võib HPA seisundi põhjustada ka takistus BH_4 sünteesi või regeneratsiooni biokeemilistes radades. Kõnealused juhtumid on PKUst oluliselt haruldasemad, kuid ka keerulisemad ravida ja sageli raskema kulu ja halvema prognoosiga. Eestis sündis 1991. aastal laps, kellel diagnoositi koostöös Leedu, Šveitsi ja Saksamaa arstidega biokeemiliselt ensüümi dihüdropteridiini reduktaasi (DHPR) puudulikkus. DHPR ensüüm vastutab BH_4 regenereerimise eest, sest see kofaktor oksüdeeritakse igakordse Phe (ja ka türosiini ning trüptofaani) hüdroksüülimise käigus. Seni oli selle DHPR puudulikkusega patsiendi haiguse täpne molekulaargeneetiline põhjus teadmata.

Käesoleva uuringu eesmärgid

1. Määrata Eesti hüperfenüülalanineemiaga patsientide genotüübid ja korreleerida need fenotüüpidega (artiklid I ja II);
2. Võrrelda *PAH* geeni variantide spektrit Eesti etniliste rühmade seas (artiklid I ja II);
3. Hinnata kõige levinuma *PAH* geeni patoloogilise variandi p.Arg408Trp geograafilist levikut Eestis (artiklid I ja II);
4. Uurida, kui korrektselt Eesti PKU patsiendid suudavad järgida dieetravi soovitusi (artikkel III);
5. Tuvastada DHPR puudulikkusega patsiendi haigust põhjustav molekulaarne muutus (artikkel IV).

Patsientide ja meetodite lühikirjeldus

Eesti PKU-ga patsientide uurimiseks loodi andmekogu, millesse hõlmati 95 teadaolevat HPA juhtumit sünniaastatega 1974 kuni 2016. Kohort jagunes kaheks: enne ja pärast Eestis vastsündinute PKU suhtes sõeluurimise sisseviimist, s.o. 1993. aastat sündinud HPA-ga patsiendid.

PAH patogeensete variantide tuvastamiseks sekveneeriti PAH geeni eksonid külgnevate piirkondadega Sangeri meetodil. Levinuima variandi p.Arg408Trp tuvastamiseks kasutati ka *PAH* geeni 12. eksoni PCR amplifitseerimist koos järgneva *Sty*I restriktsioonanalüüsiga. Lisaks oli kasutusel *PAH* geeni MLPA analüüs.

Eesti PKU patsientide genealoogilise uuringu käigus koguti andmed patogeenset *PAH* varianti p.Arg408Trp kandvate eesti rahvusest patsientide vanavanemate sünnikohtadest. Sünnikohad kanti Eesti kaardile ja potentsiaalselt haigusseoselist varianti kandvate vanavanemate hulka hinnati statistiliste meetoditega võrrelduna maakondade rahvastikutihedusega.

TÜ Kliinikumi Ühendlaboris on kasutusel alates 2010. aastast ühtne andmehaldustarkvara (e-labor), mille põhjal moodustasime andmebaasi Eesti PKU patsientidelt saadud analüüsidest ajavahemikul 2010 kuni 2018 kevadeni. Saadud andmekogumist on võimalik hinnata üksiku patsiendi ravi järgimise dünaamikat Guthrie kaartidel esitatud kuivatatud vereplekkidest ja teha üldistavaid kokkuvõtteid vanusegruppide kaupa. Phe tasemed vereplekis määrati kahe erineva meetodiga, algselt McCamani ja Robinsi kvantitatiivsel fluorimeetrilisel meetodil, alates 2015. aastast aga tandem-MS/MS abil.

