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Molecular mechanisms of PAH function in response to phenylalanine and tetrahydrobiopterin binding: implications for clinical management

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aus
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Erklärung:

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Eidesstattliche Versicherung:

Diese Dissertation wurde selbständig und ohne unerlaubte Hilfe erarbeitet.

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To All Who Participated
In This Journey

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LIST OF ORIGINAL PUBLICATIONS INCLUDED IN THIS THESIS

This thesis is based upon the following peer-reviewed original publications and the reprints were made with permission of the publisher.

Paper 1:

Activation of phenylalanine hydroxylase induces positive cooperativity toward the natural cofactor.
Gersting SW, Staudigl M, Truger MS, Messing DD, **Danecka MK**, Sommerhoff CP, Kemter KF, Muntau AC.
The Journal of Biological Chemistry, 2010; 285 (40): 30686-30697.

Paper 2:

The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response.
Staudigl M, Gersting SW, **Danecka MK**, Messing DD, Woidy M, Pinkas D, Kemter KF, Blau N, Muntau AC.
Human Molecular Genetics, 2011; 20 (13): 2628-2641.

Paper 3:

Mapping the functional landscape of frequent *phenylalanine hydroxylase* genotypes promotes personalised medicine in phenylketonuria.
Danecka MK, Woidy M, Zschocke J, Feillet F, Muntau AC, Gersting SW.
Journal of Medical Genetics, 2015; 52 (3): 175-185.

CONTRIBUTION REPORT

The work presented in this dissertation comprises part of the results of my research conducted in the lab of Professor Ania C. Muntau at the Medical Faculty of the Ludwig-Maximilians-University, Munich. The following three papers comprise the cumulative part of the doctoral thesis:

Paper 1 (Gersting *et al.*, 2010a):

This paper describes the development of an automated continuous real-time multi-well assay for PAH activity, which allowed for in-depth characterisation of BH_4-dependent PAH enzyme kinetics. I prepared *PAH* constructs for recombinant expression. I purified proteins analysed in the paper and performed PAH activity assays. Moreover, I participated in writing of the manuscript.

Paper 2 (Staudigl *et al.*, 2011):

This publication focuses on the analysis of PAH activity in the metabolic space defined as a broad range of substrate (L-phenylalanine) and cofactor (BH_4) concentrations. Thus, this work allowed for a better understanding of PAH function in the physiological, pathological and therapeutic context. Here, I prepared constructs for *PAH* recombinant expression and I purified proteins used in the assays. I prepared *PAH* constructs for generating stable HEK293 cell lines and established three cell lines for stable expression used in the paper: wild-type PAH, p.Arg261Gln and p.Tyr414Cys. I also participated in writing of the manuscript.

Paper 3 (Danecka *et al.*, 2015):

Based on the previous work, this paper characterises the function of full *PAH* genotypes. Application of PAH activity landscapes to eukaryotically expressed PAH allowed for analysing genotype specific enzyme activity in the metabolic space, *i.e.* as a function of varying substrate and cofactor concentrations. This work sets the basis for individually tailored medicine and provides a web application to assist clinicians in day-to-day care of PKU patients. In this project, I designed and carried out all laboratory experiments. In addition, I performed a comprehensive pubmed research for all available data on *PAH* genotypes. Moreover, I wrote the manuscript under the supervision of Dr. Søren W. Gersting and Prof. Dr. Ania C. Muntau.
Other co-authors contributed as follows: Mathias Woidy created 3D activity landscapes and the web application; François Feillet contributed with patient's data on *PAH* genotypes in France; Johannes Zschocke critically reviewed the manuscript.

In addition, I contributed to scientific work that relates to the topic of this dissertation but it is not included in the cumulative part of the thesis:

Loss of function in phenylketonuria is caused by impaired molecular motions and conformational instability.
Gersting SW, Kemter KF, Staudigl M, Messing DD, **Danecka MK**, Lagler FB, Sommerhoff CP, Roscher AA, Muntau AC.
The American Journal of Human Genetics, 2008; 83 (1): 5-17.
In this project, I purified recombinantly expressed PAH variants and performed proteinase K assays as well as the PAH activity assays.

***Pah*^{enu1} is a mouse model for tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and promotes analysis of the pharmacological chaperone mechanism *in vivo*.**
Gersting SW, Lagler FB, Eichinger A, Kemter KF, **Danecka MK**, Messing DD, Staudigl M, Domdey KA, Zsifkovits C, Fingerhut R, Glossmann H, Roscher AA, Muntau AC.
Human Molecular Genetics, 2010; 19 (10): 2039-2049.
For this project, I prepared constructs for eukaryotic expression of the murine PAH protein. I performed transient transfection of the murine wild-type and p.Val106Ala in the eukaryotic system followed by PAH activity assays and analysis of PAH residual protein amount. I also contributed in writing of the manuscript.

New insights into tetrahydrobiopterin pharmacodynamics from *Pah*$^{enu1/2}$, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency.
Lagler FB, Gersting SW, Zsifkovits C, Steinbacher A, Eichinger A, **Danecka MK**, Staudigl M, Fingerhut R, Glossmann H, Muntau AC.
Biochemical Pharmacology, 2010; 80 (10): 1563-1571.
I performed transient transfection of murine *Pah* constructs into COS-7 cells and performed PAH activity assays. I contributed in writing of the manuscript.

SUMMARY

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism (IEM) caused by mutations in the *phenylalanine hydroxylase* (*PAH*) gene. The molecular mechanism underlying deficiency of the PAH protein is, in most of the cases, loss of function due to protein misfolding. *PAH* mutations induce disturbed oligomerisation, decreased stability and accelerated degradation of hepatic PAH, a key enzyme in phenylalanine metabolism. Since the development of a phenylalanine-restricted diet in the 1950ies, PKU is a prototype for treatable inherited diseases. About 60 years later, the natural PAH cofactor tetrahydrobiopterin (BH_4) was shown to act as a pharmacological chaperone stabilising the misfolded PAH protein. In consequence, BH_4 (KUVAN®) was introduced to the pharmaceutical market as an alternative treatment for BH_4-responsive PAH deficiency. Therefore, PKU is also regarded as a prototype for a pharmacologically treatable protein misfolding disease.

Despite the progress in PKU therapy, knowledge on the molecular basis of PKU and the BH_4 mode of action was still incomplete. Biochemical and biophysical characterisation of purified variant PAH proteins, which were derived from patient's mutations, aimed at a better understanding of the molecular mechanisms of PAH loss of function. We showed that local side-chain replacements induce global conformational changes with negative impact on molecular motions that are essential for physiological enzyme function. The development of a continuous real-time fluorescence-based assay of PAH activity allowed for robust analysis of steady state kinetics and allosteric behaviour of recombinantly expressed PAH proteins. We identified positive cooperativity of the PAH enzyme towards BH_4, where cooperativity does not rely on the presence of phenylalanine but is determined by activating conformational rearrangements. *In vivo* investigations on the mode-of-action of BH_4 revealed differences in pharmacodynamics but not in pharmacokinetics between different strains of PAH-deficient mice (wild-type, $Pah^{enu1/1}$ and $Pah^{enu1/2}$). These observations pointed to a significant impact of the genotype on responsiveness to BH_4.

The available database information on PAH function associated with *PAH* mutations was based on non-standardised enzyme activity assays performed in different cellular systems and under different conditions usually focusing on single *PAH* mutations. These inconsistent data on PAH enzyme activity hindered robust prediction of the patient's phenotype. Furthermore, assays on single *PAH* mutations do not reflect the high allelic and phenotypic heterogeneity of PKU with 89 % of patients being compound heterozygotes. In addition, the knowledge on enzyme function and regulation in the therapeutic and pathologic metabolic context was still scarce. In order to get more insight into the interplay of the *PAH* genotype, the phenylalanine concentration and BH_4 treatment, we performed functional analyses of both, single, purified PAH variants as well as *PAH* full genotypes in the physiological, pathological and therapeutic context. The analysis of PAH activity as a function of phenylalanine and BH_4 concentrations enabled determination of the optimal working ranges of the enzyme and visualisation of differences in the regulation of PAH activity by BH_4 and phenylalanine depending on the underlying genotype. Moreover, these PAH activity landscapes allowed for setting rules for dietary regimens and pharmacological treatment based on the genotype of the patient.

Taken together, precise knowledge on the mechanism of the misfolding-induced loss of function in PAH deficiency enabled a better understanding of the molecular mode of action of pharmacological rescue of enzyme function by BH_4. We implemented the combination of genotype-specific functional analyses together with biochemical, clinical and therapeutic data of individual patients as a powerful tool for phenotype prediction and paved the way for personalised medicine strategies in phenylketonuria.

ABBREVIATIONS

1′-Oxo-PH4	1′-oxo-tetrahydropterin
2′-Oxo-PH4	2′-oxo- tetrahydropterin
3D	three dimensional
ADHD	attention deficit hyperactivity
AGMO	alkylglycerol monooxygenase
AR	aldose reductase
BBB	blood-brain barrier
BH_2	dihydrobiopterin
BH_4	6R-L-*erythro*-5,6,7,8-tetrahydrobiopterin
bp	base pair
cAMP	cyclic adenosine monophosphate
COS-7	African green monkey kidney fibroblasts-like cells
CR	carbonyl reductase
DHFR	dihydrofolate reductase
DHPR	dihydropteridine reductase
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EEG	electroencephalography
EMA	European Medicines Agency
FDA	Food and Drug Administration
GTP	guanosine triphosphate
GTPCH	GTP cyclohydrolase I
H_2NTP	7,8-dihydroneopterin triphosphate
HEK-293	human embryonal kidney cells
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPA	hyperphenylalaninemia
IAC	interallelic complementation
IARS	intrinsic autoregulatory sequence
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IEM	inborn error of metabolism
IQ	intelligence quotient
LNAA	large neutral amino acids
L-Phe	L-phenylalanine
L-Tyr	L-tyrosine
NADPH	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthase
O_2	dioxygen
OMIM	online Mendelian Inheritance in Man
PAH	phenylalanine hydroxylase
PCD	pterin-4a-carbinolamine dehydratase
Phe/Tyr ratio	phenylalanine/tyrosine ratio
PKU	phenylketonuria
PRA	predicted residual activity
PTP	6-pyruvoyl-5,6,7,8-tetrahydrobiopterin
PTPS	6-pyruvoyl-tetrahydropterin synthase
rAvPAL-PEG	PEGylated recombinant *Anabaena variabilis* phenylalanine ammonia lyase
SR	sepiapterin reductase
TH	tyrosine hydroxylase
TnT system	coupled transcription/translation system
TPH	tryptophan hydroxylase

1. INTRODUCTION

1.1. Phenylketonuria (PKU)

Phenylketonuria (PKU, OMIM 261600), an inborn error of phenylalanine metabolism, is a complex trait inherited in an autosomal recessive manner. The disorder is associated with a primary deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1) due to mutations in the *PAH* gene (GenBank: AF404777) (Scriver, 2007; Williams *et al.*, 2008; Blau *et al.*, 2010c; Mitchell *et al.*, 2011). The impaired catabolic disposal of phenylalanine caused by a complete or near-complete loss of PAH function results in an accumulation of toxic levels of the amino acid phenylalanine in body fluids and, as a consequence, leads to irreversible, profound mental disability (Mitchell *et al.*, 2011). A large number of *PAH* mutations (663 [public total] from www.hgmd.org) in combination with a high degree of compound heterozygosity (89 % of all PKU patients) (Zurflüh *et al.*, 2008) and a wide variability in distribution of common mutations between ethnic groups and geographical areas make phenylketonuria a genetic disorder with marked allelic and phenotypic heterogeneity. In addition, the genotype-phenotype correlation is weak. These specific features of PKU challenge the therapy of affected individuals and emphasise the necessity for implementation of individualised treatment options.

Epidemiology

Phenylketonuria is the most prevalent disorder of amino acid metabolism, its occurrence varies widely around the world. In the USA, the PKU prevalence is estimated as 1:15 000 live births while in Latin America it is lower and ranges from 1:25 000 to 1:50 000 (Blau *et al.*, 2010c; Blau, 2013). In Asia, the prevalence is heterogeneous (1:15 000 – 1:100 500 in China; lower than 1:200 000 in Thailand; 1:70 000 in Japan) (Williams *et al.*, 2008; Blau *et al.*, 2010c). The overall incidence of PKU in Caucasians is estimated as 1:10 000 live births. However, this number again varies among different European populations. A high number of incidents in Europe is usually linked to a high degree of consanguinity (e.g. Turkey, 1:2 600 - 1:4 000) whereas in other European countries the PKU occurrence is lower (*e.g.* Germany 1:8 000; Finland 1:100 000 – 1:200 000) (Blau *et al.*, 2010c; Mitchell *et al.*, 2011). An especially high prevalence of mild clinical phenotypes has been reported for the Spanish population (Blau *et al.*, 2010c). Africa is known to have a low rate of PKU incidents (Blau *et al.*, 2010c).

Clinical presentation

Untreated PKU results in a severe cognitive phenotype with irreversible mental disability. The clinical picture comprises a 'musty odor' to the baby's sweat and urine due to the accumulation of phenylacetate in the body, high levels of phenylalanine in blood and body fluids (HPA), growth failure, severe mental retardation, seizures, microcephaly, EEG abnormalities, ataxia, demyelination, eczema, and albinism (fair skin, fair hair, blue eyes) (Williams *et al.*, 2008; Blau *et al.*, 2010c; Feillet *et al.*, 2010; Somaraju and Merrin, 2010; van Spronsen, 2010; Mitchell *et al.*, 2011; Blau *et al.*, 2014). The progressive developmental delay may be accompanied by behavioural problems including self-harm, aggression, impulsiveness and psychosis (Blau *et al.*, 2014). Autism and attention deficit hyperactivity (ADHD) are frequent (Blau *et al.*, 2010c; Feillet *et al.*, 2010). Due to the introduction of newborn screening programs, nowadays, symptomatic PKU is rarely observed in developing countries (Mitchell *et al.*, 2011). However, even in treated PKU adults, reduced emotional wellbeing, depression, social isolation and low self-esteem have been reported (Blau *et al.*, 2010c).

PKU pathogenesis

Although *PAH* mutations disrupt phenylalanine homeostasis in the liver, the clinical effect is almost completely restricted to the brain (Williams *et al.*, 2008; Blau *et al.*, 2014). However, the aetiology of cognitive problems and anomalies in brain development still remain unclear. Yet, some mechanisms explaining the pathological changes have been proposed. First, increased concentrations of phenylalanine in blood have a competitive inhibitory effect on the transport of other amino acids (e.g. large neutral amino acids, LNAA) through the blood-brain barrier. This leads to disturbed synthesis of intracerebral proteins and alter myelin metabolism (Williams *et al.*, 2008; van Spronsen *et al.*, 2009; Anderson and Leuzzi, 2010; Blau *et al.*, 2014). In addition, reduced levels of other crucial amino acids such as tyrosine, tryptophan and methionine severely impair the correct synthesis of neurotransmitters (dopamine, noradrenalin, serotonin) resulting in behavioural problems and motor deficits (Muntau *et al.*, 2000; Feillet *et al.*, 2010). Moreover, high levels of phenylalanine in brain are believed to affect the activity of key enzymes such as pyruvate kinase, tyrosine hydroxylase, tryptophan hydroxylase and HMG-CoA reductase (van Spronsen *et al.*, 2009; Blau *et al.*, 2014).

Diagnostics and differential diagnosis

Due to the severity of untreated PKU, infants benefit from presymptomatic screening and early treatment that prevents neurological damage. In developed countries PKU is identified soon after birth by national neonatal screening programs. Blood samples (dried blood spots) are usually collected between the age of 1 and 7 days of life and the concentration of blood phenylalanine is measured using tandem mass spectrometry. Hyperphenylalaninemia (HPA, >120 µmol phenylalanine/l) accompanied by normal or reduced tyrosine concentrations (Phe/Tyr ratio >2) and normal concentrations of the remaining amino acids point to impairment of PAH function (Blau *et al.*, 2010c; Blau *et al.*, 2011). However, once HPA has been identified in the initial screen, additional testing must be performed in order to establish the definite basis of HPA. In 2-3 % of all cases elevated blood phenylalanine concentrations do not result from primary PAH deficiency but are caused by defects in genes involved in BH_4 metabolism that cause secondary PAH deficiency (BH_4-deficient HPA, OMIM 264070) (Blau *et al.*, 2010c; Blau *et al.*, 2011; Mitchell *et al.*, 2011). These are ruled out by analysis of pterins in the urine and determination of dihydropteridine reductase activity (DHPR) in erythrocytes. In the case of pathological results neurotransmitter metabolite concentrations in cerebrospinal fluid have to be assessed to specifically dissect the defect in biopterin metabolism (Mitchell *et al.*, 2011; Blau *et al.*, 2011). In addition, it has to be taken into consideration that elevated blood phenylalanine levels in the newborn may be also caused by liver dysfunction or other inborn errors of metabolism involving the liver such as galactosemia (OMIM 230400). The precise identification of the underlying genetic cause of HPA is of utmost relevance since the treatment of the affected individuals strongly differs depending on the genetic background. In the case of confirmed HPA due to PAH deficiency, molecular genetic analysis of the *PAH* gene can be performed but it is not a prerequisite for the implementation of a low-phenylalanine diet (Blau *et al.*, 2010c; Blau *et al.*, 2011; Blau *et al.*, 2014). PKU patients need regular monitoring as their therapy needs modification according to age and metabolic requirements.

1.1.1 Historical background and scientific breakthroughs in PKU

Phenylketonuria is the prototype of an inborn error of amino acid metabolism where metabolic abnormalities were shown to have severe neurological effects and that exemplified how treatment can dramatically affect the clinical manifestation of a genetic disease (Williams *et al.*, 2008; Blau *et al.*, 2010c). In addition, the history of PKU is an excellent example of how continuous efforts towards a deeper and broader understanding of the underlying molecular mechanisms may improve treatment and outcome for patients (Blau *et al.*, 2014). The milestones and breakthroughs in the history of phenylketonuria are as follows:

- 1934 - first description of PKU by the Norwegian physician Absjørn Følling. Følling examined two mentally retarded children and performed a detailed chemical analysis of their urine samples including organic extraction and purification of chemical compounds (Blau *et al.*, 2014). He linked the presence of one of the substances in the urine (phenylpyruvic acid) to the intellectual impairment of his patients and proposed the name 'imbecillitas phenylpyruvica' to describe the relation between the observed phenotype and excreted substance (Christ, 2003).
- 1935 - 1937 - Lionel Penrose introduced the name 'phenylketonuria' (Penrose, 1935; Penrose and Quastel, 1937).
- 1953 - George Jervis identified the inability to convert phenylalanine to tyrosine as a cause of phenylketonuria. This led to the recognition of PKU as a disorder with underlying enzyme deficiency (Jervis, 1953).
- 1953 - Horst Bickel developed the first phenylalanine-free protein substitute and introduced the low phenylalanine diet as a treatment option to ameliorate HPA in PKU patients and to potentially prevent mental retardation in the affected individuals (Bickel *et al.*, 1953). Since then, the phenylalanine-restricted diet became a standard treatment with greatest benefits if introduced in the newborn period.
- 1957 – Willard Centerwall developed the so-called 'diaper test' as a first screening method for PKU where the addition of ferric chloride to the wet diaper of PKU babies made the urine turn green. However, the test could only be performed at 5-6 weeks of age allowing enough time for phenylpyruvic acid to build up. This was too late to prevent the irreversible damage in the first 6 weeks of life (Centerwall, 1957; Centerwall *et al.*, 1960).
- 1958 – 1963 - Robert Guthrie developed a simple and inexpensive screening test using dried blood spots on filter cards. This allowed for early diagnosis, treatment and prevention of neurological damage in PKU (Guthrie and Susi, 1963).
- 1957 – Seymour Kaufman discovered the PAH enzyme (Kaufman, 1957).
- 1958 - 1963 – Seymour Kaufman discovered the PAH natural cofactor tetrahydrobiopterin (BH_4) (Kaufman, 1958; Kaufman, 1963).
- 1982 – Savio Woo cloned the human *phenylalanine hydroxylase* gene (*PAH*, GenBank cDNA sequence U49897.1) (Woo *et al.*, 1983).
- 1988 - 1993 – introduction of PKU mouse models (Bode *et al.*, 1988; McDonald *et al.*, 1990; Shedlovsky *et al.*, 1993; McDonald, 1994).
- 1996 – Ray Stevens determined the first composite three-dimensional (3D) structure of the PAH enzyme (Erlandsen *et al.*, 1997b; Erlandsen *et al.*, 1997a).
- 1990s – Charles Scriver and colleagues founded the first online database gathering information on *PAH* gene, mutations, polymorphisms, genotypes and published *in vitro* studies on the PAH protein (www.pahdb.mcgill.ca) (Hoang *et al.*, 1996).
- 1999 – Kure observed that pharmacological doses of BH_4 reduced blood phenylalanine concentrations in 4 patients with PAH deficiency (Kure *et al.*, 1999).
- 2002 – Muntau showed in a clinical study that BH_4 reduces blood phenylalanine concentrations, rescues *in vivo* PAH enzyme activity and increases dietary phenylalanine tolerance in the majority of PAH-deficient patients with residual enzyme activity. Therefore, pharmacological treatment with BH_4 was proposed as an alternative to the phenylalanine-restricted diet. A new PKU phenotype emerged: BH_4-responsive PAH deficiency (Muntau *et al.*, 2002).
- 2005 – Phase I clinical studies (PKU-005) on sapropterin dihydrochloride tablets (the synthetic form of BH_4) were initiated in accordance with the ICH E6 guideline on Good Clinical Practice (EMA. CHMP Assessment Report for Kuvan, 2008).
- 2007 – 2008 – KUVAN® (active substance: sapropterin dihydrochloride, 6R-BH_4 stereoisomer) was approved by the FDA and the EMA as an orphan drug to treat

patients with BH_4-responsive PAH deficiency (Levy *et al.*, 2007; Feillet *et al.*, 2008; Lee *et al.*, 2008; Trefz *et al.*, 2009a).

1.1.2 Classification

A classification of the severity of the disease is needed for predicting the putative course of the disease and to assist the patient with optimal treatment options. Establishing a reliable clinical and biochemical classification is easier in diseases where biological markers are known. However, in many cases, classification procedures may be associate with pitfalls and have to be carried out due diligence, especially when a biomarker is associated with different diseases and/or phenotypes.

PKU classification

In PKU, blood phenylalanine concentrations in untreated individuals serve as biological marker and therefore PKU classification was, for a long time, mainly based on the degree of blood phenylalanine elevation. Although accepted worldwide, this classification is not fully accurate. This is due to the fact that phenylalanine concentrations are usually measured in newborns (samples are collected between first and seventh day of life) at the time when phenylalanine concentrations may not yet have reached highest values. Additional factors that may affect phenylalanine read-outs are neonatal catabolism, the diet received at the time of blood sampling and the time when the newborn was delivered since preterm babies do not have fully developed metabolic pathways (Blau, 2006; Blau *et al.*, 2011; Blau *et al.*, 2014). Therefore, to improve PKU classification of biochemical severity, daily phenylalanine tolerance has been established as an additional, stable parameter (Blau *et al.*, 2011). It describes the daily dietary phenylalanine intake, which is tolerated by the organism without leading to increased blood phenylalanine levels. It is of particular importance to determine the phenylalanine tolerance under well-standardised conditions using very precise dietary protocols (Blau *et al.*, 2011). PKU classification based on the two parameters, pretreatment blood phenylalanine concentration and dietary phenylalanine tolerance, is summarised in **Table 1**.

Table 1. Classification of the primary hyperphenylalaninemias.

	Blood phenylalanine values before treatment	Dietary phenylalanine tolerance
Classical PKU/Type I	> 20 mg/dl (> 1200 µmol/l)	< 20 mg/kg/day
Mild PKU/Type II	> 10 mg/dl (> 600 µmol/l)	20-50 mg/kg/day
Persistent HPA/Type III	< 10 mg/dl (< 600 µmol/l)	> 50 mg/kg/day
Atypical PKU	2.5 - 20 mg/dl (150 - 1200 µmol/l)	variable
Healthy individual	50-120 µmol/l	not restricted

The table was adapted from Muntau *et al.*, 2000; Blau *et al.*, 2010c; Blau *et al.*, 2011; Mitchell *et al.*, 2011

An additional parameter, which proved to be clinically useful for discrimination between the mild and severe forms of PKU is the phenylalanine to tyrosine (Phe/Tyr) ratio (Eastman *et al.*, 2000; Zytkovicz *et al.*, 2001; Ceglarek *et al.*, 2002; Blau, 2006; Contreras *et al.*, 2015). It was shown that this parameter may improve the specificity and the positive predictive value for the diagnosis of severe PKU and reduce the number of false positives (Chace *et al.*, 1998; Ceglarek *et al.*, 2002). Due to that fact the Phe/Tyr ratio was successfully introduced by the New England Newborn Screening Program (Zytkovicz *et al.*, 2001) where patients presenting the mean value of the Phe/Tyr ratio of ≥ 5 accompanied by mean phenylalanine levels of 477 µmol/l or greater were considered as

severe classical PKU whereas patients with mean values of Phe/Tyr ratio of ≥ 1.5 and mean phenylalanine values of 215 µmol/l were classified as having mild forms of PKU. Although the clinical relevance of the Phe/Tyr ratio as a routine biomarker still needs further evaluation (Singh *et al.*, 2014), recent recommendations for diagnostic testing propose quantification and evaluation of phenylalanine level, Phe/Tyr ratio and the complete amino acid profile (Vockley *et al.*, 2014).
All in all, there is a constant need to improve the current classification in order to better capture intricacies of the PKU trait. In particular, classification of PKU on the basis of blood phenylalanine levels does not account for the phenotype of BH_4-responsive PAH deficiency.

PKU phenotypes

PAH-deficient phenotypes vary from a mild form with a slight increase in blood phenylalanine levels to a classic phenotype with pronounced HPA.
Classical PKU is the most severe form of PAH deficiency associated with highly elevated blood phenylalanine concentrations (>1200 µmol/l), very low daily tolerance of the dietary phenylalanine intake (< 250 mg) and markedly reduced activity of the PAH enzyme. Patients presenting with classical PKU require strict dietary management in order to keep their plasma phenylalanine concentrations at a safe level. Within this group, if untreated, individuals develop profound and irreversible intellectual disability (Mitchell *et al.*, 2011).
Patients classified as **mild PKU** show blood phenylalanine levels between 600 and 1200 µmol/l and a higher daily phenylalanine tolerance (250 - 400 mg per day). Most of them are functional hemizygotes with one severe and one mild mutation. In mild PKU, a phenylalanine-restricted diet is needed to keep phenylalanine values in the physiological range.
The group of patients with **persistent HPA** with blood phenylalanine concentrations below 600 µmol/l under normal diet is often considered not to be in need of special dietary regimens. However, this is a matter of debate between experts and it is important to note that the untreated females with persistent HPA can experience maternal phenylketonuria with severe embryofetopathy (Mitchell *et al.*, 2011).
BH_4-responsive PAH deficiency emerged as a new phenotype in PKU. An initial study on 4 Japanese patients with PAH deficiency (Kure *et al.*, 1999) reported that the administration of 5-10 mg/kg body weight of BH_4 in the course of a BH_4 loading test reduced plasma phenylalanine concentrations. This phenomenon was later confirmed by different metabolic centres (Lindner *et al.*, 2001; Spaapen *et al.*, 2001; Steinfeld *et al.*, 2002), a retrospective study on BH_4 loading tests (Bernegger and Blau, 2002) and a clinical study involving a large cohort of PKU patients (Muntau *et al.*, 2002) and it was recognised that pharmacological doses of BH_4 can significantly reduce or normalise blood phenylalanine levels in PAH-deficient patients. BH_4 responsiveness is ascertained with BH_4 loading tests (see chapter 1.4.2) and a widely accepted cut-off allowing for classification of the patient as BH_4-responsive is described as at least 30 % reduction in blood phenylalanine concentration after administration of BH_4 (Muntau *et al.*, 2002). The response to pharmacological treatment depends on the blood phenylalanine level and the frequency of BH_4 responsiveness is highest in mild HPA and mild PKU where patients present some residual PAH enzyme function (Bernegger and Blau, 2002; Muntau *et al.*, 2002; Spaapen and Rubio-Gozalbo, 2003; Fiege and Blau, 2007; Dobrowolski *et al.*, 2011). Although a number of *PAH* mutations have been identified to be frequently associated with response to BH_4 treatment (Blau and Erlandsen, 2004; Zurflüh *et al.*, 2008; Dobrowolski *et al.*, 2011), *PAH* genotyping is still not sufficient to reliably predict responsiveness as other modifying factors, e.g. interallelic complementation may modulate the response (Dipple and McCabe, 2000; Spaapen and Rubio-Gozalbo, 2003; Leandro *et al.*, 2006; Trefz *et al.*, 2009b). Taken together, the discovery of BH_4-responsive individuals among PKU patients led to a new treatment option that became accessible after approval of the BH_4 compound. Nevertheless, it has to be kept in mind that depending on the genetic background of the analysed population

only a subset of about 10 % to 62 % of all patients (Bernegger and Blau, 2002; Muntau *et al.*, 2002; Lindner *et al.*, 2003; Desviat *et al.*, 2004; Matalon *et al.*, 2005; Mitchell *et al.*, 2005; Zurflüh *et al.*, 2006; Zurflüh *et al.*, 2008; Dobrowolski *et al.*, 2009; Karacić *et al.*, 2009; Muntau and Gersting, 2010; Bélanger-Quintana *et al.*, 2011; Keil *et al.*, 2013) benefit from the new drug and that patients with severe clinical phenotypes are in general not BH_4-responsive. From a clinical point of view, the clear identification and selection of BH_4-responsive patients is still challenging mainly due to a weak genotype-phenotype correlation. Appropriate testing comprises newborn screening and *PAH* genotyping, short-term BH_4 loading tests and long-term BH_4 treatment tests (Blau *et al.*, 2009; Blau *et al.*, 2010c) (see chapter 1.4.2).

Atypical PKU describes a rare situation (2-3 % of all HPA incidents) where elevated levels of blood phenylalanine are due to BH_4 deficiency. Defects in BH_4 metabolism (synthesis and recycling) show more severe progression than PAH deficiency. Moreover, treatment is substantially different. BH_4-deficient patients do not respond to low phenylalanine diet alone. Instead, only early supplementation with precursors of dopamine, serotonin and BH_4 is effective (Blau *et al.*, 2010c; Blau *et al.*, 2011; Opladen *et al.*, 2012). This underscores how important it is to always consider the differential diagnosis once HPA is confirmed.

1.1.3 Treatment options

The primary goal of PKU treatment is to achieve the best-possible neurocognitive and psychosocial development of the infant. To this end, the treatment should be introduced soon after birth and, if possible, continued throughout life according to international guidelines (Vockley *et al.*, 2014). The treatment aims to maintain blood phenylalanine concentrations within the recommended range of 120 to 360 µmol/l, although those values vary between clinics and countries and are also dependent on the age of the patient (Blau, 2010a; Mitchell *et al.*, 2011; Blau *et al.*, 2014; Longo *et al.*, 2014; Vockley *et al.*, 2014;).

Low-phenylalanine diet

Dietary restriction of phenylalanine was introduced in 1953 by Horst Bickel (Bickel *et al.*, 1953) and since then it has been a mainstay in PKU treatment. The diet is introduced immediately after PAH deficiency has been confirmed and is sufficient to protect the developing nervous system from toxic effects of HPA allowing for a near-normal cognitive development of the infant. The diet is designed in such a way that it allows to lower blood phenylalanine concentrations but at the same time provides sufficient amounts of tyrosine and nutrients required for normal growth and development of the patient (Somaraju and Merrin, 2010). All high protein food such as meat, fish, eggs, standard bread and most cheese as well as children's favourites such as chocolate and wine gums have to be excluded from the diet. Some low-protein fruit and vegetables are allowed in restricted amounts. Since this regime would not provide sufficient protein amount for normal growth, supplements of phenylalanine-free amino acids have to be taken on a daily basis (Williams *et al.*, 2008; Blau *et al.*, 2010c; van Spronsen, 2010). The diet has to be tailored to each individual taking into consideration the daily phenylalanine tolerance, age, growth and activity. Marked fluctuations in phenylalanine levels should be avoided and therefore the diet has to be accompanied by regular and frequent measurements of phenylalanine values. In general, phenylalanine concentrations of 40 – 360 µmol/l are regarded as safe (Blau *et al.*, 2010c; Mitchell *et al.*, 2011).

Nowadays, experts recommend a life-long low phenylalanine diet (Phenylketonuria, 1993; National Institutes of Health Consensus Development, 2001; Williams *et al.*, 2008; Mitchell *et al.*, 2011; Vockley *et al.*, 2014). However, strict dietary management is particularly important in early life since a significant inverse correlation between phenylalanine values and IQ has been reported in children up to 12 years where each

100 µmol/l increase in mean blood phenylalanine concentration was predicted to result in 1.3 - 3.1 point reduction in IQ (Waisbren *et al.*, 2007; Feillet *et al.*, 2010; Mitchell *et al.*, 2011). On the other hand, late diagnosed young children showed marked and rapid benefit from dietary treatment although most of them retain a clearly observed degree of intellectual disability and almost all display significant learning problems (Blau *et al.*, 2014). In adulthood some relaxation of the diet is possible, however, there is no consensus to which extent the diet can be omitted and therefore adult patients require an individual management (Feillet *et al.*, 2010). Adherence to the phenylalanine-restricted diet is especially important for women who plan a pregnancy since high phenylalanine levels are teratogenic and are often associated with an increased risk for a miscarriage (Williams *et al.*, 2008; Mitchell *et al.*, 2011; Waisbren *et al.*, 2015). In addition, maternal phenylalanine concentrations around 900 µmol/l lead to mental disability, microcephaly and congenital heart disease in the offspring and values of around 360 µmol/l may still result in reduced cognitive ability and facial dysmorphisms (Blau *et al.*, 2010c). Thus, a strict dietary treatment has to be introduced before conception and continued during the pregnancy. Moreover, during pregnancy PAH-deficient women should undergo continuous nutritional guidance in order to ensure an adequate energy intake and normal weight gain and at the same time maintain low plasma phenylalanine concentrations.

Although PKU patients clearly benefit from dietary treatment, the long-term adherence to the diet is poor since, at first sight easy to handle, the dietary regime is burdensome and associated with social repercussions. During infancy the adherence to the diet is in general good, mostly due to parents control. However, as children get older and are not allowed to choose their food randomly as their peers normally would, adherence to the diet deteriorates. In addition, the difficulties linked to the dietary regime, the unpleasant taste of amino-acids supplements, psychosocial and emotional factors, and lack of reimbursement for low-protein special foods result in a poor compliance (Blau *et al.*, 2009; Blau *et al.*, 2010c; Blau, 2013). In consequence, by late adolescence and adulthood, at least 75 % of PKU patients do not adhere to the dietary treatment (Koch *et al.*, 2002; Walter *et al.*, 2002; Somaraju and Merrin, 2010; Mitchell *et al.*, 2011). The discontinuation of dietary management in adolescence is not life-threatening. However, it leads to subtle but measurable deficits in neuropsychological functioning during adult life including reduced attention span, slow motor reaction time, slow information-processing abilities, increased muscle tone, low bone mineral content (Mitchell *et al.*, 2011).

BH_4 treatment

For a detailed description of BH_4 treatment refer to chapter 1.4.2.

Apart from diet and BH_4 treatment, there are other alternative forms of therapy, which are under investigation and/or in clinical trials. These include transplantation of isolated liver cells, orthotopic liver transplantation, enzyme replacement therapy, the use of glycomacropeptides or large neutral amino acids (LNAA) supplementation.

Enzyme replacement therapy

The main goal of PAH enzyme replacement therapy is to efficiently deliver the whole functional enzyme into the organism. Since the purification of recombinantly expressed human PAH in a large scale is difficult due to its instability, complex activity and BH_4 requirement, there was a need for an alternative. PAL (phenylalanine ammonia lyase; EC 4.3.1.5) is a non-mammalian enzyme that converts phenylalanine to two non-toxic metabolites: ammonia and trans-cinnamic acid and it does not require a cofactor for its full function (Mitchell *et al.*, 2011; Bélanger-Quintana et al., 2011). The original *PAL* gene from *Anabaena varabilis* was genetically modified, recombinantly expressed in *E. coli* and subsequently modified with polyethylene glycol (PEG) to reduce immunogeneity (Gamez *et al.*, 2007; Sarkissian *et al.*, 2008). This procedure resulted in the so-called

rAvPAL-PEG. Preclinical studies have shown promising results using PAL in the PKU mouse model (Sarkissian *et al.*, 2008; Sarkissian *et al.*, 2011) with decreasing blood phenylalanine levels followed by alleviation of the PKU phenotype. An additional improvement came with the development of the oral form of PEGylated PAL (Kang *et al.*, 2010). Recently, first-in-human trials were performed on a group of 25 adult classical PKU patients in order to investigate the safety, tolerability and efficacy of a single-dose rAvPAL-PEG (Longo *et al.*, 2014; van Spronsen and Derks, 2014). The drug was shown to be overall well tolerable and significantly reduced phenylalanine blood concentrations at a dose of 0.1 mg/kg body weight. Only minor adverse effects were reported such as injection-site bruising, rash, pain, swelling or muscle spasms. However, still additional studies are needed to in-depth analyse the observed immunological response and the appearance of anti-PEG antibodies as this might be the limiting factor for the implementation of repeated administrations of the rAvPAL-PEG.

Gene therapy approaches

Gene therapy for PKU has been so far studied only in orthologous mouse models. The trials using an adeno-associated virus vector directed to the liver led to a correction of hyperphenylalaninemia without side effects for up to one year (Oh *et al.*, 2004; Ding *et al.*, 2006; Harding *et al.*, 2006). In addition, the study of Rebuffat *et al.*, 2010 has shown that intramuscular injection of *PAH* gene vectors in Pah-deficient mice resulted in restoration of hepatic PAH activity. However, in order to introduce this treatment into clinical routine, there is still a need to optimise gene delivery and safety of the gene therapy (Brunetti-Pierri, 2008; Suda *et al.*, 2009). Recently, the transplantation of liver cells with fully active PAH enzyme emerged as a potential alternative approach (Blau *et al.*, 2010c).

Large neutral aminoacids (LNAA)

Phenylalanine is known to compete with LNAA for transport across the blood-brain barrier. The supplementation with LNAA showed to reduce phenylalanine uptake into the brain despite consistently high blood phenylalanine levels (Pietz *et al.*, 1999; Moats *et al.*, 2003). On the other hand, since a similar transporter for LNAA exists in the intestine, the supplementation with a special mixture of amino acids and LNAA was shown to decrease blood phenylalanine concentrations by 39 % and had a beneficial effect on executive function (Matalon *et al.*, 2006; Matalon *et al.*, 2007). Therefore, in future, this treatment option may be used to help to protect brain from toxic effects of phenylalanine, improve the concentration level in nonadherent adults and potentially allow for diet relaxation.

1.2. Human phenylalanine hydroxylase (PAH)

Phenylalanine hydroxylase is a cytosolic enzyme mainly expressed in the liver (0.1 -0.3 % of total liver protein) (Blau, 2006). Lower but still significant amounts of the PAH enzyme are expressed in human kidneys and, to a much lower extent, in the pancreas. Expression of the enzyme in the human brain was postulated but has still not been proven (Blau, 2006; Donlon *et al.*, 2008).

PAH belongs to the family of biopterin-dependent aromatic amino acid hydroxylases including tryptophan hydroxylase (TPH; EC 1.14.16.4) and tyrosine hydroxylase (TH; EC 1.14.16.2). The aromatic amino acid hydroxylases show a high degree of sequence similarity (approximately 65 % sequence identity and 80 % sequence homology of the catalytic domains) and are structurally and functionally related enzymes. PAH, TPH and TH catalyse rate-limiting steps in neurotransmitter synthesis pathways and depend on a non-heme iron atom and the cofactor, BH_4 (Flatmark and Stevens, 1999).

1.2.1 The *PAH* gene

The human *phenylalanine hydroxylase* locus (*PAH*; GeneBank accession number AF404777 and cDNA Reference Sequence No. U49897.1) maps to chromosome 12 (band region 12q23.21) (Lidsky *et al.*, 1985). The *PAH* gene consists of 13 exons (that constitute less than 3 % of the genomic *PAH* sequence) and 12 introns (Woo *et al.*, 1983). The shortest exon is 57 bp long (exon 9), while exon 13 is the longest one and spans over 892 bp. The shortest and the longest introns comprise 556 bp (intron 10) and 17 874 bp (intron 2), respectively. The genomic *PAH* sequence is rich in GC regions (40.7 %) and this content is slightly above the mean value for human genes (37 – 38 %) (Donlon *et al.*, 2008).

To date, a total of 663 mutations have been identified in the *PAH* gene (www.hgmd.cf.ac.uk: data taken from public total, accession date 3rd April 2015) and this number is still increasing. The vast majority of these mutations are missense (approximately 62 %) followed by splice variants (14 %), small deletions (11 %), and nonsense mutations (5 %). Small insertions, small indels, gross deletions, gross insertions/duplications and complex rearrangements together account for about 8 % of all *PAH* mutations (combined data from www.pahdb.mcgill.ca and www.hgmd.cf.ac.uk and Blau *et al.*, 2014). *PAH* mutations are spread throughout the whole gene sequence, however, the highest frequency of mutations was found in exon 7 (Bjørgo *et al.*, 1998) as well as in exons 6 and 11 (Blau *et al.*, 2014). Most of the mutations map to the catalytic domain (61.2 %), followed by the regulatory (16.8 %) and oligomerisation (5.2 %) domains (Blau *et al.*, 2014).

Interestingly, although hundreds of mutations have been reported and the occurrence of the mutations depends on the ethnic background of a certain population, only few alleles are relatively common and account for the majority of disease-causing *PAH* mutations while the rest of the mutations is rare (Donlon *et al.*, 2008). Previously, 29 mutations have been identified as being frequent in the European population (Zschocke, 2003).

1.2.2 The PAH protein structure

The mammalian PAH monomer consists of 452 amino acids, shows a molecular weight of 52 kDa and structurally consists of three domains (**Figure 1**).

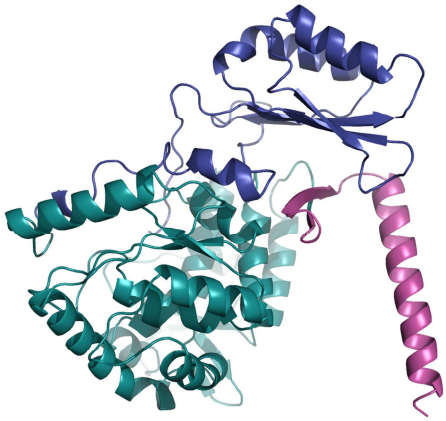

Figure 1. Cartoon-view of the crystal structure of a PAH monomer. The N-terminal domain is marked in dark blue, the catalytic domain is shown in dark green while the C-terminal domain is shown in magenta.

The N-terminal regulatory domain (residues 1-142) includes a short intrinsic autoregulatory sequence (IARS, residues 1 – 33, Kobe *et al.*, 1999) as well as a phosphorylation site at Ser16 (Pey *et al.*, 2004b; Flydal and Martinez, 2013). The IARS extends across the active site of the catalytic domain (Kobe *et al.*, 1999) thus restricting the access of the substrate and the cofactor to the active site (Flydal and Martinez, 2013). In addition, the 1,2-dihydroxypropyl side chain of the BH_4 molecule interacts with the

backbone of Ser23 of the IARS domain. This interaction is crucial for inducing the conformational changes associated with the inhibitory regulatory effect of the BH_4 cofactor (Teigen and Martinez, 2003). The flexible regulatory domain is also important for other regulatory properties of the PAH enzyme such as the activation by the substrate phenylalanine. Yet, it is still not clear whether this domain possesses an allosteric binding site for phenylalanine (Fitzpatrick, 2012; Flydal and Martinez, 2013). Interestingly, the low sequence homology in this protein region among aromatic amino acids hydroxylases may explain differences in the regulatory properties between phenylalanine, tryptophan, and tyrosine hydroxylases (Blau, 2006; Fitzpatrick, 1999).

The catalytic domain (residues 143-410) comprises the active site with binding sites for phenylalanine, BH_4 and the ferrous iron atom (Erlandsen *et al.*, 1998).

The C-terminal oligomerisation domain (residues 411-452) comprises a dimerisation motif (411-424) and a tetramerisation motif (428-452), which is a 40 Å long helix responsible for assembling the PAH tetramer via hydrophobic coiled-coil interactions of four monomers (Flydal and Martinez, 2013). Therefore, the tetramerisation region is considered to be important for transferring the cooperative conformational changes from one dimer to the other (Fusetti *et al.*, 1998).

At physiological conditions, the PAH enzyme exists in a pH-dependent and substrate-dependent equilibrium of homotetramers and homodimers (Martinez *et al.*, 1995; Gjetting *et al.*, 2001b; Gjetting *et al.*, 2001a; Erlandsen *et al.*, 2003) with the tetramer being a fully active, functional unit. The PAH tetramer is constructed as a 'dimer of dimers' where dimers are assembled asymmetrically through C-terminal tetramerisation motifs (**Figure 2**).