Eestis 1991. aastal sündinud DHPR puudulikkusega HPA patsient sai diagnoosi biokeemiliste meetodite abil Šveitsis, Zürichis, ja seda ensüümi kodeeriva *QDPR* geeni eksoonsed alad sekveneeriti korduvalt Sangeri meetodiga, tehti karüotüpiseerimine, mRNA analüüs vanematelt, eksoomi sekveneerimine. Lõpliku vastuse saamiseks tehti täisgenoomi sekveneerimine (GS) ning andmeanalüüs MIT ja Harvardi Ülikooli Broad Instituudi Genoomika platvormil.

Peamised tulemused ja järeldused

1. Eesti HPA-ga patsientide kohta avaldasime artiklid aastatel 1996 ja 2018, millega anname ülevaate meie patsientide kogumist kahe aastakümne pikkuse intervalliga. Meie kohordi suurus aastal 1996 oli 34 patsienti ja 2018. aastal 94 patsienti.
   1.1. Eesti HPA-ga patsientidel on suhteliselt homogeenne kogum *PAH* patogeenseid variante. Kõige levinum variant on p.Arg408Trp (80.4%), mis on omane Ida-Euroopale ja hõlmab meil suurima osakaalu võrreldes teiste seni kirjeldatud populatsioonidega. See ülekaalukus oli isegi kõrgem 1996. aastal avaldatud töö andmetel (84%).
   1.2. PAH geeni patogeensete variantide spekter Eestis laienes 17-ni võrreldes 1996. aasta uuringuga, mil tuvastati kokku 6 varianti.

1.3. Valdav enamik Eesti PKU patsiente on klassikalise PKU fenotüübiga (87%), mida iseloomustab madal tolerants Phe suhtes toidus ja kõrged ravieelsed Phe tasemed.
1.4. Uuendatud, 94 patsiendist koosnevas kohordis on ainult neli patsienti, kes saavad BH4 ravi, kahel on PKU pehme vorm mõõduka Phe taseme tõusuga ja neli patsienti, kelle HPA fenotüüp on niivõrd madala Phe tõusuga, et nad ei vaja ravi Phe taseme alandamiseks.

2. PKU levimus Eesti etniliste rühmade seas pole eristatav; eestlaste ja slaavi (vene, ukraina) taustaga patsientide osakaal on proportsioonis üldiste etniliste osakaaludega riigis.

3. Eestlastest PKU patsientide PAH kõige sagedama patoloogilise variandi p.Arg408Trp esivanemate päritolu uurimine paigutas kõnealuse variandi lähtepiirkonna Kagu- ja Lõuna-Eestisse, eelkõige Põlva, Võru ja Valga maakondadesse ning eriti varasemasse Petseri maakonda.

4. Eesti PKU patsientide dieetravi järgimine sarnaneb teistes riikides kirjeldatud olukorraga.
4.1. Varajases lapseeas on tüüpiline Phe tasemete edukas hoidmine ja patsientide seisundi püsiv järgimine (väheste eranditega üksikute perekondade puhul).
4.2. Olukord halveneb algkoolieas, mil analüüside väärtuste mediaan ületab riiklikutes soovitustes kehtestatu 57% patsientide puhul.
4.3. Dieetravi järgimine paraneb teismeliseeas (43%) ja jääb sarnaseks täiskasvanutel.
4.4. Osa hilisdiagnoosiga, eri tasemel puudeastmega patsientide dieet on eriti korraliku kontrolli all.
4.5. SA TÜ Kliinikumi laboriinfosüsteem (e-labor) võimaldab nii iga patsiendi analüüside tulemuste individuaalset jälgimist kui ka üldistatud ülevaate saamist eri vanuserühmadest.

5. Biokeemiliselt diagnoositud DHPR puudulikkusega patsienti uuriti mitmete molekulaarsete ja tsütoloogiliste meetoditega, kuid haigust põhjustav molekulaarne hälve leiti kogu genoomi sekveneerimisel.
5.1. Genoomi sekveneerimine tuvastas homosügootse 9-Mb inversiooni 4. kromosoomis, mis tõi kaasa struktuurse variandi murdekohtadega: Chr4(GRCh38):g.17505522 *QDPR* geeni 2. intronis ja *ACOX3* geeni 8. intronis: Chr4(GRCh38):g.8398067.
5.2. Leitud struktuurne variant on varasemalt kirjeldamata ja on selge põhjus DHPR aktiivsuse kadumiseks.