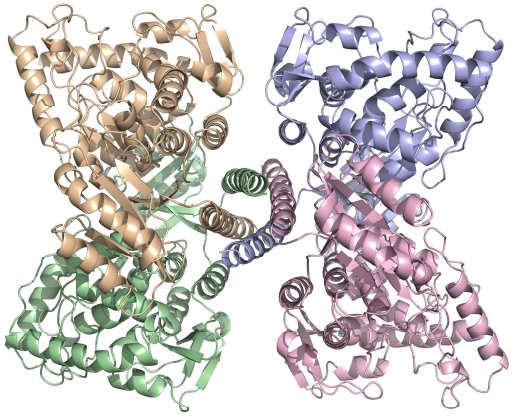

Figure 2. The 3D structure of the human PAH tetramer. Four PAH monomers are assembled asymmetrically through the C-terminal coiled-coil motifs. The 3D model was created using unpublished data obtained from crystallisation of the full-length human PAH by our research group in collaboration with Professor Patrick Cramer.

To date, only composite models of tetrameric PAH are available. These are created using truncated structures of human and rat PAH apoenzyme as well as the structures obtained after crystallisation of the enzyme with inhibitors or substrate analogues (Erlandsen *et al.*, 1998; Erlandsen *et al.*, 2000; Andersen *et al.*, 2001; Andersen *et al.*, 2002; Andersen *et al.*, 2003). In cooperation with the laboratory of Professor Patrick Cramer at the Gene Centre in Munich we recently solved the crystal structure of full-length human phenylalanine hydroxylase with a resolution of 4.6 Å. The analysis of this structure revealed new insights into behaviour and function of the enzyme. We observed a pronounced asymmetry between two dimer interfaces and a different orientation of the

tetramerisation motifs than previously reported. This structure also allowed for identifying the functional network of *PAH* mutations involved in BH_4 responsiveness (unpublished data and personal communication S.W. Gersting).

1.2.3 PAH function and catalytic activity (phenylalanine hydroxylating system)

Phenylalanine hydroxylase catalyses the rate-limiting step in the catabolic pathway of the essential amino acid phenylalanine. Under physiological conditions almost 75 % of the dietary intake of phenylalanine is catabolised and hydroxylated to tyrosine (Scriver and Waters, 1999; Underhaug *et al.*, 2012; Flydal and Martinez, 2013), whereas the remaining portion is used in anabolic processes of protein production.
The hydroxylation reaction requires, besides the PAH enzyme, two additional substrates, dioxygen (O_2) and the enzyme's natural cofactor, BH_4 ((6R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin) as a physiological electron donor (Haavik *et al.*, 1986; Kappock and Caradonna, 1996; Flatmark and Stevens, 1999; Fitzpatrick, 2012). During the reaction, a hydroxyl group is added to the phenyl group of phenylalanine forming tyrosine and at the same time BH_4 is oxidised to dihydrobiopterin (BH_2). In the next step, BH_2 is regenerated to BH_4 by dihydropteridin reductase (DHPR), whereas the product of the reaction, tyrosine, is used in the synthesis of neurotransmitters, melanin and thyroid hormones (**Figure 3**).
The enzymatic activity of human PAH is tightly regulated in order to maintain the homeostasis between the degradation of excess phenylalanine and the preservation of a certain phenylalanine and tyrosine pool critical for anabolic processes (Flydal and Martinez, 2013; Pey, 2013). The specific regulation of mammalian PAH function is mediated by three main factors: concentrations of the phenylalanine substrate and the cofactor tetrahydrobiopterin and by phosphorylation at residue Ser16 (Fitzpatrick, 2012; Flydal and Martinez, 2013). The pronounced flexibility of the PAH quaternary structure through which the regulatory properties are exerted is an essential basis for PAH regulation (Flydal and Martinez, 2013).

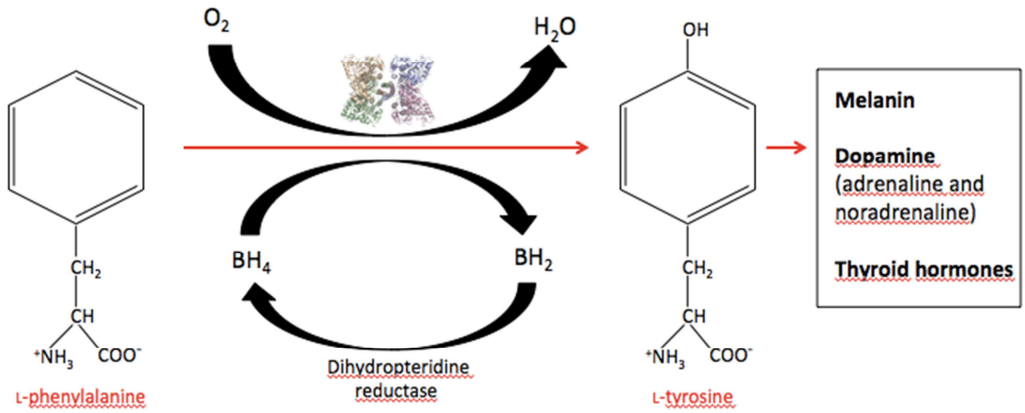

Figure 3. Overview on the reaction catalysed by PAH. The reaction requires dioxygen and BH_4 as electron donors. During the reaction, BH_4 is oxidised to BH_2, which is then regenerated to BH_4. The product of the reaction, tyrosine, is a precursor of neurotransmitters (dopamine, adrenaline, noradrenaline) and hormones (triiodothyronine and thyroxine) as well as pigments (melanin).

The substrate phenylalanine acts as a positive allosteric effector inducing in a positive cooperative manner the catalytically active form of PAH and increasing the specific activity of the enzyme by several fold (Martinez *et al.*, 1995; Fitzpatrick, 2012). Both activation and cooperativity are related to gross conformational changes occurring in the PAH protein upon substrate binding (Kleppe *et al.*, 1999; Fitzpatrick, 2012; unpublished data). These include the displacement of the autoregulatory sequence and a series of conformational changes leading to propagation of the activating process throughout the PAH tetramer (Thórólfsson *et al.*, 2003). The current model explaining this allosteric

regulation via the substrate describes the transition of the PAH protein from a low active, tense conformation (*t*-state) to a more active and high-affinity relaxed form (*r*-state). This hypothesis is supported by experimental evidence obtained using dynamic light scattering and tryptophan scans (Thórólfsson *et al.*, 2003; Kemsley *et al.*, 2003; Stokka *et al.*, 2004; Fitzpatrick, 2012; Flydal and Martinez, 2013).
The cofactor BH_4 acts as a negative effector that inhibits PAH function leading to a formation of an inactive dead-end PAH-BH_4 complex and stabilisation of the *t*-state (Phillips *et al.*, 1983; Kaufman, 1993; Thöny *et al.*, 2000; Gersting *et al.*, 2010a; Underhaug *et al.*, 2012; Flydal and Martinez, 2013). The inhibitory regulatory effect of the BH_4 cofactor is exerted by the interaction of the 1,2-dihydroxypropyl side-chain of the BH_4 molecule with residue Ser23 in the IARS sequence of the N-terminal domain (Teigen and Martinez, 2003). This interaction triggers a series of changes in the hydrogen bonding network between residue Ser23, Glu21 and residue Tyr377 and, in consequence, leads to rearrangement of the N-terminal domain and the closure of the active site. The formation of a PAH-BH_4 complex results in concordant decreased concentrations of both free BH_4 and free PAH enzyme because concentrations of BH_4 and PAH subunits in the liver (8-9 µM) are approximately equimolar. In this case, phenylalanine simultaneously controls the availability of BH_4 and the amount of active PAH by regulating the activation of PAH (Mitnaul and Shiman, 1995; Thöny *et al.*, 2000). Accordingly, there are two significant pools of BH_4 found in hepatocytes: metabolically active, free BH_4 and the inactive form of the cofactor bound to the enzyme (Mitnaul and Shiman, 1995).
The posttranslational PAH phosphorylation at Ser16 is mediated by cAMP and a Ca^{2+}/calmodulin dependent protein kinase (Kaufman, 1993; Fitzpatrick, 2012) and it results in an increase of both, PAH activity and the apparent affinity for the substrate (Døskeland *et al.*, 1996; Miranda *et al.*, 2002). The phosphorylation rate was shown *in vitro* to be increased in the presence of the substrate and decreased in the presence of BH_4 (Kaufman, 1993; Døskeland *et al.*, 1996). Similarly to phenylalanine, phosphorylation leads to a rearrangement and reorientation of the regulatory domain opening the active site (Fitzpatrick, 2012).
Taken together, all regulatory mechanisms of PAH activity are mediated by small molecule binding or phosphorylation and structurally exerted by a number of conformational changes within the protein subunits and subsequently in the PAH homotetramer.

1.3. Mechanisms of loss of function in PAH deficiency

PAH deficiency is due to mutations in the human *PAH* gene and, as described in chapter 1.2.1, more than 663 variants have been reported so far (www.hgmd.cf.ac.uk: data taken from public total, accession date 3rd April 2015). Modification of the variant PAH protein function strongly depends on the nature of the DNA alteration. In the last years, missense mutation-induced protein misfolding was identified as one of the main molecular mechanisms causing loss of function of the PAH enzyme (Waters *et al.*, 1998a; Waters *et al.*, 1998b; Waters *et al.*, 1999; Waters *et al.*, 2000; Gamez *et al.*, 2000; Pey *et al.*, 2003; Gersting *et al.*, 2008).

1.3.1 Effects of nonsense and splicing mutations in the *PAH* gene

Nonsense and splicing mutations can lead to various alterations of the DNA primary sequence including the introduction of a premature STOP codon, frameshifts as well as insertions or deletions of one or multiple nucleotides. In most cases, this has devastating consequences for the coded product leading to the formation of a truncated or incomplete and therefore non-functional protein.
In PKU, splice and nonsense variants, deletions, insertions and indels account for about 40 % of all *PAH* mutations listed in databases (Blau *et al.*, 2014). In most cases, homozygosity for one of these mutations leads to a near-complete loss of enzyme function. Consequently, carriers of these mutations in the homozygous state generally

suffer from a severe PKU phenotype and mostly do not respond to pharmacological treatment with the cofactor BH_4.

1.3.2 Effects of missense mutations in the *PAH* gene

In general, missense mutations lead to single amino acid substitutions in the translated polypeptide chain and they can induce diverse pathogenic processes that can disturb the protein on many different levels.

Effects of missense PAH mutations on PAH catalytic and regulatory properties

In vitro analyses of several *PAH* missense mutations revealed that amino acid substitution may alter kinetic properties of the mutant enzyme. This may result in decreased values of the enzyme's maximum activity (V_{max}), decreased affinity for the substrate ($[S]_{0.5}$) and/or decreased affinity for the cofactor ($[C]_{0.5}$) (Blau, 2006). *PAH* missense mutations that influence kinetic and regulatory parameters of the PAH protein are located not only in the active site (Asp143, Glu280, Pro281, Gly247) but also in positions distant from it (Arg252, Ala259, Val388, Arg408) (Kobe *et al.*, 1999; Erlandsen and Stevens, 1999; Gersting *et al.*, 2008).
One of the *PAH* mutation affecting PAH kinetic parameters, rigorously studied in several *in vitro* systems, was p.Asp143Gly (Knappskog *et al.*, 1996a; Erlandsen and Stevens, 1999). The residue Asp143 is located at the entrance to the active site and therefore may control the access of the substrate and cofactor to the active core. Indeed, studies confirmed p.Asp143Gly being a pure kinetic variant with relatively high enzyme activity but strongly reduced affinities for substrate phenylalanine and cofactor BH_4. In addition, unaffected PAH oligomerisation was reported for this PAH variant.
Another PAH mutant, which was reported as a kinetic variant is p.Val388Met (Leandro *et al.*, 2000). Studies showed reduced catalytic activity when expressed in the eukaryotic system. Moreover, data on purified variant PAH pointed to a strongly decreased affinity of the enzyme to the cofactor and to a lower extent to substrate phenylalanine. However, for this variant also accelerated protein degradation pointing to alterations of protein folding was reported (Gamez *et al.*, 2000).
Further evaluation of *PAH* variants suggested that mutations can abolish or significantly reduce phenylalanine-binding (Gjetting *et al.*, 2001b) to the putative substrate binding site in the N-terminal domain (Hufton *et al.*, 1995; Kappock and Caradonna, 1996; Kobe *et al.*, 1999). In addition, many structural hypothesis exist suggesting missense mutation-induced conformational changes of the structure of the active site and changes in the electrostatic potential of the active site. Furthermore, it is also proposed that some of the PAH mutations (Tyr337, Arg252) can affect the interaction between Ser23 and Asp27 in the IARS sequence thus having an impact on the regulatory properties of the enzyme (Erlandsen and Stevens, 1999).

Effects of missense PAH mutations on folding, assembly and thermodynamic stability of the protein

Despite having a direct influence on catalytic properties of the enzyme, missense mutations can also have a major impact on structure and conformation of the affected protein. In addition, they can disturb protein interaction with proteins of the *protein homeostasis network* and thus disturb the *homeostasis boundaries* of the affected protein (Wiseman *et al.*, 2007; Powers *et al.*, 2009; Pey, 2013). As a consequence, the availability of properly folded and functional protein is reduced. This condition is referred to as a protein misfolding disease with loss of function, also known as a conformational disease. This particular molecular phenotype is often observed in inborn errors of metabolism such as PAH deficiency (Bross *et al.*, 1999; Muntau *et al.*, 2014).
Under physiological conditions, each protein has a certain set of values of energetic parameters that allow for its proper folding and full activity *e.g.* thermodynamic stability and folding/misfolding/unfolding rates (Balch *et al.*, 2008; Pey, 2013). In addition, to achieve its native state, each protein also has to interact with the so-called *cellular*

protein homeostasis network, which includes all factors responsible for correct protein synthesis, folding, trafficking and degradation such as molecular chaperones, native states ligands, heat shock proteins as well as unfolded protein response or antioxidant response (Powers *et al.*, 2009). The components of the *protein homeostasis network* as well as their concentrations may vary depending on the cell type and cell function (Powers *et al.*, 2009). Nevertheless, all elements of the *protein homeostasis network* have to work in a very complex and well-orchestrated manner allowing for preservation of the efficient function of the protein even under unfavourable conditions such as environmental stress or genetic factors (Pey, 2013). The folding energetics, which a protein must possess in order to fulfil its biological function, in the context of a given *homeostasis network* capacity in a certain cell is described as *protein homeostasis boundaries* (Powers *et al.*, 2009; Pey, 2013). In this view, stress factors such as missense mutations may challenge protein folding by changing protein energetics or they may affect protein-protein interactions necessary for maintaining proper function of the protein. As a result, the affected protein is pushed outside of its *homeostasis boundaries*. In consequence, this leads to global destabilisation of the protein, reduced thermodynamic stability of the functional native state, a decreased rate of correct folding, an increased propensity to unfold, impaired assembly and degradation (Waters, 2001; Powers *et al.*, 2009; Pey, 2013) (**Figure 4**).

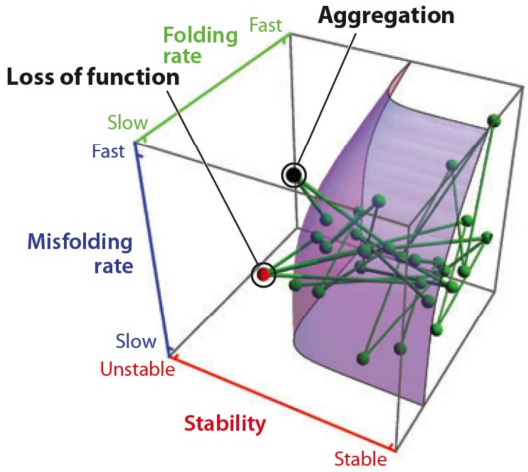

Figure 4. Graphic representation of the so-called '*protein homeostasis boundary*'. In their native state and under favourable environmental conditions proteins are described by their specific energetic parameters (folding rate, misfolding rate and thermodynamic stability). When challenged, proteins may fall outside their homeostasis boundary (represented as violet area). This can result in a disease phenotype of loss of function or aggregation (shown by red and black circles). Figure taken from Powers *et al.*, 2009.

In PAH deficiency, the molecular mechanism of loss of function due to protein misfolding was demonstrated to be associated with disturbed oligomerisation, decreased proteolytic and thermodynamic stability, increased susceptibility towards aggregation, accelerated degradation or reduced folding efficiency (Knappskog *et al.*, 1996b; Eiken *et al.*, 1996; Waters *et al.*, 1999; Waters *et al.*, 2000; Gamez *et al.*, 2000; Waters, 2001; Gjetting *et al.*, 2001a; Waters, 2003; Pey *et al.*, 2003; Pey *et al.*, 2007; Martinez *et al.*, 2008; Gersting *et al.*, 2008; Muntau and Gersting, 2010; Cerreto *et al.*, 2011; Gomes, 2012; Pey, 2013; Muntau *et al.*, 2014). Recently, more efforts have been made to further clarify the molecular basis of functional impairment in PAH deficiency. It was shown that *PAH* missense mutations affect enzyme allostery rather than enzyme activity (Gersting *et al.*, 2008). This suggested altered protein conformation and impaired molecular motions needed for promoting regulatory processes such as activation and inhibition throughout the oligomeric PAH structure. However, all pathological processes induced by *PAH* mutations are likely to be mutually dependent and a combined action of all disturbances leads to loss of PAH enzyme function and alters the phenylalanine hydroxylation system (Waters *et al.*, 2000; Gjetting *et al.*, 2001a; Muntau and Gersting, 2010).

1.4. Tetrahydrobiopterin (BH_4)

Tetrahydrobiopterin (BH_4, 6R-L-*erythro*-5,6,7,8-tetrahydrobiopterin) is an essential cofactor for various processes and enzymes (Thöny *et al.*, 2000). In the late 1950ies BH_4 was recognised to be an essential cofactor for the phenylalanine hydroxylase enzyme (Kaufman, 1958; Kaufman, 1963). Subsequently, two other amino acids hydroxylases, tryptophan hydroxylase (TPH, EC 1.14.16.4) (Friedman *et al.*, 1972) and tyrosine hydroxylase (TH, EC 1.14.16.2) (Nagatsu *et al.*, 1964; Shiman *et al.*, 1971; Blau, 2006) were shown to have a strong requirement for BH_4 to exert their catalytic function. Furthermore, BH_4 was found to be an important cofactor also for alkylglycerol monooxygenase (AGMO, EC 1.14.16.5) (Tietz *et al.*, 1964) and all three isoforms of nitric oxide synthase (NOS, EC 1.14.13.39) (Kwon *et al.*, 1989; Tayeh and Marletta, 1989; Mayer *et al.*, 1991; Gorren and Mayer, 2002). Therefore, tetrahydrobiopterin has multiple roles in human biochemistry. On the one hand, BH_4 is involved in the conversion of the amino acids phenylalanine, tyrosine and tryptophan to the various neurotransmitters dopamine, serotonin, melatonin, norepinephrine (noradrenaline) and epinephrine (adrenaline). On the other hand, since BH_4 is involved in production of nitric oxide, it was suggested that the cofactor may play a role in cardiovascular diseases (Werner *et al.*, 2011). Consistently, depletion of BH_4 was shown to contribute to vascular pathophysiology, hypertension, cardiac hypertrophy and ischemia physiology (Moens and Kass, 2006; Moens and Kass, 2007; Werner *et al.*, 2011). In addition, reduced concentrations of BH_4 were reported in several neurological diseases including Alzheimer's disease, Parkinson's disease, autism and depression (Thöny *et al.*, 2000; Foxton *et al.*, 2007). Supplementation with exogenous BH_4 turned out to improve endothelial function in smokers and in patients with hypercholesterolemia, coronary artery disease, and diabetics (Moens and Kass, 2006). Because of its pivotal role in the physiological processes described above, BH_4 is an emerging therapeutic agent in the fields of inborn errors of metabolism and cardiovascular diseases.

1.4.1 BH_4 structure and synthesis

BH_4 is a heterocyclic compound composed of a pterin ring and a 1,2-dihydroxypropyl side chain (**Figure 5**). Tetrahydrobiopterin is very unstable and in aqueous solutions it undergoes auto-oxidation forming inactive 7,8-dihydrobiopterin (BH_2) (Thöny *et al.*, 2000; Moens and Kass, 2006).

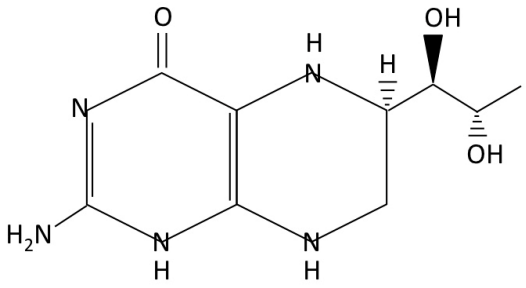

Figure 5. Chemical structure of tetrahydrobiopterin. BH_4 is composed of a pterin ring and a 1,2-dihydroxypropyl side chain.

In vivo, BH_4 is synthesised either *de novo* or regenerated via the so-called *salvage pathway* (Blau, 2006; Werner *et al.*, 2011). *De novo* BH_4 biosynthesis (**Figure 6**) is carried out by three key enzymes: guanidine triphosphate cyclohydrolase (GTP cyclohydrolase I; GTPCH; EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS, EC 4.6.1.10) and sepiapterin reductase (SR, EC 1.1.1.153) (Thöny *et al.*, 2000; Werner *et al.*, 2011). In the first step guanosine triphosphate (GTP) is converted to the unstable intermediate 7,8-dihydroneopterin triphosphate (H_2NTP). This reaction is carried out by the rate-limiting GTPCH enzyme, which is strongly regulated at both the transcriptional

(cytokines, calcium, endotoxins) and post-translational level where BH_4 itself serves as an inhibitor of GTPCH activity. Interestingly, phenylalanine displays a positive feedback on GTPCH function. Next, H_2NTP is converted by PTPS to the second unstable intermediate 6-pyruvoyl-5,6,7,8-tetrahydrobiopterin (PTP) in a Mg^{2+} and Zn^{2+} dependent reaction. The final step is the NADPH-dependent reduction of PTP catalysed by the homodimeric enzyme sepiapterin reductase to the final product, BH_4 (Thöny *et al.*, 2000; Blau, 2006; Werner *et al.*, 2011).

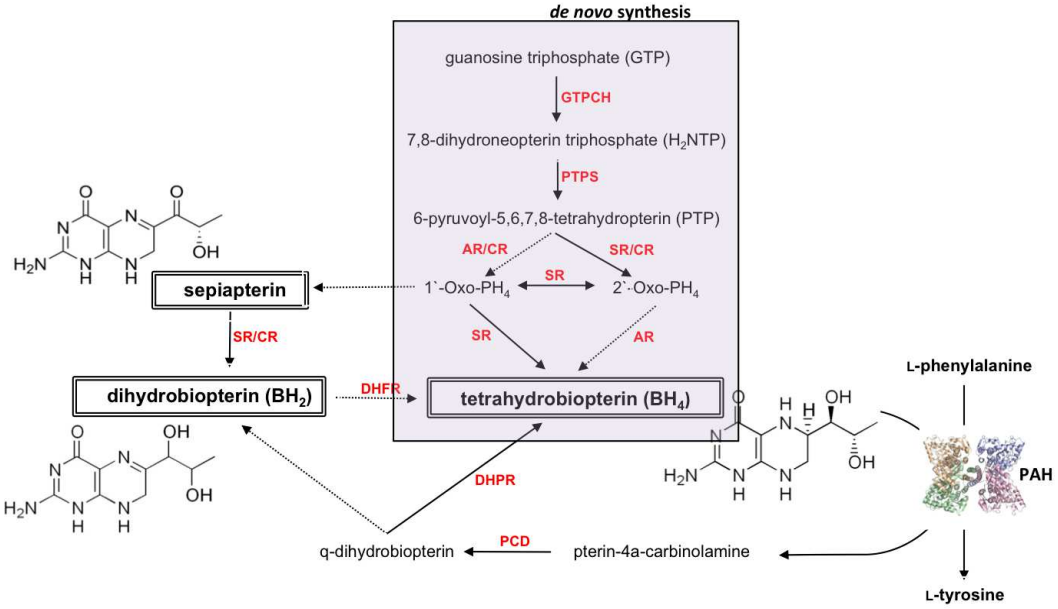

Figure 6. Schematic representation of *de novo* BH_4 biosynthesis and the *salvage pathway*. The *de novo* pathway is marked by the box and it comprises reactions catalysed by GTPCH (GTP cyclohydrolase I), PTPS (6-pyruvoyl-tetrahydropterin synthase), SR (sepiapterin reductase) and alternatively by CR (carbonyl reductase) and AR (aldose reductase). The conversion of PTP to BH_4 occurs via two additional intermediates: 1'-Oxo-PH_4 (1'-oxo-tetrahydropterin) and 2'-Oxo-PH_4 (2'-oxo- tetrahydropterin). The *salvage pathway* regenerates the oxidised form of BH_4 (by-product of the phenylalanine hydroxylation reaction) in a chain of reactions catalysed by PCD (pterin-4a-carbinolamine dehydratase) and DHPR (dihydropteridine reductase) or DHFR (dihydrofolate reductase). Alternatively, BH_4 is produced from sepiapterin using SR (sepiapterin reductase), and alternatively carbonyl reductase (CR). Adapted from Blau, 2006 and Werner *et al.*, 2011

The *salvage pathway* (**Figure 6**) regenerates BH_4 from its oxidised forms (7,8-dihydrobiopterin and sepiapterin) in two independent NADPH-dependent reactions catalysed mainly by sepiapterin reductase (SR), dihydrofolate reductase (DHFR, EC 1.5.1.3) and dihydropteridine reductase (DHPR, EC 1.5.1.34). The alternative way of BH_4 regeneration is crucial in phenylalanine metabolism since it ensures a continuous supply of the cofactor and prevents accumulation of harmful intermediates produced after BH_4 oxidation. Although important, the reaction is not sufficient to fully compensate for the defects in tetrahydrobiopterin biosynthesis or recycling (Thöny *et al.*, 2000; Moens and Kass, 2006).

1.4.2 Pharmacological treatment of PAH-deficient patients with BH_4

The discovery of the phenotype of BH_4-responsive PAH deficiency (see 1.1.2) prompted a clinical study in our laboratory. This work demonstrated that i) the majority of patients with mild PAH deficiency phenotypes respond to pharmacological treatment with BH_4, ii) BH_4 rescues *in vivo* phenylalanine oxidation and iii) increases dietary phenylalanine tolerance in these patients (Muntau *et al.*, 2002). In consequence, a synthetic form of BH_4 (a dihydrochloride salt formulation of the biologically active 6R-diastereoisomer of BH_4; sapropterin dihydrochloride) was approved in the USA and Canada in 2007 (FDA) and in Europe in 2008 (EMA) as an orphan drug to treat BH_4-responsive PAH deficiency (Levy *et al.*, 2007; Feillet *et al.*, 2008; Lee *et al.*, 2008). The goal of this treatment

strategy is to enhance the activity of the variant PAH enzyme and thereby restore the phenylalanine metabolism, to decrease plasma phenylalanine concentrations, decrease phenylalanine accumulation and, in turn, increase daily phenylalanine intake (Blau, 2010a; Muntau *et al.*, 2014). BH_4 treatment requires combined efforts and excellent teamwork of metabolic paediatricians, dietitians, biochmists as well as the parents of PKU children. In addition, many parameters show interindividual variability and thus have to be considered on a personalised basis. These concern the type of BH_4-response such as the patient being a fast or a slow responder or the dosage needed as well as the desired target blood phenylalanine concentration. Therefore, the supplementation with BH_4 has to be individually tailored and adjusted to the patient's need.

Genotyping

The attitude towards *PAH* genotyping on a routine basis is different from country to country and even from centre to centre within one country. In Germany, about 50 % of all patients are genotyped immediately after newborn screening and subsequent confirmation of diagnosis (Blau *et al.*, 2010c). Many mutations have been linked to BH_4-responsiveness because they occurred in homozygous or functional hemizygous patients, who were clearly shown to respond to the drug (Bernegger and Blau, 2002; Muntau *et al.*, 2002; Blau and Erlandsen, 2004; Desviat *et al.*, 2004; Zurflüh *et al.*, 2008). However, it has to be taken into consideration that genotype-phenotype correlation in patients with PAH deficiency is not always reliable and a number of missense *PAH* mutations and resulting *PAH* genotypes are considered as inconsistently linked to clinical phenotypes (Kayaalp *et al.*, 1997; Guldberg *et al.*, 1998a; Scriver and Waters, 1999; Muntau *et al.*, 2002; Pey *et al.*, 2003; Fiori *et al.*, 2005; Leuzzi *et al.*, 2006; Pey *et al.*, 2007; Yildirim *et al.*, 2007; Zurflüh *et al.*, 2008; Santos *et al.*, 2010; Sarkissian *et al.*, 2012b; Blau *et al.*, 2014). The information on BH_4 responsive and unresponsive *PAH* mutations and genotypes as well as patient's data is available on BIOPKU and PAHdb databases (www.biopku.org; http://www.pahdb.mcgill.ca; Blau *et al.*, 2014; Wettstein *et al.*, 2015).

Short-term BH_4 loading tests

After identification of the patient by newborn screening and confirmation of hyperphenylalaninemia by an independent laboratory method, a short-term BH_4 loading test is usually performed. It assesses blood phenylalanine concentrations after a single dose of 20 mg/kg body weight BH_4 over a period of 24 to 72 hours. Patients who respond to the drug with a decrease in phenylalanine concentrations of at least 30 % suffer from BH_4-responsive PAH deficiency or one of the very rare forms of primary BH_4 deficiency (Blau, 2010a; Blau *et al.*, 2010c; Blau *et al.*, 2011). The latter in general show a very rapid and pronounced decrease of phenylalanine concentrations and additionally show abnormal pterins in the urine. In the presence of PAH deficiency results can differ depending on the underlying genotype or the metabolic state at the time of testing (Staudigl *et al.*, 2011; Elsas *et al.*, 2011). False negative test results exclusively based on short-term BH_4 loading tests may irretrievably exclude the patient from pharmacological treatment because obtained results will not be questioned by physicians dealing with the patient in future. This leads to the necessity to perform an additional long-term BH_4 treatment test in those patients with a genotype compatible with BH_4-responsiveness and ambiguous or negative test results upon short-term tests.

Long-term BH_4 treatment test

In Europe, KUVAN® is approved for patients older than 4 years (Blau, 2010a). It is thus at the discretion of the physician and of the patient's parents, whether a long-term BH_4 treatment test will be performed already in the newborn period or later. Most clinical centres carry it out at the age of 4 years in order to conclusively answer the question whether the patient is eligible for a pharmacological treatment (Prof. Ania C. Muntau, personal communication). Different to the short-term loading test, the efficacy endpoint

of the long-term treatment test is not the blood phenylalanine concentration, but dietary phenylalanine tolerance (Leuders *et al.*, 2014). During the treatment period, blood phenylalanine concentrations are kept constant at the therapeutic target level of 42 to 240 µmol/l and the phenylalanine supply is stepwise raised. The increase is stopped, when blood phenylalanine concentrations are constantly at the upper limit of the target range. The individual benefit of the patient from the pharmacological treatment is defined as the difference between the tolerance without BH_4 and the maximum phenylalanine tolerance under BH_4 treatment. The treatment is only continued, when the increase in tolerance is considered clinically relevant at an individual basis.

Long-term BH_4 treatment

Long-term BH_4 treatment (KUVAN®) is implemented in a patient that has undergone the diagnostic procedures described above and was considered to benefit either by optimised long-term blood phenylalanine concentrations, the predictor for neurocognitive outcome, or by significant increase in phenylalanine tolerance, or a combination of the two. The drug is available as soluble tablets containing 100 mg of the active ingredient, sapropterin dihydrochloride (EMA. Kuvan EU Summary of Product Characteristics, 2008; Blau, 2010a; Blau, 2013). In addition, the product contains mannitol, anhydrous calcium hydrogen phosphate, crospovidone type A, ascorbic acid, sodium stearyl fumarate, and riboflavin. From all additional components, ascorbic acid is of specific importance for the treatment since it protects BH_4 from undue oxidation (EMA. Kuvan EU Summary of Product Characteristics, 2008; Blau, 2010a). The daily dosage has to be individually adjusted according to the patient's residual PAH activity and dietary phenylalanine supply. In general, the treatment is started with 10 mg/kg body weight as single daily dose, this can be titrated to a maximum of 20 mg/kg body weight, if needed (EMA. Kuvan EU Summary of Product Characteristics, 2008). In practice, a single dose of the drug is sufficient to maintain stable blood phenylalanine concentrations over a 24 h period without substantial increase in phenylalanine concentration after food intake (personal communication A. Muntau). PKU patients who respond to BH_4 treatment can be divided into two groups. The first group of patients can completely discontinue the low phenylalanine diet and be treated with sapropterin dihydrochloride only, whereas the second group needs a combination of sapropterin dihydrochloride supplementation together with a diet, which allows for a higher content in natural protein depending on the degree of response to BH_4. Therefore, the supplementation with sapropterin dihydrochloride reduces nutritional deficiencies, significantly improves quality of life and, in turn, allows for better adherence to the treatment (EMA. Kuvan EU Summary of Product Characteristics, 2008; Blau, 2010a, Keil *et al.*, 2013; Trefz *et al.*, 2015).
The most frequent adverse events reported during the phase III clinical studies conducted prior approval of sapropterin dihydrochloride were headache, vomiting, cough, rash, upper abdominal pain, diarrhoea, nausea, and rhinorrhoea (Blau, 2010a; Burton *et al.*, 2011; Blau, 2013). In clinical routine, serious side effects are very rare and generally dose-dependent (Trefz *et al.*, 2015).

Post approval activities

After KUVAN® was approved, a multinational drug registry study was designed to analyse and collect the information on long-term outcomes of PKU patients treated with sapropterin dihydrochloride (The Kuvan Adult Maternal and Pediatric European Registry; KAMPER) (Blau, 2010a; Trefz *et al.*, 2015). The study was started in 2009 and is planned to be terminated in 2024 (15-years study). Data on BH_4-responsive patients are collected in more than 100 centres in 11 European countries. The aim of the study is to assess the overall long-term safety of the treatment as well as to analyse the safety in specific groups of patients such as children below 4 years of age, pregnant women and elder patients (Blau, 2010a).

1.4.3 BH_4 as a pharmacological chaperone

Since the introduction of BH_4 treatment for PAH-deficient patients many studies have been conducted in order to analyse and elucidate the BH_4 mode of action. Although the mechanisms of responsiveness to BH_4 are multifactorial (Erlandsen *et al.*, 2004; Pey *et al.*, 2004a; Okano *et al.*, 2007; Gersting *et al.*, 2008; Lagler *et al.*, 2010; Staudigl *et al.*, 2011; Sarkissian *et al.*, 2012a), it is commonly accepted that BH_4 exerts its function as a pharmacological chaperone stabilising misfolded PAH variants (Erlandsen *et al.*, 2004; Pey *et al.*, 2004a; Perez *et al.*, 2005; Aguado *et al.*, 2006; Martinez *et al.*, 2008; Gersting *et al.*, 2010b; Muntau and Gersting, 2010; Sarkissian *et al.*, 2012a; Underhaug *et al.*, 2012; Pey, 2013).

Pharmacological chaperones

The concept of a pharmacological chaperone was introduced at the end of the last century when it was shown that the imino sugar migalastat rescues the activity of α-galactosidase A in Fabry disease (Fan *et al.*, 1999). Since then only two compounds have been approved on the market as pharmacological chaperones: sapropterin dihydrochloride for PKU treatment (Burnett, 2007; Levy et al., 2007; Thompson, 2008) and tafamidis for the treatment of transthyretin-related hereditary amyloidosis (Bulawa *et al.*, 2012).

A pharmacological chaperone is a small molecule often resembling natural ligands of the target protein (*e.g.* protein cofactors, agonists, antagonists, competitive inhibitors) that binds specifically and reversibly to the misfolded target and rescues protein function by improving protein folding and by stabilising the protein structure (Leandro and Gomes, 2008; Muntau and Gersting, 2010; Underhaug *et al.*, 2012; Muntau *et al.*, 2014). The correction of misfolding by pharmacological chaperones can be exerted in four ways: i) the altered protein can be thermodynamically stabilised ii) the folding rate can be increased by pharmacological chaperones that stabilises the folding transition states iii) the misfolding rate can be decreased by stabilisation of the native state of the protein and iv) the combination of all mechanisms. In consequence, the defective protein can regain optimal values for correct overall protein function and go back to its specific *homeostasis boundries* (**Figure 7**) (Powers *et al.*, 2009). The stabilising process induced by a pharmacological chaperone is often achieved at the cost of a certain degree of inhibition of protein function (Fan, 2008; Muntau *et al.*, 2014). Yet, this inhibition may be overruled by an increase of the amount of functional protein within the cell (Fan, 2008; Muntau and Gersting, 2010; Muntau *et al.*, 2014).

There are defined rules that a molecule has to fulfill in order to be regarded as an active pharmacological chaperone. First, the pharmacological chaperone has to bind reversibly to the target protein with high affinity thus ascertaining its usage at low dosages. Second, binding of the molecule has to induce conformational changes of the target protein structure. Third, inhibitory compounds are more likely to exert their chaperone role at higher efficacy than nonspecific stabilisers. In addition, the targeted variant protein has to show instability, however, must still posses some residual activity. Last but not least, the pharmacological chaperone is only effective when a small change in the target protein can make a big difference concerning the phenotypic outcome rescuing loss of function of the target protein (Underhaug *et al.*, 2012; Muntau *et al.*, 2014).

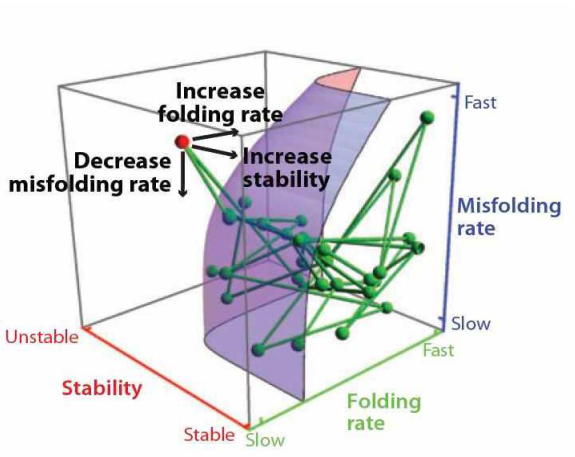

Figure 7. Effects of pharmacological chaperones on the misfolded protein. The affected protein (*e.g.* due to missense mutations) is pushed outside its homeostasis boundary (red circle). The pharmacological chaperone binds to the misfolded protein increasing thermal and conformational stability of the protein. As a result, the protein may be pushed back to the near-optimal setting rescuing the loss of function genotype. Taken from Powers *et al.*, 2009.

The pharmacological chaperone effect of BH_4

Experimental studies using purified PAH proteins revealed stabilisation of the misfolded protein by BH_4 against denaturation and degradation (Doskeland and Flatmark, 1996; Erlandsen *et al.*, 2004; Pey *et al.*, 2004a; Perez *et al.*, 2005; Aguado *et al.*, 2006; Gersting *et al.*, 2010b; Muntau and Gersting, 2010). In line with *in vitro* studies, pharmacological doses of the BH_4 cofactor were shown to stabilise wild-type PAH levels in mouse livers (Thöny *et al.*, 2004; Scavelli *et al.*, 2005). Studies on the *Pah*^{enu1} mouse model for BH_4-responsive PAH deficiency demonstrated that the loss of function phenotype results from a loss of PAH that is a consequence of protein misfolding and aggregation followed by accelerated degradation (Gersting *et al.*, 2010b). BH_4 alleviates this pathophysiological triad by conformational stabilisation of the protein leading to an increase in the effective functional PAH concentration and to a correction of the loss of function phenotype. Moreover, data obtained *in vitro* and *in vivo* showed that BH_4 works in a selective manner confined to the pathological metabolic state. Recent studies suggest that the observed rescue of PAH activity upon BH_4 treatment requires a well balanced metabolic environment of the phenylalanine substrate and the BH_4 cofactor (Gersting *et al.*, 2010b; Muntau and Gersting, 2010; Staudigl *et al.*, 2011; Sarkissian *et al.*, 2012b; Pey, 2013; Muntau *et al.*, 2014). Moreover, the underlying genotype (Staudigl *et al.*, 2011) as well as ubiquitin-dependent degradation (Sarkissian *et al.*, 2012a) of PAH variants also play an important role.

The success of the use of the natural compound BH_4 to correct the loss of function phenotype in PAH deficiency triggered studies to identify BH_4 derivatives or new molecules that could efficiently stabilise the PAH enzyme but show more favourable pharmacokinetic and pharmacodynamic properties as well as improved synthesis, bioavailability and stability. To date, four compounds, 5-benzylhydantoin, 6-amino-5-(benzylamino)-uracil, 3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one and 5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one have been demonstrated to improve PAH stability, increase *in vivo* phenylalanine oxidation and reduce blood phenylalanine levels (Pey *et al.*, 2008; Santos-Sierra *et al.*, 2012).

1.5. Genotype-phenotype correlation in phenylketonuria

After many diseases have been identified as simple monogenic disorders with mendelian type of inheritance, systematic mutation analyses have been prompted in order to establish genotype-phenotype correlations and to provide reliable predictions of the

course of the disease and ease management decisions. However, most studies failed to establish robust genotype-phenotype correlations. This is due to the fact that phenotypic manifestation is often modified and influenced by many additional genetic, epistatic and environmental factors as well as differences in the homeostasis and metabolism of individual patients (Dipple and McCabe, 2000; Scriver, 2007; Elsas *et al.*, 2011; Pey, 2013).

In PKU, as in many other monogenic diseases, the primary goal of genotyping followed by research on genotype-phenotype correlations was to determine whether genotype analysis could be sufficient to properly predict the severity and the course of the disease (Guldberg *et al.*, 1998a). Many researchers additionally hoped that genotyping would allow for predicting the type and level of responsiveness to BH_4 supplementation as this would allow for straightforward introduction of adequate treatment without performing additional clinical tests (Blau *et al.*, 2011; Mitchell *et al.*, 2011). Indeed, the first publications analysing these aspects were promising (Okano *et al.*, 1991; Guldberg *et al.*, 1998a; Blau *et al.*, 2011). However, more intricate *in vitro* studies (Staudigl *et al.*, 2011) and studies on larger groups of PKU patients (Sarkissian *et al.*, 2012b) demonstrated that the genotype in many cases does not reliably predict BH_4 responsiveness. This is mainly due to the fact that PKU is a complex disease arising from the genotype (underlying mutations), the enzymatic phenotype (PAH enzyme activity), the metabolic phenotype (blood phenylalanine levels), the cognitive phenotype (IQ, mental retardation, impaired cognitive development) and the therapeutic phenotype (BH_4 treatment). Furthermore, distinct modulators (discussed below and summarised in **Table 2**) may affect each of these levels and by this weaker genotype-phenotype correlation (Scriver and Waters, 1999; Waters, 2001; Santos *et al.*, 2010; Mitchell *et al.*, 2011).

The PAH genotype

Phenylketonuria due to deficiency of PAH is characterised by high allelic heterogeneity with > 660 mutations (www.hgmd.cf.ac.uk) affecting the *PAH* gene. In addition, about 89 % of PKU patients are compound heterozygous (Zurflüh *et al.*, 2008). Especially these two genetic factors make the prediction of genotype-phenotype correlations in phenylketonuria extremely difficult (Guldberg *et al.*, 1998b; Guldberg *et al.*, 1998a; Waters *et al.*, 1998b; Mirisola *et al.*, 2001; Pey et *al.*, 2003; Rivera *et al.*, 2011). In addition, the possibility of having more than two mutations on the homologous alleles has also to be kept in mind. In line with this statement, the necessity to perform complete sequencing of the whole gene has to be taken into consideration as introduction of additional mutations can further add to alteration of the gene product (Scriver and Waters, 1999).

Disconcordances at the level of the enzymatic phenotype

The function of variant PAH enzymes can be modulated in various ways. As discussed in chapter 1.3.2 *PAH* mutations can have an unpredictable impact on the speed and effectiveness of protein translation, regulatory properties, kinetic parameters, capacity of protein folding, thermodynamic stability, protein conformation and the quaternary structure of the enzyme. All this may render the protein prone to aggregation and accelerated degradation and in turn may lead to a decreased abundance of the intracellular effective protein amount. On the other hand, inter-individual variability in the quality and quantity of proteolytic and folding systems as well as chaperone function may play an important role and further add to inconsistencies of genotype-phenotype correlations (Scriver and Waters, 1999; Waters, 2001; Mitchell *et al.*, 2011). One of the modulators of protein function is interallelic complementation (IAC). IAC is a well-known phenomenon in human genetics contributing to genotype-phenotype correlation especially in compound heterozygosity. The theory of interallelic complementation originates from work of Brenner (Brenner, 1959) and Fincham (Fincham and Coddington, 1963; Fincham and Stadler, 1965) and refers to the specific protein-protein interactions occurring in multimeric proteins where the hybrid is assembled from

products of two mutated alleles. Complementation at the level of protein is usually considered in terms of enzyme activity. Mutant proteins in the homomultimeric state, which originates from protein subunits encoded by one mutant allele, show either decreased or abolished activity while those defects may be mutually corrected in heteromultimers, composed of subunits encoded by both mutant alleles as well as the wild-type allele, resulting in a partial restoration of protein function. The phenomenon was explained in 1960s by the '*good corrects bad*' hypothesis, which implies stabilisation of a misfolded monomer in the presence of the unaltered wild-type monomer (Crick and Orgel, 1964). This theory was further expanded to allosteric enzymes, which are characterised by a very delicate balance between their active and inactive state. Accordingly, a mutation within such a protein, not necessarily directly associated with a ligand binding site, may result in trapping the enzyme in one state or another. In this case, disturbed balance may be either corrected or restored by another mutant. This is known as the '*bad corrects bad*' hypothesis (Monod, 1965; Monod *et al.*, 1965; McGavin, 1968). A number of *PAH* compound heterozygous genotypes were analysed in a prokaryotic expression system showing negative IAC (Kaufman *et al.*, 1975; Waters *et al.*, 2001; Waters, 2001; Leandro *et al.*, 2006; Leandro *et al.*, 2011). Therefore, IAC may explain why compound heterozygous genotypes consisting of two mutations with similar severity may induce milder or more severe phenotypes than predicted by each of the mutations.

Table 2. Factors affecting reliable prediction of genotype-phenotype correlations in phenylketonuria

	Affected unit	**Modyfying factors**
Genotype (allelic interaction)	*PAH* gene	High allelic heterogeneity Compound heterozygosity Additional mutations in *cis*
Phenotype		
PAH protein	PAH polypeptide	Translation, chaperones, proteases
Enzymatic phenotype	PAH tetramer Hydroxylation reaction	Interallelic complementation Protein stability Tetramer assembly Synthesis and recycling of the cofactor
Metabolic phenotype	Phenylalanine homeostasis	Phenylalanine intake Phenylalanine disposal Intestinal absorption
Cognitive phenotype	IQ and executive function	Function of the blood-brain barrier Expression of certain transporters *e.g.* for LNAA
Therapeutic phenotype	Treatment	Diet only, diet in combination with BH_4 treatment, supplementation with BH_4 only, no treatment
Additional factors	Calculation of *in vitro* PAH enzyme activity Estimation of BH_4-responsiveness	Different transfection methods and protocols for PAH activity assays Protocol differences for BH_4 loading tests performed in clinical centres

Table adapted and modified from Scriver and Waters, 1999.

Disconcordances at the level of the metabolic phenotype

Measures of the metabolic phenotype are blood phenylalanine values and dietary tolerance for the phenylalanine intake. Since this metabolic homeostasis is a complex process, inter-individual differences in the intestinal absorption, hepatic uptake and the

disposal of amino acid by hydroxylation, transamination and decarboxylation may in part account for discrepancies observed (Treacy *et al.*, 1996; Scriver and Waters, 1999; Fiege *et al.*, 2004).

Disconcordances at the level of the cognitive phenotype

Studies have shown that IQ scores in untreated patients or even in siblings carrying the same *PAH* genotype might show significant differences (Ramus *et al.*, 1993; Scriver and Waters, 1999). Moreover, even when the metabolic phenotype in two individuals is similar, the cognitive phenotypes might be diverse (Moller *et al.*, 1998; Weglage *et al.*, 1998). This discordance may be explained by differences in the function of the blood-brain barrier (BBB) leading to two different kinetics of phenylalanine uptake through the BBB and this can subsequently modulate the phenylalanine content in brain (Moller *et al.*, 1998; Scriver and Waters, 1999; Mitchell *et al.*, 2011).

Additional factors hampering reliable genotype-phenotype correlation in phenylketonuria

Although *PAH* mutations can be classified based on the level of PAH function as severe (very low enzyme activity) or mild (substantial residual enzyme activity) different individuals, even siblings, may show great differences of the disease phenotype (Dipple and McCabe, 2000; Waters, 2001). Also in the case of homozygous or functional hemizygous PKU patients, where the prediction should be quite straightforward, discrepancies exist (Kayaalp *et al.*, 1997; Guldberg *et al.*, 1998a; Scriver and Waters, 1999; Muntau *et al.*, 2002; Fiori *et al.*, 2005; Leuzzi *et al.*, 2006; Yildirim *et al.*, 2007; Zurflüh *et al.*, 2008; Santos *et al.*, 2010; Sarkissian *et al.*, 2012b; Blau *et al.*, 2014).
This might be the result of the fact that all existing information on the effect of *PAH* mutations on PAH function was generated by *in vitro* studies performed using purified PAH protein or eukaryotically expressed PAH. There are no data available on *in vivo* variant PAH function since a liver biopsy is considered as an unjustified invasive intervention (Blau, 2006). Therefore, currently used data on enzyme activity and residual protein amount originates from PAH proteins expressed using different expression methods, vectors and expression systems (e.g. *E.coli*, COS-7, HEK293, TnT system). Moreover, methods for measurement of PAH activity may vary (*e.g.* incubation time and the cofactor used). All this may lead to overestimation of *in vitro* activities, variability in measured *in vitro* PAH activity even for single mutations as well as incorrect calculation of "predicted residual activity" (PRA) for compound heterozygotes where PRA is expressed as the mean of monoallelic *in vitro* enzyme activities for two mutations constituting the *PAH* genotype.
As to estimation of BH_4-responsiveness, many efforts have been made to identify BH_4-responsive genotypes and mutations. Genotype analysis of a large group of PKU patients listed in BIOPKU database (Zurflüh *et al.*, 2008) allowed for identification of the so-called most common responsive *PAH* mutations (p.Ala403Val, p.Arg261Gln, p.Tyr414Cys, p.Ala300Ser, p.Val245Ala, p.Leu48Ser, p.Glu390Gly and p.Arg241Cys) (Wettstein *et al.*, 2015). However, frequent inconsistencies as to BH_4-responsiveness even in the patients with the same genotype were observed in the presence of p.Leu48Ser, p.Ile65Thr, p.Arg158Gln, p.Arg261Gln, p.Tyr414Cys, p.Glu390Gly, p.Val388Met (Kayaalp *et al.*, 1997; Muntau *et al.*, 2002; Fiori *et al.*, 2005; Leuzzi *et al.*, 2006; Yildirim *et al.*, 2007; Zurflüh *et al.*, 2008; Santos *et al.*, 2010; Sarkissian *et al.*, 2012b; Blau *et al.*, 2014). In addition, discrepancies in the PKU phenotype were reported even among individuals bearing two null mutations (p.[Arg252Trp];[Pro281Leu], p.[Arg261Stop];[Pro281Leu], p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln], p.[Arg408Trp];[Pro281Leu]) for which the expected classical PKU was not observed or BH_4-responsiveness has been reported (Santos *et al.*, 2010; Leuders *et al.*, 2014). In the same study, the general hypothesis that the presence of the less severe allele determines the severity of the disease (Guldberg *et al.*, 1998b; Guldberg *et al.*, 1998a; Santos *et al.*, 2010) was not confirmed. These inconsistencies may result from molecular modifying

factors described above, however, weak genotype-phenotype correlation may also result from differences in BH_4 loading test protocols (Fiege and Blau, 2007; Sarkissian *et al.*, 2012b). This includes the dosages of BH_4 used, the initial phenylalanine values of the patients at the beginning of the test, dietary fluctuations during the loading tests and the duration of the test (Zurflüh *et al.*, 2008; Sarkissian *et al.*, 2012b; Leuders *et al.*, 2014). Moreover, there are reports on patients showing BH_4-responsiveness in a single loading test but when the patients were exposed to long-term treatment no response was observed (Zurflüh *et al.*, 2008; Leuders *et al.*, 2014).

Taken together, further research is needed for in-depth analysis of variant PAH function as prerequisite for solid phenotype-genotype correlations in phenylketonuria. However, many *in vivo* factors modulate the behaviour of the endogenous PAH protein in a complex way (*e.g.* interallelic complementation, metabolic environment). Their careful consideration in experimental set-ups may foster a reliable transfer of results obtained by *in vitro* studies into the *in vivo* situation.

2. AIMS OF THIS WORK

In the past 15 years, huge effort has been undertaken to elucidate the molecular mechanisms underlying phenylketonuria and successful pharmacological treatment of PAH deficiency by BH_4. Missense mutations in the *PAH* gene were shown to induce protein misfolding with loss of function of variant PAH proteins. Conformational stabilisation of misfolded variant PAH was dissected as the mode-of-action of pharmacological doses of BH_4. Despite this progress there was still a significant gap of knowledge regarding PAH function *in vivo*. In particular, our understanding of PAH function in relation to the patient's metabolic state and BH_4-therapy was incomplete.
We addressed these questions in three scientific projects that comprise the cumulative part of this thesis. The first one focused on fine-mapping of BH_4-dependent PAH enzyme kinetic parameters. Here, we aimed to develop a robust continuous assay for high-throughput measurement of PAH activity and to improve our understanding of BH_4 binding-induced molecular motions driving PAH kinetics. Based on the novel assay, in the second project, we aimed to analyse the impact of varying substrate and cofactor concentrations on PAH function of recombinantly expressed and purified wild-type and variant PAH. In addition, we aimed to retrace experimental findings *in vitro* with patient data derived from oral BH_4 loading tests. The third project aimed to establish a cell-based assay of PAH function that reliably mimics the patient's situation *in vivo*. This comprised mapping of the country-specific distribution of *PAH* genotypes in Europe and Middle East, genotype-specific PAH functional analyses, and investigations of PAH function in the pathological metabolic context additionally addressing the impact of pharmacological doses of BH_4.

3. CUMULATIVE THESIS: Summary of Published Results

3.1. PAPER 1: Activation of phenylalanine hydroxylase induces positive cooperativity toward the natural cofactor.

Gersting SW, Staudigl M, Truger MS, Messing DD, **Danecka MK**, Sommerhoff CP, Kemter KF, Muntau AC.
The Journal of Biological Chemistry, 2010; 285 (40): 30686-30697.

The function of tetrameric PAH is tightly and complexly regulated by the substrate, L-phenylalanine, the cofactor, BH_4, as well as by phosphorylation. These regulatory mechanisms require the PAH enzyme to be highly flexible in order to transmit reversible conformational changes occurring upon binding of enzyme's effectors throughout the protein. In addition, it has been previously shown that alterations in enzyme kinetic parameters of variant PAH reflect mutation-induced structural alterations. Thus, in-depth evaluation of kinetics and allostery of PAH protein appeared to be useful when assessing to which extent local single amino acid replacement lead to global conformational alterations compromising enzyme function. Yet, current standard PAH activity assays are laborious, time consuming and designed as end-point measurements. Therefore, we aimed to develop an automated continuous real-time assay of PAH activity for robust though comprehensive analyses of steady state enzyme kinetics.
Application of the fluorescence-based multi-well assay revealed an initial high activity burst phase followed by a linear steady-state rate of the enzymatic reaction for activated (pre-incubated with L-phenylalanine) wild-type PAH enzyme. In contrast, the non-activated PAH exhibited an initial lag-phase followed by a linear phase of product (L-Tyr) formation. Further analysis of L-phenylalanine dependent wild-type PAH kinetics showed the well-known allosteric behaviour for the activated PAH enzyme whereas binding of phenylalanine to the non-activated enzyme was found to be non-cooperative. These kinetic data were in line with the kinetic data obtained from discontinuous assays. Noteworthy, the analysis of BH_4-dependent kinetics led to previously unknown findings. Although non-activated wild-type PAH followed a non-cooperative Michaelis-Menten kinetic model, the activated enzyme clearly showed a sigmoidal, allosteric behaviour. This provided evidence for BH_4-dependent cooperativity with PAH activation as a prerequisite. To investigate whether the shift in enzyme kinetics from Michaelis-Menten to the Hill model is due to the presence of the L-phenylalanine substrate itself or it rather depends on conformational changes and rearrangements within the protein, three pre-activated genetics PAH variants (human p.Arg68Ser, truncated human 103-427 PAH and murine p.Val106Ala) were analysed and their behaviour was compared to that of the wild-type PAH. Pre-incubation of the wild-type enzyme with phenylalanine led to a series of structural and functional changes as seen by a red-shifted and enhanced tryptophan emission, a right-shifted thermal denaturation profile, increased V_{max} and the switch from non-cooperative to positive cooperative kinetics accounting for activation. Data on variant PAH provided evidence that BH_4-dependent positive cooperativity of the activated enzyme does not rely on the presence of L-Phe but is determined by activating conformational rearrangements.
Taken together, our new continuous multi-well PAH activity assay proved to be faster and more efficient but as precise and accurate in evaluating PAH kinetics and allostery as the standard methods. This technique revealed a previously unknown positive cooperativity of pre-activated PAH towards BH_4 that relies on activating conformational rearrangements. Moreover, we showed that the presence of L-Phe affects the BH_4-dependent kinetic properties of PAH protein. These findings may thus have implications for an individualised therapy of patients with PAH deficiency. In addition, our data supported the notion that the patient's metabolic state may have a greater impact on conformation and function of the PAH enzyme that it was previously appreciated.

3.2. PAPER 2: The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response.

Staudigl M, Gersting, SW, **Danecka MK**, Messing DD, Woidy M, Pinkas D, Kemter KF, Blau N, Muntau AC.
Human Molecular Genetics, 2011; 20 (13): 2628-2641.

The discovery of a pharmacological treatment for phenylketonuria raised new questions about function and dysfunction of phenylalanine hydroxylase. In addition, *in vitro* data as well as observations made in daily clinical practice suggested that the influence of substrate and cofactor concentrations on PAH enzyme function and on the response to the pharmacological treatment with BH_4 might be of even more functional and therapeutic relevance than previously estimated. Moreover, the information on the behaviour of the PAH enzyme in the pathological and therapeutic setting is still scarce.
In order to investigate the interdependent effects of substrate and cofactor on PAH enzyme kinetics of different *PAH* variants we i) adapted our continuous real-time activity assay to cover an expanded range of phenylalanine and BH_4 concentrations; ii) depicted obtained results as three-dimensional color-coded activity landscapes; iii) described the optimal working ranges for purified wild-type and variant PAH; iv) substantiated our findings in an eukaryotic expression system, and v) translated *in vitro* observations into the human situation by analysing literature data on the results of oral BH_4 loading tests from PAH-deficient patients.
The analysis of the wild-type PAH kinetics under physiological to supraphysiological concentrations of phenylalanine and BH_4 allowed for depicting the optimal working range for the enzyme (252 – 2026 µM L-Phe; 44-306 µM BH_4) as well as the peak activity (561 µM L-Phe; 125 µM BH_4). The data provided evidence that phenylalanine concentrations for the optimal working range of the wild-type PAH enzyme were lower whereas BH_4 concentrations were higher than previously assumed. Moreover, it was shown that with increasing phenylalanine concentrations more BH_4 is needed to maintain the same level of PAH activity. In addition, a mutual interdependence of cofactor and substrate inhibition was found. Next, activity landscapes of eight purified PAH variants were performed and the results were compared with those obtained for the wild-type. The presence of *PAH* missense mutations led to various alterations with respect to the extension and position of optimal working ranges of activity landscapes. The observed differences in the patterns of activity landscapes included broadening or narrowing of the working optima, shifts of the peak activities towards higher or lower phenylalanine concentrations and/or towards cofactor concentrations. This comprehensive analysis of PAH activity landscapes revealed that differences in the regulation of PAH activity by BH_4 and L-phenylalanine *in vitro* strongly depend on the analysed variant and therefore, *in vivo,* on the underlying genotype. In the next step, activity landscapes were in part reinvestigated in the eukaryotic system with stable expression of the wild-type and two PAH variants. The kinetic behaviours of all PAH proteins expressed in eukaryotic cells were in line with key findings of the activity landscapes obtained using purified PAH. The *in vitro* observations were transferred to the human situation by analysing literature data derived from BH_4-loading tests performed in PAH-deficient patients. This allowed to further substantiate that enzyme function in the individual patient at a given time point is the resultant of the metabolic state and the dosage of cofactor treatment both in turn determined by the underlying *PAH* genotype.
Taken together, our approach allowed for detailed visualisation and better understanding of conditions corresponding to those occurring in physiological and pathological situations in PKU patients and in the therapeutic context upon cofactor treatment. In addition, our findings may set the basis for a mutation-dependent individualised dietary treatment of PKU patients. Moreover, this work pinpoints the importance of genotyping PKU patients and underscores the need for more personalised testing procedures.

3.3. PAPER 3: Mapping the functional landscape of frequent phenylalanine hydroxylase genotypes promotes personalised medicine in phenylketonuria.

Danecka MK, Woidy M, Zschocke J, Feillet F, Muntau AC, Gersting, SW.
Journal of Medical Genetics, 2015; 52 (3): 175-185.

Phenylketonuria is characterised by pronounced allelic heterogeneity with a high number of *PAH* gene mutations, high incidence of compound heterozygosity and the variability in distribution of common mutations between ethnic groups and geographical regions. In addition, a continuum of residual *in vivo* PAH activity, depending on the underlying mutations, results in phenotypic heterogeneity where the clinical picture ranges from mild HPA to severe classical PKU. The discovery of a BH_4-responsive phenotype further adds to the phenotypic variability of PAH deficiency. Many efforts have been undertaken to characterise PAH function and to establish robust genotype-phenotype relationships. Yet, available data on residual PAH activity is restricted to single mutations that were expressed in different cellular systems and assayed by means of diverse enzyme assay protocols. Moreover, non-invasive functional assays, for example, in blood cells, are not feasible because metabolic PAH function is restricted to the liver and liver biopsies are considered as unjustified interventions. Thus, present *in vitro* data on PAH activity of single variant PAH proteins do not reliably reflect PAH function of majority of the PKU patients who show compound heterozygosity. Therefore a significant gap of knowledge concerns the enzymatic function of PAH reflecting full *PAH* genotypes. In addition, our previous work revealed that PAH activity also depends on the metabolic phenotype and a potential pharmacologic administration of BH_4. Furthermore, although public databases (PAHdb, BIOPKU) provide general information on *PAH* mutations and associated phenotypes there is no database access on occurrence and frequency of full *PKU* genotypes.
In order to provide genotype-specific data on PAH function and to develop a consistent model assessing PAH activity arising from two different alleles we i) mapped the most common *PAH* genotypes in Europe and Middle East; ii) determined PAH residual function of the 30 most frequent homozygous and compound heterozygous genotypes in a cell based model; iii) presented data as three-dimensional PAH activity landscapes and iv) assessed the PAH working optima of full *PAH* genotypes.
First, a comprehensive literature search was performed in order to determine the frequency and region-specific distribution of *PAH* genotypes. Due to a shared ethnic background the research focused on Europe and the Middle East. Data from 20 countries spanning from Portugal to Iran were selected resulting in a study population of 3066 PKU patients displaying 690 different genotypes based on 264 mutations. Overall, the type and frequency of single *PAH* mutations in our study population matched well with frequencies described in the databases. The two predominant *PAH* mutations were p. Gln355_Tyr356insGlyLeuGln and p.Arg408Trp and both showed a region-specific distribution. The majority of genotypes was compound heterozygous (91 %), which is in line with literature data. The 30 most frequent genotypes, which encode expressible PAH proteins from both alleles, covered 55 % of patients in our study population.
Residual function of wild-type and the 30 most frequent PAH genotypes was assessed in COS-7 cells transiently expressing variant PAH encoded by both alleles of the respective *PAH* genotypes. PAH activity was determined at broad ranges of cofactor and substrate concentrations mimicking alteration in the metabolic state as well as a pharmacological treatment with BH_4. The activity landscape of wild-type PAH showed peak enzyme activity at 431 µM L-phenylalanine and 130 µM BH_4, which was in line with data previously obtained for purified PAH. At L-phenylalanine concentrations above the therapeutic threshold of 360 µM the enzyme showed a need for increased BH_4 concentrations to maintain the same level of activity. Residual PAH activity ranged from 1.7 % up to 11 % for all full *PAH* genotypes. We classified *PAH* genotypes into three

groups depending on the residual activity: < 3.5 % (group 1), ≥ 3.5 % and < 5 % (group 2) and ≥ 5 % (group 3). Next, we related residual activities with patterns of the activity landscapes and phenotypic data from PKU patients. Group 1 genotypes showed very low activity with no defined peak of enzyme activity. Clinical phenotypes retrieved from the BIOPKU database showed a consistent association with BH_4 non-responsive classical PKU. In group 2, enzyme activities were still low, however, an area of peak activity mapping to the area of wild-type PAH was delimitable. This group was associated with phenotypic heterogeneity with both mild and classical PKU phenotypes and a significant share of patients responding to BH_4. Genotypes from group 3 showed considerable residual activities with delimitable peak areas with two exceptions. However, the position and shape of areas of peak enzyme activity showed large variations within this group. Some genotypes induced a left shift of the peak activity, which mapped to significantly lower phenylalanine concentrations than observed for the wild-type. On the other hand, some genotypes induced a right-shift of peak enzyme activity towards higher phenylalanine concentrations. Interestingly, all but one of these genotypes contained the mutation p.Arg261Gln. A broadening of the optimal working range was observed for four genotypes and three out of four contained p.Arg261Gln. On the other hand, a narrow optimal working range was identified in eight genotypes with p.Leu48Ser and p.Arg408Trp being leading mutations in this group. Six genotypes were identified as being associated with a shift of peak activity towards higher BH_4 concentrations. Most of these genotypes contained mutations associated with inconsistent genotype-phenotype correlations (p.Leu48Ser, p.Ile65Thr, p.Ala300Ser). Group 3 genotypes with a defined area of peak activity were predominantly associated with MHP or mild PKU and high response rates to BH_4 cofactor treatment. Only five genotypes in this group showed unresponsiveness to BH_4 treatment and predominance of classical PKU. Interestingly, three of these genotypes contained p. Gln355_Tyr356insGlyLeuGln and three genotypes were associated with peak activities at increased cofactor concentrations.

The analysis of full *PAH* genotypes allowed for the recognition of four orienteering rules, which may set the basis for personalised medicine strategies: i) low residual activity is associated with mostly BH_4 unresponsive classical PKU ii) a lack of a defined area of peak activity leads to a loss of response to BH_4 iii) the occurrence of clinically inconsistent phenotypes with low rates of BH_4 response may be linked to a need for higher BH_4 dosages iv) high residual activity, a defined peak of activity and normal need for BH_4 are associated with mostly responsive mild PKU or mild HPA. Additionally, the observed changes in patterns of the PAH activity landscapes allowed for identifying hypotheses concerning dietary treatment of PAH patients. Patients carrying genotypes that induced a right-shift *in vitro* may be associated with higher phenylalanine tolerance, hence, allowing for a less restrictive regime of the low-phenylalanine diet. On the other hand, carriers of genotypes with narrow working optima and/or shift of the activity landscapes towards lower substrate values *in vitro*, may need particularly strict dietary control. These concepts have to be confirmed by clinical studies that are currently planned. In addition, some genotypes induced an increased need for BH_4. This may have implications on the pharmacological treatment of patients carrying these genotypes as well as on BH_4 response tests. BH_4 dosages used in standard tests may be insufficient to achieve an increase in enzyme activity and a decrease in phenylalanine concentrations.

In conclusion, our work exemplifies how application of molecular genetics may translate into medical practice. It gives new significance to gene analysis of the *PAH* locus in clinical routine and shows the importance of considering functional analysis of full genotypes. The generation of activity landscapes in the metabolic and therapeutic space provided clinically relevant new insights into genotype-related impaired PAH function beyond the known link between residual activity and clinical phenotype. In the presence of specific genotypes we observed different patterns of activity landscape architecture and established orienteering rules for their interpretation. In addition, the work provides a web-based tool to assist clinicians in clinical care of PKU patients. Furthermore, we showed that analysis of genotype-related PAH activity landscapes together with available information on clinical phenotypes may significantly improve clinical management.

Therefore, in future this approach may set the basis for implementation of personalised medicine strategies concerning both, the dietary regimes and pharmacological treatment of PKU patients.

4. DISCUSSION AND OUTLOOK

In 1999, the oral administration of BH_4 was reported to decrease pathologic blood phenylalanine concentrations in a group of PAH-deficient PKU patients. Applicability of this observation to clinical practice was substantiated in a clinical trial study carried out in our centre as well as by numerous case reports and retrospective studies, where predominantly mild phenotypes of PAH deficiency responded to BH_4 and treatment was associated with enhanced PAH enzyme function *in vivo*. Since then, the field of phenylketonuria research focused on the molecular mechanisms underlying BH_4 responsiveness as well as on the mode of action of the BH_4 molecule. Many *in vitro* studies, including work performed in our laboratory, have shown that BH_4 acts as a pharmacological chaperone stabilising the PAH protein and therefore correcting the loss of function phenotype. In 2007 and 2008, the synthetic form of BH_4 (sapropterin dihydrochloride, KUVAN®) was approved by the FDA and the EMA, respectively, as an orphan drug to treat BH_4-responsive PAH deficiency. This new treatment significantly improved the quality of life of PAH-deficient patients responding to BH_4. In many cases, the pharmacological therapy allowed to relax or completely terminate the burdensome phenylalanine-restricted diet. Yet, there is still need for improvement in the pharmacological treatment of PKU patients. First, current therapeutic protocols are not personalised and not always maximum efficacy of the drug is achieved in the individual patient. Second, there is a demand to develop derivatives of the approved drug with better bioavailability, enhanced pharmacodynamics and improved pharmacokinetics. Third, there is a high need to identify novel compounds with pharmacological chaperone effect that would allow for an enlargement of the target group including PKU patients that are not yet amenable to pharmacological treatment.

Among the challenges in the clinical management of PKU patients, the low reliability in prediction of the clinical course, even if genotyping is performed, as well as contradictory data on PAH function associated with single *PAH* mutations, which are derived from databases or case reports, may further complicate pharmacological treatment. These difficulties and pitfalls were, at least in part, due to high allelic heterogeneity and compound heterozygosity among PKU patients, inconsistent phenotypes, the lack of models to establish robust genotype-phenotype correlations and the absence of *in vitro* data on PAH activity of full *PAH* genotypes. Moreover, there was a significant gap of knowledge regarding PAH function in the metabolic context of the individual patient. Differences between medical centres regarding BH_4 loading test protocols additionally added to misperceptions regarding BH_4-responsive and non-responsive *PAH* genotypes. As a consequence, these uncertainties resulted in persistent questioning of the necessity of genotyping of PAH-deficient patients and hindered the progress of the development of personalised treatment strategies in PKU.

Therefore, we sought to generate data on PAH function associated with individual genotypes that reflect the situation *in vivo*. In particular, we aimed to further improve the understanding of the interplay between the phenylalanine substrate, the BH_4 cofactor/drug, the genotype underlying PAH deficiency and PAH enzyme function. We designed a three-step research project where we first developed a continuous fluorescence-based PAH activity assay, which allowed for measuring PAH kinetics in a robust and efficient way. Our assay was then applied to analyse PAH function of recombinantly expressed and purified wild-type and variant PAH proteins at a broad range of phenylalanine and BH_4 concentrations. In the final step, we adapted and modified the PAH activity assay enabling the determination of PAH function of full *PAH* genotypes in a cell based model. This model allowed mimicking the pathological (phenylalanine) and therapeutic (BH_4) states as observed in PKU patients.

The application of the real-time PAH activity assay revealed a previously not described positive cooperativity of the PAH protein towards the cofactor BH_4. In-depth spectroscopic analyses uncovered PAH activating conformational changes as a prerequisite for BH_4-dependent positive cooperativity. In addition, we showed that the presence of phenylalanine affects the BH_4-dependent kinetic properties of PAH. These

results supported the notion that a patient's metabolic state may have a significant effect on the interplay of the BH_4 drug and its target. In other words, the metabolic control of blood phenylalanine levels interferes with the effect of BH_4 on conformation and function of the PAH enzyme.

Next, high-throughput analyses assessed enzyme activity of recombinantly expressed PAH at a broad range of substrate and cofactor concentrations. This 96-well based method enabled the establishment of three dimensional PAH activity landscapes. Here, we determined the optimal working ranges for the wild-type and PAH variants. Furthermore, the landscapes revealed important differences in the regulation of PAH activity by BH_4 and phenylalanine and helped to visualise the interdependence of substrate and cofactor concentrations on variant PAH enzyme activity. Interestingly, we showed that phenylalanine concentrations for optimal working range of PAH are lower whereas BH_4 concentrations are higher than it was previously assumed. From the physiological point of view, maximum PAH activity at lower phenylalanine concentrations seem to be reasonable since liver phenylalanine concentrations are expected to reach levels up to 500 µM under physiological conditions rather than values of 1000 µM, which are used in standard PAH activity assays. A combination of data derived from PAH activity landscapes with data on BH_4 loading tests performed in PAH-deficient patients led to the conclusion that the outcome of a BH_4 loading test may much more vary in function of individual test circumstances than previously assumed. In particular, the level of blood phenylalanine may have a significant effect on the test outcome.

As a third step, we generated activity landscapes of full *PAH* genotypes, expressed in eukaryotic cells under conditions that mimic the patients' metabolic or therapeutic states. The validity of this method may be substantiated by the fact that observations on homozygous genotypes were in line with our previous results on purified variant PAH proteins reflecting single mutations. Several patterns of changes in the architecture of PAH activity landscapes were observed. We combined data from our functional analyses with biochemical, clinical, and therapeutic data derived from PKU patients. This approach allowed for generating hypotheses on the mechanisms behind individual genotypes. In particular, these observations may be of practical relevance for the clinical management of patients and we established orienteering rules for the implementation of personalised medicine strategies in PAH deficient patients. As an example, a shift in maximum PAH activity towards higher BH_4 concentrations observed for some genotypes may indicate a higher need for BH_4 to achieve optimal enzymatic phenylalanine turnover in the patient. As a consequence, in order to avoid false negative results with respect to the drug response, which is defined as the maximum drop in blood phenylalanine levels, BH_4 loading tests could be performed at higher doses of BH_4 or at an increased duration time of the test. On the other hand, our data may indicate that genotypes associated with residual function of less than 3.5 % or associated with a complete loss of a delimitable area of peak activity are associated with non-responsiveness to BH_4. In these cases, extended testing of patients that were non-responsive to BH_4 using standard protocols could be avoided. The observation of a shift of peak activity towards higher phenylalanine concentrations may exemplify how the analysis of genotype-specific PAH activity landscapes could aid in the dietary management of PKU patients. Patients carrying genotypes associated with peak PAH activity at phenylalanine concentrations above the therapeutic target may exhibit suboptimal residual PAH activity in the target range. Given that an increase in blood phenylalanine would be associated with an increase in PAH function, shifting the therapeutic target towards higher phenylalanine values may be associated with a significant increase in phenylalanine tolerance. In addition, we categorised *PAH* genotypes with respect to the associated functional parameters derived from PAH activity landscapes. This allowed to establish genotype-phenotype correlations with respect to the biochemical phenotype (blood phenylalanine) and the pharmacological phenotype (response to BH_4-treatment). However, the establishment of a robust model that would allow for reliable predictions requires the analysis of a higher number of PAH activity landscapes, a project that is in progress in

our laboratory. In order to further increase the evidence of our *in vitro* data, patient studies may address efficiency of BH_4 treatment as well as validity of BH_4 loading tests under consideration of individual genotypes and of varying blood phenylalanine concentrations. Moreover, varying BH_4 concentrations, the effect of BH_4 in healthy individuals, the time to onset of action and the effect duration of the drug will be tested in more detail.

In conclusion, our work pinpoints the significance of genotyping PKU patients in clinical routine and shows the importance of considering functional analysis of full *PAH* genotypes. In addition, our findings underscore the need for more personalised testing procedures addressing individual patient characteristics, the metabolic state and the dosage of the test compound in order to reliably identify BH_4 responsive PAH-deficient patients. Furthermore, our study may set the basis for genotype-specific personalised PKU therapy with respect to both, pharmacological treatment with BH_4 and phenylalanine restricted dietary regimens.

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6. CUMULATIVE THESIS: Publications

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Activation of Phenylalanine Hydroxylase Induces Positive Cooperativity toward the Natural Cofactor*[S]

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Protein misfolding with loss-of-function of the enzyme phenylalanine hydroxylase (PAH) is the molecular basis of phenylketonuria in many individuals carrying missense mutations in the *PAH* gene. PAH is complexly regulated by its substrate L-Phenylalanine and its natural cofactor 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). Sapropterin dihydrochloride, the synthetic form of BH_4, was recently approved as the first pharmacological chaperone to correct the loss-of-function phenotype. However, current knowledge about enzyme function and regulation in the therapeutic setting is scarce. This illustrates the need for comprehensive analyses of steady state kinetics and allostery beyond single residual enzyme activity determinations to retrace the structural impact of missense mutations on the phenylalanine hydroxylating system. Current standard PAH activity assays are either indirect (NADH) or discontinuous due to substrate and product separation before detection. We developed an automated fluorescence-based continuous real-time PAH activity assay that proved to be faster and more efficient but as precise and accurate as standard methods. Wild-type PAH kinetic analyses using the new assay revealed cooperativity of activated PAH toward BH_4, a previously unknown finding. Analyses of structurally preactivated variants substantiated BH_4-dependent cooperativity of the activated enzyme that does not rely on the presence of L-Phenylalanine but is determined by activating conformational rearrangements. These findings may have implications for an individualized therapy, as they support the hypothesis that the patient's metabolic state has a more significant effect on the interplay of the drug and the conformation and function of the target protein than currently appreciated.

Phenylalanine hydroxylase (PAH[4]; EC 1.14.16.1) is a non-heme iron monoxygenase that catalyzes the hydroxylation of the substrate L-Phe to L-Tyr in the presence of its natural cofactor 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) and molecular dioxygen. Mutations in the *PAH* gene can lead to protein misfolding with loss of function and subsequently to phenylketonuria ([MIM 261600]), the most common inborn error of amino acid metabolism in European-descended populations (1, 2). Pharmacological doses of BH_4 can correct protein misfolding in a significant number of patients with PAH deficiency, and sapropterin dihydrochloride, the synthetic form of the natural PAH cofactor, was recently approved as the first pharmacological chaperone to treat phenylketonuria patients (3–5).

The enzyme is a homotetramer built as a dimer of dimers with each subunit consisting of an N-terminal regulatory domain (residues 1–142), a catalytic domain (residues 143–410), and a C-terminal oligomerization domain (residues 411–452). Elaborate functional and kinetic studies have revealed complex enzyme regulation by its substrate and cofactor as well as by phosphorylation (6–8). Binding of the substrate induces a catalytically competent (activated) enzyme, whereas binding of BH_4 leads to formation of an inactive dead-end PAH-BH_4 complex (9–12). These regulatory mechanisms require reversible conformational changes that are transmitted throughout the enzyme upon binding of BH_4 and L-Phe (13). Structural analyses of BH_4 binding revealed that the cofactor interacts with the N-terminal autoregulatory sequence and the pterin binding loop, leading to stabilizing hydrogen bonds and to formation of a binary enzyme-BH_4 complex (14). The largest conformational changes were observed upon binding of L-Phe, where local changes at the active site are propagated globally through hinge-bending motions in the catalytic domain, also altering the position and orientation of bound BH_4 (13, 15) and of the regulatory domain (16).

Analyses of the effects of missense mutations in the *PAH* gene on PAH enzyme kinetic properties have shown that residual enzyme activity generally is high, yet allostery is often disturbed (17–19), with reduced cooperativity for substrate binding, decreased substrate activation, or altered affinity to the substrate and the cofactor. Thus, the evaluation of kinetics and allostery can help to assess to which extent local single amino acid replacements lead to global conformational alterations compromising enzyme function. In this context, comprehensive steady state kinetic analyses beyond single determination of residual enzyme activity are needed to retrace the structural impact of missense mutations on the phenylalanine hydroxylating system. Yet, the current standard activity assays requiring substrate and product separation before L-Tyr detection by radioactivity or fluorescence signals (20–22) are laborious and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials and Methods, Tables 1 and 2, and Figs. 1 and 2.

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[2] Part of an M.D. thesis to be submitted at Ludwig-Maximilians-University, Munich, Germany.

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[4] The abbreviations used are: PAH, phenylalanine hydroxylase; IFE, inner filter effect; BH_4, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine.

time consuming. In addition, designed as end-point measurements, these discontinuous assays assume a linear range of activity for the time period chosen, although variations of temperature and pH as well as concentrations of enzyme, substrate, and cofactor can dramatically change the linearity of a reaction over the fixed time window (23). Therefore, we aimed to develop an automated continuous real-time assay of PAH activity. Our new fluorescence-based multi-well assay has given rise to the possibility of evaluating PAH kinetics and allostery faster and more efficiently but as precisely and accurately as the standard methods. Surprisingly, by application of this technique, the data obtained for BH_4-dependent PAH kinetics did not fit to the well accepted model of a single hyperbolic function (Michaelis-Menten kinetic model). Instead, a good fit was found using a sigmoidal binding curve (Hill kinetic model). Although positive cooperativity for the binding of L-Phe has been extensively studied (24, 25), cooperativity toward BH_4 has not been described to date. However, cofactor-dependent kinetic studies were routinely performed using the non-activated PAH enzyme (19, 26), whereas an L-Phe preincubated (activated) enzyme was applied in our experiments.

Thus, we aimed to characterize BH_4-dependent PAH kinetics in more detail and to investigate whether activation of PAH is a prerequisite for the positive cooperativity observed. Real-time fluorescence kinetic analyses using L-Phe-activated and non-activated PAH were performed. Furthermore, genetic variants of PAH, which are structurally preactivated by single amino acid replacements, were analyzed. To discriminate between non-cooperative and cooperative enzyme kinetics, in-depth model comparisons by nonlinear regression analysis were conducted with fitting of the data to the Michaelis-Menten or the Hill kinetic model.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant PAH Enzymes—The cDNA of human phenylalanine hydroxylase (EST clone obtained from Imagines, formerly RZPD, Germany) was cloned into the pMAL-c2E and pMAL-c2X expression vectors (New England Biolabs) encoding an N-terminal maltose-binding protein (MBP) tag and enterokinase or Factor Xa cleavage sites, respectively. *PAH* mutants were constructed by site-directed mutagenesis as described (17). *Escherichia coli* DH5α were transformed with the expression vector for wild-type and mutant MBP-PAH fusion proteins. Proteins were purified by affinity chromatography (MBP Trap, GE Healthcare) followed by size-exclusion chromatography using a HiLoad 26/60 Superdex 200 column (GE Healthcare) on an ÄKTAxpress system as previously described (17). The isolated tetrameric fusion proteins were collected, and protein concentrations were determined spectrophotometrically using $\epsilon_{280\ (1\ mg/ml)} = 1.63$. Tetrameric fusion protein was cleaved by factor Xa (10 units of factor Xa:1 mg of fusion protein) at 4 °C for 16 h and isolated by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare). Protein concentrations of the cleaved tetrameric PAH were determined spectrophotometrically using $\epsilon_{280\ (1\ mg/ml)} = 1.0$.

Verifying Direct Fluorescence Detection of Enzymatic L-Tyr Production—To verify the spectral separation needed for the direct in-well fluorescence detection of enzymatic L-Tyr production, L-Tyr (0–150 μM) (Sigma) in 17 mM NaHepes, pH 7.3, was added to all wells of a 96-well plate (NUNC F96) containing a reaction mixture with 1 mg/ml catalase (Sigma), 10 μM ferrous ammonium sulfate (Fe^{2+}) (Fluka), and L-Phe (0–1000 μM) (Sigma) yet lacking the apoenzyme and BH_4. L-Tyr fluorescence intensity was subsequently measured using a fluorescence photometer (FLUOstar OPTIMA, BMG Labtech) at an excitation wavelength of 274 nm and an emission wavelength of 304 nm. Individual experiments were assayed as triplicates. All concentrations mentioned refer to a final volume of 204 μl.

Quantification of L-Tyr Production—For the quantification of L-Tyr production, standards consisting of L-Tyr (0–463 μM) and L-Phe (547 μM) in 17 mM NaHepes, pH 7.3, 1 mg/ml catalase, and 10 μM ferrous ammonium sulfate were measured before each experiment using the fluorescence photometer (excitation 274 nm, emission 304 nm). Individual experiments were assayed as triplicates before enzyme kinetic measurements on each experimental day. All concentrations mentioned refer to a final volume of 204 μl.

Analysis of the Inner Filter Effect (IFE) of BH_4—A 96-well plate was prepared with L-Tyr (0–150 μM) in 17 mM NaHepes, pH 7.3, and a standard reaction mixture containing the standard concentration of 1 mM L-Phe in 17 mM NaHepes, pH 7.3, 1 mg/ml catalase, 10 μM ferrous ammonium sulfate, and 15 mM NaHepes, pH 7.0. Subsequent to injection of BH_4 (0–125 μM) (6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin, Schircks Laboratories) stabilized in 2 mM dithiothreitol (DTT) (Fluka), L-Tyr fluorescence intensity was measured using the fluorescence photometer (excitation 274 nm, emission 304 nm). The IFE of BH_4 was corrected by defining a correction factor for each BH_4 concentration added to the reaction mixture: $q = f$ (at each $[BH_4]$)/f (fluorophore alone), where f is the fluorescence intensity, and q is the correction factor for the substrate concentration (27, 28). All measurements were assayed as triplicates before enzyme kinetic measurements on each experimental day. All concentrations mentioned refer to a final volume of 204 μl.

Time-dependent Enzyme Activity Measurement—For time-dependent enzyme activity measurements, the assay was performed with and without preincubation of the enzyme with 1 mM L-Phe. A reaction buffer containing 1 mg/ml catalase, 10 μM ferrous ammonium sulfate, and the tetrameric MBP-PAH fusion protein (0.01 mg/ml) was prepared. After preincubation with 1 mM L-Phe in 22.35 mM NaHepes, pH 7.3, for 5 min at 25 °C, the reaction was initiated by the addition of 75 μM BH_4 stabilized in 2 mM DTT. For enzyme activity measurements without L-Phe preincubation, the reaction was initiated by simultaneous injection of 1 mM L-Phe and 75 μM BH_4.

Time-dependent substrate production was assessed by detection of the increase in L-Tyr fluorescence intensity at an excitation wavelength of 274 nm and an emission wavelength of 304 nm using a fluorescence spectrophotometer (Cary Eclipse, Varian). All concentrations mentioned refer to the final concentration in a 204-μl reaction mixture.

Multiwell Enzyme Activity Assay with and without L-Phe Preactivation—For PAH activity measurement, L-Phe in 22.35 mM NaHepes, pH 7.3, was added to 12 wells of a 96-well plate with varying L-Phe concentrations (0–1000 μM) or at a constant L-Phe concentration (1 mM) using the injection system of a fluorescence photometer. A reaction buffer containing 1 mg/ml catalase, 10 μM ferrous ammonium sulfate, and the tetrameric MBP-PAH fusion protein (0.01 mg/ml) was prepared and injected in all 12 wells. After preincubation with L-Phe for 5 min at 25 °C, the reaction was initiated by the addition of BH_4 stabilized in DTT for a final concentration of 75 μM BH_4 with varying L-Phe concentrations (0–1000 μM) or varying BH_4 concentrations (0–125 μM) at 1 L-Phe concentration (1 mM) and 2 mM DTT.

For enzyme activity measurements without L-Phe preincubation, the reaction buffer was prepared and injected to 12 wells. The reaction was initiated by simultaneous injection of varying L-Phe concentrations (0–1000 μM) and 1 BH_4 concentration (75 μM) or 1 L-Phe concentration (1 mM) and varying BH_4 concentrations (0–125 μM).

Steady state kinetics of PAH were determined at 25 °C and a 60-s measurement time per well. Substrate production was assessed by detection of the increase in L-Tyr fluorescence intensity at an excitation wavelength of 274 nm and an emission wavelength of 304 nm using a fluorescence photometer (FLUOstar OPTIMA, BMG Labtech) and assayed as duplicates on 3 consecutive days. Fluorescence intensity signals were corrected for the quenching effect of BH_4. All concentrations mentioned refer to the final concentration in a 204 μl reaction mixture.

For all enzyme activity measurements, fluorescence intensity was recorded and, after subtraction of the blank reaction, converted to enzyme activity units (nmol Tyr/min × mg protein) using the standard curve obtained by L-Tyr concentration measurements. Data were analyzed by nonlinear regression analysis using the single hyperbolic model (Michaelis-Menten kinetic model),

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

where v is the observed rate of enzyme catalysis, V_{max} is the maximum rate of enzyme catalysis, $[S]$ is the substrate concentration, and K_m is the substrate concentration at which $V_{max}/2$ is reached and the sigmoidal kinetic model (Hill kinetic model),

$$v = \frac{V_{max}}{\left(1 + \left[\frac{EC_{50}}{[S]}\right]^h\right)} \quad \text{(Eq. 2)}$$

where v is the observed rate of enzyme catalysis, V_{max} is the maximum rate of enzyme catalysis, $[S]$ is the substrate concentration, EC_{50} is the substrate concentration at which $V_{max}/2$ is reached, and h is the Hill coefficient (GraphPad Prism 4.0c). Values are given as the mean ± S.E. of three independent experiments. The coefficient of variation was determined as the ratio of the S.D. to the mean value. Comparison of model fitting was performed using the F-test (GraphPad Prism QuickCal), residuals of values, the S.D. of the residuals ($S_{y.x}$), the runs test, and the square of residuals (R^2) (see supplemental Tables S1 and S2 and Fig. S2) (29–34).