ACKNOWLEDGEMENTS

First of all, my deepest gratitude goes to Professor Katrin Õunap, who accepted the challenge of welcoming me to PhD studies at the Department of Clinical Genetics. Her straightforward willingness to conduct my pathway during the highs and lows of these years is of utmost importance for achieving the results of this work.

I am very grateful to my second supervisor, Senior Researcher Dr. Kersti Lilleväli for all the efforts to establish the conditions to perform the studies and for the support in various fields we had to cross.

I sincerely thank Professor Eero Vasar for the opportunity of continuing my studies at the Department of Physiology in the Curriculum of Neurosciences.

This study would not have been possible without the knowledge and views on genetics and biomedical sciences provided by Professor Andres Metspalu during my studies in the 1990s, which became the firm basis for further work.

I am very thankful to the referees, Dr. Eve Õiglane-Šlik and Dr. Elin Org for the wise and useful recommendations with the thesis.

My very special thanks go to Dr Sander Pajusalu for his helpfulness and extraordinary skills of working with genome data.

I express my gratefulness to Mari-Anne Philips, whose suggestions have become an important turning point for achieving the upcoming goals.

It would be unimaginable I could have succeeded with this study if I did not have my family, who have been unintendedly suffering from deprivation from my humble person all this time.

My sincere gratitude goes to all of my co-authors: Katrin Õunap, Andres Metspalu, Karit Reinson, Kai Muru, Kristi Rähn, Ülle Murumets, Tõnu Möls, Siret Saarsalu, Kadi Künnapas, Tiina Kahre, Sander Pajusalu, Monica H. Wojcik, Julia Goodrich, Ryan L. Collins, Pille Tammur, Nenad Blau, and Kersti Lilleväli.

My best regards go to my fellow PhD students, especially Sander, Karit and Maria, who have been excellent examples of wisdom, hard-working nature and commitment to be followed.

I thank Karl Räim, Pille Kool and Uku Lilleväli for their help with statistical analyses and formatting.

My deepest thanks go to Professor Scott F. Gilbert for the linguistic revision of my thesis.

It has been very important that I have spent my working days together with my good colleagues in the Department of Clinical Genetics and Metabolic laboratory as well as in the Department of Physiology.

This study has been dedicated to Kertu, whose life has been the reason for leading my life and research to this pathway and setting up the goals of the study.

PUBLICATIONS

CURRICULUM VITAE

Name: Hardo Lilleväli
Date of birth: July 24, 1967, Tallinn, Estonia
Citizenship: Estonian
Address: 2 L. Puusepa Street, 51014, Tartu, Estonia
Phone: + 372 7 319 485
E-mail: hardo.lillevali@kliinikum.ee; hardo@ut.ee

Education and employment:

1974–1985	Tallinn Secondary School No 44
1985–1992	University of Tartu, Faculty of Biology and Geography, biologist
1985–1987	compulsory military service in the military forces of the USSR
1992–1995	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology, MSc studies (M.Sc. in molecular biology)
1995–2000	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology, PhD studies (molecular biology)
2000–2005	freelance patent expert, translator
2005–2007	Estonian Ministry of Environment, Department of Nature Conservation chief officer (genetically modified organisms)
2006–2009	University of Tartu, Faculty of Science and Technology, Institute of Technology, University of Tartu, Expert in Biotechnology
2009–2013	University of Tartu, Support Units, Office of Research and Development, Intellectual Property Manager
2013–2014	Estonian Ministry of Education and Research, Research Policy Department, chief expert, adviser
2015–…	Tartu University Hospital, United Laboratories of Tartu University Hospital, laboratory specialist
2015–2020	University of Tartu, Faculty of Medicine, PhD studies, Programme of Neurosciences