Tryptophan Fluorescence Measurements—For tryptophan fluorescence emission scans, wild-type PAH, dimeric PAH 103–427, and variant PAH R68S were diluted to 11 μM subunits PAH (0.6 μg/μl) in 20 mM NaHepes and 200 mM NaCl, pH 7.0, containing 10 μM ferrous ammonium sulfate and 2 mM DTT. Fluorescence measurements were performed using a fluorescence spectrophotometer (Cary Eclipse, Varian) at an excitation wavelength of 295 nm with excitation and emission slits set to 2.5 and 5 nm, respectively.

Differential Scanning Fluorimetry—Differential scanning fluorimetry analyses were performed on a Cary Eclipse fluorescence spectrophotometer equipped with a temperature-controlled Peltier multicell holder (Varian). Denaturation of 6 μM MBP-PAH subunits diluted in 20 mM NaHepes and 200 mM NaCl, pH 7.0, containing 2 mM DTT was performed by scanning a temperature range of 25 to 70 °C at a rate of 1.2 °C/min. In the cases indicated, L-Phe was added to a final concentration of 1 mM. Changes in 8-anilino-1-naphtalenesulfonic acid fluorescence emission (Sigma) were monitored at 500 nm (excitation 395 nm, slit widths 5.0/10.0 nm). The phase transitions of three to eight independent experiments were determined, and the respective transition midpoints were calculated using the Boltzmann sigmoidal equation. Transition midpoints for wild-type and variant PAH with and without L-Phe were plotted and compared using a paired *t* test.

RESULTS

Direct Fluorescence Detection of Enzymatic L-Tyr Production—To date measurement of PAH activity is routinely performed using a standard reaction mixture containing the apoenzyme, Fe^{2+}, L-Phe, and BH_4 followed by time-consuming chromatographic separation of substrate and product. Yet differences in the fluorescence properties of the aromatic amino acids L-Phe and L-Tyr, such as emission and excitation wavelengths as well as the quantum yield, would allow for spectral separation of these substances even in a mixed solution.

To determine spectral separation of the two substances, we assessed fluorescence signal intensities of varying L-Tyr concentrations at 304 nm (35), the L-Tyr emission wavelength, as a function of increasing L-Phe concentrations. We showed that direct in-well detection of L-Tyr was unaffected by the various L-Phe concentrations used in our assay (Fig. 1*A* and supplemental Fig. 1*A*). This was true for all L-Tyr concentrations expected in the following enzyme kinetic measurements. However, L-Tyr fluorescence signal intensities decreased with increasing BH_4 concentrations (supplemental Fig. 1*B*), suggesting an IFE of BH_4 on L-Tyr excitation and emission. Therefore, we determined specific correction factors on the basis of the factorial decrease of signal intensity for every BH_4 concentration added to account for the IFE (Fig. 1*B*) (27, 28). Evaluation of the IFE of BH_4 and calculation of the correction factor for each BH_4 concentration added to the reaction mixture was performed before each enzyme kinetic measurement (Fig. 1*B*).

The analysis of steady state enzyme kinetics using direct in-well detection of L-Tyr production revealed a time-dependent change of enzyme activity upon the addition of BH_4, with an

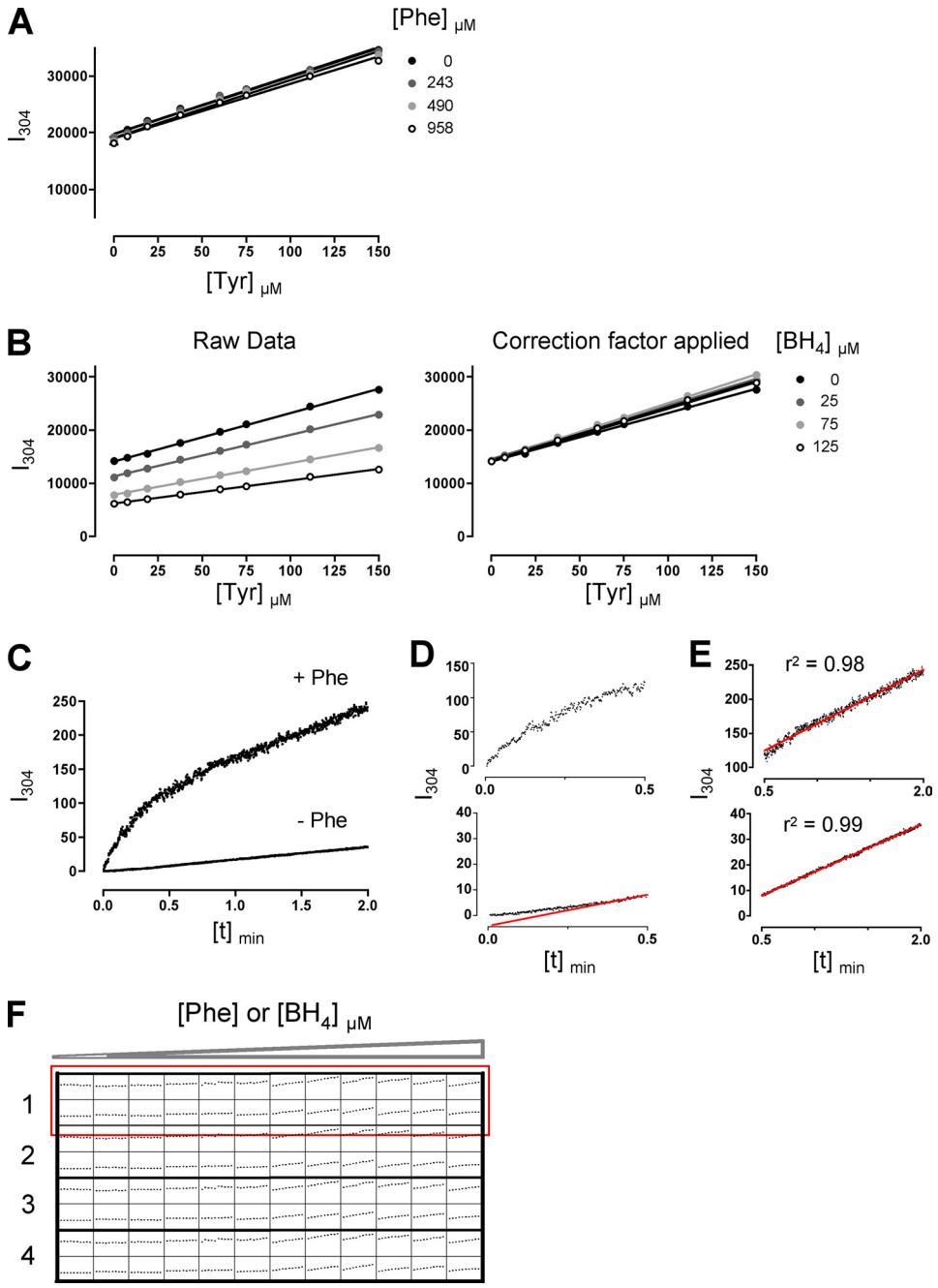

FIGURE 1. **A novel continuous assay for the measurement of PAH activity.** *A*, fluorescence intensity (I_{304}) of L-Tyr concentrations (0–150 μM) with increasing L-Phe concentrations (0, 243, 490, and 958 μM) is shown. L-Tyr fluorescence intensity was not influenced by increasing L-Phe concentrations, confirming spectral separation of the two substances in one mixed solution. Values are given as the mean ± S.E. of three independent measurements. *B*, quenching of L-Tyr fluorescence intensity by BH_4 is shown. Measurement of L-Tyr (0–150 μM) subsequent to the addition of increasing BH_4 concentrations (0, 25, 75, and 125 μM), revealed an inner filter effect of BH_4 on L-Tyr excitation and emission (*left panel*). For each BH_4 concentration used in the assay, a correction factor was calculated according to the factorial decrease in signal intensity to account for the inner filter effect (*right panel*). Values are given as the mean ± S.E. of three independent measurements. *C*, continuous measurement of time-dependent wild-type PAH kinetics with and without preincubation of the enzyme with 1 mM L-Phe are shown. *D*, L-Phe preincubation (activation) led to burst-phase kinetics within the first 30 s of the reaction (*top*) followed by a linear phase of L-Tyr production. Without L-Phe preincubation, an initial lag-phase before steady state enzyme kinetics was found (*bottom*, a *red line* was used to guide the eye). *E*, the time frame chosen for the measurement of steady state enzyme kinetics between 30 and 120 s showed a linear rate of reaction (*top*, with L-Phe preincubation; *bottom*, without L-Phe preincubation). *F*, a 96-well plate for sequential measurement of PAH enzyme kinetics is shown. Direct in-well measurements of enzyme kinetics of up to four different PAH enzymes (*numbers 1–4*) were performed by the sequential analysis of 2 rows, consisting of 24 wells (*red box*). Each row contained the PAH enzyme varying substrate concentrations (0–1 mM) and one cofactor concentration (75 μM) or varying cofactor concentrations (0–125 μM) and one substrate concentration (1 mM). Repeated cycles allowed kinetic measurements of 24 wells over a time period of 60 s.

initial high activity burst phase (Fig. 1*C* and Fig. 1*D*, *top*) followed by a linear steady state rate of catalysis for the L-Phe preincubated (activated) enzyme (Fig. 1*C* and Fig. 1*E*, *top*). In contrast, the non-activated enzyme showed an initial lag-phase (Fig. 1*C* and Fig. 1*D*, *bottom*) before a linear phase of L-Tyr production (Fig. 1*C* and Fig. 1*E*, *bottom*).

In addition, time-dependent initial velocity measurements require substrate turnover to remain less than 10% that of the

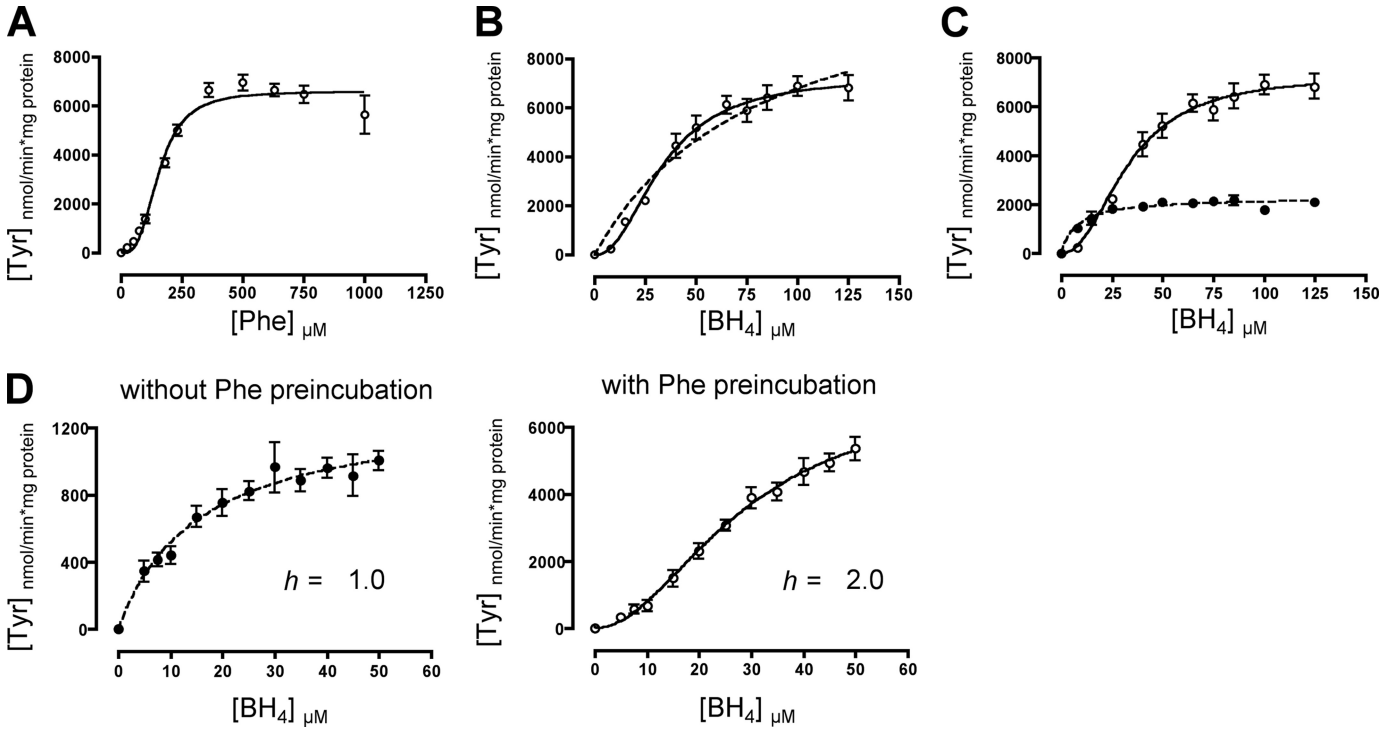

FIGURE 2. **Measurements of wild-type PAH kinetics.** *A*, reaction rates at variable L-Phe concentrations (0–1 mM) and one BH_4 concentration (75 μM) are shown. Before initiation of the reaction by BH_4, the enzyme was preincubated for 5 min at 25 °C with L-Phe to activate the enzyme. Nonlinear regression analysis was performed using the Hill equation. *B* and *C*, reaction rates at variable BH_4 concentrations (0–125 μM) and one L-Phe concentration (1 mM) are shown. *B*, data obtained for the L-Phe preincubated (activated) enzyme were evaluated using the Michaelis-Menten equation (*dashed line*) and the Hill equation (*solid line*). *C*, a comparison of enzyme kinetics measured using the non-activated (●) and the activated (○) wild-type PAH is shown. The non-activated enzyme showed non-cooperative binding of BH_4. The activated enzyme indicated positive cooperativity for the binding of BH_4. *D*, enzyme kinetics of non-activated and activated PAH at variable BH_4 concentrations (0–50 μM) and one L-Phe concentration (1 mM) are shown. Data obtained for the non-activated enzyme were fit to the Michaelis-Menten equation (*left panel*). Data of the activated enzyme followed Hill kinetics (*right panel*). For all enzyme activity measurements, fluorescence intensity was recorded and after subtraction of the blank reaction converted to enzyme activity units (nmol L-Tyr/min × mg protein) using the standard curve obtained by L-Tyr concentration measurements. Values are given as the mean ± S.E. of three independent experiments.

substrate concentrations added to the reaction mixture (23). A time frame of 60 s for measurement of steady state enzyme kinetics starting 30 s after the addition of BH_4 proved to best fulfill the criteria of linearity and limited substrate turnover.

To allow for an accurate and efficient performance of the enzyme kinetic assay, liquid handling and signal detection were automated using a multi-well fluorescence detection device with an integrated injection system. Various substrate or cofactor concentrations were injected for L-Phe or BH_4-dependent kinetics, respectively, and process automation enabled sequential duplicate measurements of up to four different PAH enzymes, resulting in reduced time for experimental preparation and procedure (Fig. 1*F*).

Thus, real-time measurement of PAH product formation revealed that direct in-well detection of L-Tyr during the catalytic reaction without prior separation of substrate and product is feasible when BH_4 quenching is taken into account. In addition, real-time kinetics give more insights into both pre-steady state and steady state kinetics of phenylalanine hydroxylation, allowing thorough analysis of PAH enzyme kinetics.

A Continuous PAH Activity Assay Reveals BH_4-dependent Cooperativity—The newly developed continuous assay was used to determine enzyme kinetic parameters at varying substrate concentrations (L-Phe, 1–1000 μM) and a constant cofactor concentration (BH_4, 75 μM) or at varying cofactor concentrations (BH_4, 0–125 μM) and a constant substrate concentration (L-Phe, 1 mM).

Measurement of L-Phe-dependent PAH kinetics showed sigmoidal behavior for the activated enzyme (Fig. 2*A*) as previously described (24, 36–38). Enzyme kinetic parameters were calculated by nonlinear regression analysis using the Hill equation, accounting for substrate cooperativity and compared with the results of a standard discontinuous PAH activity assay (Table 1) (17). Values for V_{max} were substantially higher when determined by the continuous assay (6598 nmol Tyr/min × mg protein) as compared with the discontinuous assay (3470 nmol Tyr/min × mg protein). However, apparent affinity to the substrate ($S_{0.5}$ 156 μM), cooperativity (Hill coefficient, h_{Phe} 3.0), and substrate activation (activation-fold 2.8) showed virtually identical results in both experiments (discontinuous assay; $S_{0.5}$ 155 μM, h_{Phe} 3.0, activation-fold 3.0). As expected from previous studies using recombinant human PAH (26, 37) and the rat enzyme (36), enzyme kinetic parameters obtained without L-Phe preincubation gave different results (Table 1). V_{max} was markedly lower (2533 nmol Tyr/min × mg protein), and the apparent affinity of the enzyme to L-Phe (K_m 318 μM) was reduced. In addition, binding of L-Phe to the non-activated enzyme was found to be non-cooperative (h_{Phe} 1.0). This is in concordance with studies using surface plasmon resonance (25). The enzyme kinetic parameters determined for both acti-

TABLE 1

Comparison of L-Phe-dependent enzyme kinetic parameters obtained by standard PAH activity assay and direct in-well detection of L-Tyr production

Steady state kinetic parameters of WT MBP-PAH fusion protein are shown. Apparent affinities for L-Phe ($S_{0.5}$, K_m, and the Hill-coefficient (h_{Phe}) as a measure of cooperativity are shown. Measurements were performed with (+) and without (−) L-Phe preincubation of the enzyme. Enzyme kinetic parameters were determined at variable L-Phe concentrations (0–1000 μM) and standard BH_4 concentrations (75 μM). CV, coefficient of variation, defined as the ratio of the S.D. to the mean value.

	L-Phe preincubation	V_{max}	CV	$S_{0.5}$	CV	K_m	h_{Phe}	Activation fold[a]
		nmol L-Tyr/min × mg protein	%	μM	%	μM		
WT[b]	−	495[c]		318[c]			1.5[c]	—
	+	1550[c]		154[c]			2.2[c]	3.1[c]
	+	3470 ± 75[d]	2	155 ± 6[d]	4		3.0[d]	3.0[d]
WT[e]	−	2533 ± 217		—		318 ± 68	1.0	—
	+	6598 ± 190	3	156 ± 9	6		3.0	2.8

[a] Fold increase in PAH activity by L-Phe preincubation calculated at the standard L-Phe (1 mM) and BH_4 (75 μM) concentrations.
[b] Measurement by standard discontinuous PAH activity assay (HPLC and fluorimetric detection). Values are given as the mean ± S.E. of three independent experiments.
[c] From Knappskog *et al.* (37); activation fold was calculated from V_{max}.
[d] From Gersting *et al.* (17).
[e] Measurement by continuous PAH activity assay (direct in-well fluorescence detection). Values are given as the mean ± S.E. of four independent experiments.

TABLE 2

Comparison of BH_4-dependent enzyme kinetic parameters obtained by standard PAH activity assay and direct in-well detection of L-Tyr production

Steady state kinetic parameters of WT MBP-PAH fusion protein are shown. Apparent affinities for BH_4 ($C_{0.5}$) and the Hill-coefficient (h_{BH4}) as a measure of cooperativity are shown. Measurements were performed with (+) and without (−) L-Phe preincubation of the enzyme. Enzyme kinetic parameters were determined at variable BH_4 concentrations (0–125 μM) and standard L-Phe concentrations (1 mM). CV, coefficient of variation, defined as the ratio of the S.D. to the mean value.

	L-Phe preincubation	V_{max}	CV	K_m	CV	$C_{0.5}$	CV	h_{BH4}
		nmol L-Tyr/min × mg protein	%	μM	%	μM	%	
WT[a]	+	3425 ± 139[b]	4	24 ± 3[b]	12.5	—		1.0
WT[c]	−	2277 ± 84		8 ± 1		—		1.0
	+	7288 ± 282	4	—		33 ± 2	6	2.2

[a] Measurement by standard discontinuous PAH activity assay (HPLC and fluorimetric detection). Values are given as the mean ± S.E. of three independent experiments.
[b] From Gersting *et al.* (17).
[c] Measurement by continuous PAH activity assay (direct in-well fluorescence detection). Values are given as mean ± S.E. of four independent experiments.

vated and non-activated PAH were well comparable with the data found in previous studies (Table 1) (17, 37).

Surprisingly, the data obtained for BH_4-dependent PAH kinetics did not fit to the well accepted Michaelis-Menten kinetic model. Instead, the data showed a sigmoidal behavior, indicating BH_4-dependent cooperativity (Fig. 2*B*). Although BH_4-dependent kinetics was as yet mainly determined using the non-activated enzyme (19, 26), we conducted the assay utilizing the L-Phe-preincubated (activated) enzyme. To examine whether the Hill kinetic model describing BH_4-dependent kinetic parameters depends on the activation state of the enzyme, the assay was run with and without prior incubation of the enzyme by L-Phe, and the results were compared with data from the literature (Table 2). Similar to L-Phe-dependent enzyme kinetics, V_{max} of the activated enzyme was markedly lower in the discontinuous assay (3425 nmol Tyr/min × mg protein) when compared with the continuous assay (7288 nmol Tyr/min × mg protein), but the values for the apparent affinity to the ligand were comparable for both methods used (K_m 24 μM; $C_{0.5}$ 33 μM) (Table 2). Although the analysis of the non-activated enzyme showed a reduction in V_{max} (2277 nmol Tyr/min × mg protein), an increased apparent affinity to BH_4 (K_m 8 μM) was observed. In addition, steady state kinetic analysis of activated PAH indicated BH_4-dependent-positive cooperativity (Hill coefficient, h_{BH4} 2.2), whereas Michaelis-Menten kinetics for the non-activated PAH was confirmed (Fig. 2*C*). To obtain a better resolution of the range that best discriminates between both kinetic models, the assay was repeated in the limits of 0–50 μM BH_4 (Fig. 2*D*). The data obtained for the activated enzyme clearly followed Hill kinetics (h_{BH4} 2.0). This was in contrast to the non-activated enzyme, where nonlinear regression analysis showed hyperbolic kinetics following the Michaelis-Menten kinetic model (h_{BH4} 1.0).

The enzyme kinetic parameters determined for L-Phe- and BH_4-dependent enzyme kinetics were well comparable with previous studies using the standard discontinuous assay (17, 19), confirming the accuracy of our newly developed continuous assay. Furthermore, we aimed to determine the assay precision and calculated the coefficient of variation for all enzyme kinetic parameters of the activated enzyme, comparing the standard discontinuous with the continuous assay. The coefficients of variation for both L-Phe- and BH_4-dependent kinetic parameters were similar for the two assays, revealing equal precision in enzyme kinetic measurements (Tables 1 and 2).

In conclusion, allosteric parameters obtained using the newly developed PAH activity assay were well comparable with results from standard discontinuous assays. The assay accuracy as well as precision confirmed the suitability of this method for the evaluation of enzyme kinetic parameters of PAH. In addition, PAH kinetic analysis using the continuous assay gave evidence for BH_4-dependent cooperativity with PAH activation as a prerequisite.

BH_4-dependent Cooperativity Relies on an Activated Structural Conformation of PAH—Three variant PAH enzymes, R68S, V106A, and the dimeric double-truncated form 103–427 (19, 37, 39, 40), were used to characterize the interrelation of enzyme activation and BH_4-dependent cooperativity in more detail. In particular, we aimed to investigate whether the shift in enzyme kinetics from Michaelis-Menten to the Hill kinetic model depends on the presence of the L-Phe substrate itself or on structural attributes of the activated enzyme. It is known that substrate activation induces conformational changes (16,

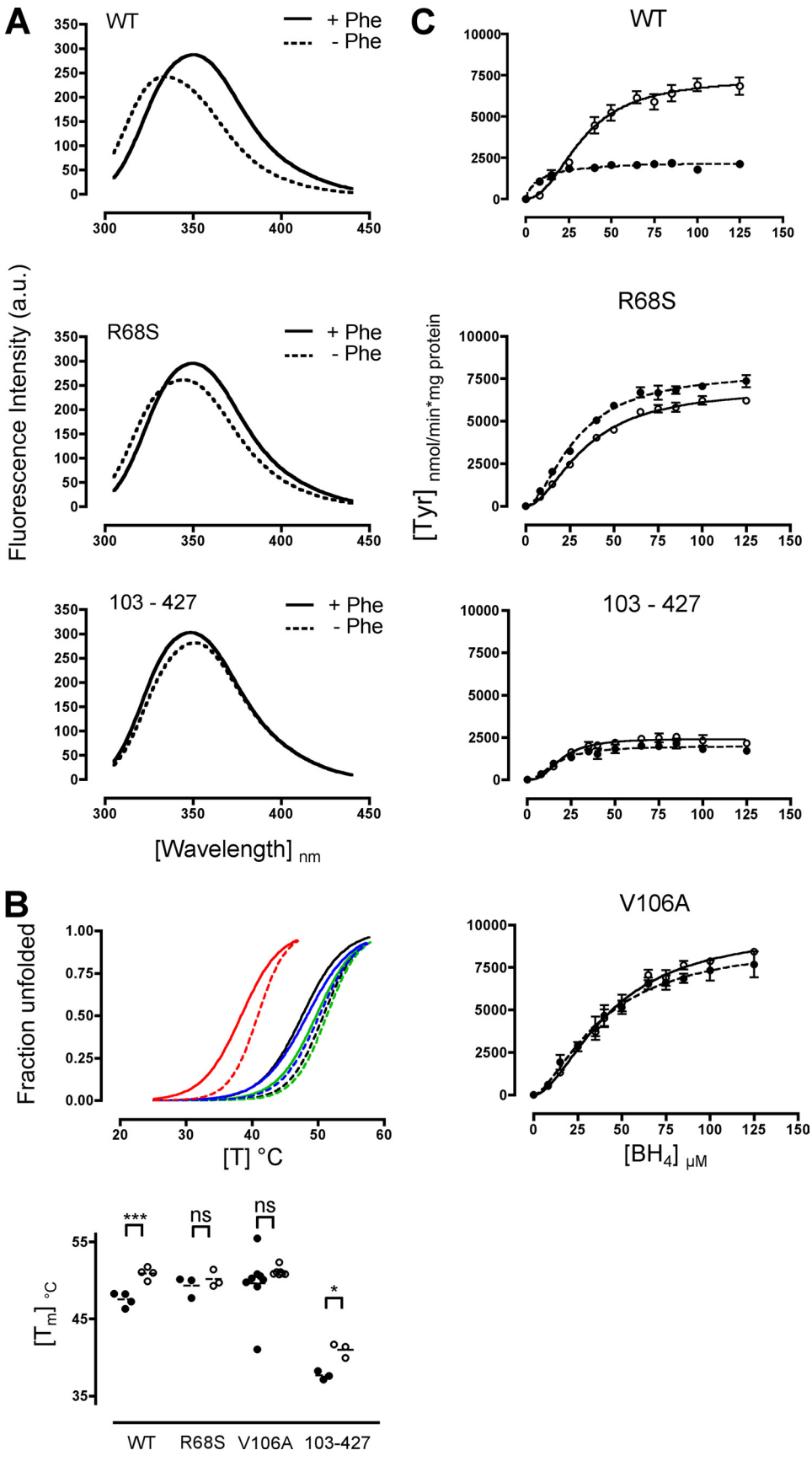

TABLE 3

Trp emission scans of PAH cleaved by factor Xa

Tryptophan fluorescence emission spectra were obtained with and without L-Phe (1 mM) preincubation of the cleaved WT PAH and preactivated variants. Fluorescence measurements were performed using a fluorescence spectrophotometer at an excitation wavelength of 295 nm with excitation and emission slits set to 2.5 and 5 nm, respectively. a.u., arbitrary units.

	Without L-Phe preincubation		With L-Phe preincubation	
	Wavelength	Fluorescence intensity	Wavelength	Fluorescence intensity
	nm	*a.u.*	*nm*	*a.u.*
WT	340	247	351	296
R68S	345	266	351	304
103–427	350	288	350	310

TABLE 4

Mean transition midpoints of thermal denaturation assays

Calculation of transition midpoints from differential scanning fluorimetry of WT PAH and preactivated variants with and without 1 mM L-Phe. Transition midpoints were calculated using the Boltzmann sigmoidal equation. Transition midpoints are given as the mean ± S.E. of three to four for V106A eight independent experiments. Mean transition midpoints with and without L-Phe were compared using a paired *t* test, two-tailed. NS, not significant.

	T_m		*p* value
	Without L-Phe preincubation	With L-Phe preincubation	
	℃		
WT	47.49 ± 0.46	50.90 ± 0.38	$p = 0.0006$
R68S	49.28 ± 0.78	50.15 ± 0.64	NS
V106A	49.63 ± 1.40	51.11 ± 0.21	NS
103–427	37.68 ± 0.32	40.99 ± 0.54	$p = 0.0345$

37, 41–45) that convert the enzyme from a low activity T-state to a high activity R-state (Monod Wyman Changeux model) (46, 47). This is accompanied by an increase in quantum yield and a red-shifted emission maximum of the Trp-120 residue (48, 49). To determine the level of preactivation, structural attributes of the variants were compared with wild-type PAH. Spectral differences with and without preincubation by the substrate were utilized to assess the activation state.

Tryptophan emission scans of wild-type PAH and the variants R68S and 103–427 were performed. As expected, wild-type PAH revealed a red shift in the emission maximum (340 to 351 nm) and an increase in intrinsic tryptophan fluorescence (247–296 arbitrary units) upon the addition of L-Phe (Fig. 3*A*) (Table 3). Both variants showed a red-shifted emission maximum in the absence of L-Phe, which was more pronounced for the dimeric variant. Upon L-Phe preincubation, R68S yielded an emission maximum of the activated wild-type (351 nm), whereas the emission maximum of the dimeric PAH remained unchanged (350 nm). Both variants showed an increase in quantum yield but to a lesser extent than observed for wild-type PAH. Furthermore, we determined activation-induced structural rearrangements for all three variants by thermal unfolding using temperature-dependent differential scanning fluorimetry (Fig. 3*B*). Although the addition of L-Phe induced a highly significant increase in the transition midpoint for wild-type PAH ($p = 0.0006$), no significant increase was found for the variants R68S and V106A (Fig. 3*B*) (Table 4). These variants already showed increased transition midpoints at the activated wild-type level even without L-Phe preincubation. The dimeric PAH 103–427, although showing markedly decreased transition midpoints in general, revealed a significant increase when L-Phe was added ($p = 0.0345$). Taken together, all variants displayed structural characteristics that are indicative of mutation-induced conformational preactivation but to different extents.

According to the results on structural preactivation, evaluation of the activation-fold determined by the continuous assay (Fig. 3*C*) (Table 5) confirmed functional preactivation of the variants without L-Phe preincubation (R68S, 0.9; V106A, 1.0; 103–427, 1.3). Nonlinear regression analysis of BH_4-dependent kinetics of all three preactivated variants followed the Hill model as shown for activated wild-type PAH. Although distinctly positive, the Hill-coefficients ranging from 1.6 to 2.1 were lower than that determined for activated wild-type PAH (*h*, 2.2). The dimeric variant 103–427 showed a V_{max} (1980 nmol Tyr/min × mg protein) comparable with that of the non-activated wild-type PAH, which did not change markedly upon L-Phe preincubation (2421 nmol Tyr/min × mg protein). This is in contrast to an increase by 3.2-fold observed for wild-type PAH preincubated with L-Phe. However, for the variants R68S and V106A, a V_{max} comparable with the activated wild-type PAH was found without L-Phe preincubation, and no further increase was measured when the substrate was present. R68S and V106A without L-Phe preincubation showed lower cofactor affinities than the non-activated wild-type PAH; however, the values were at the same level as determined for the L-Phe preincubated wild-type PAH. $C_{0.5}$ of the dimeric PAH 103–427 was 2-fold higher as compared with the non-activated wild-type PAH. Notably, no marked changes in sigmoidal behavior and kinetic parameters were observed for all variants irrespective of whether they were preincubated by L-Phe (Fig. 3*C*) (Table 5). In summary, analyses of structurally preactivated variants substantiated BH_4-dependent positive cooperativity of

FIGURE 3. **Determining the activated structural and functional conformation.** *A*, intrinsic tryptophan fluorescence emission spectra of the Factor Xa cleaved wild-type PAH, variant PAH R68S, and 103–427 are shown. Fluorescence emission spectra were acquired in the absence (*dashed line*) or presence (*solid line*) of 1 mM L-Phe. The excitation wavelength for Trp fluorescence measurements was 295 nm, with an excitation and emission slit of 2.5 and 5 nm, respectively. *a.u.*, arbitrary units. *B*, differential scanning fluorimetry of the wild-type PAH and variant PAH R68S, V106A, and 103–427 fusion protein are shown. Denaturation of PAH was monitored by scanning a temperature range of 25 to 70 °C at a rate of 1.2 °C/min. Changes in 8-anilino-1-naphtalenesulfonic acid fluorescence emission were monitored at 500 nm (excitation 395 nm, slit widths 5.0/10.0 nm). The fraction unfolded of three to four independent experiments for wild-type, R68S, and 104–427 and seven to eight independent experiments for V106A without L-Phe (*solid line*) and with 1 mM L-Phe (*dashed line*) were determined (*top panel*; wild-type (*black*), R68S (*blue*), V106A (*green*), and 103–427 (*red*)), and the respective transition midpoints were calculated using the Boltzmann sigmoidal equation. For comparison of the transition midpoints, a paired *t* test, two-tailed, was used. Transition midpoints for wild-type and variant PAH, with (○) and without (●) L-Phe preincubation were plotted and compared (*bottom panel*) (*ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *C*, enzyme activity measured for the wild-type PAH and the preactivated variants R68S, V106A, and 103–427 without preincubation (●) and with preincubation of the enzyme (○) with 1 mM L-Phe before initiation of the reaction by the addition of BH_4. Data obtained for the non-preincubated and preincubated enzymes followed the Hill kinetic model as shown for the activated wild-type PAH. For all enzyme activity measurements, fluorescence intensity was recorded and after subtraction of the blank reaction converted to enzyme activity units (nmol of L-Tyr/min × mg protein) using the standard curve obtained by L-Tyr concentration measurements. Values are given as the mean ± S.E. of three independent experiments.

TABLE 5

Comparison of BH_4-dependent enzyme kinetic parameters of variant PAH proteins with and without L-Phe preincubation

Steady state kinetic parameters of variant MBP-PAH fusion proteins were determined by direct in-well activity measurements. Apparent affinities for BH_4 ($C_{0.5}$) and the Hill-coefficient (h) as a measure of cooperativity are shown. Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-Phe concentrations (1 mM) with and without preincubation of the enzyme with L-Phe (1 mM).

	Without L-Phe preincubation				With L-Phe preincubation			
	V_{max} [a]	K_m	$C_{0.5}$ [a]	h_{BH_4}	V_{max} [a]	$C_{0.5}$ [a]	h_{BH_4}	Activation fold [b]
	nmol L-Tyr/min × mg protein	μM	μM		*nmol L-Tyr/min × mg protein*	μM		
WT	2277 ± 84	8 ± 1	—	1.0	7288 ± 282	33 ± 2	2.2	2.8
R68S	7928 ± 232	—	29 ± 1	1.8	6940 ± 225	34 ± 2	1.8	0.9
V106A	9041 ± 489	—	40 ± 3	1.6	9773 ± 534	43 ± 4	1.8	1.0
103–427	1980 ± 100	—	16 ± 2	2.1	2421 ± 73	19 ± 1	2.6	1.3

[a] Values are given as the mean ± S.E. of three independent measurements.
[b] Fold increase in PAH activity by L-Phe preincubation calculated at the standard L-Phe (1 mM) and BH_4 (75 μM) concentrations.

the activated enzyme, where the kinetic model does not rely on the presence of L-Phe but is determined by activating conformational rearrangements.

Evaluation of Model Fitting—To validate the experimental data on conditions that determine PAH cooperativity, an extended evaluation of model fitting by nonlinear regression analysis of BH_4-dependent kinetics was performed. The test parameters goodness of fit (R^2), root mean square ($S_{y.x}$), runs test, and residuals of values were compared, and an F-test was run to discriminate between the two nested kinetic models Michaelis-Menten and Hill for activated and non-activated PAH, respectively.

First we analyzed non-activated PAH. A simple calculation of goodness of fit (R^2) did not allow for distinction between the two models used for data analyses (R^2_{MM} 0.97; R^2_H 0.97) (supplemental Table 1). The residuals of values (supplemental Fig. 2) and, thus, the S.D. of the residuals also showed no marked improvement of data description by the more complicated Hill equation. Although the runs test showed a marginally significant deviation of the data from the Michaelis-Menten model ($p = 0.048$), the F-test proved the simpler Michaelis-Menten kinetics to be the correct model for data analysis of BH_4-dependent kinetics of non-activated PAH (F ratio 2.86; $p = 0.125$) (Table 6). This was confirmed by refined analysis of BH_4-dependent kinetics in the limits of 0–50 μM. As before, R^2, $S_{y.x}$, and the residuals of values did not allow for a clear distinction between the two models (supplemental Table 1). However, no significant deviation of the data from any of the two equations could be determined by the runs test. Yet again, the F-test revealed that the data were best described by the simpler Michaelis-Menten kinetic model (F ratio 0.58; $p = 0.463$) (Table 6).

In contrast to non-activated PAH, enzyme activation by L-Phe now resulted in marked differences of the two models even by goodness of fit (R^2_{MM} 0.96; R^2_H 0.99) (supplemental Table 1) (Table 6). In addition, evaluation of the data by the Hill equation revealed a decrease of $S_{y.x}$ by ~2-fold, and the residuals of values showed less fluctuation (supplemental Fig. 2). The runs test showed no significant deviation from any of the two models used, but comparison by the F-test resulted in a highly significant p value (F ratio 274.28; $p < 0.0001$) (Table 6) favoring Hill kinetics. Detailed analysis at low BH_4 concentrations (0–50 μM) additionally uncovered a significant deviation of the data from Michaelis-Menten kinetics in the runs test ($p = 0.024$) (Table 6) (supplemental Table 1 and Fig. 2). Taken together, the evaluation of model fitting provided clear evidence for BH_4-dependent positive cooperativity of activated PAH, whereas the BH_4-dependent kinetics of the non-activated enzyme followed the non-cooperative Michaelis-Menten model.

TABLE 6

Comparison of two nested non-linear regression models

F-test for two nested models after measurement of wild-type PAH kinetics without and with preincubation of the enzyme with L-Phe. Enzyme kinetics were measured with L-Phe (1 mM) and BH_4 (0–125 μM and 0–50 μM). The F-test assumes that the Michaelis-Menten equation is a simpler case of the Hill equation. If the simpler model is correct, the F ratio is near 1.0. To verify the correctness of the more complicated model (if the F ratio is >1.0), the p value is calculated. If the p value is less than the traditional significance level of 5%, it can be concluded that the data do not randomly fit to the more complicated model but fit significantly better to Hill than to Michaelis-Menten kinetics.

BH_4	F-test		
	L-Phe preincubation	F ratio	p value
μM			
0–125	−	2.86	0.125
0–125	+	274.28	<0.0001
0–50	−	0.58	0.463
0–50	+	62.79	<0.0001

Next, we aimed to learn whether the presence of the L-Phe substrate has an impact on the kinetic model beyond L-Phe induced conformational changes upon PAH activation. To validate structural preactivation of R68S, V106A, and of the dimeric double-truncated 103–427 PAH, we first analyzed whether BH_4-dependent kinetic data would fit significantly better to the more complex Hill equation even without L-Phe preincubation. Indeed, we identified an increase in R^2, a more than 2-fold decrease in $S_{y.x}$, and a significant deviation of the Michaelis-Menten model in the runs test for the variants R68S and V106A (supplemental Table 2). Only the dimeric 103–427 showed a less pronounced reduction in $S_{y.x}$ and no discrimination between the two models in the runs test. In all cases fluctuation of the residuals of values was lower using the Hill equation (supplemental Fig. 2). This was in line with the results obtained by the F-test ($p < 0.01$) (Table 7). Hence, the kinetic data fit significantly better to the more complicated Hill equation, indicating substrate-independent structural preactivation. Second, model fitting of the structurally activated variants was compared with and without prior incubation with L-Phe in the next step. All in all, the presence of L-Phe only negligibly changed the test results. Only for the dimeric 103–427, $S_{y.x}$ was markedly lower upon evaluation by sigmoidal kinetics, and the runs test classified Michaelis-Menten as an incorrect

TABLE 7

Comparison of two nested non-linear regression models with and without preincubation of preactivated variants

F-test for two nested models after measurement of enzyme kinetics without and with preincubation of the enzyme with L-Phe. Enzyme kinetics were measured with L-Phe (1 mM) and BH_4 (0–125 μM). The F-test assumes that the Michaelis-Menten equation is a simpler case of the Hill equation. If the simpler model is correct, the F ratio is near 1.0. To verify the correctness of the more complicated model (if the F ratio is >1.0), the *p* value is calculated. If the *p* value is less than the traditional significance level of 5%, it can be concluded that the data do not randomly fit to the more complicated model but fit significantly better to Hill than to Michaelis-Menten kinetics.

	F-test		
	L-Phe preincubation	F ratio	*p* value
R68S	−	58.02	<0.0001
R68S	+	69.63	<0.0001
V106A	−	24.00	<0.001
V106A	+	35.44	<0.001
103–427	−	10.66	<0.01
103–427	+	40.23	<0.001

model (supplemental Table 2). Again, all data provided evidence for a correct data description by the Hill kinetic model (supplemental Table 2 and Fig. 2) (Table 7).

In summary, extended analysis and comparison of the two non-linear regression models Michaelis-Menten and Hill revealed non-cooperative kinetics of the non-activated enzyme, whereas activation of the enzyme clearly induced cooperativity. Moreover, L-Phe preincubation did not have a significant impact on the kinetic model of structurally activated variant PAH.

DISCUSSION

In this study we established a new method for the evaluation of PAH kinetic parameters, allowing for real-time detection of enzyme activity. Continuous assays are the safest means of determining reaction velocity from the slope of a plot of signal *versus* time (23). However, many assays used for the evaluation of PAH enzyme kinetics are discontinuous due to chromatographic separation of the substrate from the product before product detection. We aimed to establish the measurement of L-Tyr production without separation from the substrate L-Phe. The spectral properties of the aromatic amino acids and differences in quantum yield allowed for direct detection of L-Tyr uninfluenced by L-Phe concentrations. However, the IFE of BH_4 at the excitation and emission maxima of L-Tyr (274 and 304 nm, respectively) had to be taken into account. By analyzing the IFE of BH_4 on L-Tyr fluorescence in the entire range of concentrations used in the assay, a concentration-dependent correction factor was defined (27, 28). This facilitated accurate and precise quantification of L-Tyr product formation inside the assay reaction mixture. In addition, all substances required to perform the activity assay were applied by means of an integrated injection system. Although equally precise, this led to marked time reduction in sample preparation. Furthermore, sequential duplicate measurements subsequent to sample injections of up to four different PAH proteins in a 96-well format substantially increased the throughput of enzyme kinetics analyses. The addition of various substrate and cofactor concentrations as well as their injection at different time-points proved this method to be very flexible, allowing for numerous assay conditions in one single run.

By applying the new technique, we observed burst-phase kinetics of preactivated and lag-phase kinetics of non-activated PAH. The discontinuous assays used previously measured product formation from the initiation of the reaction to a defined end point, and burst- and lag-phase kinetics were not taken into consideration. However, pre-steady state kinetics should not be ignored as they may lead to erroneous interpretations regarding the existence of cooperativity (50). A complete model describing the enzyme reaction is a prerequisite for comprehensive understanding of the mechanisms involved in pre-steady state kinetics. Assumptions to approach such a model have been made using a prokaryotic monomeric PAH (51). Yet this enzyme lacks the regulatory properties of the oligomeric multidomain human protein and is, therefore, not applicable to human PAH. Therefore, we decided to assess steady state kinetics at a 1-min time frame after the burst-phase and provided a linear rate of reaction velocity for the non-activated and the activated enzyme while remaining within 10% of substrate turnover.

The data of continuous measurements were compared with our results obtained previously by the discontinuous assay as well as to data described in the literature. Differences in V_{max} between the discontinuous and the continuous assay were found. These variations may be due to the different methods used, yet additional aspects such as different time frames for measurement of steady state kinetics and the improvement of protein purification methods in our laboratory within the last years play an important role and may further explain these findings. Notably, large differences in enzyme activities, ranging up to 7-fold, can also be found in the literature (16, 37, 52, 53). However, kinetic parameters describing apparent affinity and enzyme allostery ($S_{0.5}$, $K_m/C_{0.5}$, Hill coefficient (*h*), and activation-fold) were similar to results obtained using discontinuous assays and to results previously described (17, 37), confirming the accuracy of our newly developed continuous assay. Furthermore, calculation of the coefficient of variation proved the new assay to be as precise as the standard discontinuous assays applied.

Interestingly, data points did not fit to the Michaelis-Menten kinetic model when the method was applied to determine BH_4-dependent enzyme kinetics. Instead, a good fit was found applying the Hill kinetic model. All previous studies using different methods had shown hyperbolic non-cooperative binding kinetics of the cofactor to PAH (17, 19, 25, 53). When applying the continuous assay, BH_4-dependent kinetics of wild-type PAH revealed positive cooperativity. However, our experiments were performed with an L-Phe-preincubated (activated) enzyme, whereas in most of the previous studies the non-activated enzyme had been used. To elucidate whether PAH activation determines cofactor dependent cooperativity, we compared enzyme kinetics of non-activated and of activated PAH. In agreement with previous findings, the continuous assay without L-Phe preincubation resulted in hyperbolic binding kinetics of BH_4. Together with the observed BH_4-dependent cooperativity of activated PAH, these results suggested that cooperativity of BH_4 depends on an activated state of the enzyme.

Thus, we investigated whether the shift in enzyme kinetics from Michaelis-Menten to the Hill kinetic model depends on

the presence of the L-Phe substrate itself or on structural attributes of the activated enzyme. This was dissected by means of conformationally preactivated genetic variants of PAH (R68S, V106A, dimeric 103–427). Using two different spectroscopic techniques we analyzed local and global effects on protein structure by mutation/truncation or by L-Phe, respectively, and correlated this with enzyme kinetic parameters. Preincubation of wild-type PAH with L-Phe leads to a series of structural and functional changes resulting in enzyme activation (16, 37, 41–45). On the structural level these include a red-shifted and enhanced tryptophan emission and a right-shifted thermal denaturation profile. On the functional level an increase in V_{max}, a decreased apparent affinity, and a switch from non-cooperative to positive cooperative kinetics accounted for activation. All three variants displayed characteristics of structural and functional preactivation but to varying degrees. Preactivation of the variants was reflected by the activation-fold with values ranging from 0.9 to 1.3. Furthermore, all variants displayed clear positive cooperativity without prior incubation with the substrate. In the presence of L-Phe some selective structural changes for single variants were observed, *i.e.* a minor shift in tryptophan emission for R68S and a significantly enhanced transition midpoint of the thermal denaturation for 103–427. However, no variant showed decisive changes in enzyme kinetic parameters upon L-Phe preincubation. This observation held true irrespective of whether the parameter was at the same level as activated wild-type PAH. These data provide evidence that the variants are activated at a structural level and that L-Phe does not have any additional effect on their activity and cooperativity. We conclude that the conformation associated with preactivation accounts for positive cooperativity where L-Phe induces activating conformational changes that in turn lead to allostery. The addition of the substrate to the assay, however, does not induce cooperativity by itself.

Mathematical analyses of the data obtained by enzyme kinetic measurements were used to substantiate our findings on the comparison of the kinetic models (29, 34, 54–58). A simple calculation of best-fit parameters for enzyme kinetic data (R^2, root mean square, runs test) did not always allow for a clear distinction between the Michaelis-Menten and the Hill model. However, the application of an F-test made evident to which biological mechanism the kinetic data are linked with highest probability (29–32). Even though a more complex model like the Hill equation would always fit the experimental data better than a simpler model, the F-test revealed that kinetics of non-preincubated wild-type PAH were not described significantly better by this model. However, analysis of the L-Phe-activated enzyme gave a significant p value in the F-test for the Hill equation and, thus, proved positive cooperativity. These findings were corroborated when enzyme kinetic analyses were focused on the area of distinct sigmoidality, *i.e.* on the range of 0 to 50 μM BH_4. Statistical analyses of model fitting for preactivated PAH variants verified BH_4-dependent cooperativity even without prior incubation with L-Phe. Taken together, mathematical analyses of the cofactor-dependent enzyme kinetic data confirmed that the non-activated enzyme follows Michaelis-Menten kinetics, whereas the activated enzyme shows cooperativity.

Cooperativity of PAH to the L-Phe substrate is reflected by a Hill coefficient of >3.0 and has previously been described to be propagated throughout the whole tetramer (59). Alterations in the orientation of the oligomerization domain transfer cooperative activating conformational changes from one to the other dimer. This requires a switch between a low affinity "T-state" conformation to a high affinity "R-state" conformation at elevated L-Phe concentrations following the model proposed by Monod, Wyman, and Changeux. The Hill coefficients of BH_4-dependent kinetics determined for the L-Phe preincubated (activated) wild-type PAH and the preactivated variants in this study were ~2.0. The current study may, thus, allow speculating that enzyme activation leads to positive cooperativity in BH_4 binding. The dissimilar Hill coefficients for substrate and cofactor binding may also show that cooperative binding of BH_4 follows different conformational alterations propagating cooperativity than found for L-Phe binding. This may explain that positive cooperativity was not only observed for the tetrameric enzyme but also for the truncated PAH 103–427 that lacks the regulatory and the oligomerization domain and, thus, only exists in dimeric form. However, whether there is cooperative behavior of PAH upon BH_4 binding in the strict mechanistic sense or hysteresis leading to cooperative kinetics could not be fully elucidated. Further crystallization and NMR studies are needed for a thorough understanding of the impact of L-Phe and BH_4 binding on cooperative allosteric changes in PAH structure.

In conclusion, the development of a novel method for real-time measurement of PAH activity provided accurate, fast, and efficient PAH enzyme kinetic measurements in a 96-well format. Application of this method for wild-type PAH revealed BH_4-dependent positive cooperativity previously not described. Spectroscopic assessment of activating conformational changes and statistical evaluation of model-fitting disclosed PAH activation as a prerequisite for BH_4-dependent positive cooperativity. BH_4 has recently been approved as a pharmacological chaperone drug in the treatment of phenylketonuria. We showed that the presence of L-Phe affects the BH_4-dependent kinetic properties of PAH. These findings may, thus, have implications for an individualized therapy, as they support the hypothesis that patient metabolic state may have a more significant effect on the interplay of the drug and the conformation and function of the target protein than currently appreciated.

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SUPPLEMENTAL MATERIALS AND METHODS

Real-time multi-well kinetic analysis. The application of the enzyme reaction mixture to a 96-well plate with varying substrate concentrations was automated using a multi-well fluorescence detection device with integrated injection system (FLUOstar OPTIMA, BMG Labtech). The reaction was initiated by sequential injection of BH_4 with and without simultaneous injection of L-Phe, allowing the determination of L-Tyr production in all wells after the following scheme. Following the injection of BH_4, L-Phe and a final mixture of the samples, 2 rows consisting of 24 wells were measured in repeated cycles over a time period of 60 seconds (Fig. 1F), the time frame evaluated to be within the linear range of action for the enzyme concentration used (Fig. 1E).

Defining the protein concentration. Enzyme activity was measured using the assay described under standard conditions with 1 mM L-Phe and 75 µM BH_4, with varying protein concentrations (0.005-3 mg/ml). Production of L-Tyr was measured directly over a time period of 10 minutes. Initial velocity was determined as the time period in which less than 10 % of the substrate is utilized. For all protein concentrations, steady state kinetics occurred 30 seconds after initiation of the reaction and burst-phase kinetics. With decreasing protein concentrations, steady state kinetics were linear over increasing periods of time, yet sensitivity for the measurement of L-Tyr production decreased. All measurements were assayed as triplicates and all concentrations refer to a final volume of 204 µl.
A final protein concentration of 0.01 mg/ml with an optimal time range of 60 seconds for linear range of reaction was chosen for all activity assays (Fig. 1E).

FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1. **Verifying spectral separation and the inner filter effect of BH_4.** *A,* To verify spectral separation needed for the direct in-well fluorescence detection of enzymatic L-Tyr production, 190 µM L-Tyr were measured at the L-Tyr excitation wavelength 304 nm as a function of increasing L-Phe concentrations. L-Tyr fluorescence signal intensities uninfluenced by increasing L-Phe concentrations are shown. *B,* A decrease in L-Tyr fluorescence intensity with increasing BH_4 concentrations, as applied in the BH_4-dependent enzyme activity assay, is shown. Specific correction factors were determined on the basis of the factorial decrease of signal intensity for every BH_4 concentration added to the reaction mixture to account for its inner filter effect.

SUPPLEMENTAL FIGURE 2. **Comparison of non-linear regression model fitting by the Michaelis-Menten and the Hill kinetic model.** Residuals of values calculated using the Michaelis-Menten and Hill-equation for non-linear regression analysis of the enzyme kinetic data obtained for the wild-type PAH, R68S, V106A and the dimeric PAH 103-427, without (-) and with (+) preincubation of the enzyme with 1 mM L-Phe, are shown. Enzyme kinetics were determined with varying BH_4 concentrations (0-50 and 0-125 µM) and one L-Phe concentration (1 mM). The residuals of values were used as the primary criteria for selecting one model over the other. In case of indifferent residuals of values, the simpler Michaelis-Menten kinetic model was chosen for data-fitting because a more complex model like the Hill equation would always fit the experimental data better than a simpler model. A good model fitting reduces the residuals to zero. The model best describing the enzyme kinetic data obtained is shown in green. While BH_4-dependent enzyme kinetics of the non-activated wild-type PAH were best described by the Michaelis-Menten equation, the activated wild-type PAH and the preactivated variants followed the Hill kinetic model.

SUPPLEMENTAL TABLE 1. Comparison of two nested non-linear regression models

		Henri-Michaelis-Menten-Equation			Hill-Equation		
[BH_4] μM	L-Phe preincubation	R^2	Sy.x	Runs	R^2	Sy.x	Runs
0-125	-	0.9660	127.5	3[§] (P = 0.048)	0.9742	117.8	8 n.s.
0-125	+	0.9609	559.2	5 n.s.	0.9943	225.6	7 n.s.
0-50	-	0.9802	46.60	6 n.s.	0.9813	47.75	10 n.s.
0-50	+	0.9806	287.0	3[§] (P = 0.024)	0.9973	112.1	6 n.s.

Analysis of goodness of fit, Sy.x and runs test for two nested models after measurement of wild-type PAH kinetics without and with preincubation of the enzyme with L-Phe. Enzyme kinetics were measured with L-Phe (1 mM) and BH_4 (0-125 μM and 0-50 μM).

[§] If the data were randomly scattered above and below the curve, there is less than 5% chance of observing so few runs. The data systematically deviate from the curve. Most likely, the data were fit to the wrong equation. $P < 0.05$

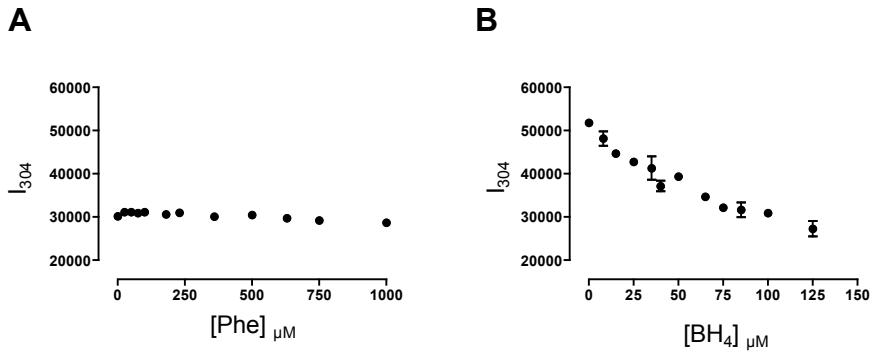
SUPPLEMENTAL TABLE 2. Comparison of two nested non-linear regression models with and without preincubation of preactivated variants

		Henri-Michaelis-Menten-Equation			Hill-Equation		
	L-Phe preincubation	R^2	Sy.x	Runs	R^2	Sy.x	Runs
R68S	-	0.9788	412.4	3[§] (P = 0.048)	0.9972	160.3	5 n.s.
R68S	+	0.9762	385.1	3[§] (P = 0.047)	0.9973	138.2	9 n.s.
V106A	-	0.9850	341.4	3[§] (P = 0.024)	0.9956	195.2	9 n.s.
V106A	+	0.9790	454.7	3[§] (P = 0.024)	0.9954	224.9	7 n.s.
103-427	-	0.9231	197.4	5 n.s.	0.9628	144.8	7 n.s.
103-427	+	0.9177	265.0	3[§] (P = 0.033)	0.9836	124.6	7 n.s.

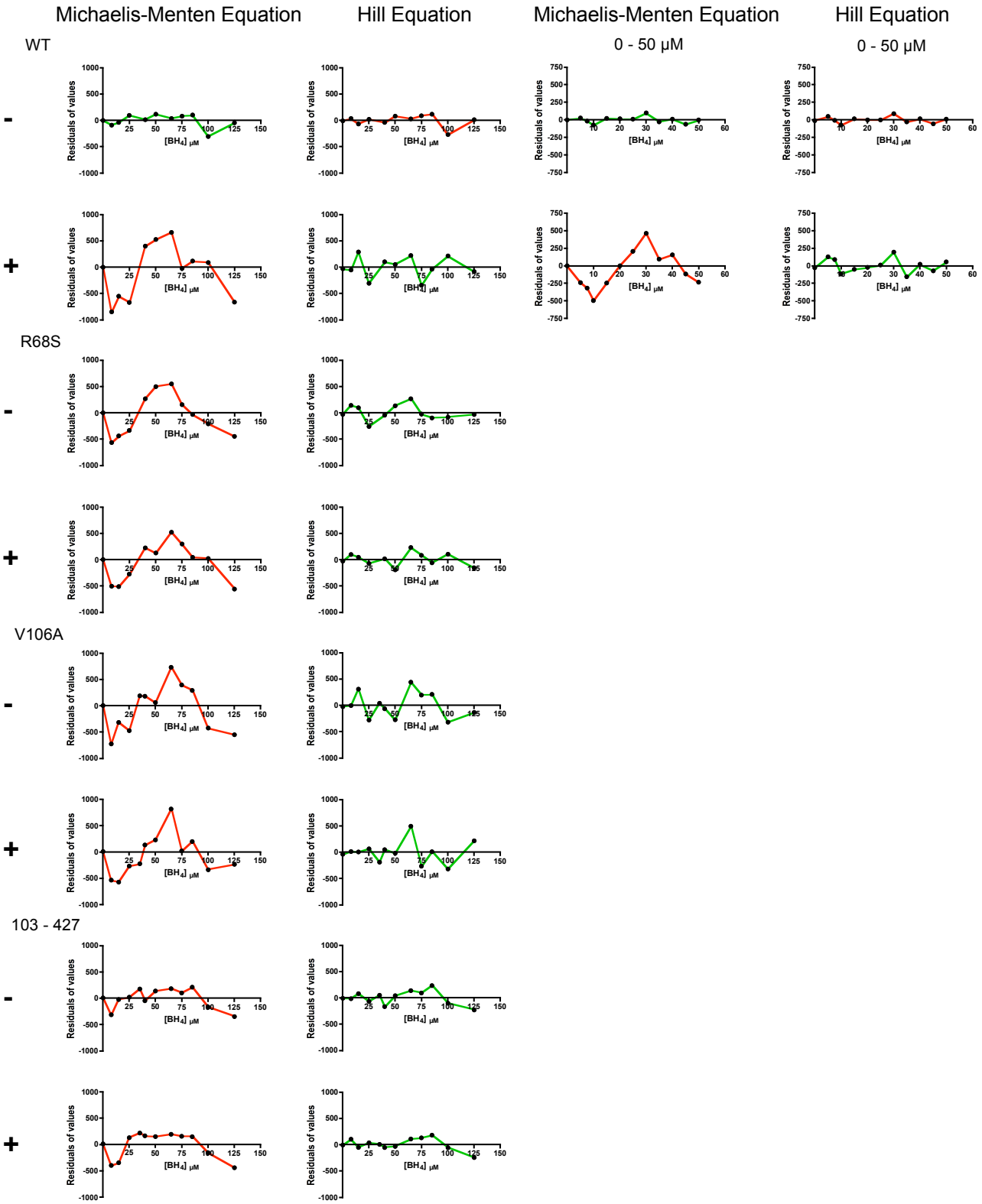
Analysis of goodness of fit, Sy.x and runs test for two nested models after measurement of enzyme kinetics without and with preincubation of the enzyme with L-Phe. Enzyme kinetics were measured with L-Phe (1 mM) and BH_4 (0-125 μM).

[§] If the data were randomly scattered above and below the curve, there is less than 5% chance of observing so few runs. The data systematically deviate from the curve. Most likely, the data were fit to the wrong equation. $P < 0.05$

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



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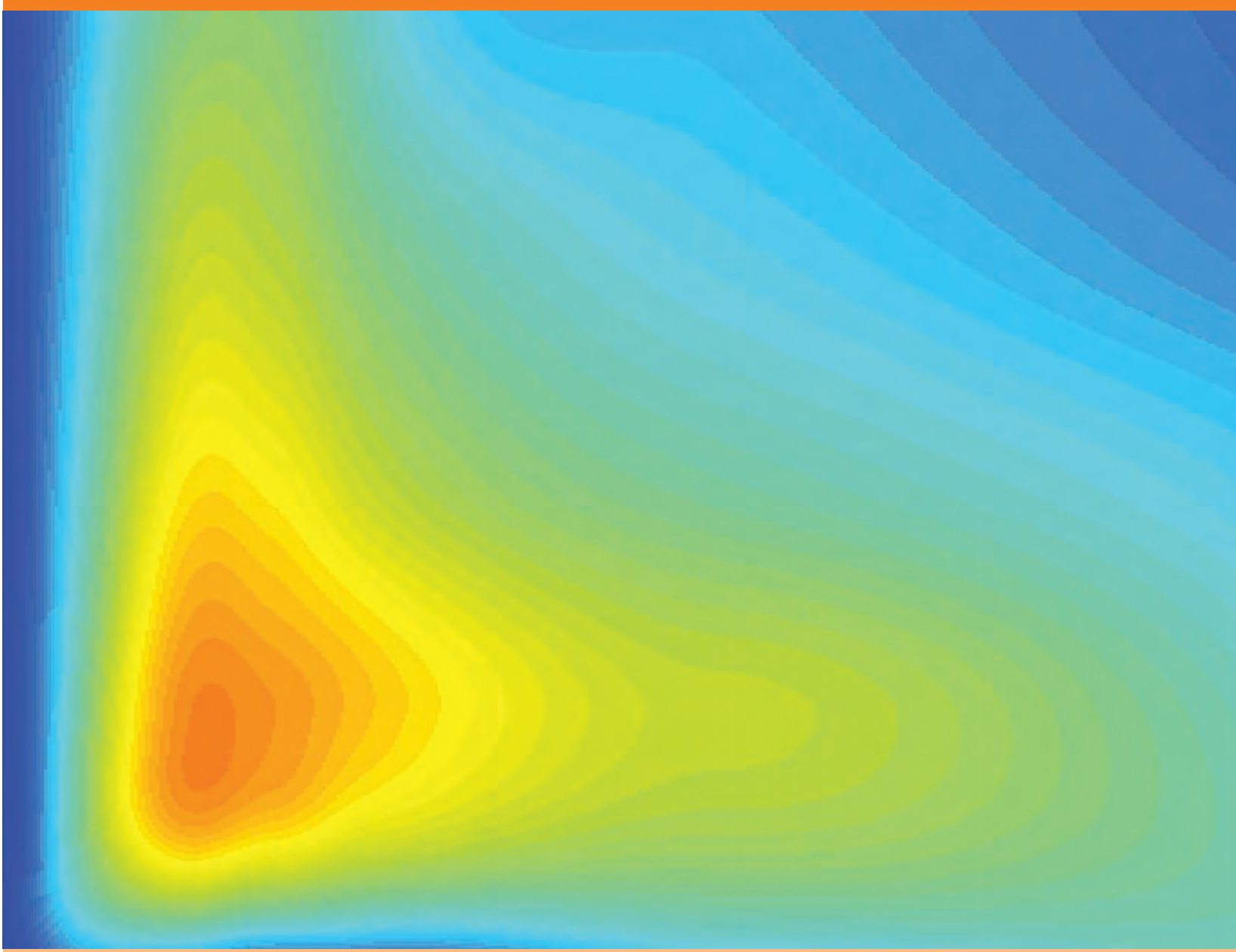

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The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response

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The discovery of a pharmacological treatment for phenylketonuria (PKU) raised new questions about function and dysfunction of phenylalanine hydroxylase (PAH), the enzyme deficient in this disease. To investigate the interdependence of the genotype, the metabolic state (phenylalanine substrate) and treatment (BH_4 cofactor) in the context of enzyme function *in vitro* and *in vivo*, we (i) used a fluorescence-based method for fast enzyme kinetic analyses at an expanded range of phenylalanine and BH_4 concentrations, (ii) depicted PAH function as activity landscapes, (iii) retraced the analyses in eukaryotic cells, and (iv) translated this into the human system by analyzing the outcome of oral BH_4 loading tests. PAH activity landscapes uncovered the optimal working range of recombinant wild-type PAH and provided new insights into PAH kinetics. They demonstrated how mutations might alter enzyme function in the space of varying substrate and cofactor concentrations. Experiments in eukaryotic cells revealed that the availability of the active PAH enzyme depends on the phenylalanine-to-BH_4 ratio. Finally, evaluation of data from BH_4 loading tests indicated that the patient's genotype influences the impact of the metabolic state on drug response. The results allowed for visualization and a better understanding of PAH function in the physiological and pathological state as well as in the therapeutic context of cofactor treatment. Moreover, our data underscore the need for more personalized procedures to safely identify and treat patients with BH_4-responsive PAH deficiency.

INTRODUCTION

Phenylketonuria (PKU; OMIM #261600), the most common inborn error of amino acid metabolism, is an autosomal recessive disorder caused by phenylalanine hydroxylase (PAH) deficiency (PAH; EC 1.14.16.1) (1). Currently, 627 different disease-causing mutations in the *PAH* gene are known (www.pahdb.mcgill.ca; www.hgmd.org) and some of these were shown to lead to protein misfolding with loss of function (2–4).

Pharmacological doses of 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4), the enzyme's natural cofactor, can reduce blood phenylalanine concentrations (5–10) and increase phenylalanine oxidation rates *in vivo* (11) in patients with PAH deficiency without any evidence of cofactor deficiency. The compound was shown to rescue the biochemical phenotype by correcting PAH misfolding and was thus classified as a pharmacological chaperone (4,12). Following these studies, sapropterin dihydrochloride, the synthetic form of the natural PAH cofactor, was approved as an orphan drug to alleviate or even replace burdensome dietary treatment in a significant share of patients with PKU due to PAH deficiency (13–16).

However, not all patients show BH_4 responsiveness. Since the introduction of sapropterin dihydrochloride as a pharmacological treatment, many attempts to predict BH_4 responsiveness from a patient's genotype were made (17–19).

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Combined evidence seems to support the view that residual enzyme activity of individual mutations may be a parameter that—to some extent—allows for discrimination of responders and non-responders (19–22). In two studies performed on Croatian and Turkish populations, calculation of mean *in vitro* residual enzyme activity of the two PAH variants arising from both alleles led to the identification of some responders with high accuracy, whereas patients with two fully inactive alleles were found to be always non-responders (19,22). Yet, in many instances, no clear genotype–phenotype correlation is found pointing to contributing factors such as the patient's age or initial blood phenylalanine concentrations (17,19). Marked inconsistencies as to BH_4 responsiveness were observed for two of the most common PKU mutations associated with this particular phenotype, R261Q and Y414C (17) and for R252W, L48S and R241C homozygous genotypes (22). In addition, interpretation of genotype effects is hampered by the fact that >80% of BH_4 responders are compound heterozygous (17).

Hydroxylation of the substrate L-phenylalanine to the product L-tyrosine with the use of the natural cofactor BH_4 and molecular oxygen is a complexly regulated catalytic mechanism. While L-phenylalanine induces activating conformational rearrangements, BH_4 leads to the formation of an inactive dead-end PAH–BH_4 complex (23–26). Recent studies unraveled new aspects concerning the interplay of phenylalanine and BH_4 having an impact on enzyme kinetics as well as on drug response. Adoption of an enzyme activity assay using a newly developed fluorescence-based real-time PAH activity assay revealed cooperativity of recombinant PAH towards the BH_4 cofactor. This was restricted to the phenylalanine substrate-activated state of the enzyme indicating that conformational rearrangements of the PAH protein induce cooperative binding (27). Moreover, investigations of the BH_4 effect in two different mouse models for BH_4-responsive PAH deficiency provided evidence that the response to BH_4 in terms of rescue of enzyme function by increasing the effective intracellular PAH amount also depends on phenylalanine concentrations and on the underlying genotype (12). These results suggested that the influence of substrate and cofactor concentrations in the presence of a certain genotype on enzyme function and on the response to the pharmacological chaperone BH_4 might be of even more functional and therapeutic relevance than previously estimated. In addition, the BH_4 loading test routinely used worldwide to assess BH_4 responsiveness in PAH deficiency (28,29) was shown to result in a number of inconsistencies that are still not well understood. In some but not all cases, this may be due to inadequate BH_4 dosage or to initial blood phenylalanine concentrations near to the physiological state. Unfortunately, this may lead to false negative results precluding cofactor treatment and thus increasing burden of treatment in some BH_4-responsive patients.

To address these issues, (i) we adapted our new fluorescence-based method for fast enzyme kinetic analyses to cover an expanded range of phenylalanine and BH_4 concentrations when compared with previous analytical setups enabling the investigation of the mutual impact of substrate and cofactor on PAH enzyme kinetics, (ii) we depicted these data as activity landscapes uncovering the optimal working range of recombinant wild-type and mutated PAH, (iii) we retraced these analyses in a eukaryotic cell culture system, revealing that the availability of the active PAH enzyme depends on both the metabolic state and drug dosage, and (iv) we translated this into the human system by analyzing the effect of the genotype, phenylalanine concentrations and the BH_4 dosage applied on the results of oral BH_4 loading tests from PAH-deficient patients.

RESULTS

Expanded insights into wild-type PAH kinetics unraveling the mutual impact of substrate and cofactor concentrations

In order to investigate the interdependence of L-phenylalanine and BH_4 in PAH enzyme kinetics, we adapted a newly developed fluorescence-based real-time activity assay (27) to simultaneously analyze the effect of a wide range of substrate and cofactor concentrations on PAH activity. Process automation now allowed for continuous measurement of tyrosine product formation over time in one single operation consisting of six sequential runs for all 96 wells (Fig. 1A). The assay was expanded to cover the space of 0 to 4000 μM L-phenylalanine and 0–500 μM BH_4 (Fig. 1B). First, we validated the data obtained with the new method by comparison with previous findings using a standard high-performance liquid chromatography (HPLC) based discontinuous assay (2). Data points determined at either varying L-phenylalanine concentrations (0–1000 μM) and one BH_4 concentration (75 μM) or at one L-phenylalanine concentration (1000 μM) and varying BH_4 concentrations (0–125 μM), respectively, were used to calculate enzyme kinetic parameters. Prior to calculation, an *F*-test was used to decide whether the Michaelis–Menten or the Hill kinetic model was more appropriate (27). Both L-phenylalanine and BH_4-dependent PAH enzyme kinetics showed clear data-fitting to the Hill equation (Fig. 1C), as previously described for the L-phenylalanine-activated enzyme (27). Although V_{max} values for L-phenylalanine and BH_4-dependent kinetics were higher in the new assay, allosteric parameters, i.e. apparent substrate affinity, the Hill coefficient and apparent cofactor affinity, were similar in both assays (Table 1).

We depicted the data analyzed by non-linear and polynomial regression fitting as three-dimensional landscapes of enzyme activity (30). This enabled a visual representation of the mutual impact of substrate (*x*-axis) and cofactor concentrations (*y*-axis) on PAH kinetics (color code) representing functional conditions of the PAH enzyme. Wild-type PAH showed a peak maximum enzyme activity at 575 μM L-phenylalanine and 125 μM BH_4, respectively (Fig. 1D). The analysis of PAH enzyme kinetics at BH_4 and L-phenylalanine concentrations extended to supraphysiological levels led to a number of interesting observations. High PAH enzyme activity was determined at a surprisingly wide range of substrate and cofactor concentrations. The optimal working range reflected by PAH enzyme activity in the boundaries of $[S]_{0.5}$ to K_i for the substrate and $[C]_{0.5}$ to K_i for the cofactor spanned from 252 to 2026 μM L-phenylalanine and from 44 to 306 μM BH_4 (Table 2). At L-phenylalanine concentrations above 561 μM, we observed the well-known substrate inhibition of enzyme activity (30,31). Notably, at cofactor

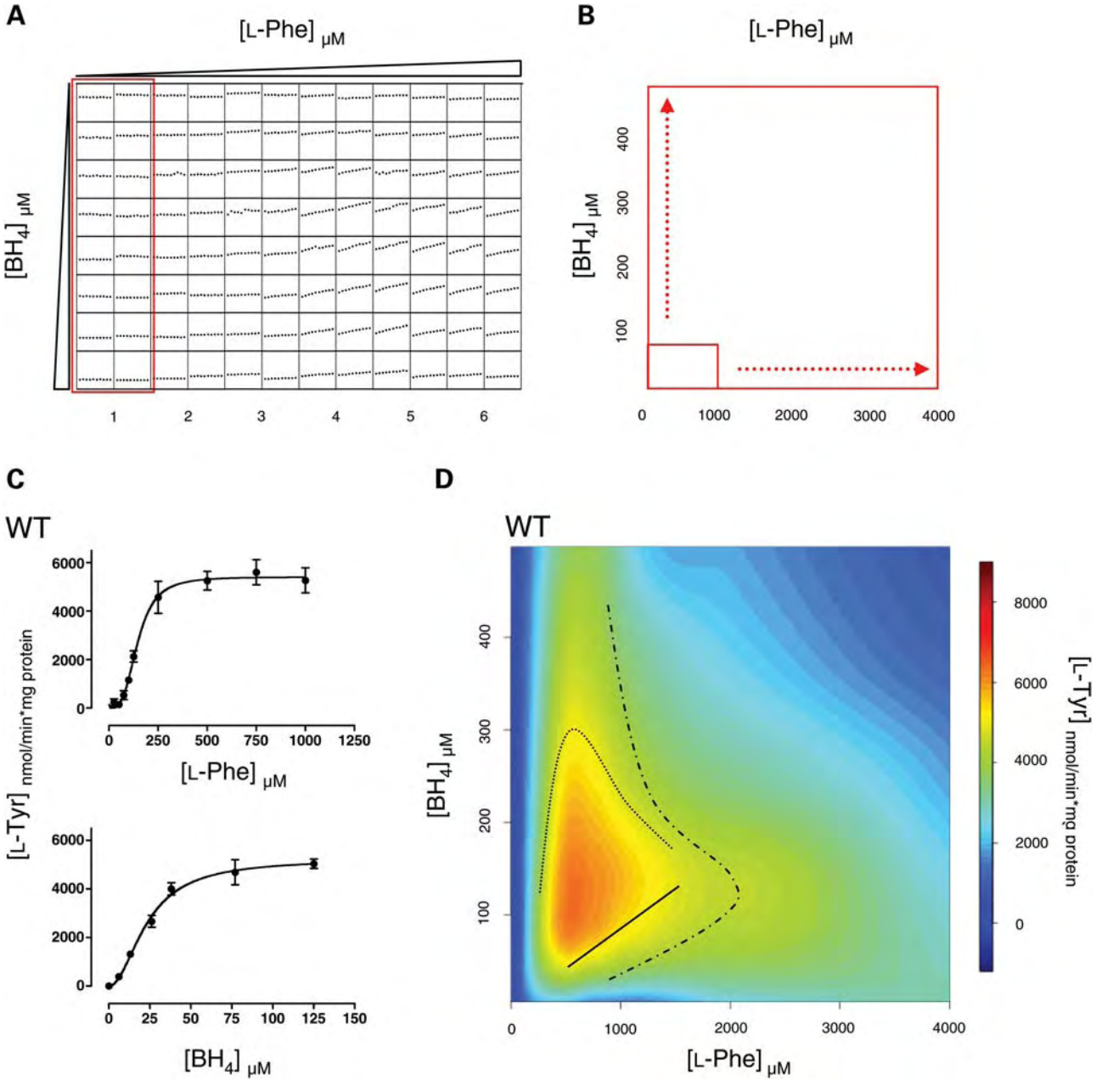

Figure 1. Optimal working range of wild-type PAH activity. (**A**) Scheme of sequential measurements of PAH enzyme kinetics in a 96-well plate. One sequence consists of two columns (red box). In each column, cofactor concentrations (0–500 µM) were varied at a fixed substrate concentration (0–4000 µM), respectively. Repeated cycles allowed for kinetic measurements of 16 wells over a time period of 90 s. (**B**) Extension of substrate and cofactor concentrations. The range of L-phenylalanine and BH_4 concentrations was expanded from standard conditions (BH_4, 75 µM; L-phenylalanine 1000 µM, red box) to 500 µM BH_4 and 4000 µM L-phenylalanine (arrows), respectively. (**C**) Cooperativity of PAH towards substrate and cofactor. Pre-activated PAH showed sigmoidal behavior for L-phenylalanine- (*upper panel*) and BH_4-dependent (*lower panel*) PAH enzyme kinetics. (**D**) Activity landscape of human wild-type PAH. Data for PAH enzyme activity assayed at varying L-phenylalanine and BH_4 concentrations were interpolated and depicted by a color code. The dot-and-dash line represents K_i for substrate inhibition at varying cofactor concentrations, the dotted line represents K_i for cofactor inhibition at varying substrate concentrations. With increasing substrate concentrations, more BH_4 is needed to maintain the same level of enzyme activity (solid line).

concentrations above 108 µM cofactor inhibition occurred. In addition, a mutual interdependence of both inhibitory events was found. These observations represent previously unknown findings. Over and above that, at L-phenylalanine concentrations within the range naturally occurring in the pathological state (500–1500 µM), the enzyme requires more BH_4 with increasing L-phenylalanine concentrations to maintain the same level of enzyme activity.

Taken together, the considerable extension of analysis conditions and the evaluation of data by compiling activity landscapes provided new insights into PAH kinetics. It allowed for a precise evaluation of peak PAH enzyme activity and the

Table 1. Comparison of enzyme kinetic parameters of human wild-type MBP–PAH at standard L-phenylalanine (1000 μM) and BH_4 (75 μM) concentrations

	L-phenylalanine[a]			BH_4[b]			
	V_{max} (nmol L-tyrosine/min × mg protein)	$[S]_{0.5}$ (μM)	h_{Phe}	V_{max} (nmol L-tyrosine/min × mg protein)	$[C]_{0.5}$ (μM)	K_m (μM)	h_{BH4}
Continuous assay	5407 ± 210	145 ± 11	3.3	5222 ± 286	23 ± 2		2.0
Discontinuous assay	3470 ± 75	155 ± 6	3.0	3425 ± 139		24 ± 3	

Steady-state kinetic parameters of wild-type MBP–PAH fusion proteins. V_{max} and the apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}$, K_m) as well as the Hill coefficient (h) as a measure of cooperativity are shown. Enzyme kinetic parameters were determined from enzyme activities measured using the newly developed fluorescence-based continuous assay and compared with enzyme activities measured by the standard HPLC-based discontinuous assay (2). Data were analyzed using the F-test for model comparison and the kinetic parameters of the best-fitting model (cooperative versus non-cooperative) were calculated. Values are given as mean ± SEM of three independent measurements.

[a]Enzyme kinetic parameters determined at variable L-phenylalanine concentrations (0–1000 μM) and standard BH_4 concentration (75 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

[b]Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-phenylalanine concentration (1000 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

Table 2. Peak enzyme activity and range of substrate and cofactor concentrations of wild-type and variant PAH

	Peak activity (nmol L-tyrosine/min × mg protein)	Residual activity (%)	L-phenylalanine concentration at peak activity (μM)	$[S]_{0.5}$–K_i[§]	BH_4 concentration at peak activity (μM)	$[C]_{0.5}$–K_i or K_m–K_i[§]
WT	6370	100	561	252–2026	108	44–306
F39L	5865	92	622	187–2275	143	35–331
I65T	3533	55	612	254–2075	135	29–322
R261Q	2654	42	842	344–2825	149	41–321
P275L	5215	82	1293	238–1980	334	71–435
P314S	1956	31	612	76–1043	80	42–218
V388M	6355	100	591	201–1933	105	47–296
Y414C[a]	3106	49	591	124–1048[a]	105	43–274
Y417H	5206	82	471	147–1501	92	63–262

Peak enzyme activity of variant tetrameric MBP–PAH fusion proteins measured by direct in-well activity measurements. The apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}/K_m$) as well as K_i for substrate and cofactor inhibition were calculated based on non-linear regression analysis at L-phenylalanine and BH_4 concentrations of peak enzyme activity.

[§]K_i calculated at peak L-phenylalanine and BH_4 concentrations using the Boltzman-sigmoidal equation.

[a]Calculation of K_i at a range of L-phenylalanine 0–1624 μM.

optimal working range spanning a wide area of substrate and cofactor concentrations. Moreover, cofactor inhibition as well as a mutual impact of substrate and cofactor concentrations on PAH activity have been identified.

Activity landscapes: the effect of *PAH* mutations on PAH enzyme kinetics

Variant PAH proteins harboring mutations mapping to the regulatory (F39L, I65T), the catalytic (R216Q, P275L, P314S, V388M) and the dimerization motif of the oligomerization domain (Y414C, Y417H) of the enzyme were analyzed. The results from kinetic measurements were depicted as activity landscapes and compared with those from wild-type PAH (Fig. 2). For each variant analyzed, enzyme kinetic parameters and peak enzyme activities with their corresponding L-phenylalanine or BH_4 concentrations were determined (Tables 2 and 3). The area of optimal enzyme activity defined as the range $[S]_{0.5}$ to K_i for the substrate and $[C]_{0.5}$ to K_i for the cofactor, respectively, was calculated (Table 2).

The variant proteins bearing the mutation F39L or I65T, both located in the regulatory domain, showed at first glance a landscape pattern comparable with that of the wild-type. Besides enzyme activity (F39L, 92%; I65T, 55%), the variants displayed unaffected enzyme kinetic parameters as well as similar effects of substrate and cofactor inhibition as found for the wild-type enzyme. However, a more detailed analysis revealed that peak PAH activities were found at similar substrate concentrations, but at ~1.3-fold higher cofactor concentrations when compared with the wild-type. This indicates that in the presence of these mutations, more cofactor is needed to achieve optimal PAH function.

All mutations mapping to the catalytic domain induced marked alterations of activity landscapes. For the variant R261Q, the area of substantial PAH activity was much larger than for the wild-type, but residual enzyme activity was reduced to 42%. The concentrations of substrate and cofactor needed to achieve peak PAH activity were shifted to higher values (L-phenylalanine, 1.5-fold; BH_4, 1.4-fold). We observed reduced affinity to the substrate by a factor of 0.5 ($[S]_{0.5}$ 282 μM). Binding of BH_4 followed Michaelis–Menten kinetics representing a loss of cooperativity that occurred with reduced affinity (K_m 66 μM). Broadening of the landscape resulted from a significant shift of enzyme inhibition. Notably, the variant did not display cooperative binding of BH_4 when activated. Taken together, the unique feature of

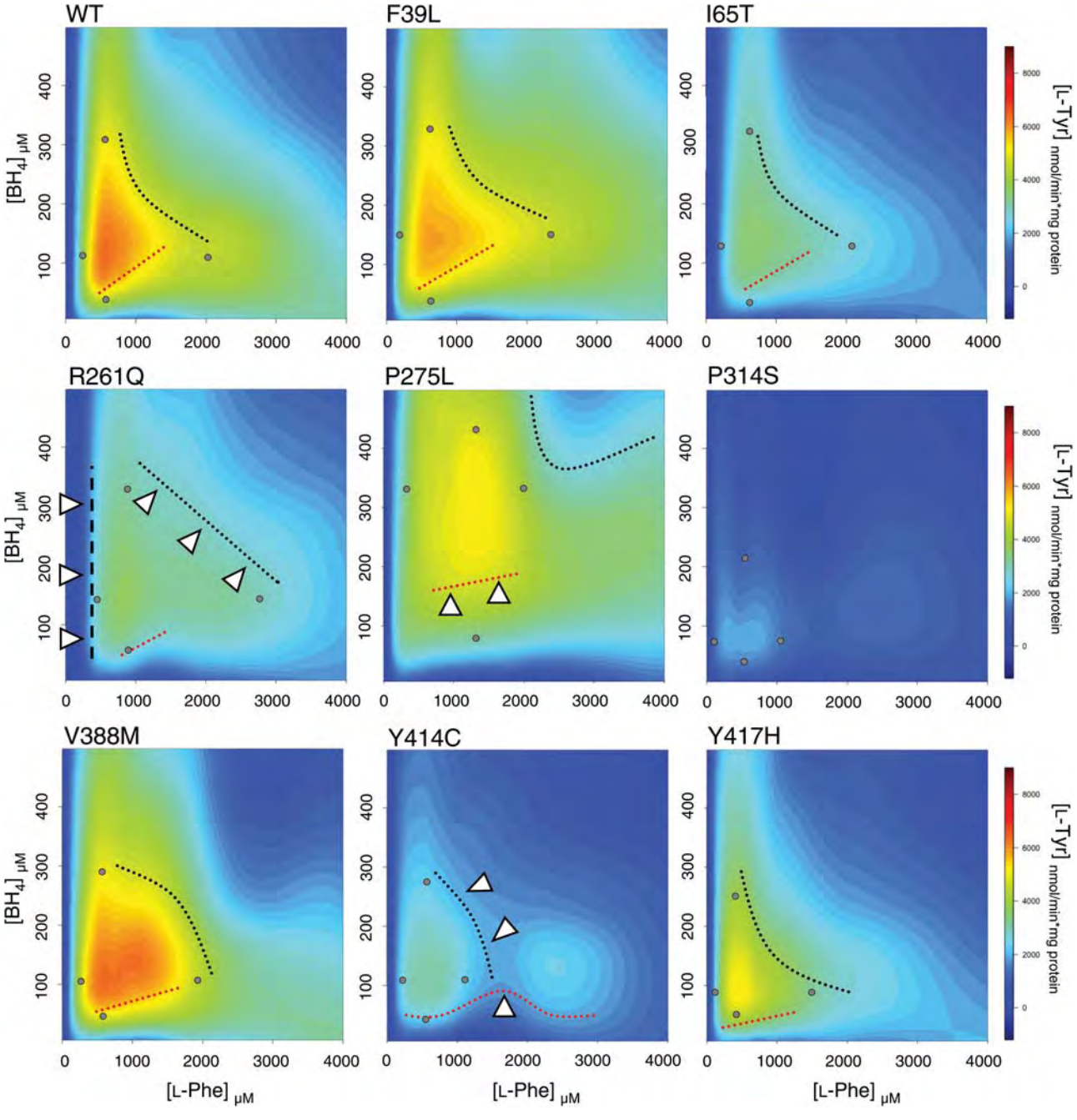

Figure 2. Activity landscapes of recombinant wild-type and variant PAH. The interpolated specific enzyme activities are color-coded and given as a function of different L-phenylalanine and BH_4 concentrations. Substrate and cofactor inhibition are depicted as a summation line (black dotted line). Positions of $S_{0.5}$ and K_i for L-phenylalanine and $C_{0.5}$ and K_i for BH_4, respectively, are indicated at peak enzyme activities (open circles). Marked changes in the activity landscape of variant PAH when compared with the wild-type (WT) are highlighted (open triangles) and BH_4 concentrations needed to maintain the same enzyme activity with increasing L-phenylalanine concentrations are shown (red dotted line).

the R261Q variant was the right shift of PAH enzyme activity towards higher L-phenylalanine concentrations indicating a reduced affinity of this variant to its substrate and that the enzyme displays low activity at L-phenylalanine concentrations below 600 µmol/l. At these substrate concentrations, even very high BH_4 doses would not produce any response in PAH activity. The presence of the mutation P275L resulted in an enzyme with overall high residual enzyme activity (82%), yet with a shift of peak enzyme activity to 2.3-fold higher L-phenylalanine and 3-fold higher BH_4 concentrations (L-phenylalanine 1293 µM, BH_4 334 µM) than the wild-type. In comparison to the wild-type, the enzyme had a substantially higher need for BH_4 to achieve the optimal working range. As a consequence, substrate and cofactor inhibition was almost abolished. The variant protein bearing the mutation P314S showed a severe loss in residual enzyme activity (31%) with

Table 3. Enzyme kinetic parameters at standard L-phenylalanine (1000 μM) and BH_4 (75 μM) concentrations calculated from activity landscapes

	L-phenylalanine[a]			BH_4[b]			
	V_{max} (nmol L-tyrosine/min × mg protein)	$[S]_{0.5}$ (μM)	h_{Phe}	V_{max} (nmol L-tyrosine/min × mg protein)	$[C]_{0.5}$ (μM)	K_m (μM)	h_{BH4}
WT	5407 ± 210	145 ± 11	3.3	5222 ± 286	23 ± 2		2.0
F39L	4961 ± 342	115 ± 15	2.7	5669 ± 311	36 ± 3		1.7
I65T	3166 ± 96	161 ± 10	2.7	3277 ± 196	27 ± 3		1.7
R261Q	3041 ± 128	282 ± 18	3.4	4693 ± 598		66 ± 17	
P275L	3204 ± 126	112 ± 8	2.6	3022 ± 179	36 ± 3		2.8
P314S	1650 ± 91	76 ± 9	1.8	991 ± 25	24 ± 1		5.2
V388M	5639 ± 112	140 ± 6	2.7	5895 ± 236	26 ± 2		1.8
Y414C	2895 ± 206	120 ± 17	2.4	2148 ± 203	28 ± 4		2.6
Y417H	4434 ± 247	111 ± 10	3.6	3011 ± 93	23 ± 1		2.5

Steady-state kinetic parameters of variant tetrameric MBP–PAH fusion proteins determined by direct in-well activity measurements. Data were analyzed using the *F*-test for model comparison and the kinetic parameters of the best-fitting model (cooperative versus non-cooperative) were calculated. V_{max} and the apparent affinities for L-phenylalanine ($S_{0.5}$) and BH_4 ($C_{0.5}$, K_m) as well as the Hill coefficient (h) as a measure of cooperativity are shown. Values are given as mean ± SEM of three independent measurements.