Scientific work and professional organizations

Research fields:	Clinical genetics, metabolomics, laboratory diagnostics.
Membership:	Member of the Estonian Society of Medical Genetics

List of publications

1. **Lilleväli H***, Pajusalu S*, Wojcik MH, Goodrich J, Collins RL, Murumets Ü, Tammur P, Blau N, Lilleväli K, Õunap K. Genome sequencing identifies a homozygous inversion disrupting *QDPR* as a cause for dihydropteridine reductase deficiency. Molecular Genetics & Genomic Medicine, 2020: e1154. doi:10.1002/mgg3.1154.
2. Muru K, Reinson K, Künnapas K, **Lilleväli H**, Nochi Z, Mosegaard S, Pajusalu S, Olsen RKJ, Õunap K. FLAD1-associated multiple acyl-CoA dehydrogenase deficiency identified by newborn screening. Mol Genet Genomic Med. 2019 Sep;7(9):e915. doi: 10.1002/mgg3.915. Epub 2019 Aug 8.
3. **Lilleväli H**, Reinson K, Muru K, Saarsalu S, Künnapas K, Kahre T, Murumets Ü, Õunap K. The evaluation of phenylalanine levels in Estonian phenylketonuria patients during eight years by electronic laboratory records. Mol Genet Metab Rep. 2019 Mar 23;19:100467. doi: 10.1016/j.ymgmr. 2019.100467. eCollection 2019 Jun.
4. **Lilleväli H**, Reinson K, Muru K, Simenson K, Murumets Ü, Möls T, Õunap K. Hyperphenylalaninaemias in Estonia: Genotype-Phenotype Correlation and Comparative Overview of the Patient Cohort Before and After Nation-Wide Neonatal Screening. JIMD Rep. 2018;40:39–45. doi: 10.1007/8904_ 2017_61. Epub 2017 Sep 28.
5. **Lilleväli H**, Margus K, Õunap K, Metspalu A. Mutation 985A>G in the MCAD gene shows low incidence in Estonian population. Hum Mutat. 2000 Mar;15(3):293–4.
6. Õunap K, **Lilleväli H**, Metspalu A, Lipping-Sitska M. Development of the phenylketonuria screening programme in Estonia. J Med Screen. 1998;5(1): 22–3. PubMed PMID: 9575455.
7. **Lilleväli H**, Õunap K, Metspalu A. Phenylalanine hydroxylase gene mutation R408W is present on 84% of Estonian phenylketonuria chromosomes. Eur J Hum Genet. 1996;4(5):296–300.
8. Õunap K, **Lilleväli H**, Klaassen T, Metspalu A, Sitska M. The incidence and characterization of phenylketonuric patients in Estonia. J Inherit Metab Dis. 1996;19(3):381–2.

ELULOOKIRJELDUS

Nimi: Hardo Lilleväli
Sünniaeg: 24. juuli 1967, Tallinn, Eesti
Kodakondsus: Eesti
Aadress: L. Puusepa 2, 51014, Tartu
Telefon: + 372 7 319 485
E-post: hardo.lillevali@kliinikum.ee; hardo@ut.ee

Haridus- ja ametikäik:

1974–1985	Tallinna 44. Keskkool (Mustamäe Gümnaasium)
1985–1992	Tartu Ülikool, Bioloogia-geograafiateaduskond, Molekulaar- ja rakubioloogia instituut, bioloog
1985–1987	sundajateenistus NSVL relvajõududes
1992–1995	Tartu Ülikool, Bioloogia-geograafiateaduskond, Molekulaar- ja rakubioloogia instituut, magistriõpe (M.Sc. molekulaarbioloogia, teaduskraad)
1995–2000	Tartu Ülikool, Bioloogia-geograafiateaduskond, Molekulaar- ja rakubioloogia instituut, doktoriõpe (molekulaarbioloogia)
2000–2005	FIE Hardo Lilleväli (patendiuuringud, tõlked)
2005–2007	Keskkonnaministeerium, looduskaitse osakond, peaspetsialist (geneetiliselt muundatud organismid)
2006–2009	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tartu Ülikooli Tehnoloogiainstituut, biotehnoloogia ekspert
2009–2013	Tartu Ülikool, Tugiüksused, teadus- ja arendusosakond, intellektuaalomandi peaspetsialist
2013–2014	Haridus- ja Teadusministeerium, teadusosakond, peaekspert, nõunik
2015–…	SA Tartu Ülikooli Kliinikum, Tartu Ülikooli Kliinikumi ühendlabor, laborispetsialist
2015–2020	Tartu Ülikool, arstiteaduskond, doktoriõpe (neuroteaduste õppekava)

Teadus- ja erialane tegevus

Uurimisvaldkonnad:	Kliiniline geneetika, metaboloomika, laboridiagnostika.
Liikmelisus:	Eesti Meditsiinigeneetika Seltsi liige

Publikatsioonide nimekiri

1. **Lilleväli H***, Pajusalu S*, Wojcik MH, Goodrich J, Collins RL, Murumets Ü, Tammur P, Blau N, Lilleväli K, Õunap K. Genome sequencing identifies a homozygous inversion disrupting *QDPR* as a cause for dihydropteridine reductase deficiency. Molecular Genetics & Genomic Medicine, 2020: e1154. doi:10.1002/mgg3.1154
2. Muru K, Reinson K, Künnapas K, **Lilleväli H**, Nochi Z, Mosegaard S, Pajusalu S, Olsen RKJ, Õunap K. FLAD1-associated multiple acyl-CoA dehydrogenase deficiency identified by newborn screening. Mol Genet Genomic Med. 2019 Sep;7(9):e915. doi: 10.1002/mgg3.915. Epub 2019 Aug 8.
3. **Lilleväli H**, Reinson K, Muru K, Saarsalu S, Künnapas K, Kahre T, Murumets Ü, Õunap K. The evaluation of phenylalanine levels in Estonian phenylketonuria patients during eight years by electronic laboratory records. Mol Genet Metab Rep. 2019 Mar 23;19:100467. doi: 10.1016/j.ymgmr.2019.100467. eCollection 2019 Jun.
4. **Lilleväli H**, Reinson K, Muru K, Simenson K, Murumets Ü, Möls T, Õunap K. Hyperphenylalaninaemias in Estonia: Genotype-Phenotype Correlation and Comparative Overview of the Patient Cohort Before and After Nation-Wide Neonatal Screening. JIMD Rep. 2018;40:39–45. doi: 10.1007/8904_2017_61. Epub 2017 Sep 28.
5. **Lilleväli H**, Margus K, Õunap K, Metspalu A. Mutation 985A>G in the MCAD gene shows low incidence in Estonian population. Hum Mutat. 2000 Mar;15(3):293–4.
6. Õunap K, **Lilleväli H**, Metspalu A, Lipping-Sitska M. Development of the phenylketonuria screening programme in Estonia. J Med Screen. 1998;5(1): 22–3. PubMed PMID: 9575455.
7. **Lilleväli H**, Õunap K, Metspalu A. Phenylalanine hydroxylase gene mutation R408W is present on 84% of Estonian phenylketonuria chromosomes. Eur J Hum Genet. 1996;4(5):296–300.
8. Õunap K, **Lilleväli H**, Klaassen T, Metspalu A, Sitska M. The incidence and characterization of phenylketonuric patients in Estonia. J Inherit Metab Dis. 1996;19(3):381–2.