[a]Enzyme kinetic parameters determined at variable L-phenylalanine concentrations (0–1000 μM) and standard BH_4 concentration (75 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

[b]Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-phenylalanine concentration (1000 μM) with pre-incubation of the enzyme by L-phenylalanine (1000 μM).

a narrow optimal working range that was shifted towards lower L-phenylalanine as well as BH_4 concentrations. In addition, enzyme kinetics revealed a significantly increased apparent affinity to the substrate with marked reduction in cooperativity. In contrast to previous findings (3,32), where the variant protein V388M was described as a K_m variant with reduced affinity to the cofactor, we detected high residual enzyme activity over an expanded range of substrate and cofactor concentrations. Residual PAH activity was 100% and peak catalysis (L-phenylalanine 591 μM, BH_4 105 μM) as well as effects of substrate and cofactor inhibition were similar to that of the wild-type enzyme.

Mutations mapping to the oligomerization domain, Y414C and Y417H, showed a narrowed optimal working range that was shifted to lower substrate concentrations. However, this was less pronounced for the milder mutation Y417H (82% residual enzyme activity) when compared with Y414C (49%). On the other hand, Y417H needed less BH_4 to achieve the area of optimal function. Interestingly, different to all variants analyzed, Y414C showed two peaks of high enzyme activity.

Taken together, we identified many similarities between the activity landscapes of the wild-type and variant PAH proteins, showing generally high residual enzyme activity as well as substrate and cofactor inhibition. However, these activity landscapes also revealed important differences in the regulation of PAH activity by BH_4 and L-phenylalanine and helped to visualize the interdependence of substrate and cofactor concentrations on variant PAH enzyme activity.

Cofactor action on PAH function in respect of kinetic behavior and the chaperone effect

We used two different approaches to analyze different aspects of cofactor action on the wild-type, R261Q and Y414C PAH enzyme in HEK293 cells. First, we in part retraced the activity landscapes analyzing the kinetic behavior by assaying enzyme activity of cell lysates at different L-phenylalanine concentrations (0–1000 μM) while keeping the BH_4 concentration constant (75 μM) (Fig. 3A). Secondly, we analyzed the chaperone effect of the BH_4 cofactor at different phenylalanine and BH_4 concentrations after a 72 h incubation.

As expected, the wild-type protein showed the highest enzyme activity of all three proteins analyzed with a peak activity at about 250 μM L-phenylalanine. The latter finding differed from the peak activity observed for recombinant PAH at ~500 μM. As seen for prokaryotic PAH, a further increase in L-phenylalanine concentrations resulted in substrate inhibition. The variant R261Q displayed ~50% residual enzyme activity and a lower slope of the curve that leveled off at a plateau. Reduced enzyme function, which was shifted toward higher substrate concentrations with a broadened working range, substantiated results from activity landscapes. Residual enzyme activity of eukaryotic Y414C was substantially lower than the specific activity of the corresponding recombinant variant. In addition to peak activity at 180 μM L-phenylalanine, a second slight incline of activity was observed at high L-phenylalanine concentrations (1000 μM). Although generally shifted toward lower substrate concentrations, this is in line with the two peaks observed in the activity landscape.

Next, we aimed to elucidate the long-term chaperone effect of varying intracellular phenylalanine and BH_4 concentrations on PAH function. The determination of enzyme activity at standard conditions after previous incubation of cells with different substrate and cofactor concentrations assays the availability of functionally active PAH. For this purpose, stably transfected cells were cultivated with L-phenylalanine concentrations of 91, 500 or 1200 μM and BH_4 concentrations of 0, 40 or 75 μM for 72 h. First, cells were cultivated at L-phenylalanine levels representing the physiological state (91 μM), mild PKU (500 μM) or classical PKU (1200 μM) without the addition of BH_4 to the medium (Fig. 3B). Wild-type PAH activity showed a trend towards an increase only at clearly pathological L-phenylalanine concentrations. For R261Q, a steady but statistically not significant increase in enzyme activity was seen

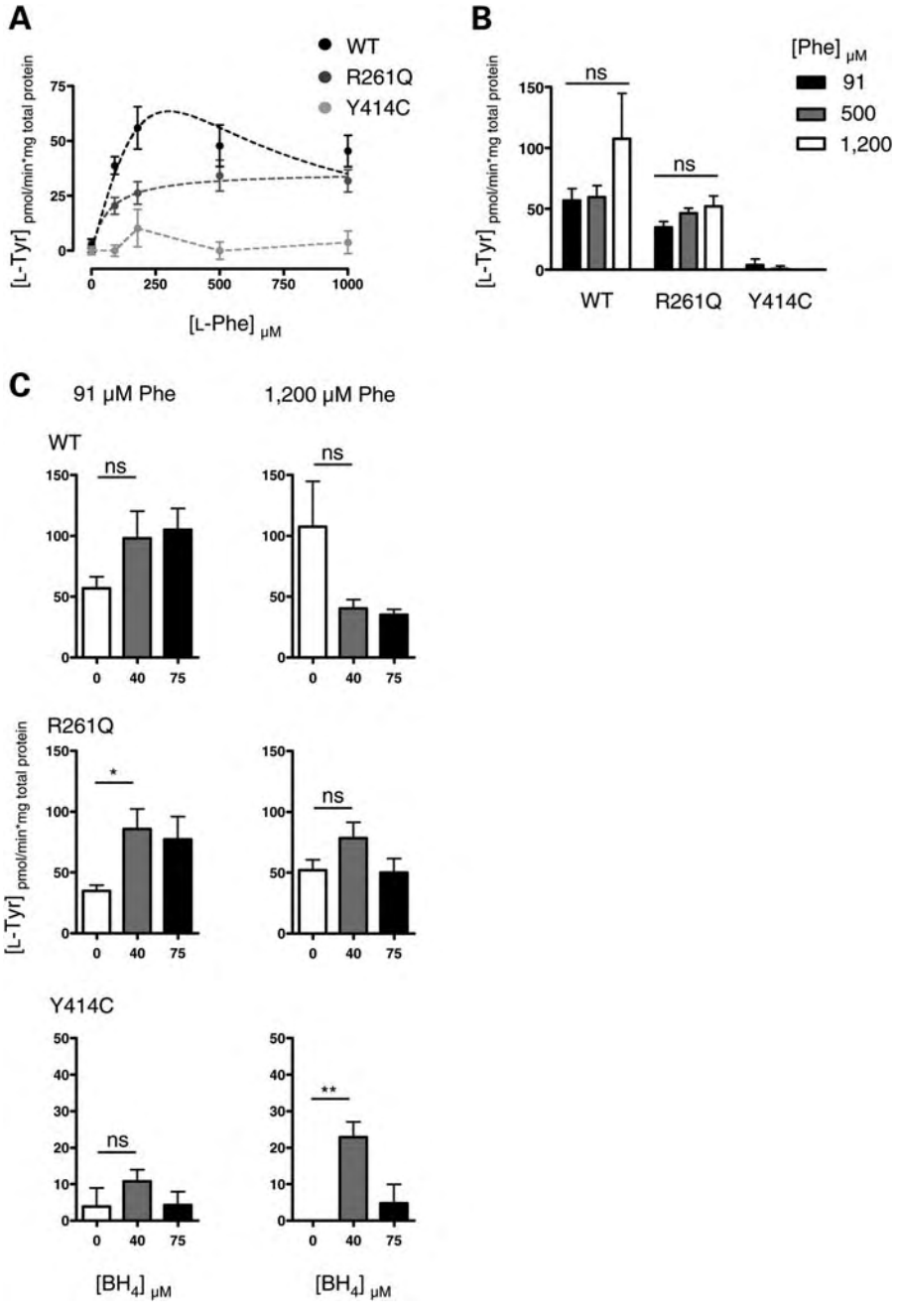

Figure 3. Cofactor action on PAH function in respect of kinetic behavior and the chaperone effect. (**A**) Enzyme activity of eukaryotic expressed PAH assayed at varying substrate concentrations. Enzyme activities in cell lysates of HEK293 cells stably transfected with wild-type PAH, R261Q or Y414C were determined at varying L-phenylalanine concentrations (0–1000 µM) and a fixed BH_4 concentration (75 µM). (**B**) Effect of varying intracellular phenylalanine concentrations on functionally active PAH. PAH enzyme activity in HEK293 cell lysates expressing wild-type PAH, R261Q or Y414C was determined at standard assay concentrations (1000 µM L-phenylalanine and 75 µM BH_4) after prior incubation with different L-phenylalanine concentrations (black, 91 µM; dark gray, 500 µM; white, 1200 µM) for 72 h. (**C**) Mutual impact of varying substrate and cofactor concentrations on functionally active PAH. Cells stably expressing wild-type PAH, R261Q or Y414C were cultivated at different phenylalanine (left column, 91 µM; right column, 1200 µM) and BH_4 concentrations (white, 0; dark gray, 40 µM; and black, 75 µM). PAH enzyme activities were analyzed at standard assay conditions. PAH activities are given as mean ± SEM of three independent experiments. Significant differences in enzyme activities were calculated using one-way ANOVA and Dunnett's multiple comparison test (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$).

upon increasing substrate concentrations in the medium (35 ± 5 to 52 ± 9 pmol L-tyrosine/min × mg total protein). For Y414C, residual activity was very low without any effect of increasing L-phenylalanine concentrations.

To further evaluate the mutual impact of the substrate and the cofactor on enzyme activity in the eukaryotic system, 40 or 75 µM BH_4 were added to the medium at L-phenylalanine concentrations of 91 or 1200 µM, respectively (Fig. 3C).

Interestingly, at physiological L-phenylalanine concentrations (91 μM), the addition of 40 and 75 μM BH_4 induced a trend towards increased enzyme activity of wild-type PAH (57 ± 9 to 98 ± 22 and 105 ± 18 pmol L-tyrosine/min × mg total protein), whereas the opposite effect was observed at elevated L-phenylalanine concentrations (1200 μM L-phenylalanine, 108 ± 37 to 40 ± 7 and 35 ± 5 pmol L-tyrosine/min × mg total protein). For the variant R261Q, the addition of 40 μM BH_4 at physiological L-phenylalanine concentrations resulted in a significant increase in enzyme activity (35 ± 5 to 86 ± 17 pmol L-tyrosine/min × mg total protein), but the inhibitory effect at elevated L-phenylalanine concentrations as seen for the wild-type was not observed. At physiological L-phenylalanine concentrations, the variant Y414C showed low residual enzyme activity with only a minor increase upon the addition of 40 μM BH_4. Interestingly, at high L-phenylalanine concentrations (1200 μM), the addition of 40 but not 75 μM BH_4 led to a significant increase in residual enzyme activity (23 ± 4 pmol L-tyrosine/min × mg total protein) achieving as much as 40% of the wild-type level.

Taken together, findings from the prokaryotic system depicted by activity landscapes were substantiated in the eukaryotic environment. Also in this system probing lysates of cultured cells, the mutual impact of different L-phenylalanine and BH_4 concentrations on enzyme activity varied among different PAH variants with substrate inhibition for the wild-type as well as constant activity levels for R261Q also at very high L-phenylalanine concentrations. Residual enzyme activity of Y414C was substantially lower in cell culture when compared with the specific activity of the recombinant protein, but enzyme function was rescued by the addition of BH_4. This may point to impaired protein stability in the eukaryotic environment and a stabilizing pharmacological chaperone effect by BH_4.

The mutual impact of substrate and cofactor concentrations on results from BH_4 loading tests performed in PAH deficient patients

So far, data pointed to a simultaneous dependency of PAH function from available substrate and cofactor concentrations. After having confirmed results from the prokaryotic system in the eukaryotic system, we aimed to investigate whether our observations may be transferred to the human situation by analyzing data from BH_4-loading tests performed in patients with PAH deficiency. To address this issue, we collected data of patients homozygous or functionally hemizygous for the mutations F39L, I65T, R261Q or Y414C, that underwent a BH_4-loading test with a dose of 20 mg/kg body weight and a duration of at least 24 h from the literature (19,33–37) and from the BIOPKU database (www.biopku.org). First, we compiled the course of blood phenylalanine values within 24 h after a single dose of BH_4 as a function of initial blood phenylalanine concentrations. For all mutations analyzed, different blood phenylalanine concentrations at the beginning of the test led to differences in the extent of BH_4 responsiveness, i.e. the percent decrease in blood phenylalanine after drug administration. Patients carrying the mutations F39L, I65T and Y414C showed a peak level of BH_4 responsiveness below an initial blood phenylalanine concentration of 500 μM (Fig. 4A). In the presence of F39L and I65T increasing initial blood phenylalanine concentrations were associated with a decrease in BH_4 responsiveness. While patients with F39L still displayed positive response at 1000 μM phenylalanine (>30% decrease in blood phenylalanine), those carrying I65T did not show a drug response when initial blood phenylalanine concentrations were >800 μM. The mutation Y414C led to inconsistent response to BH_4 with maximum responsiveness at initial blood phenylalanine concentrations up to 750 μM phenylalanine and at 1000 μM phenylalanine. In general, patients bearing the mutation Y414C showed a high degree in BH_4 responsiveness with the lowest response remaining within the range of 30% decrease in blood phenylalanine.

Patients bearing the mutation R261Q showed strong inconsistencies in BH_4 responsiveness with some patients displaying high levels of responsiveness (>60% decrease of blood phenylalanine) and others no response at all (Fig. 4B). To investigate the basis of these inconsistencies, we analyzed the impact of both the genotype on BH_4 response and of phenylalanine concentrations on different genotypes comprising the R261Q mutation. The decrease in blood phenylalanine concentrations was significantly stronger in individuals carrying the R261Q mutation in the homozygous state (median, 37.5%) than those carrying it in the functional hemizygous state (median, 6.5%). However, among homozygous patients, some were responders and some were not, whereas all functionally hemizygous patients had to be classified as non-responders. Interestingly, the level of initial blood phenylalanine did not allow differentiating either homozygous responders from homozygous non-responders or homozygotes from functional hemizygotes.

In addition to the influence of initial blood phenylalanine concentrations on BH_4 response, we analyzed the impact of the genotype on BH_4 dose response to investigate the optimal PAH working range *in vivo*. Literature data providing information on BH_4 dosage revealed clear differences for patients homozygous for R261Q, patients homozygous for Y414C and those compound heterozygous for these two mutations (Fig. 4C). In the presence of the mutation R261Q, the percent decrease in blood phenylalanine levels remained nearly unchanged for the range of BH_4 dosages between 10 and 30 mg/kg body weight. In contrast, patients bearing the mutation Y414C showed a gain in BH_4 response with increasing BH_4 doses (5–20 mg/kg body weight). Interestingly, patients compound heterozygous for R261Q and Y414C showed an intermediate response with respect to the BH_4 dosage administered (10–20 mg/kg body weight) when compared with patients homozygous for these mutations.

As a conclusion, *in vivo* PAH activity is a function of the phenylalanine substrate and the BH_4 cofactor as well as the patient's genotype. Hence, enzyme function in the individual patient at a given time point is the resultant of the metabolic state and the dosage of cofactor treatment both in turn determined by the underlying mutations.

DISCUSSION

Regulation of PAH activity is essentially governed by the abundance of the phenylalanine substrate and the BH_4

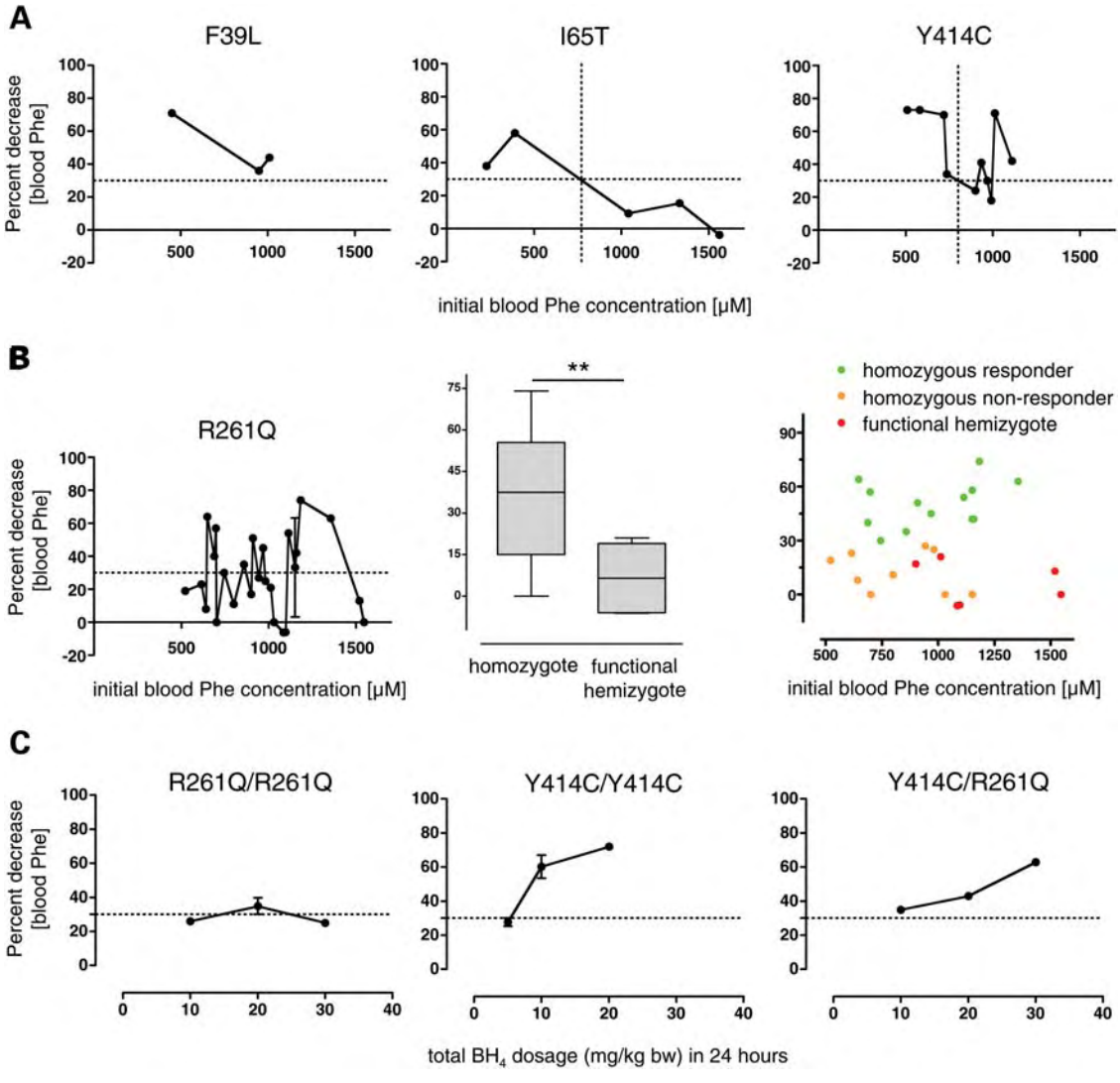

Figure 4. Evaluation of BH_4 responsiveness in PAH-deficient patients. (**A**) The impact of the genotype and of initial blood phenylalanine concentrations on BH_4 response. The graphs show the percent decrease in blood phenylalanine levels 24 h after a single dose of BH_4 (20 mg/kg bw) in patients homozygous or functional hemizygous for the mutations F39L ($n = 3$), I65T ($n = 5$) or Y414C ($n = 10$) as a function of different blood phenylalanine values at the beginning of the test. The horizontal dashed line indicates 30% decrease in blood phenylalanine concentrations, the arbitrary criterion of BH_4 responsiveness. The vertical dashed line shows the initial blood phenylalanine concentration, above which BH_4 leads to a blood phenylalanine decrease of $<30\%$. (**B**) BH_4 responsiveness in patients carrying the mutation R261Q ($n = 28$). (Left) Percent decrease in blood phenylalanine 24 h after a single dose of BH_4 (20 mg/kg bw) as a function of initial blood phenylalanine values in patients homozygous or functional hemizygous for the R261Q mutation. (Middle) Percent decrease in blood phenylalanine 24 h after a single dose of BH_4 (20 mg/kg bw) in patients carrying the R261Q mutation in the homozygous state ($n = 22$) in comparison to functional hemizygotes ($n = 6$). The boxes show the interquartile ranges (25th to 75th percentiles), the horizontal black bars represent the median, the bars indicate the range. Significance is indicated ($^{**}P < 0.01$, unpaired Student's *t*-test). (Right) Percent decrease in blood phenylalanine concentrations in function of initial blood phenylalanine values in homozygous responders ($n = 13$) and non-responders ($n = 9$) as well as functional hemizygous patients ($n = 6$) displaying the mutation R261Q. (**C**) The impact of the genotype on BH_4 dose response. Percent decrease in blood phenylalanine 24 h after a single application of BH_4 in different dosages in patients homozygous for R261Q ($n = 24$) (*left*), homozygous for Y414C ($n = 10$) (*middle*) or compound heterozygous for R261Q/Y414C ($n = 3$) (*right*).

cofactor. The supply of substrate, e.g. upon food intake, induces enzyme activation and subsequently full catalytic activity. In contrast, at low blood phenylalanine levels, e.g. under fasting conditions, BH_4-induced PAH inhibition prevents from undue elimination of the essential amino acid phenylalanine. Thus, it is the ratio of phenylalanine to BH_4 that determines activation and inhibition of the enzyme. However, the concentration of BH_4 in the liver cell is held rather constant, whereas phenylalanine levels undergo substantial fluctuations in function of the metabolic state and—in the presence of PAH deficiency—of the underlying genotype. While treating patients with BH_4 we intervene in this system without disposing of profound knowledge concerning the effect of shifts of the substrate-to-cofactor ratio. In the work presented here, we first wanted to apply the newly developed real-time fluorescence-based PAH enzyme activity assay to identify the optimal working range of the enzyme with respect to these substances. Secondly, we aimed to analyze

the influence of changes in the phenylalanine-to-BH_4 ratio on PAH function of the wild-type and variant enzymes.

Analyses of wild-type PAH revealed two interesting new findings. In addition to the well-known enzyme inhibition by the substrate, cofactor inhibition was identified by the extension of the enzyme activity assay to much higher cofactor concentrations. Moreover, we learned that with increasing phenylalanine concentrations, more BH_4 is needed to maintain the same level of PAH activity. Still, it has to be considered that the full range of substrate and cofactor concentrations applied in our novel assay provided new insights into theoretical aspects of PAH enzymology, but it is well beyond physiological levels. However, our approach allowed for detailed visualization and better understanding of conditions corresponding to those occurring at the edges of classical pathological situations in PKU patients and in the therapeutic context upon cofactor treatment.

The optimal working range of the wild-type enzyme occurred at phenylalanine concentrations of 250–500 μM. From a physiological point of view, this appears reasonable, since upon food intake liver phenylalanine concentrations are expected to reach levels up to 500 μM rather than around 1000 μM, the phenylalanine concentration at which standard PAH enzyme activity assays are performed (2,38). Our results showed that the optimal BH_4 concentration for PAH enzyme activity is ∼100 μM, while standard PAH activity assays are run at 75 μM BH_4 (2,38). In any case, the physiological cellular BH_4 concentration in the liver is by far lower (∼8.5 μM) (26), implying that the cell has always to be considered BH_4 deficient in view of the enzymatic task. Yet, in light of the inhibitory potential of BH_4 on the enzyme, a cellular cofactor concentration significantly below the K_m value of 23 μM reduces inhibition when activity is needed, i.e. at phenylalanine concentrations above the physiological range. The trade-off between these two tasks is balanced by a 6-fold higher affinity of the enzyme toward the cofactor (K_m, 23 μM) than toward the substrate ($S_{0.5}$, 145 μM). As a consequence, PAH binds BH_4 and phenylalanine at a ratio of 0.5 at 100 μM blood phenylalanine (inhibition) when compared with 0.09 at 561 μM blood phenylalanine (no inhibition). These theoretical assumptions are corroborated by previous *in vivo* ^{13}C-phenylalanine oxidation tests performed in a PKU mouse model (39). In the euphenylalaninemic state, a hypothetical BH_4 deficiency is overruled by an inhibitory effect following the application of BH_4 in wild-type animals. However, in hyperphenylalaninemia phenotypes, the administration of BH_4 leads to an immediate increase in ^{13}C-phenylalanine oxidation. In view of a time to effect of $<$5 min, this has to be considered independent of a pharmacological chaperone effect and rather points to compensation of BH_4 deficiency in this metabolic state. Comparable studies in humans addressing phenylalanine concentrations, BH_4 concentrations, the effect of BH_4 in healthy individuals, time to onset of action and effect duration would be of interest, but have not yet been performed.

Next, we investigated the effect of selected missense mutations in the *PAH* gene on the optimal working range of the enzyme. In general, activity landscapes of the wild-type and variant PAH proteins displayed comparable patterns with rather high residual enzyme activity and a limited area of maximum activity. More detailed analyses, however, revealed various alterations with respect to the extension and position of the optimal working range in the coordinates of substrate and cofactor concentrations. Most variant proteins were in need of more BH_4 to achieve peak activity (F39L, I65T, R261Q, P275L, Y417H, Y414C). For two variants (R261Q and P275L), maximum activity was determined at markedly higher phenylalanine concentrations (842 and 1293 μM). In contrast, three variants (P314S, Y414C and Y417H) presented a narrowed optimal working range that was shifted to lower phenylalanine concentrations when compared with the wild-type.

Two of these mutations, R261Q and Y414C, are frequent, but inconsistently associated with BH_4 responsiveness (17,34–37). The analysis of activity landscapes provided first evidence for an impact of the metabolic state on variant PAH function. R261Q displayed marked reduction in enzyme activity at phenylalanine concentrations in the therapeutic range below 240 μM giving rise to the hypothesis that patients bearing this mutation in the homozygous or functional hemizygous state would not benefit from a restrictive dietary regime. On the other hand, the mutation Y414C that induces a shift of activity to lower phenylalanine concentrations would require a rather strict metabolic adjustment with low blood phenylalanine values to achieve optimized PAH activity. Thus, observations from the analysis of activity landscapes could be of importance for a deeper understanding of inconsistent results from BH_4 loading tests or some disappointing experiences upon BH_4 treatment of our patients. To perform a further step in this direction, we carried out cell culture experiments and analyzed data from BH_4 loading tests performed in PKU patients.

Data extracted from activity landscapes were reproduced in the setting of stably transfected cells. Having gained insights into the mutual impact of varying substrate and cofactor concentrations on PAH activity, we were then interested in answering the question of how changes in the metabolic state would affect the effective PAH concentration, that is, the intracellular amount of functional PAH enzyme available for phenylalanine conversion. Since BH_4 has been classified as a pharmacological chaperone, i.e. a stabilizing compound that helps to overcome PAH degradation in the cell, it is expected to raise the amount of PAH in cell culture. In addition, we had previously shown that the effective PAH concentration is influenced by changes in the phenylalanine-to-BH_4 ratio in the mouse (12). To address these issues in cell culture, we mimicked physiological (euphenylalaninemic) and pathologic (hyperphenylalaninemic) conditions representing classical PKU. The supplementation of BH_4 induced diverse results in the presence of R261Q and Y414C, respectively. BH_4 was beneficial for catalytic PAH function of the R261Q variant particularly at low phenylalanine levels, whereas a significant increase in PAH enzyme activity reflecting an increase in the effective PAH amount was observed for Y414C under PKU conditions. Interestingly, the therapeutic range for BH_4 was narrow for Y414C, a finding confirming the observations from the activity landscapes.

As a next step, we aimed to verify the clinical relevance of our findings and analyzed the effect of different substrate

and cofactor concentrations on the outcome of single dose BH_4-loading tests in individuals carrying different *PAH* genotypes. In clinical routine, the initial phenylalanine concentration at the beginning of the BH_4-loading test is not expected to significantly affect the outcome of the test. In general, only a minimum phenylalanine concentration of 400 μM is considered to be required for reliable test results. Surprisingly, data from BH_4 loading tests available in the BIOPKU database and in the literature (19,33–37,40) does not confirm this view. We learned that patients carrying one of the mutations F39L, I65T or Y414C in either a homozygous or a functional hemizygous state show substantially different responses to the BH_4-loading test in function of the phenylalanine concentration at the beginning of the test. For example, in presence of the mutation I65T, the response may vary from 60% at 500 μM phenylalanine to 0% at 1500 μM blood phenylalanine. This is a new finding that may undermine our trust in current BH_4-loading test protocols. Our results may, for instance, allow for the hypothesis that patients carrying the I65T mutation are at risk to show false negative test results at phenylalanine concentrations >750 μM. In the case of the R261Q mutation, phenylalanine concentrations did not significantly influence test results (Fig. 4B). However, the kind of genotype significantly affected BH_4 responsiveness with carriers of the R261Q mutation in the homozygous state showing a higher response (37.5% decrease in phenylalanine after BH_4 loading) than individuals with the mutation in the functional hemizygous state (6.5% decrease). Further analysis revealed that none of the patients carrying the R261Q mutation in combination with a null mutation met the criterion of BH_4 responsiveness of 30% decrease of phenylalanine concentrations, whereas 12 out of 21 patients with a homozygous genotype did and 9 out of 21 patients did not. Similar observations were recently reported in 27 Turkish PKU patients with a homozygous R261Q genotype and variable clinical phenotypes (11% mild hyperphenylalaninemia, 67% mild PKU, 22% classic PKU), from which only 39.1% were BH_4 responsive (22). Taken together, our results show that the outcome of a BH_4-loading test may much more vary in function of individual test circumstances than previously assumed. Unfortunately, it has to be expected that this is true for a number of mutations and in view of the lifelong consequences for our patients arising from the initial classification of being a responder or not it has to be emphasized that with the knowledge available today results from single-loading tests are not sufficient to determine BH_4 responsiveness in patients with PAH deficiency.

Last, we analyzed the effect of BH_4 dosage on the results of single BH_4-loading tests. Since ~10 years, a dosage of 20 mg/kg body weight is internationally recommended (11,28,29,41). However, in some centers and countries, this recommendation may not be followed. In homozygous R261Q patients, the dosage of BH_4 (10, 20 and 30 mg/kg body weight) did not seem to influence test results (26, 27 and 25% decrease, respectively). In presence of the Y414C mutation, increasing BH_4 dosages increased BH_4 responsiveness in terms of percent decrease of blood phenylalanine after BH_4 application. Interestingly, compound heterozygous patients carrying both the R261Q and the Y414C mutation also showed a dose dependency of response, but to a lower extent than homozygous Y414C patients.

In summary, we developed a rapid PAH enzyme activity assay allowing for a much higher throughput than previous assays and for detailed analysis of a broad range of substrate and cofactor concentrations on PAH enzyme kinetics. This enabled new insights into optimal PAH working range at physiological, pathological and therapeutic conditions. As to enzyme kinetics, two main conclusions can be drawn from our experimental work: phenylalanine concentrations for optimal working range of PAH are lower, whereas BH_4 concentrations for optimal PAH activity are higher than previously assumed. The validity of our observations was substantiated and expanded by the fact that we were able to translate data from the prokaryotic system into the eukaryotic cell culture system and into patient data. Of relevance for the clinical context, we revealed a significant impact of the genotype, substrate concentrations and BH_4 dosage on the assessment of BH_4 responsiveness.

Since the discovery of the pharmacological effect of BH_4 in patients with PAH deficiency (5), the scientific community discusses possible mechanisms of BH_4 responsiveness in PKU. The initial concept was kinetic action, in particular the hypothesis that *PAH* gene mutations lead to decreased affinity of the variant protein to the cofactor, that is overcome by the administration of pharmacological doses of BH_4. Kinetic studies using the recombinant PAH protein revealed that this is true only in rare instances (2,32,42). Subsequent work moved the concept away from kinetic effects toward the view of BH_4 acting as a molecular chaperone by increasing the stability of partially misfolded PAH proteins and by this the effective intracellular concentration of functional PAH enzyme (3,4,12). A deeper view into PAH enzyme kinetics using a technology that allows for the analysis of a broad range of substrate and cofactor concentrations on PAH activity now showed that besides the indubitable chaperone effect, kinetic aspects also have to be taken into account. Thus, we may now put forward the view of both concepts being of relevance for the diagnosis and the treatment of patients with PAH deficiency. The diagnostic loading test with BH_4 or long-term BH_4 treatment has to be seen in the light of the fact that short-term supply of BH_4 can compensate for latent BH_4 deficiency as to optimal catalytic function (kinetic effect), whereas long-term treatment with pharmacological doses of BH_4 increases the stability of PAH and by this the amount of metabolically active enzyme (chaperone effect). In addition, individual mutations may shift the impact of one or the other therapeutic effect.

For daily clinical routine, this underscores the need for even more standardized and at the same time individualized test procedures including detailed documentation of phenylalanine concentrations before the BH_4 load and the awareness that the metabolic status of the patient will influence the outcome of the test. In non-responders with suggestive genotypes, repetition of the loading test at different initial phenylalanine concentrations may help rule out false negative results. Moreover, we suggest to combine short-term BH_4 loading tests (assessment of kinetic effects) and long-term BH_4 treatment

tests (assessment of chaperone effects) with *in vivo* ^{13}C-phenylalanine oxidation tests (assessment of the effect of BH_4 on *in vivo* PAH enzyme activity) (11,43). The test is non-invasive, innocuous, easy to perform and may add important information about an individual's response to the drug at a functional level.

In conclusion, our work pinpoints the importance of genotyping PKU patients even in clinical routine and underscores the need for more personalized testing procedures addressing individual patient characteristics, the metabolic state and the dosage of the test compound to safely identify BH_4 responsiveness in PAH-deficient patients.

MATERIALS AND METHODS

Patients and mutations

Mutations previously identified in BH_4 responsive patients (4,11) were analyzed in terms of the effect of various substrate (L-phenylalanine) and cofactor (BH_4) concentrations on PAH enzyme activity. The mutations mapped to the regulatory domain (F39L, I65T), the catalytic domain (R261Q, P275L, P314S, V388M) or to the dimerization motif of the oligomerization domain (Y414C, Y417H). Forty-six patients homozygous and functional hemizygous for the mutations F39L ($n = 3$), I65T ($n = 5$), R261Q ($n = 28$) and Y414C ($n = 10$) were identified performing a comprehensive literature survey and by extracting data from the BIOPKU database (www.biopku.org). Patients were included in the analysis, when data on a BH_4-loading test using 20 mg/kg body weight and blood phenylalanine concentrations over a period of at least 24 h were available. In addition, the effect of different BH_4 dosages, ranging from 5 to 30 mg/kg body weight, on the course of blood phenylalanine concentrations was analyzed (16,19,34,35,37).

Expression and purification of recombinant PAH proteins

The cDNA of human PAH (EST clone obtained from imaGenes, formerly RZPD, Germany) was cloned into the prokaryotic expression vector pMAL-c2E (New England Biolabs) encoding an N-terminal maltose-binding protein (MBP) tag. *PAH* mutants were constructed by site-directed mutagenesis as previously described (2). Expression plasmids containing the wild-type *PAH* and variants were transformed to *Escherichia coli* DH5α. Expressed proteins were purified by affinity chromatography (MBPTrap, GE Healthcare) followed by size-exclusion chromatography using a HiLoad 26/60 Superdex 200 column (GE Healthcare) on an ÄKTAxpress system (2). Obtained tetramers of the fusion proteins were collected and protein concentrations were determined spectrophotometrically using $\varepsilon_{280\ (1mg/ml)} = 1.63$.

PAH activity assay

Enzyme activity of the recombinantly expressed PAH. The multi-well PAH activity assay and data evaluation were performed as previously described (27) with modifications. L-phenylalanine and 22.35 mM Na HEPES, pH 7.3, were added to all wells of a 96-well plate with different volumes. This resulted in 12 columns of varying L-phenylalanine concentrations (0–4000 μM). A reaction buffer containing 1 mg/ml catalase (Sigma-Aldrich), 10 μM ferrous ammonium sulfate (Sigma-Aldrich) and the tetrameric MBP–PAH fusion protein (0.01 mg/ml) was prepared and injected in all 96 wells. After pre-incubation with L-phenylalanine for 5–20 min, the reaction was initiated by the addition of variable concentrations of BH_4 (6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin, Cayman Chemical) (0–500 μM) stabilized in 100 mM dithiothreitol (DTT; Fluka Chemie). PAH activity was determined at 25°C and 90 s measurement time per well. Using sets of 16 wells and 10 measurement cycles per set, total measurement time for all 96 wells was 22 min. Substrate production was measured by the detection of the increase in L-tyrosine fluorescence intensity, at an excitation wavelength of 274 nm and an emission wavelength of 304 nm, using a fluorescence photometer (FLUOstar OPTIMA, BMG Labtech) and assayed as triplicates. Measured fluorescence intensity signals were corrected by the inner filter effect of BH_4 for every BH_4 concentration added. Enzyme activity measurements were quantified by the measurement of L-tyrosine standards (0–200 μM) before each experiment, and fluorescence intensity was converted to enzyme activity units (nmol L-tyrosine/min × mg protein). Data were analyzed by non-linear regression analysis using the Michaelis–Menten or the Hill kinetic model after comparison of model-fitting using the *F*-test (GraphPad Prism 4.0c) (27). All concentrations mentioned refer to the final concentration in a 202 μl reaction mixture.

Standard PAH activity assay of eukaryotic expressed PAH. PAH enzyme activity was determined as previously described (2,39) with modifications. Twenty microliters of total lysates obtained from cell culture were pre-incubated with 1000 μM L-phenylalanine and 1 mg/ml catalase (Sigma-Aldrich) for 5 min (25°C) in 15 mM Na HEPES pH 7.3, followed by 1 min incubation with 10 μM ferrous ammonium sulfate (Sigma-Aldrich). The reaction was initiated by the addition of 75 μM BH_4 stabilized in 2 mM DTT, carried out for 60 min at 25°C and stopped by acetic acid followed by 10 min incubation at 95°C. All concentrations mentioned refer to the final concentration in a 100 μl reaction mixture. The amount of L-tyrosine production was measured and quantified by HPLC, assayed as duplicates. Three independent experiments were performed.

PAH activity landscapes

The data set of multi-well enzyme activity assayed in a 12 × 8 matrix corresponding to 12 different L-phenylalanine concentrations ranging from 0 to 4000 μM (columns) at 8 BH_4 concentrations ranging from 0 to 500 μM (rows) was loaded into non-linear regression analysis software (GraphPad Prism 4.0c). A non-linear regression analysis was performed for each column of the data matrix in order to extend the sparse data set for BH_4 concentrations from 8 measured to 400 newly calculated values following a substrate inhibition curve. This resulted in a 12 × 400 matrix of activity values. For further calculation of the data and for the creation of landscapes, this data matrix was exported to the free software

package R (www.r-project.org). In order to draw a smooth surface of the landscape, we used the function *interp.loess* from additional R package *tgp* (http://cran.r-project.org/web/packages/tgp/index.html), which interpolates between two data points by using local polynomial regression fitting to find a function between them. This resulted in an increase in data from an originally measured 12 × 8 (96-well format) over a 12 × 400 to a 400 × 400 data set. This grid was then depicted as a smooth landscape plot using the function *image.plot* from package fields (http://cran.r-project.org/web/packages/fields/index.html). To facilitate calculation of landscapes, a script was written accepting comma-separated files and automatically coloring landscapes depending on the measured and interpolated fluorescence intensities.

Stable expression of PAH in HEK293

Stably transfected cells were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. The Flp-In-293 cell line was maintained in basic DMEM (PAA Laboratories) supplemented with L-glutamine, high glucose (4.5 g/l), 10% fetal bovine serum (GIBCO), 1% antibiotics (Antibiotic-Antimycotic; PAA) and 100 μg/ml Zeocin (Invitrogen). Cells were stably transfected with pEF5/FRT/V5-DEST cDNA constructs coding for the wild-type, R261Q or Y414C PAH, respectively. Positive clones were selected and maintained in medium containing 150 μg/ml hygromycin B (Invitrogen).

For all further experiments, cells were cultured for 72 h under three different conditions: (i) basic RPMI 1640 medium (91 μM phenylalanine, PAA Laboratories) supplemented with stable glutamine, 10% fetal bovine serum (GIBCO), 1% antibiotics (Antibiotic-Antimycotic; PAA) and 150 μg/ml of hygromycin B, (ii) basic medium (as described above) with 500 μM phenylalanine, and (iii) basic medium with 1200 μM phenylalanine. Additionally, culture conditions were modified by adding 40 or 75 μM BH_4, respectively. Culture medium was changed every 24 h. Cells were harvested and lysed by three freeze-thaw cycles in a Tris–KCl lysis buffer (0.03 M Tris, 0.2 M KCl, pH 7.2) containing protease inhibitors (Roche), followed by 20 min centrifugation at 3000 rcf, 4°C. Recovered supernatants were subsequently used for activity assays.

Statistics

Group mean values were compared by Student's unpaired two-tailed *t*-test. Eukaryotic PAH activities following BH_4 treatment were analyzed by one-way ANOVA and Dunnett's post-test. Statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software).

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ORIGINAL ARTICLE

Mapping the functional landscape of frequent *phenylalanine hydroxylase* (*PAH*) genotypes promotes personalised medicine in phenylketonuria

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ABSTRACT

Background In phenylketonuria, genetic heterogeneity, frequent compound heterozygosity, and the lack of functional data for *phenylalanine hydroxylase* genotypes hamper reliable phenotype prediction and individualised treatment.

Methods A literature search revealed 690 different *phenylalanine hydroxylase* genotypes in 3066 phenylketonuria patients from Europe and the Middle East. We determined phenylalanine hydroxylase function of 30 frequent homozygous and compound heterozygous genotypes covering 55% of the study population, generated activity landscapes, and assessed the phenylalanine hydroxylase working range in the metabolic (phenylalanine) and therapeutic (tetrahydrobiopterin) space.

Results Shared patterns in genotype-specific functional landscapes were linked to biochemical and pharmacological phenotypes, where (1) residual activity below 3.5% was associated with classical phenylketonuria unresponsive to pharmacological treatment; (2) lack of defined peak activity induced loss of response to tetrahydrobiopterin; (3) a higher cofactor need was linked to inconsistent clinical phenotypes and low rates of tetrahydrobiopterin response; and (4) residual activity above 5%, a defined peak of activity, and a normal cofactor need were associated with pharmacologically treatable mild phenotypes. In addition, we provide a web application for retrieving country-specific information on genotypes and genotype-specific phenylalanine hydroxylase function that warrants continuous extension, updates, and research on demand.

Conclusions The combination of genotype-specific functional analyses with biochemical, clinical, and therapeutic data of individual patients may serve as a powerful tool to enable phenotype prediction and to establish personalised medicine strategies for dietary regimens and pharmacological treatment in phenylketonuria.

INTRODUCTION

The large number of *phenylalanine hydroxylase* (*PAH*) mutations (625 entries (public total), http://www.hgmd.org), the high incidence of compound heterozygosity, and the variability in distribution of common mutations between ethnic groups and geographical areas makes phenylketonuria (PKU, OMIM #261600) a genetic disease with pronounced allelic heterogeneity. PKU caused by deficiency of the PAH enzyme[1 2] has an overall incidence of 1:10 000 in European descendants, which varies considerably among different populations.[3 4]

The clinical phenotype of PKU is driven by mutation-induced loss of PAH function. A continuum of residual in vivo PAH enzyme activity determines the clinical picture, ranging from severe classical PKU via mild PKU to mild hyperphenylalaninaemia (MHP) with increasing activity and decreasing blood phenylalanine concentrations from severe to mild.[5 6] In addition to standard treatment using a phenylalanine-restricted diet, a significant proportion of patients is amenable to pharmacological treatment with the PAH cofactor tetrahydrobiopterin (BH_4).[7 8] This constitutes a superordinate phenotype that requires residual PAH activity[9 10] and further adds to the phenotypic variability of PAH deficiency.[11]

Mutations in the *PAH* gene can induce PAH loss-of-function by different molecular mechanisms. Splicing mutations, nonsense mutations, and out-of-frame indels lead to a complete loss of the PAH protein, whereas missense mutations as well as some in-frame indels still lead to the production of variant PAH proteins. Loss of function of variant PAH is a consequence of loss of specific enzymatic activity and/or loss of effective intracellular PAH protein amount caused by protein misfolding.[12–16]

Databases such as *PAH*db (http://www.pahdb.mcgill.ca) and BIOPKU (http://www.biopku.org) provide general information on *PAH* mutations and associated phenotypes; however, there is no database access on occurrence and frequency of genotypes in individual countries. A significant gap of knowledge concerns the enzymatic function associated with *PAH* genotypes. Available data on residual PAH enzyme activity is limited to single mutations expressed in different cellular systems using diverse enzyme assay protocols.[5 13 16–19] Non-invasive functional assays, for example, in blood cells, are not feasible because metabolic PAH function is restricted to the liver. Therefore, PAH activity is commonly determined by means of experimental in vitro systems. Since calculated enzyme activities based on heterogeneous data on single mutations do not reliably reflect residual activity in the patient, a consistent model system to assess PAH enzyme function arising from two different alleles reflecting the most common genotypes is needed. In addition, previous work has revealed that PAH function depends on the metabolic phenotype and a potential pharmacologic

administration of BH_4.[20] Genotype-specific data on PAH function assessed at different substrate and cofactor concentrations may thus assist physicians in managing the dietary treatment and in predicting the probability of pharmacological response in clinical practice. In order to provide this information, we (1) mapped the most common reported *PAH* genotypes in Europe and Middle East; (2) determined PAH residual function of frequent homozygous and compound heterozygous genotypes in a cell based model; (3) generated activity landscapes; and (4) assessed the PAH working range.

This study provides the first comprehensive dataset on PAH function arising from the most common genotypes carried by PKU patients in Europe and the Middle East. PAH enzyme activity assessed at a wide range of phenylalanine and BH_4 concentrations mirrors metabolic and therapeutic conditions and thus provides information on the optimal range of substantial PAH enzyme function in PKU patients. This may allow for genotype-specific optimisation of phenylalanine restricted dietary regimens and treatment with BH_4.

METHODS

Data sources for *PAH* genotypes and phenotypes

Data from 24 publications reporting *PAH* genotypes in 19 countries in Europe and the Middle East and unpublished data from German and French medical centres were analysed (see online supplementary table S1). Types and frequencies of mutations were obtained from the Human Gene Mutation Database (http://www.hgmd.org), the *PAH*db (http://www.pahdb.mcgill.ca) and the BIOPKU database (http://www.biopku.org). Data on PKU clinical phenotypes and BH_4 responsiveness were obtained from BIOPKU.

Assay of PAH activity

In this study, we analysed 30 *PAH* genotypes consisting of 18 *PAH* missense mutations and one intronic mutation c.1066-11G>A (previously known as IVS10-11G>A),[16] which results in the insertion of three amino acids (p.Gln355_Tyr356insGlyLeuGln) (see online supplementary table S2). Constructs carrying *PAH* mutations were co-expressed in COS-7 cells reflecting *PAH* genotypes. The multi-well PAH activity assay for determination of activity landscapes from eukaryotically expressed PAH was performed as previously described for recombinantly expressed and purified PAH[20] with modifications. Varying volumes of a 5 mM L-phenylalanine (Sigma-Aldrich) solution and 22.35 mM NaHEPES, pH 7.3, were injected (FLUOstar OPTIMA, BMG Labtech) into all wells of a 96-well plate. This resulted in 12 columns of varying L-phenylalanine concentrations (0–4000 μM). A reaction buffer containing 1 mg/mL catalase (Sigma-Aldrich), 10 μM ferrous ammonium sulfate (Sigma-Aldrich), and 20 μL protein lysate per well was subsequently injected in all 96 wells. After pre-incubation for 5 min, the reaction was triggered by the addition of variable concentrations of BH_4 (*6R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin, Schircks Laboratories) (0–500 μM, rows of the 96-well plate) stabilised in 100 mM dithiothreitol (DTT, Sigma-Aldrich). The PAH activity assay was incubated at 25°C for 15 min and stopped by acetic acid. All concentrations mentioned refer to the final concentration in a 200 μL reaction mixture. The formation of the L-Tyr product was quantified by high performance liquid chromatography (HPLC) using a Hypersil ODS-2 column (ThermoScientific). All PAH activity landscapes were assayed in three independent experiments and the data were combined. Genotypes were divided into three groups based on PAH activity determined at the area of peak activity (group 1, PAH residual activity <3.5%; group 2, ≥3.5% and <5%; group 3 ≥5%). If no area of peak activity was delimitable, activity at standard conditions (L-phenylalanine, 1 mM; BH_4, 75 μM) was used for classification.

PAH activity landscapes

PAH activity landscapes were generated as previously described[20] with modifications. The dataset obtained from the multi-well PAH activity assayed in a 12×8 matrix corresponding to 12 different L-phenylalanine concentrations (0–4000 μM; columns) at eight BH_4 concentrations (0–500 μM; rows) was loaded into an analysis software (GraphPad Prism 5.0a). Non-linear regression analysis allowed for expanding the dataset for BH_4 concentrations from eight measured to 200 interpolated values following a substrate inhibition curve. This resulted in a 12×200 matrix of activity values. For the generation of landscapes, the data matrix was exported to the free software package *R* (http://www.r-project.org). In order to draw a smooth surface of the landscape, the function *interp.loess* from an additional *R* package *tgp* (http://cran.r-project.org/web/packages/tgp/index.html) was applied, which interpolates between two data points by using local polynomial regression fitting to find a function between them. This resulted in a matrix of 400×400 data points, which was then depicted as a smooth landscape plot using the function *image.plot* from package fields (http://cran.r-project.org/web/packages/fields/index.html). To facilitate calculation of landscapes, a web application (http://pah-activitylandscapes.org/calcLandscapes.php) was set up accepting comma-separated files and automatically colouring landscapes depending on the measured and interpolated enzyme activity values.

Web application

An interactive website provides country- and region-specific information on *PAH* genotypes and links genotypes to graphical representation of activity landscapes (http://pah-activitylandscapes.org). The site will be updated and users can request the generation of landscapes of additional genotypes.

More detailed information on material and methods is available in the online supplementary data.

RESULTS

PAH genotypes in Europe and the Middle East

We performed a comprehensive PubMed literature search to determine the frequency and region-specific distribution of *PAH* genotypes. The study focused on Europe and the Middle East owing to the shared ethnic background in this region and data from 20 countries spanning from Portugal to Iran were selected for evaluation (figure 1 and see online supplementary table S1). The 3066 PKU patients of this study population displayed 690 different genotypes based on 264 mutations (http://pah-activitylandscapes.org).

The type and frequency of single *PAH* mutations constituting the genotypes in our study cohort matched well with the frequency documented in gene databases (table 1), and mutations considered as prevalent in the European population[4] covered 80.3% of alleles in our cohort (see online supplementary table S2). The predominant variant p.Arg408Trp (37%) occurred in all countries except Lebanon and Cyprus, while the second most common variant, p.Gln355_Tyr356insGlyLeuGln (10.2%), did not appear in Croatian, Slovenian, and Swedish populations. A region-specific distribution was also observed for other mutations (see online supplementary figure S1).

The most common full *PAH* genotypes for eight countries are shown in figure 1. Of all genotypes analysed, 9% were homozygous, 91% compound heterozygous, and 49% of PKU patients

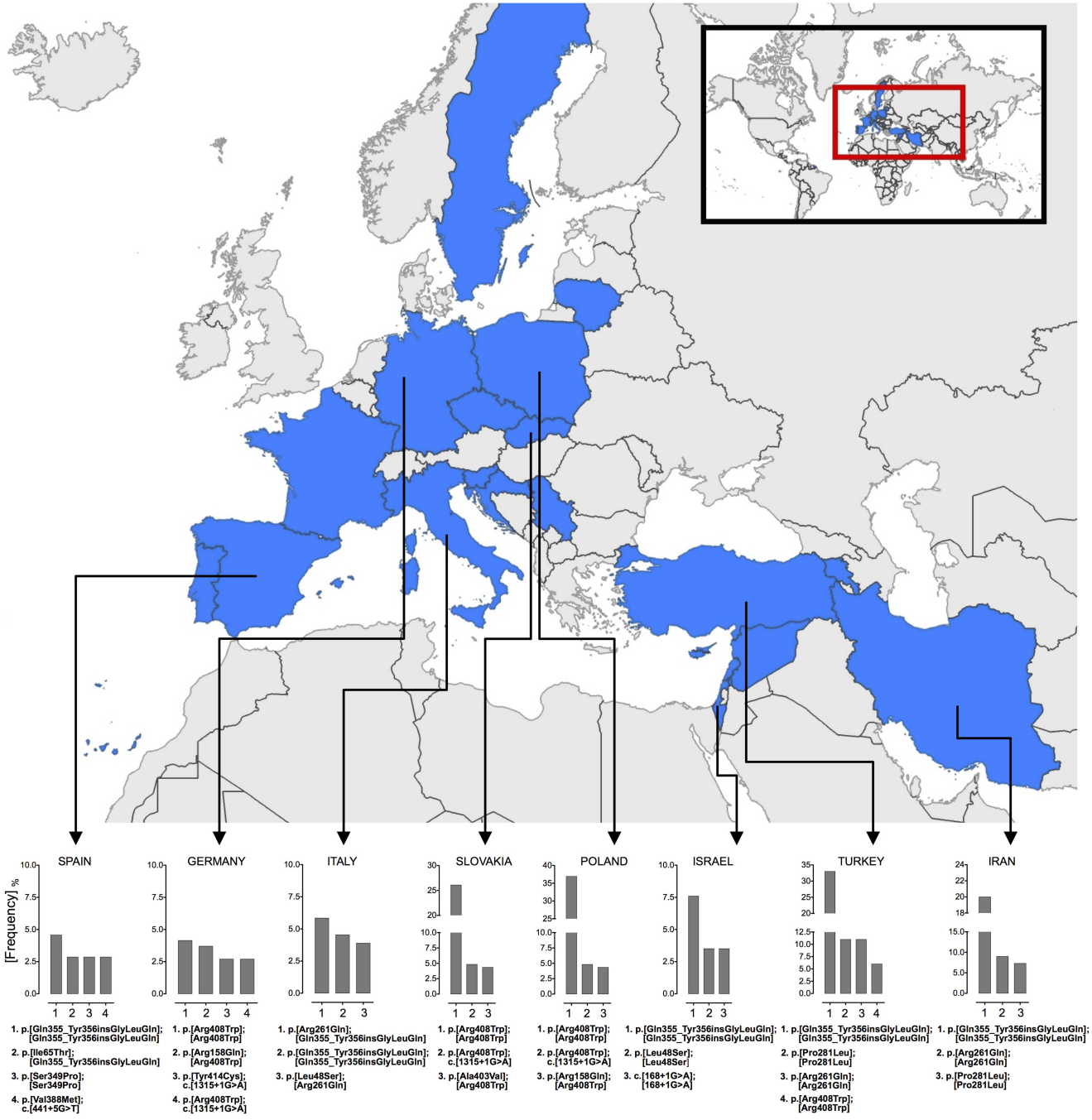

Figure 1 *Phenylalanine hydroxylase* (*PAH*) genotypes in Europe and Middle East. Countries included in the study are marked in blue. The graphs below show the frequencies of the most common *PAH* genotypes in individual countries for which data from more than 100 patients were available. In case the effect of intronic mutation on protein level is not known, the substitution at DNA level was used. The map was created using http://www.stepmap.de.

carried missense mutations on both alleles. The 30 most frequent genotypes encoding expressible PAH proteins from both alleles (table 1) covered 55% of patients in our study population (BIOPKU, 33%; *PAH*db, 18%). The frequency of these genotypes was high in Croatia (88%), Turkey (87%), and France (61%) and lower in Slovenia (11%) and Spain (10%). The genotype p.[Arg408Trp];[Arg408Trp] occurred in 21% of patients and was predominant in Northern and Eastern Europe (Lithuania, Norway, Poland, Slovakia), followed by homozygous p.Gln355_Tyr356insGlyLeuGln (5.2%), which was frequent in the Middle East (Iran, Turkey, Israel, Lebanon, Armenia). Genotype frequencies in five geographical regions (Northern Europe, Southern Europe, Western Europe, Eastern Europe, and Middle East) and country-specific frequencies are summarised in online supplementary figure S2 and are available at http://pah-activitylandscapes.org.

Residual PAH function of wild-type PAH and frequent *PAH* genotypes

Ex vivo assessment of PAH enzyme activity in liver tissue requires unjustifiable invasive procedures. Thus, we aimed to provide a cellular model that quantifies genotype-specific residual PAH function and takes into account PAH protein homeostasis and the metabolic environment. PAH activity of

Table 1 *PAH* genotypes in PKU patients from Europe and the Middle East and associated frequencies

			Frequency [%]		
	Allele 1	Allele 2	Our study*	BIOPKU†	*PAH*db†
1‡	p.Arg408Trp	p.Arg408Trp	21.0	10.7	5.0
2‡	p.Gln355_Tyr356insGlyLeuGln	p.Gln355_Tyr356insGlyLeuGln	5.2	2.3	1.5
3‡	p.Arg408Trp	p.Arg158Gln	2.9	1.7	0.8
4‡	p.Arg408Trp	p.Gln355_Tyr356insGlyLeuGln	2.8	1.5	0.2
5‡	p.Arg261Gln	p.Arg261Gln	2.1	1.7	1.7
6‡	p.Arg408Trp	p.Ala403Val	2.0	1.2	–
7	p.Arg408Trp	c.1315+1G>A	2.0	1.5	1.7
8‡	p.Arg408Trp	p.Pro281Leu	1.7	1.0	0.5
9‡	p.Arg408Trp	p.Arg261Gln	1.6	1.4	1.8
10‡	p.Pro281Leu	p.Pro281Leu	1.6	0.9	1.2
11‡	p.Arg408Trp	p.Tyr414Cys	1.4	1.1	1.2
12‡	p.Arg408Trp	p.Ala300Ser	1.3	0.8	0.2
13‡	p.Leu48Ser	p.Leu48Ser	1.1	0.8	–
14‡	p.Arg408Trp	p.Ile306Val	1.0	0.5	–
15‡	p.Arg261Gln	p.Gln355_Tyr356insGlyLeuGln	0.9	0.7	0.5
16‡	p.Arg408Trp	p.Glu390Gly	0.8	0.6	0.2
17‡	p.Arg408Trp	p.Arg252Trp	0.7	0.6	0.5
18‡	p.Arg408Trp	p.Leu48Ser	0.7	1.0	0.3
19‡	p.Arg252Trp	p.Arg252Trp	0.6	0.4	0.2
20‡	p.Arg408Trp	p.Arg297His	0.6	0.2	–
21‡	p.Pro281Leu	p.Gln355_Tyr356insGlyLeuGln	0.6	0.3	0.3
22‡	p.Ala300Ser	p.Gln355_Tyr356insGlyLeuGln	0.6	0.4	0.2
23	p.Tyr414Cys	c.1315+1G>A	0.6	0.6	1.7
24‡	c.441+5G>T	c.441+5G>T	0.5	0.2	0.2
25‡	p.Leu48Ser	p.Gln355_Tyr356insGlyLeuGln	0.5	0.4	0.3
26‡	p.Leu48Ser	p.Arg261Gln	0.5	0.5	0.2
27‡	p.Arg408Trp	p.Val245Ala	0.5	0.3	0.3
28‡	p.Leu48Ser	p.Arg158Gln	0.4	0.3	0.2
29‡	p.Ile65Thr	p.Gln355_Tyr356insGlyLeuGln	0.4	0.4	0.6
30	p.Arg408Trp	g.47563_51794del4232 g.56161_56430ins268	0.4	–	–
31‡	p.Arg408Trp	p.Ile283Phe	0.4	0.2	0.3
32‡	p.Arg408Trp	p.Val388Met	0.4	0.2	–
33‡	p.Arg408Trp	p.Arg241His	0.4	0.3	–
34	p.Arg261Gln	c.1315+1G>A	0.4	0.4	0.8
35	p.Arg408Trp	p.Phe55fs	0.4	0.4	0.2
36‡	p.Arg408Trp	p.Leu348Val	0.4	0.4	0.2
	Total number of patients (n)		3066	7453	658
	Population of patients covered by 30 genotypes (%)		55.0	32.8	18.4
	Population of patients covered by 36 genotypes (%)		59.4	35.9	22.9

The table shows the 36 most frequent genotypes in Europe and the Middle East and their relative frequencies.
*Frequencies of genotypes retrieved from the literature (PubMed search) and obtained via personal communications.
†Frequencies of genotypes retrieved from public databases: BIOPKU (query 23 April 2014) and *PAH*db (query 19 April 2014).
‡30 *PAH* genotypes encoding expressible PAH proteins from both alleles selected for experimental studies.
PAH, phenylalanine hydroxylase; PKU, phenylketonuria.

cells expressing variant PAH encoded by both alleles of individual *PAH* genotypes was determined at the physiological to pathological range of phenylalanine concentrations (25–4000 μM) as well as a physiological to supratherapeutic range of BH_4 cofactor concentrations (9–500 μM).

The activity landscape of the wild-type enzyme showed a peak maximum enzyme activity of 72 658 pmol L-Tyr/min x mg protein at 431 μM L-phenylalanine and 130 μM BH_4 (figure 2, see online supplementary figure S3 and table 2). This was in line with previous results for purified wild-type PAH protein expressed in *Escherichia coli*.[20] The optimal working range for L-phenylalanine spanned from 135 μM ($[S]_{0.5}$) to 747 μM (K_i). Thus, the PAH enzyme displayed very low activity at L-phenylalanine concentrations <120 μM and a broader range of considerable activity at higher L-phenylalanine concentrations, even >600 μM. In addition, the wild-type enzyme showed high activity at cofactor concentrations between 30 μM ($[C]_{0.5}$) and 294 μM (K_i). At L-phenylalanine concentrations above the therapeutic threshold of 360 μM[21] the enzyme showed a need for increased BH_4 concentrations to maintain the same level of activity.

Residual PAH activity of eukaryotically expressed genotypes (table 2, see online supplementary tables S3 and S4) ranged from 1.7% (p.[Arg408Trp];[Arg408Trp]) to 11% (p.[Arg261Gln];[Arg261Gln]) of wild-type PAH, which fits well with the threshold of 15% PAH activity related to metabolic decompensation.[2] Genotypes were divided into three groups: those with PAH residual activity <3.5% (group 1, n=9); those

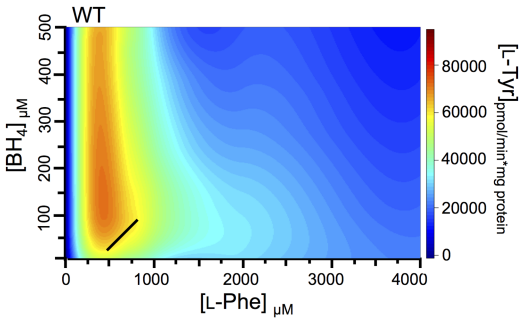

Figure 2 Phenylalanine hydroxylase (PAH) activity landscape of human wild-type (WT) PAH expressed in cell culture. Data for PAH enzyme activity assayed at varying L-phenylalanine and BH_4 concentrations were interpolated and depicted by a colour code. With increasing substrate concentrations, more BH_4 is needed to maintain the same level of enzyme activity (solid line).

with activities ≥3.5% and <5% (group 2, n=3); and those ≥5% (group 3, n=18) (table 2, figures 3–5). Group 3 contained all genotypes with mutations known to be associated with mild clinical phenotypes (p.Val245Ala, p.Ala300Ser, p.Glu390Gly, p.Ala403Val). Although p.Gln355_Tyr356insGlyLeuGln is commonly considered a null mutation,[16] it reached up to 5% of activity in the homozygous state, suggesting preservation of some residual function in patients. Taking into consideration the 30 most frequent genotypes, 37% of PKU patients in this cohort carried a genotype associated with low residual PAH function <5%, and 19% of patients can be expected to have a considerable residual enzyme function with activities >5%. Armenia, Croatia, Iran, Lithuania, Poland, Slovakia and Turkey showed a predominance of severe genotypes, whereas France, Italy, and the Czech Republic are countries where milder biochemical phenotypes are more frequent than severe ones (see online supplementary table S5).

Peak enzyme activity and optimal working range of *PAH* genotypes

We aimed to identify the metabolic context for best PAH function. Peak PAH enzyme activity— that is, the relation of optimal enzyme activity to substrate and cofactor concentrations—constitutes an important functional feature in phenylalanine metabolism and is of clinical relevance. We defined the working optimum for the PAH enzyme as the range of activity between $[S]_{0.5}$ and (K_i) for the substrate and between $[C]_{0.5}$ and (K_i) for the cofactor— that is, the ranges of all activities above 50% (table 2 and see online supplementary figure S4). Functional activity landscapes from group 1 displayed very low activity with no defined peak (figure 3). In group 2, enzyme activities were still low but an area of peak activity mapping to the area of wild-type PAH was delimitable (figure 4). Group 3 genotypes showed considerable residual activities and delimitable peak areas with the exceptions of p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln] and homozygous p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln] (figure 5). However, the position and shape of areas of peak enzyme activity showed large variation within this group. The substrate concentration at peak activity ranged from 251–882 µM for genotypes that displayed a defined peak area. For p.[Ile306Val];[Arg408Trp], p.[Ala403Val];[Arg408Trp], and p.[Tyr414Cys];[Arg408Trp] peak activity mapped to significantly lower phenylalanine concentrations than observed for the wild-type. These left-shifts were associated with higher apparent affinity of the enzymes towards the substrate, as reflected by lower values for $[S]_{0.5}$. On the other hand, p.[Arg261Gln];[Arg261Gln], p.[Arg261Gln];[Arg408Trp], p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln], and p.[Arg297His];[Arg408Trp] led to peak activities at higher phenylalanine concentrations associated with lower apparent affinity of the enzyme towards the substrate, as reflected by higher values for $[S]_{0.5}$. All but one of these genotypes contained p.Arg261Gln. All other genotypes displayed peak activities at L-phenylalanine concentrations in the wild-type range.

The working range of wild-type PAH with respect to the substrate covered an interval of >600 µM. Broadening of the working range of the PAH enzyme (interval >900 µM), denoting high residual enzyme activity over a wide range of phenylalanine concentrations, was observed for p.[Leu48Ser];[Arg261Gln], p.[Arg261Gln];[Arg261Gln], p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln], and p.[Arg297His];[Arg408Trp]. Three of these genotypes contained the allele p.Arg261Gln and 4.1% of the patients in our study population carried a genotype associated with this activity landscape feature. On the other hand, a narrow working range leading to considerable PAH activity only in a tight window of substrate concentrations (interval <460 µM) was observed for p.[Leu48Ser];[Leu48Ser], p.[Leu48Ser];[Arg158Gln], p.[Leu48Ser];[Arg408Trp], p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln], p.[Ala300Ser];[Gln355_Tyr356insGlyLeuGln], p.[Ile306Val];[Arg408Trp], p.[Ala403Val];[Arg408Trp], and p.[Tyr414Cys];[Arg408Trp], with p.Leu48Ser and p.Arg408Trp being the leading mutations in this group. These occurred in 7.7% of the patients. Genotypes showing a left-shift always showed a smaller area of enzyme activity, but not all genotypes with narrow working ranges were left-shifted. Thus, high affinity towards the substrate and a smaller area of phenylalanine-dependent activity range may be due to independent mechanisms.

The cofactor concentration at peak activity ranged from 94–409 µM. All genotypes with a residual activity ≥3.5% and peak enzyme activities at lower cofactor concentrations than the wild-type (p.[Leu48Ser];[Arg261Gln], p.[Arg261Gln];[Arg261Gln], p.[Arg261Gln];[Arg408Trp], p.[Arg297His];[Arg408Trp], p.[Glu390Gly];[Arg408Trp], p.[Ala403Val];[Arg408Trp]) included the alleles p.Arg261Gln or p.Arg408Trp. Interestingly, a shift towards lower cofactor concentrations was not associated with an increase in the apparent affinity of the enzymes towards the cofactor as reflected by comparable values for $[C]_{0.5}$. Genotypes p.[Leu48Ser];[Arg408Trp], p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln], p.[Ile65Thr];[Gln355_Tyr356insGlyLeuGln], p.[Ala300Ser];[Gln355_Tyr356insGlyLeuGln], p.[Leu48Ser];[Arg158Gln], and p.[Leu348Val];[Arg408Trp] led to peak activities at higher BH_4 concentrations. It is of note that the presence of the allele p.Gln355_Tyr356insGlyLeuGln correlated with a shift of peak activity towards higher BH_4 concentrations when residual activity is ≥5%. Most of these genotypes contained a mutation previously classified as being inconsistently linked to clinical phenotypes (p.Leu48Ser, p.Ile65Thr, p.Ala300Ser) (see online supplementary figure S5). Patients carrying these genotypes may need higher cofactor concentrations for optimal enzyme activity. All other genotypes induced peak activities at similar BH_4 concentrations than the wild-type.

Impact of PAH function on clinical phenotypes

Next, we linked functional data to clinical phenotypes retrieved from BIOPKU (see online supplementary figure S5). Group 1 genotypes with very low PAH activities lacking a well-defined peak of activity were consistently associated with BH_4 non-responsive classical PKU. One exception, p.[Arg241His];[Arg408Trp], showed a

Table 2 Residual PAH activity and optimal enzymatic working range of PAH proteins

Genotype	Group	*Peak PAH activity	Residual activity at peak (% of WT)	†L-Phenylalanine concentration at peak activity	‡$[S]_{0.5}$—K_i	§BH_4 concentration at peak activity	¶$[C]_{0.5}$—K_i
WT		72 658±13 134	100	431±23	135–747	130±11	30–294
p.[Leu48Ser];[Leu48Ser]	3	4769±411	6.6	351±3	92–533	146±11	37–463
p.[Leu48Ser];[Arg158Gln]**	2	3041±134	4.2	330±7	78–542	200±15	89–458
p.[Leu48Ser];[Arg261Gln]	3	6229±61	8.6	501±17	146–1164	100±1	31–302
p.[Leu48Ser];[Arg408Trp]	2	2534±118	3.5	351±0	90–543	268±13	33–432
p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln]	3	5468±337	7.5	391±9	103–556	235±73	32–432
p.[Ile65Thr];[Gln355_Tyr356insGlyLeuGln]	3	4978±185	6.8	441±30	119–605	409±76	43–424
p.[Arg158Gln];[Arg408Trp]††	1	2470±708	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Arg241His];[Arg408Trp]††	1	2071±156	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Val245Ala];[Arg408Trp]	3	7486±360	10	391±9	120–643	157±12	34–468
p.[Arg252Trp];[Arg252Trp]††	1	2699±74	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Arg252Trp];[Arg408Trp]††	1	2267±202	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Arg261Gln];[Arg261Gln]	3	8031±861	11	762±10	283–1314	94±2	28–249
p.[Arg261Gln];[Arg408Trp]	3	4598±351	6.3	752±6	252–1023	99±14	35–258
p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln]	3	6013±903	8.3	802±119	197–1118	149±39	32–247
p.[Pro281Leu];[Pro281Leu]††	1	2265±252	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Pro281Leu];[Arg408Trp]††	1	2297±117	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln]††	3	4163±663	5.7	n. a.	n. a.	n. a.	n. a.
p.[Ile283Phe];[Arg408Trp]††	1	2534±307	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Arg297His];[Arg408Trp]	3	7162±123	9.8	882±141	319–1938	99±11	29–219
p.[Ala300Ser];[Arg408Trp]	3	3819±375	5.2	391±4	120–615	135±11	30–461
p.[Ala300Ser];[Gln355_Tyr356insGlyLeuGln]	3	5011±439	6.9	431±11	131–576	219±70	34–429
p.[Ile306Val];[Arg408Trp]	3	4494±453	6.2	271±6	64–513	105±6	52–298
p.[Leu348Val];[Arg408Trp]	3	5700±395	7.8	511±31	156–1046	215±29	31–331
p.[Val388Met];[Arg408Trp]	3	5365±110	7.4	361±4	112–730	159±8	32–371
p.[Glu390Gly];[Arg408Trp]	3	6005±314	8.3	501±7	150–770	100±15	30–288
p.[Ala403Val];[Arg408Trp]	3	6444±1054	8.8	251±14	52–510	100±0	44–226
p.[Arg408Trp];[Arg408Trp]††	1	1273±157	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Arg408Trp];[Gln355_Tyr356insGlyLeuGln]††	1	2919±453	n. a.	n. a.	n. a.	n. a.	n. a.
p.[Tyr414Cys];[Arg408Trp]	2	3560±271	4.9	301±3	87–528	139±9	33–404
p.[Gln355_Tyr356insGlyLeuGln]; [Gln355_Tyr356insGlyLeuGln]††	3	3710±369	n. a.	n. a.	n. a.	n. a.	n. a.

The table summarises height and position of peak PAH enzyme activities and the optimal working ranges arising from group 1, 2 and group 3 *PAH* genotypes. Residual PAH activity at standard conditions is summarised in table S3.
*Peak PAH enzyme activity is given as [pmol L-tyrosine/min x mg protein]±SEM.
†L-Phenylalanine concentration at peak activity is given as [μM]±SEM.
‡$[S]_{0.5}$—K_i indicates the optimal PAH working range (>50% of the respective residual activity) as to substrate concentrations.
§BH_4 concentration at peak activity is given as [μM]±SEM.
¶$[C]_{0.5}$—K_i indicates the optimal PAH working range (>50% of the respective residual activity) as to cofactor concentrations.
**Calculated parameters refer to a peak mapping to the area of WT peak PAH activity; a second area of high activity was observed for L-phenylalanine > 2500 μM.
††Calculated parameters refer to the area of highest PAH activity; a defined peak area was not delimitable.
n. a., not applicable, no peak area was delimitable.
PAH, phenylalanine hydroxylase; WT, wild-type.

leading phenotype of mild PKU (70% of patients), yet responsiveness to BH_4 was only assessed for two cases. Group 2 genotypes, all displaying intermediate residual activity and a defined area of peak activity, showed phenotypic heterogeneity with both mild and classical PKU phenotypes and a significant share of patients responding to BH_4. Among group 3, two subgroups were analysed. Group 3 genotypes without area of peak activity (p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln], p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln]), leading to a total loss of landscape architecture, were mainly associated with classical PKU and a low response rate to BH_4 (10–25%). These also displayed rather low residual activities within this group (5.1%, 5.7%). Yet other genotypes with residual activities in the same range, but showing a defined area of peak activity, displayed higher response rates suggesting that a loss of landscape architecture is linked to a loss of BH_4 responsiveness.

Group 3 genotypes with peak activity were predominantly associated with MHP or mild PKU and high response rates to cofactor treatment. This was not the case for p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln], p.[Ile65Thr];[Gln355_Tyr356insGlyLeuGln], p.[Arg261Gln];[Arg408Trp], p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln], and p.[Leu348Val];[Arg408Trp], with the predominance of classical PKU and unresponsiveness to pharmacological treatment. Interestingly, three of these genotypes contained p.Gln355_Tyr356insGlyLeuGln and three of these genotypes were associated with peak activities at increased cofactor concentrations. Therefore, dosages used in standard response tests may not be sufficient to meet the high need for BH_4 of patients carrying one of these genotypes.

Taken together, the results from this study indicate that residual PAH activity alone is not a reliable predictor of the clinical phenotype but other factors may be taken into account.

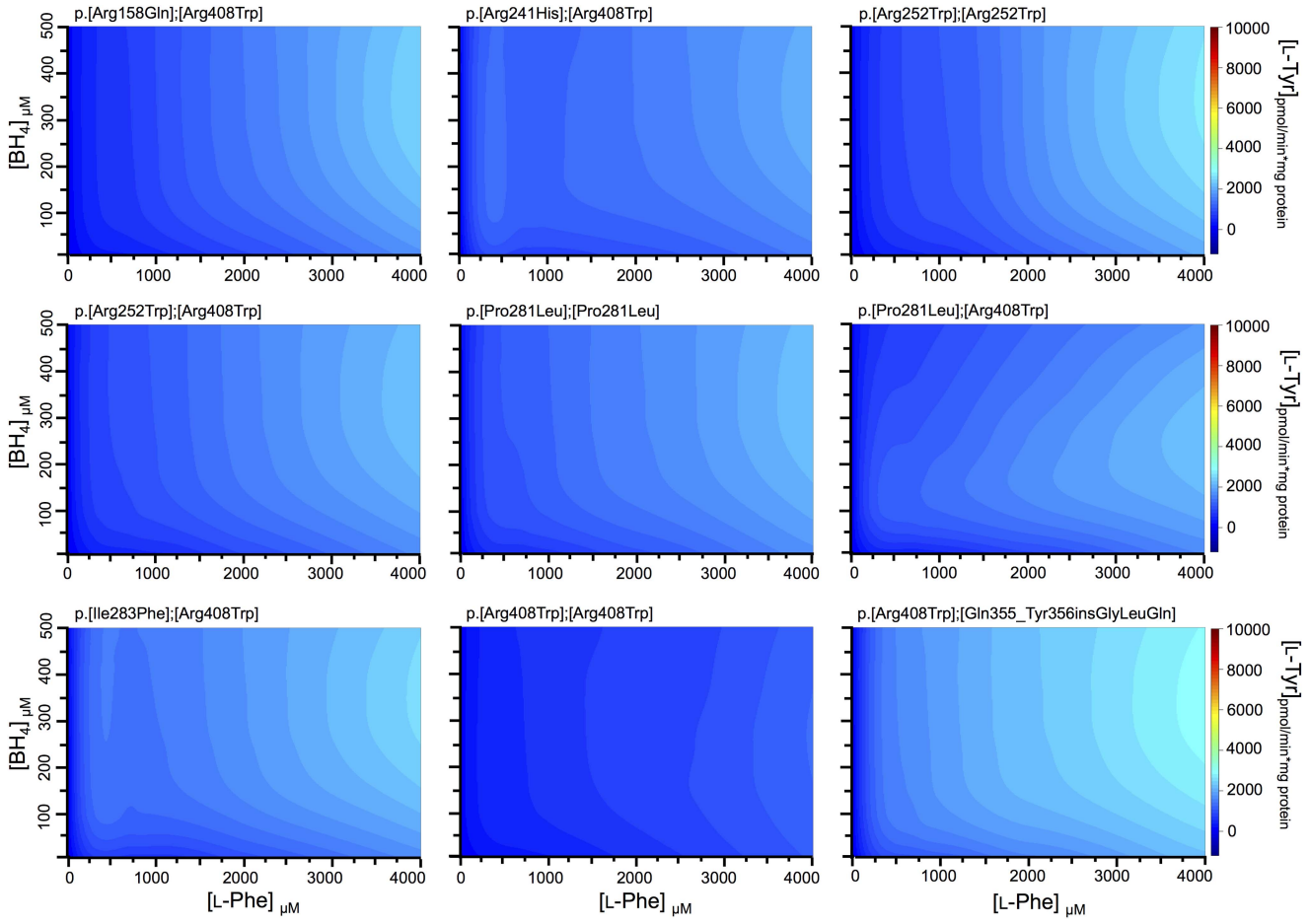

Figure 3 Group 1 phenylalanine hydroxylase (PAH) activity landscapes. The interpolated residual enzyme activities after expression of homozygous and compound heterozygous *PAH* genotypes were colour-coded and given as a function of different L-phenylalanine and BH_4 concentrations. Group 1 refers to genotypes associated with a PAH residual activity of <3.5% of the wild-type.

Our analyses led to the recognition of the following orienteering rules for the implementation of personalised medicine strategies. (1) Low residual activity is associated with mostly BH_4 unresponsive classical PKU. (2) Lack of a defined area of peak activity leads to a loss of response to BH_4. (3) The occurrence of inconsistent clinical phenotypes with low rates of BH_4 response is linked to a higher need for BH_4. (4) Residual activity >5%, a defined peak of activity, and normal need for BH_4 are associated with mostly BH_4 responsive mild PKU or MHP. Moreover, the occurrence of the allele p.Arg261Gln is often associated with inconsistent phenotypes, whereas the allele p.Gln355_Tyr356insGlyLeuGln is linked to a higher cofactor need.

DISCUSSION

The healthcare practitioner's view on genetic disease underwent change during the last decade. Genotyping has become easier and databases provide access to a wealth of data related to genetic variation. In addition, an increasing number of therapies evolved from an improved understanding of the molecular basis of maladies. After diagnosis of an inherited disease, families request reliable prediction of the clinical phenotype and the long

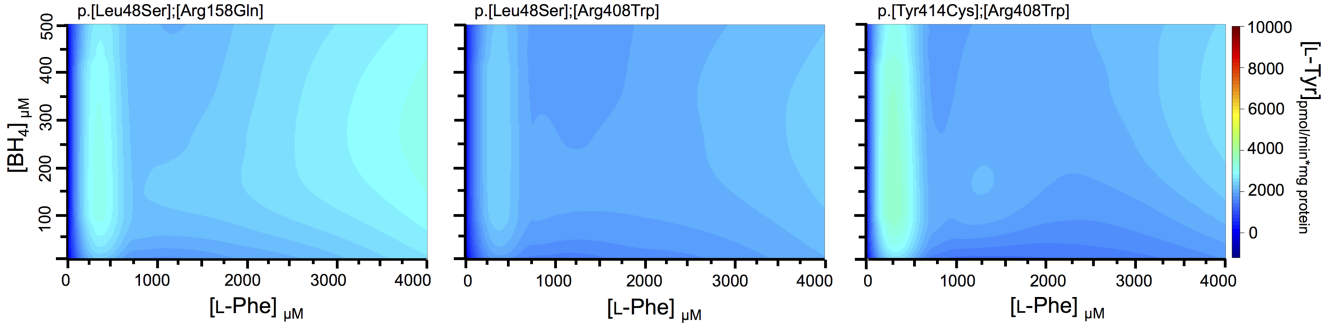

Figure 4 Group 2 phenylalanine hydroxylase (PAH) activity landscapes. The interpolated residual enzyme activities after expression of homozygous and compound heterozygous *PAH* genotypes were colour-coded and given as a function of different L-phenylalanine and BH_4 concentrations. Group 2 refers to genotypes associated with a PAH residual activity of ≥3.5% and <5% of the wild-type.

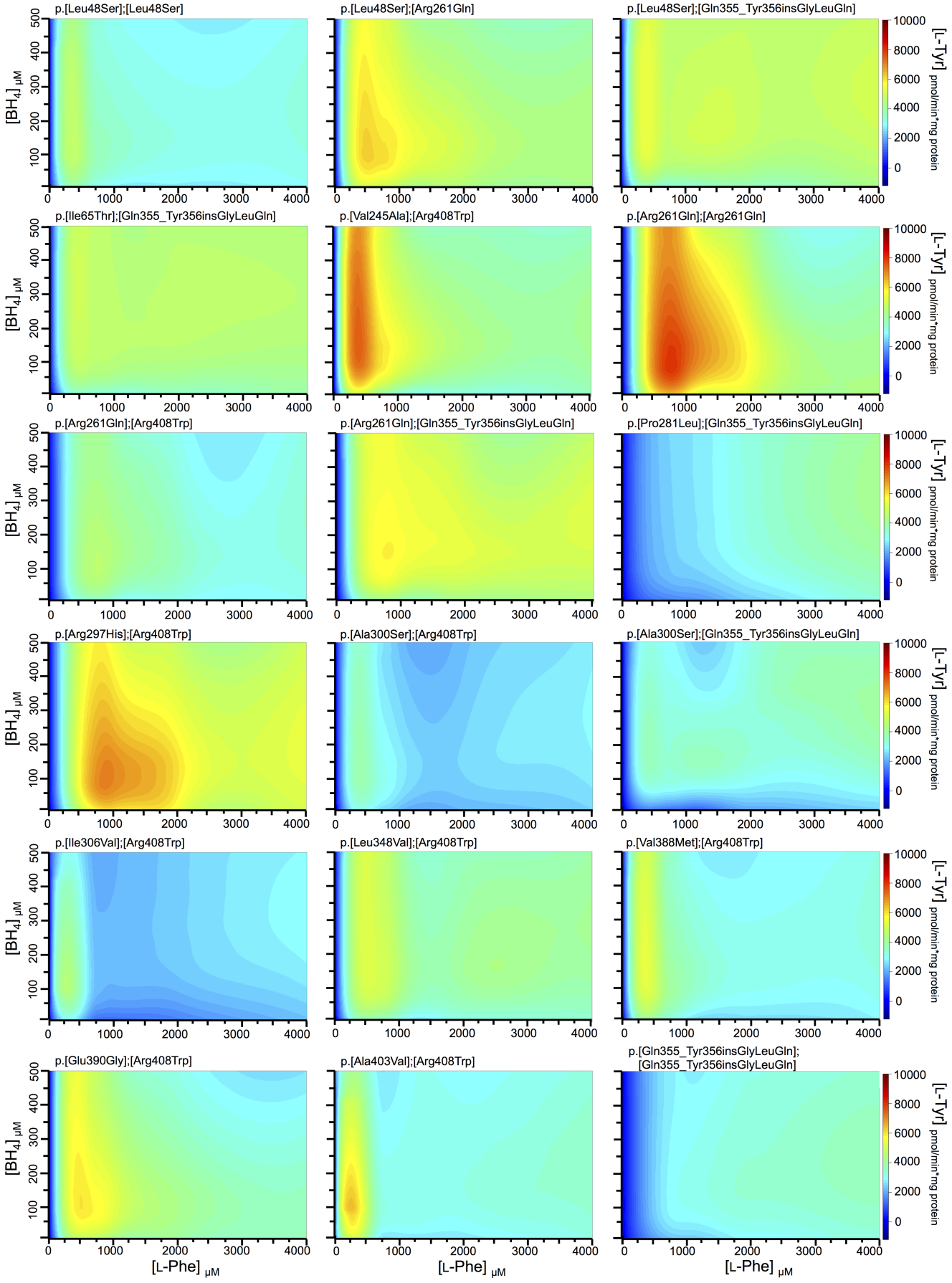

Figure 5 Group 3 phenylalanine hydroxylase (PAH) activity landscapes. The interpolated residual enzyme activities after expression of homozygous and compound heterozygous *PAH* genotypes were colour-coded and given as a function of different L-phenylalanine and BH_4 concentrations. Group 3 refers to genotypes associated with a PAH residual activity ≥5% of the wild-type.

term outcome. In the specific case of PKU, the approval of a drug providing the first pharmacological therapy for the disorder[7 22 23] added a new dimension to diagnosis and treatment. Clinical genetics at the service of patients faces the challenge of combining empirical evidence buried in databases with functional evidence from basic research. To this end, an experimental basis to assess molecular consequences of genetic alteration and to generate solid functional data considering full genotypes is required.

In this study, we aimed to provide a tool for the thorough analysis of genotype-related PAH function for PKU patients in Europe and the Middle East. A comprehensive literature search revealed that the 30 most frequent genotypes represent 55% of patients. Thus, the experimental investigation of a convenient number of genotypes can cover significant shares of a given population and thus provide meaningful information to physicians in clinical practice.

Residual enzyme activities reported for a number of single *PAH* mutations[16 24 25] do not reflect the patient's situation since values well above 50% are not expected to produce a biochemical and clinical phenotype. A study investigating in vivo PAH function provided evidence for a threshold, where enzyme activities >15% were linked to normal biochemical parameters and thus normal function.[2] Moreover, it is well known that consequences arising from full genotypes consisting of both alleles differ from the average induced by the two mutations involved and that proteins arising from different alleles influence each other—a phenomenon termed interallelic complementation.[26–28] Furthermore, although residual PAH enzyme activity assessed at fixed substrate and cofactor conditions for single mutations shows a general correlation with clinical severity and BH_4 responsiveness,[29 30] there is still significant inconsistency that hampers solid phenotype prediction in clinical care. This prompted us to develop a model system that mimics the cellular situation in carriers of both homozygous and compound heterozygous genotypes. Our system was calibrated at the equilibrium of PAH synthesis and degradation and takes into account the contributions of protein misfolding and accelerated degradation.[12 13 31] In the liver, the PAH enzyme is subjected to fluctuation of phenylalanine and, under treatment, of BH_4. In order to reflect the situation in vivo, we analysed PAH activity landscapes for full genotypes at a wide range of substrate and cofactor concentrations. These conditions assessed PAH function in the context of metabolic and therapeutic states and mirrored major molecular mechanisms underlying missense mutation-induced PKU; however, data obtained in vitro may still differ from residual activity in patients.

We observed several patterns of changes in the architecture of PAH activity landscapes and the combined evidence from functional analyses with biochemical, clinical, and therapeutic data delivered means to generate hypotheses about the mechanisms behind individual genotypes. Changes in PAH activity landscapes as regards to substrate concentrations may impact dietary management. Some genotypes (p.[Arg261Gln];[Arg261Gln], p.[Arg261Gln];[Arg408Trp], p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln], p.[Arg297His];[Arg408Trp]) induced a right-shift of the activity landscape. As a consequence, these genotypes are associated with very low enzyme activity at low phenylalanine concentrations. On the one hand, the enzyme is inactive at phenylalanine concentrations <200 μM. On the other hand, the right-shift results in good metabolic PAH function at higher phenylalanine concentrations (peak activity, ~800 μM phenylalanine). Therefore, internationally accepted target phenylalanine concentrations <360 μM in the patient may in these cases be difficult to achieve. In turn, phenylalanine tolerance may be disproportionately higher at phenylalanine concentrations slightly above the target, lowering the burden of diet. This has to be confirmed by clinical studies with appropriate analysis of dietary phenylalanine intake and blood phenylalanine concentrations. Notably, different dietary regimens leading to different metabolic states may at least in part explain the significant phenotypic variability of homozygous p.Arg261Gln and p.[Arg261Gln];[Arg408Trp] with respect to disease severity and BH_4 responsiveness (http://www.biopku.org) (see online supplementary figure S5).[20 32] In the experimental dataset presented here, the presence of the mutation p.Arg261Gln in the genotype often induced broadening of the PAH working range, with rather high enzyme activities at a wide range of phenylalanine concentrations. This may be associated with fewer phenylalanine fluctuations, known to be a major factor of brain damage.[33] Eight genotypes showed a narrow range of L-phenylalanine concentrations associated with considerable PAH activity. In three cases this was accompanied by a shift of the area of peak PAH activity below 300 μM L-phenylalanine. The carrier of such a genotype may need to keep blood phenylalanine concentrations in a tight range to optimise phenylalanine flux and could thus benefit from avoidance of phenylalanine fluctuations.