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
3. **Dzhamilja Safiulina.** The studies of mitochondria in cultured cerebellar granule neurons: characterization of mitochondrial function, volume homeostasis and interaction with neurosteroids. Tartu, 2006.
4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
6. **Annika Vaarmann**. The studies on cystatin B deficient mice: neurochemical and behavioural alterations in animal model of progressive myoclonus epilepsy of Unverricht-Lundborg type. Tartu, 2007.
7. **Urho Abramov.** Sex and environmental factors determine the behavioural phenotype of mice lacking CCK_2 receptors: implications for the behavioural studies in transgenic lines. Tartu, 2008.
8. **Hendrik Luuk.** Distribution and behavioral effects of WFS1 protein in the central nervous system. Tartu, 2009.
9. **Anne Must.** Studies on molecular genetics of male completed suicide in Estonian population. Tartu, 2009.
10. **Kaido Kurrikoff**. Involvement of cholecystokinin in chronic pain mechanisms and endogenous antinociception. Tartu, 2009.
11. **Anu Aonurm-Helm.** Depression-like phenotype and altered intracellular signalling in neural cell adhesion molecule (NCAM)-deficient mice. Tartu, 2010.
12. **Silva Sütt.** Role of endocannabinoid system and *Wfs1* in regulation of emotional behaviour: behavioural, pharmacological and genetic studies. Tartu, 2010.
13. **Mari-Anne Philips.** Characterization of *Myg1* gene and protein: expression patterns, subcellular localization, gene deficient mouse and functional polymorphisms in human. Tartu, 2010.
14. **Ranno Rätsep.** Genetics of psoriasis and vitiligo, focus on IL10 family cytokines. Tartu, 2010.
15. **Kairit Joost.** Selective screening of metabolic diseases in Estonia: the application of new diagnostic methods. Tartu, 2012, 143 p.
16. **Monika Jürgenson.** A complex phenotype in mice with partial or complete deficiency of the NCAM protein. Tartu, 2012, 117 p.

17. **Ene Reimann.** Description of the cytokines and cutaneous neuroendocrine system in the development of vitiligo. Tartu, 2012, 117 p.
18. **Jürgen Innos.** Behavioural, pharmacological and neurochemical characterisation of limbic system-associated membrane protein (LSAMP) deficient mice. Tartu, 2013, 113 p.
19. **Kaili Anier.** The role of DNA methylation in the development of cocaine-induced behavioural sensitisation. Tartu, 2013, 147 p.
20. **Maarika Liik.** Cognitive functioning, perceived cognition, subjective complaints and symptoms of depression in patients with epilepsy: neuropsychological assessment and spet brain imaging study. Tartu, 2014, 124 p.
21. **Sten Ilmjärv.** Estimating differential expression from multiple indicators. Tartu, 2015, 125 p.
22. **Paula Reemann.** The effects of microenvironment on skin cells. Tartu, 2015, 146 p.
23. **Tanel Visnapuu.** Pharmacological and behavioral characterization of the monoaminergic and GABA-ergic systems of Wfs1-deficient mice. Tartu, 2015, 107 p.
24. **Indrek Heinla.** Behavioural and genetic comparison of B6 and 129Sv mouse lines focusing on the anxiety profile and the expression of *Lsamp* gene. Tartu, 2016, 115 p.
25. **Liina Haring.** Cognitive functioning after first psychotic episode. Tartu, 2017, 146 p.
26. **Triin Tekko.** Neurodevelopmental Approach in the Study of the Function of *Wfs1* and *Lsamp*, Potential Targets in the Regulation of Emotional Behaviour. Tartu, 2018, 194 p.
27. **Alina Altpere.** Targeting of mechanisms of elevated anxiety in female Wfs1-deficient mice. Tartu, 2018, 98 p.
28. **Maarja Toots.** Pharmacological challenge in rodent models of Wolfram syndrome with emphasis on diabetic phenotype. Tartu, 2018, 114 p.
29. **Katyayani Singh.** Neuropsychiatric endophenotypes – focusing on IgLON adhesion molecules in the mouse brain. Tartu, 2019, 148 p.
30. **Kattri-Liis Eskla.** Therapeutic strategies for ischemia reperfusion injury. Tartu, 2019, 138 p.