Another group of genotypes induced changes in PAH activity landscapes as regards to cofactor concentrations that may have implications not only for pharmacological treatment with BH_4. Some genotypes showed PAH activity peaks at lower BH_4 concentrations. Among these, the presence of the p.Arg261Gln mutation was associated with inconsistent BH_4 response, whereas the mutation p.Arg408Trp led to consistent BH_4 response. An activity peak at increased BH_4 concentrations was associated with the occurrence of p.Gln355_Tyr356insGlyLeuGln. Moreover, genotypes in this group also harboured mutations typically associated with inconsistent clinical phenotypes (p.Leu48Ser, p.Ile65Thr, p.Ala300Ser). Patients carrying these genotypes may need more BH_4 to achieve their optimal enzyme function. As a consequence BH_4 dosages used in standard BH_4 response tests may be insufficient to achieve an increase of enzyme activity and decrease of phenylalanine concentrations, the endpoint in response tests. Interestingly, genotypes with loss of landscape architecture p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln], p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln] also contained p.Gln355_Tyr356insGlyLeuGln and are associated with severe BH_4 non-responsive phenotypes. The apparent affinity of PAH to BH_4 in eukaryotic cells is ~30 μM and peak PAH activity was reached at 130 μM in our setting, which is in line with values obtained from recombinant PAH.[20] In addition, liver cells keep BH_4 at a concentration of about 9 μM.[34] Therefore, the PAH system suffers from a constant shortage of BH_4. Oxidative stress[35] or other environmental factors with negative impact on BH_4 availability may trigger PAH deficiency and thus may further aggravate clinical phenotypes. Taken together, alterations in BH_4 demand may add to the molecular mechanisms underlying phenotypic variability related to both disease severity and BH_4 responsiveness.

Our results confirm the frequent observation that residual PAH enzyme activity is not only the determinant as to the severity of the biochemical phenotype but also a prerequisite for BH_4 response and thus correlates with it.[9] However, this correlation is not absolute. For instance, in group 3 genotypes with residual enzyme activity >5%, we observed a significant proportion of genotypes associated with little or no BH_4 response. On the one hand, they were associated with relatively low residual activity

within this group; on the other hand, lack of response to cofactor treatment was linked to a higher cofactor need. The definition of three groups provides a classification of genotypes with different overall PAH enzyme activity that allows for a global estimation of the severity of the disease. Beyond that, our study revealed that PAH activity landscapes not only provide information on the quantitative value of residual activity but also on the position of enzyme activity in the metabolic and therapeutic space, and that this is another important determinant of the phenotype.

Inconsistent phenotypes in individuals carrying identical genotypes are well known in clinical practice. In Leuders *et al*,[36] five patients carrying the genotype p.[Leu48Ser];[Leu48Ser] all showed responsiveness at a low BH_4 dosage. In databases, four cases with non-responsive PAH deficiency (9%) and four cases with slow response to BH_4 (9%) are reported for individuals carrying this genotype (see online supplementary figure S5). These observations, however, are not necessarily in contradiction to each other. We observed a higher need for BH_4 to achieve peak activity for three genotypes containing the mutation p.Leu48Ser. Notably, Leuders *et al*[36] tested BH_4 response after 7 days of treatment. Repeated administration of BH_4 may lead to constantly elevated concentrations of cellular BH_4 and thus provide the variant PAH protein with a sufficient amount of cofactor, which may explain the consistent positive response. On the other hand, inconsistent BH_4 responsiveness has previously been reported for genotypes containing the p.Arg261Gln mutation.[37 38] Following our observation of a right-shifted area of peak activity with respect to phenylalanine values for p.[Arg261Gln];[Arg408Trp], the variability in the phenotype of patients carrying this genotype may, at least in part, be due to fluctuations in substrate concentrations, particularly with regard to blood phenylalanine concentrations at the time of the BH_4 loading test. As a conclusion for these observations, the assessment of PAH activity landscapes testing PAH function revealed how varying metabolic and therapeutic conditions may contribute to variability in the clinical phenotype. Nevertheless, other genetic and non-genetic factors[39] may also influence phenylalanine clearance and thus the clinical phenotype in PKU patients. Among these, differences in the disposal of excess phenylalanine by transamination when the hydroxylation reaction is blocked, variation in the carrier-mediated uptake of phenylalanine by the brain and the liver, and environmental factors such as oxidative stress are the best recognised modifiers to date.[25 33 35 40–42]

In conclusion, this work may exemplify how application of molecular genetics translates into medical practice. It gives new significance to gene analysis of the *PAH* locus in clinical routine and shows the importance of considering full genotypes. The analysis of activity landscapes in the metabolic and therapeutic space provided clinically relevant new insights into genotype-related impaired PAH function beyond the known link between residual activity and clinical phenotype. In the presence of specific genotypes we observed different patterns of activity landscape architecture and established orienteering rules for their interpretation. In addition, the work provides a web-based tool to assist clinicians in clinical care of PKU patients (http://pah-activitylandscapes.org). The web application allows for retrieving country- and region-specific information on genotypes and their related PAH function. Its sustainability and increasing representativeness will be ensured by continuous extension and updates as well as research on demand delivering new PAH activity landscapes upon request.

The analysis of individual genotype-related PAH activity landscapes together with the information provided on genotype-related clinical phenotypes may permit improved long term phenotype prediction and the implementation of personalised medicine strategies for dietary regimens and pharmacological treatment.

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Contributors ACM, FF and JZ contributed data on genotypes. ACM, MKD and SWG designed the study. MKD and MW performed functional analyses and generated activity landscapes. ACM, MKD and SWG analysed data and wrote the manuscript. JZ contributed to the final version of the manuscript.

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Competing interests ACM and FF are members of Merck Serono advisory boards and ACM is currently conducting research sponsored by this company. SWG is currently conducting research sponsored by Merck Serono. The research presented in this specific manuscript was not sponsored by the company.

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Supplemental Data

Figure S1

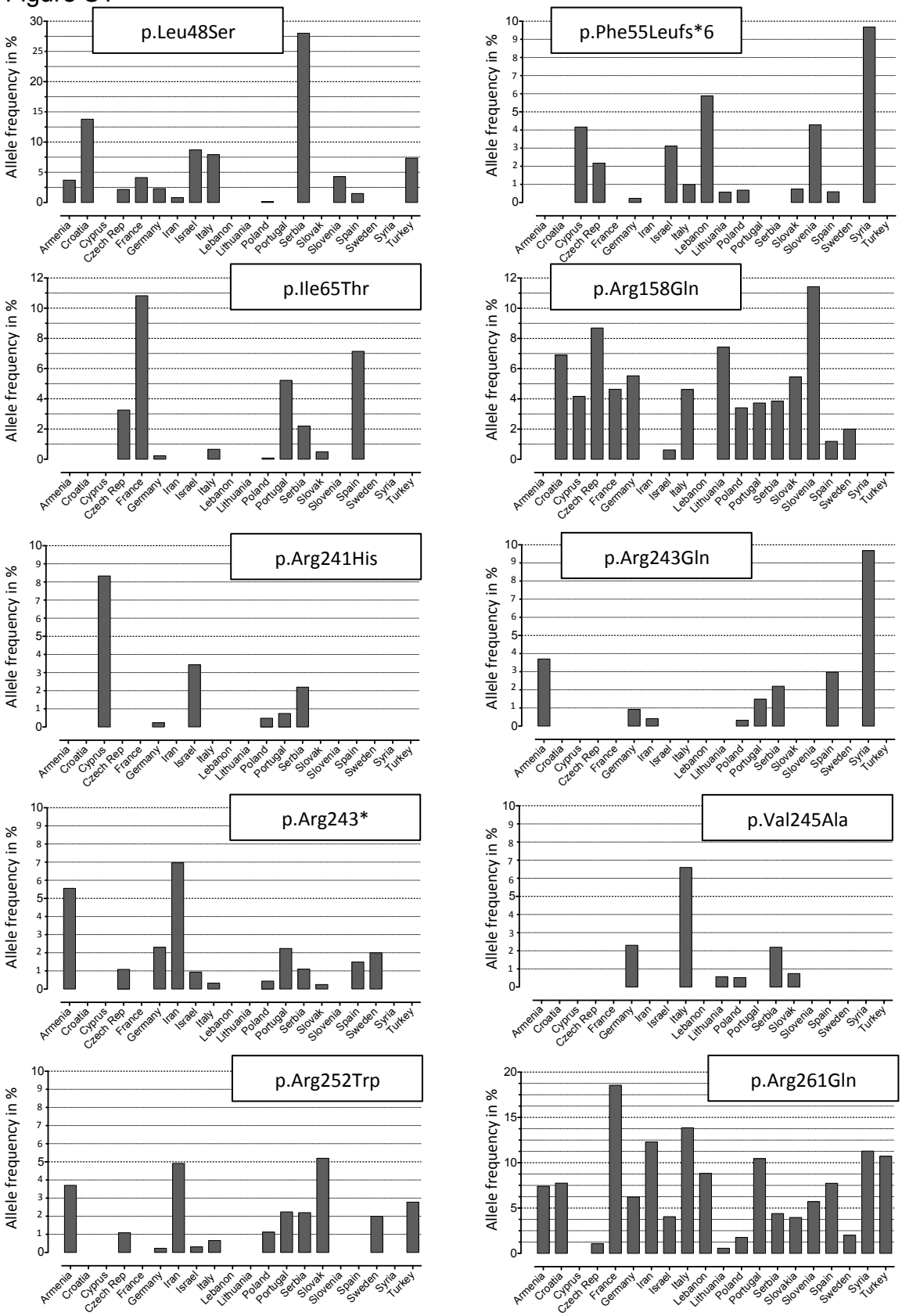

Figure S1

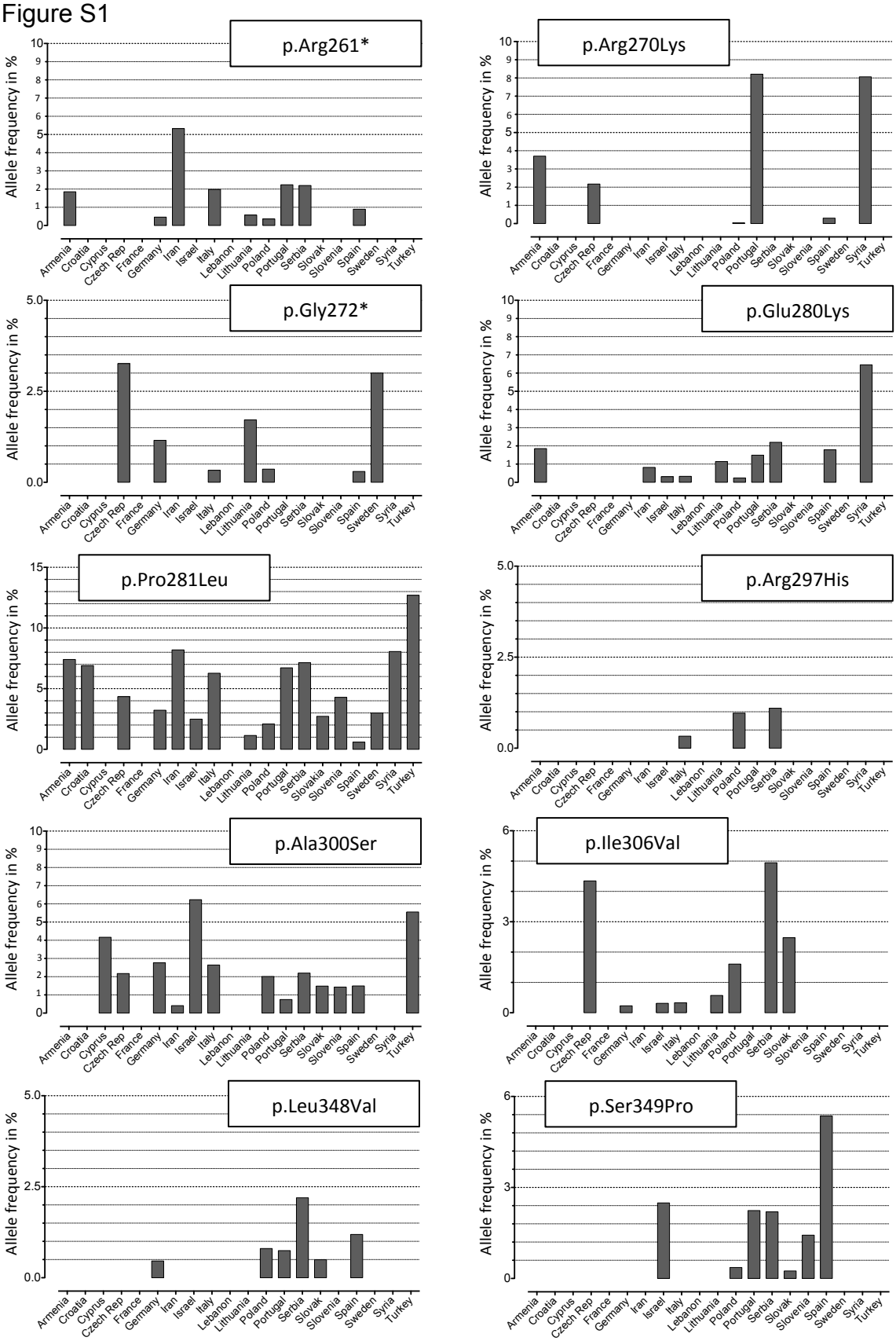
p.Arg261*
p.Arg270Lys
p.Gly272*
p.Glu280Lys
p.Pro281Leu
p.Arg297His
p.Ala300Ser
p.Ile306Val
p.Leu348Val
p.Ser349Pro
Allele frequency in %
Armenia
Croatia
Cyprus
Czech Rep
France
Germany
Iran
Israel
Italy
Lebanon
Lithuania
Poland
Portugal
Serbia
Slovak
Slovenia
Spain
Sweden
Syria
Turkey

Figure S1

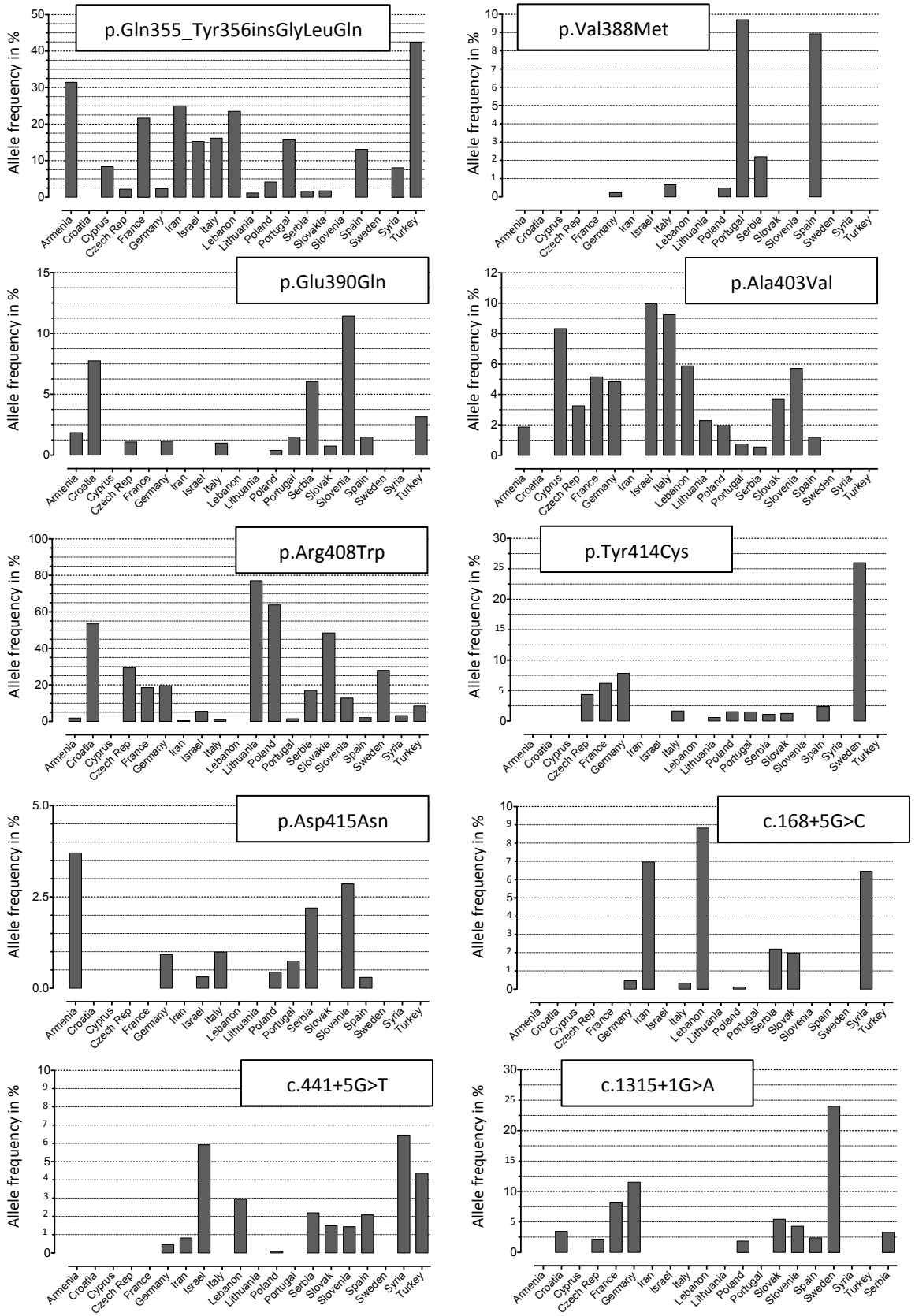
p.Gln355_Tyr356insGlyLeuGln
p.Val388Met
p.Glu390Gln
p.Ala403Val
p.Arg408Trp
p.Tyr414Cys
p.Asp415Asn
c.168+5G>C
c.441+5G>T
c.1315+1G>A
Allele frequency in %
Armenia
Croatia
Cyprus
Czech Rep
France
Germany
Iran
Israel
Italy
Lebanon
Lithuania
Poland
Portugal
Serbia
Slovakia
Slovak
Slovenia
Spain
Sweden
Syria
Turkey

Figure S1 Country-wise distribution of 30 most common *PAH* mutations identified in European and Middle East cohort of PKU patients. A comprehensive literature research and personal communications were the data sources for information on *PAH* genotypes. The frequency and the country-wise distribution of the underlying mutations is shown. Each mutation showed a unique distribution pattern.

Figure S2

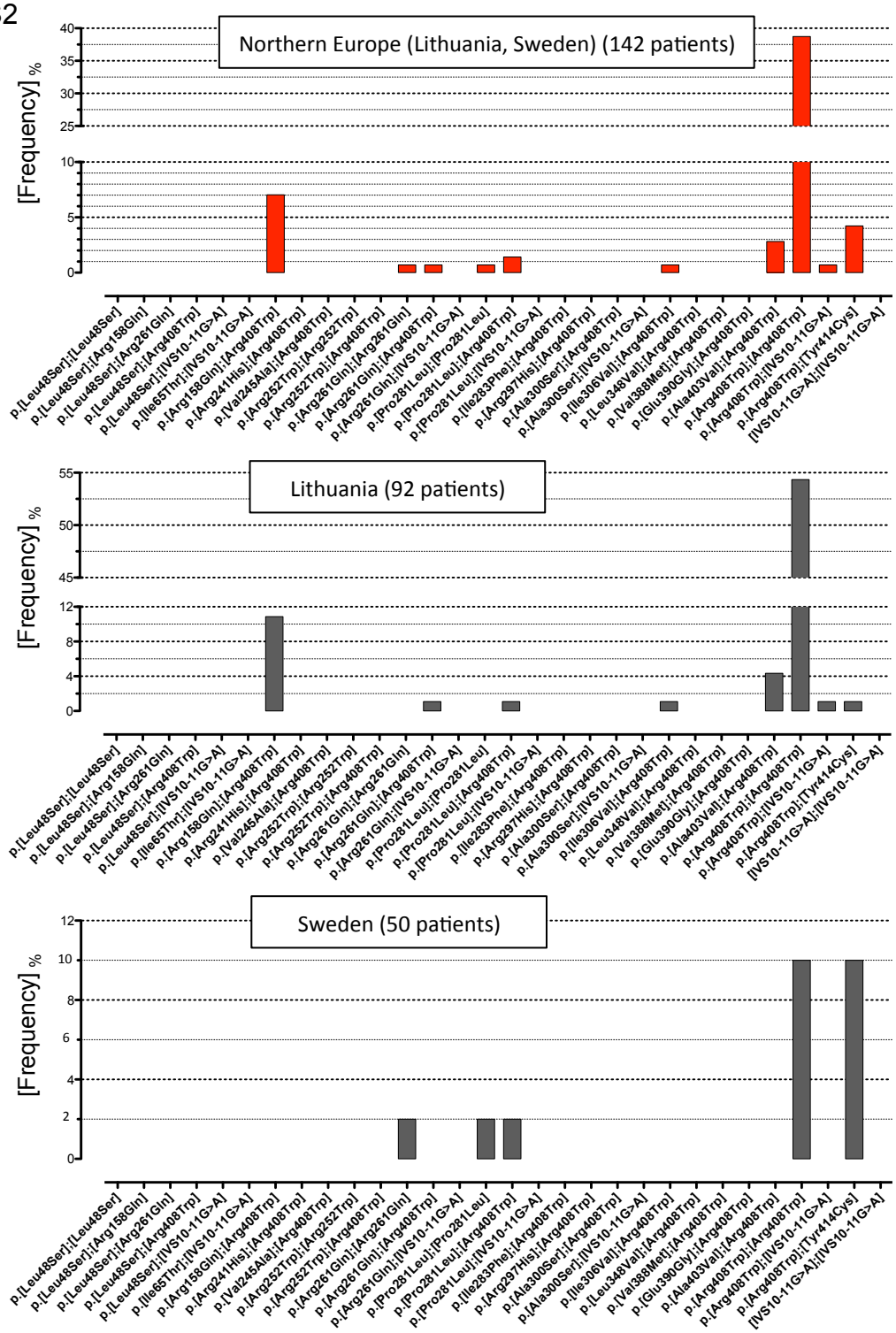
Northern Europe (Lithuania, Sweden) (142 patients)
[Frequency] %
Lithuania (92 patients)
[Frequency] %
Sweden (50 patients)
[Frequency] %
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

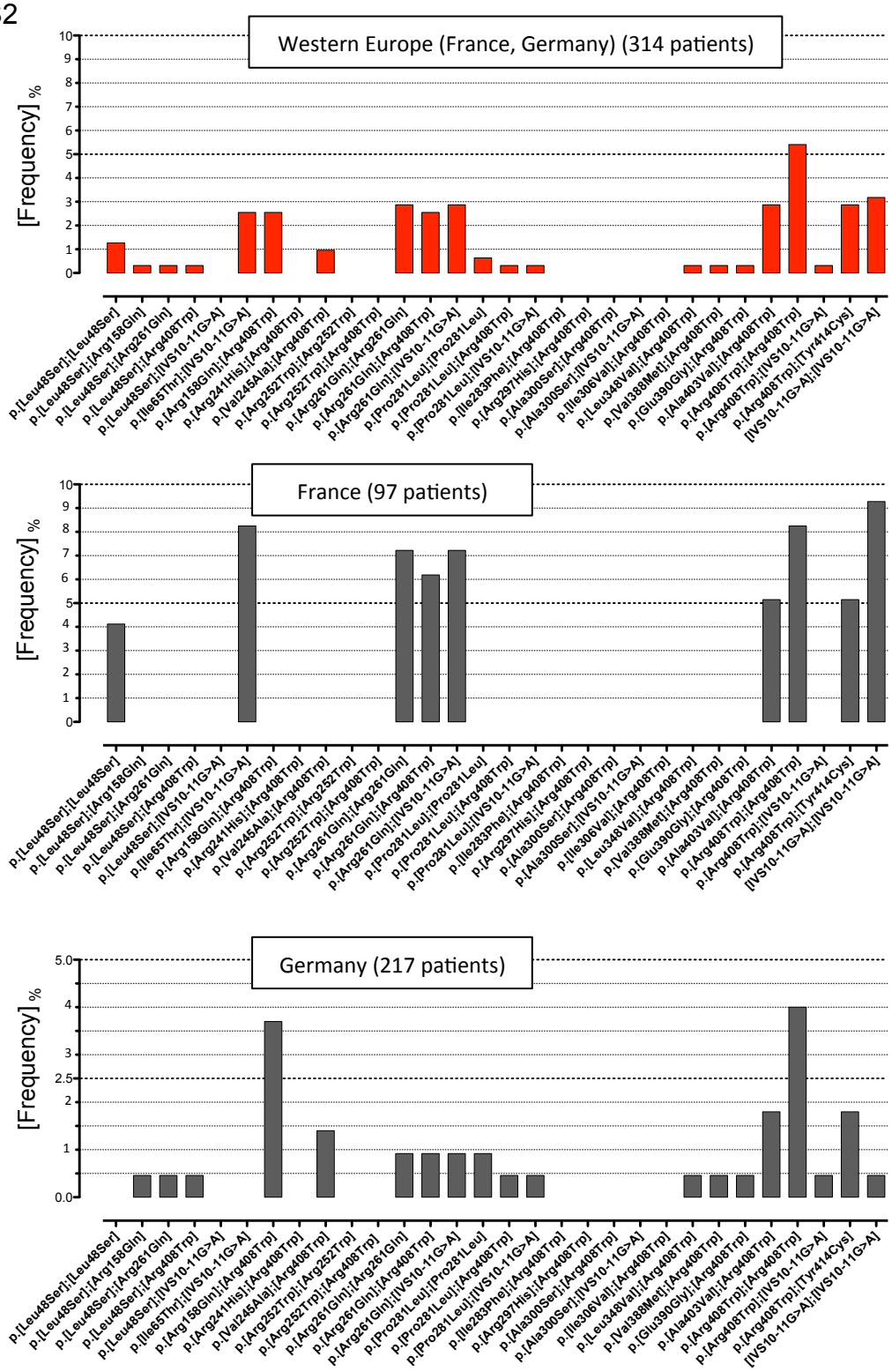
Western Europe (France, Germany) (314 patients)
[Frequency] %
France (97 patients)
[Frequency] %
Germany (217 patients)
[Frequency] %
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

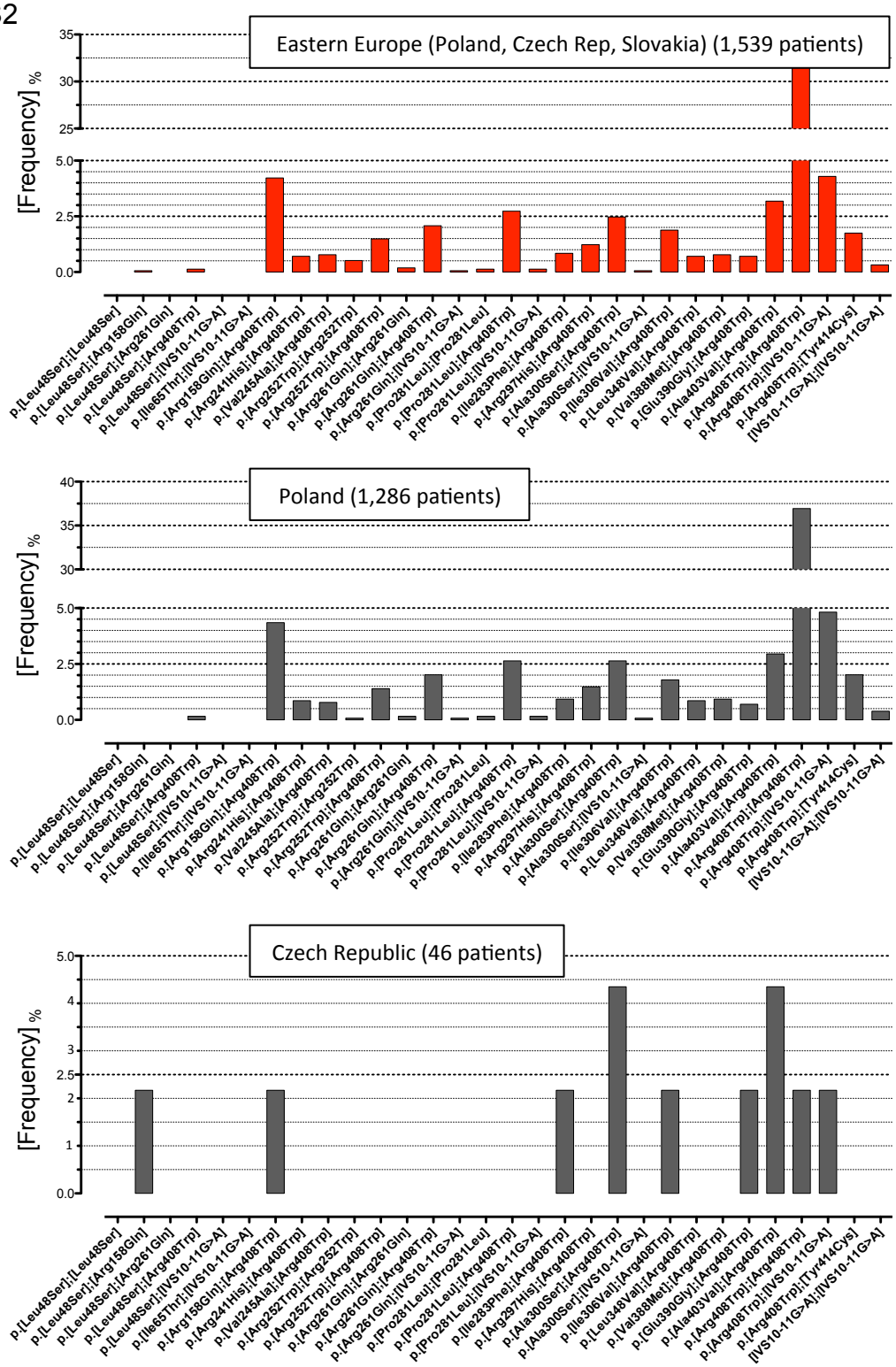
Eastern Europe (Poland, Czech Rep, Slovakia) (1,539 patients)
[Frequency] %
Poland (1,286 patients)
[Frequency] %
Czech Republic (46 patients)
[Frequency] %
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

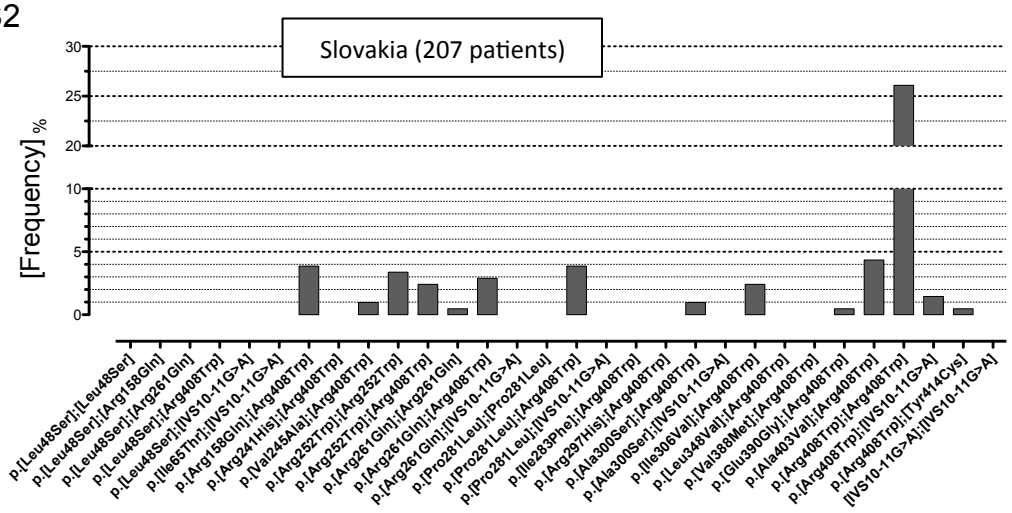
Slovakia (207 patients)
[Frequency] %
30
25
20
10
5
0
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

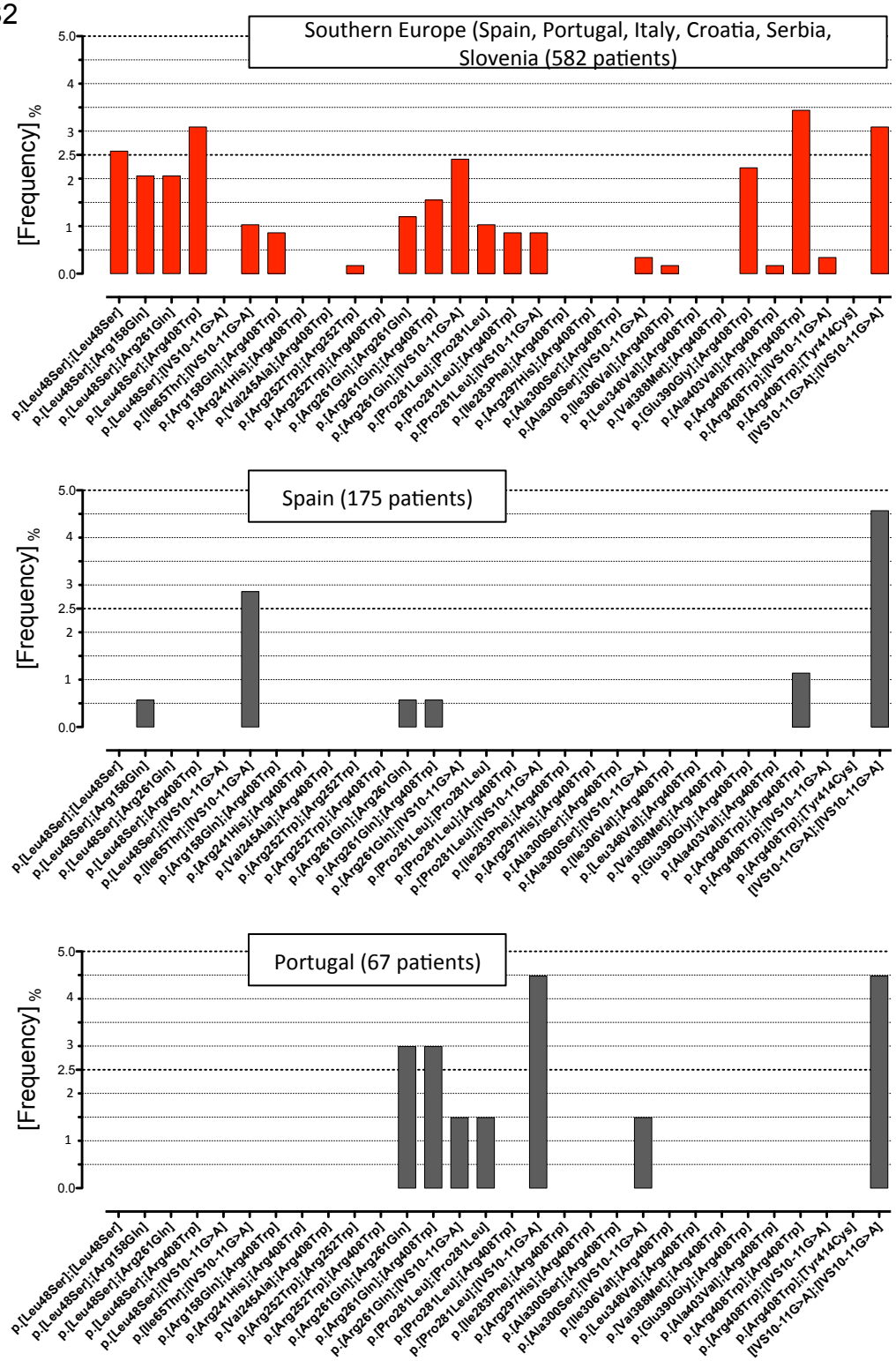
Southern Europe (Spain, Portugal, Italy, Croatia, Serbia, Slovenia (582 patients)
Spain (175 patients)
Portugal (67 patients)
[Frequency] %
5.0
4
3
2.5
2
1
0.0
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

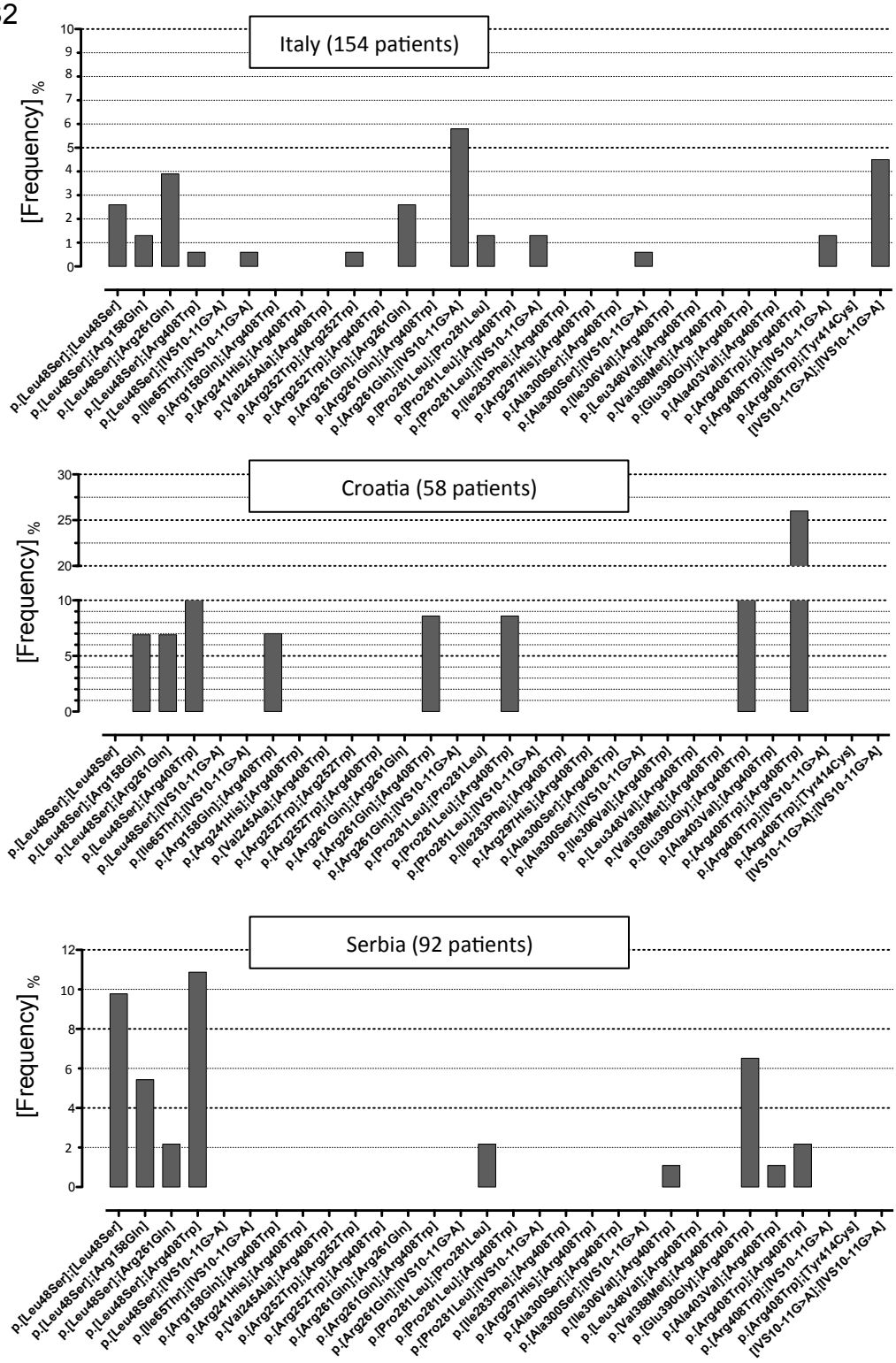
Italy (154 patients)
[Frequency] %
Croatia (58 patients)
[Frequency] %
Serbia (92 patients)
[Frequency] %
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

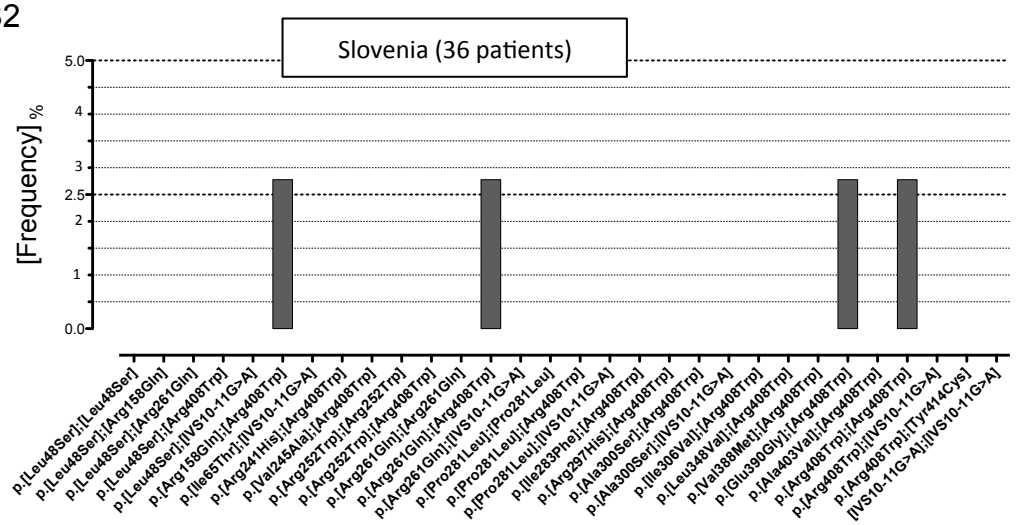
Slovenia (36 patients)
[Frequency] %
5.0
4
3
2.5
2
1
0.0
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Arg158Gln];[IVS10-11G>A]
p.[Leu48Ser];[Arg408Trp]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

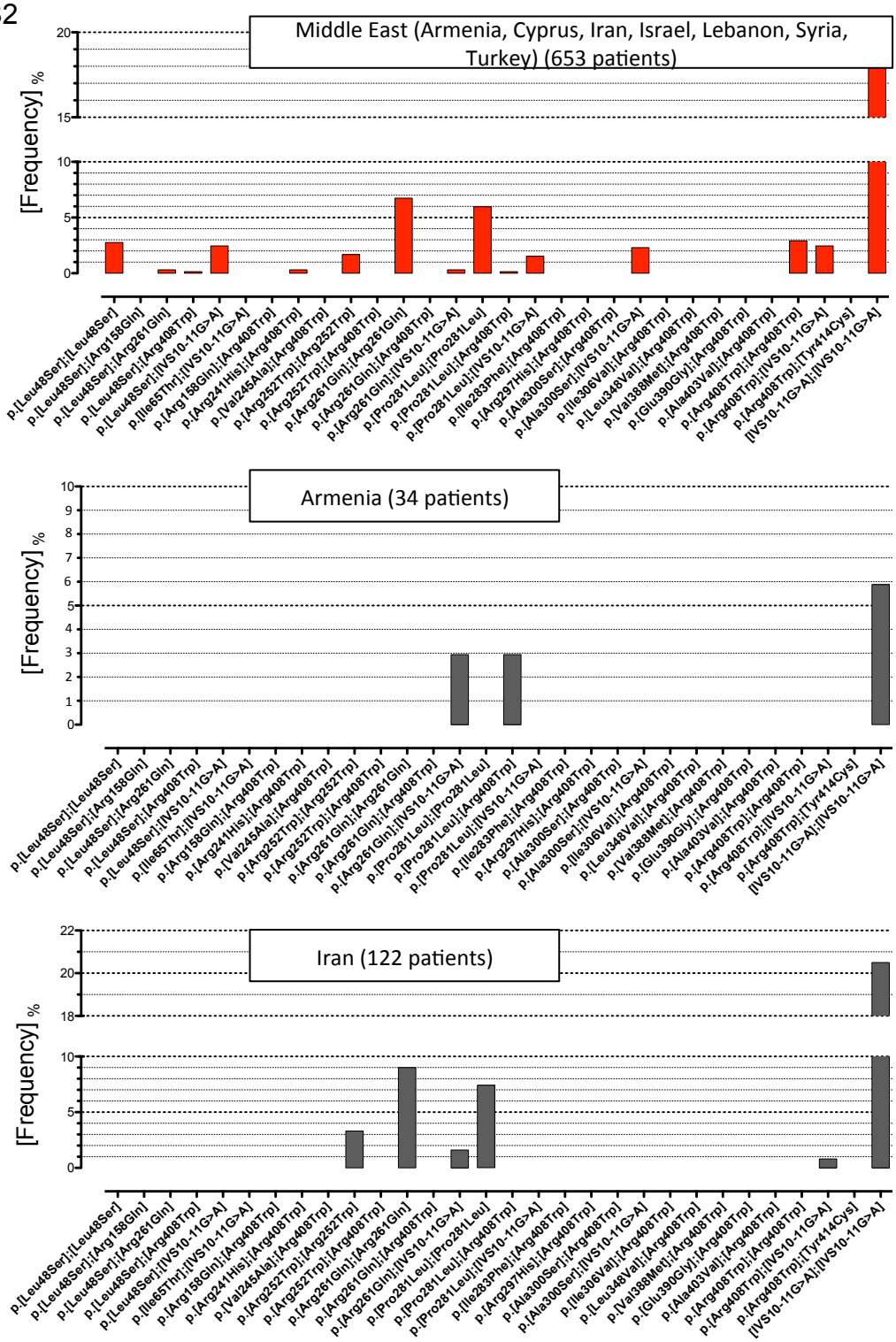
Middle East (Armenia, Cyprus, Iran, Israel, Lebanon, Syria, Turkey) (653 patients)
[Frequency] %
Armenia (34 patients)
[Frequency] %
Iran (122 patients)
[Frequency] %
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

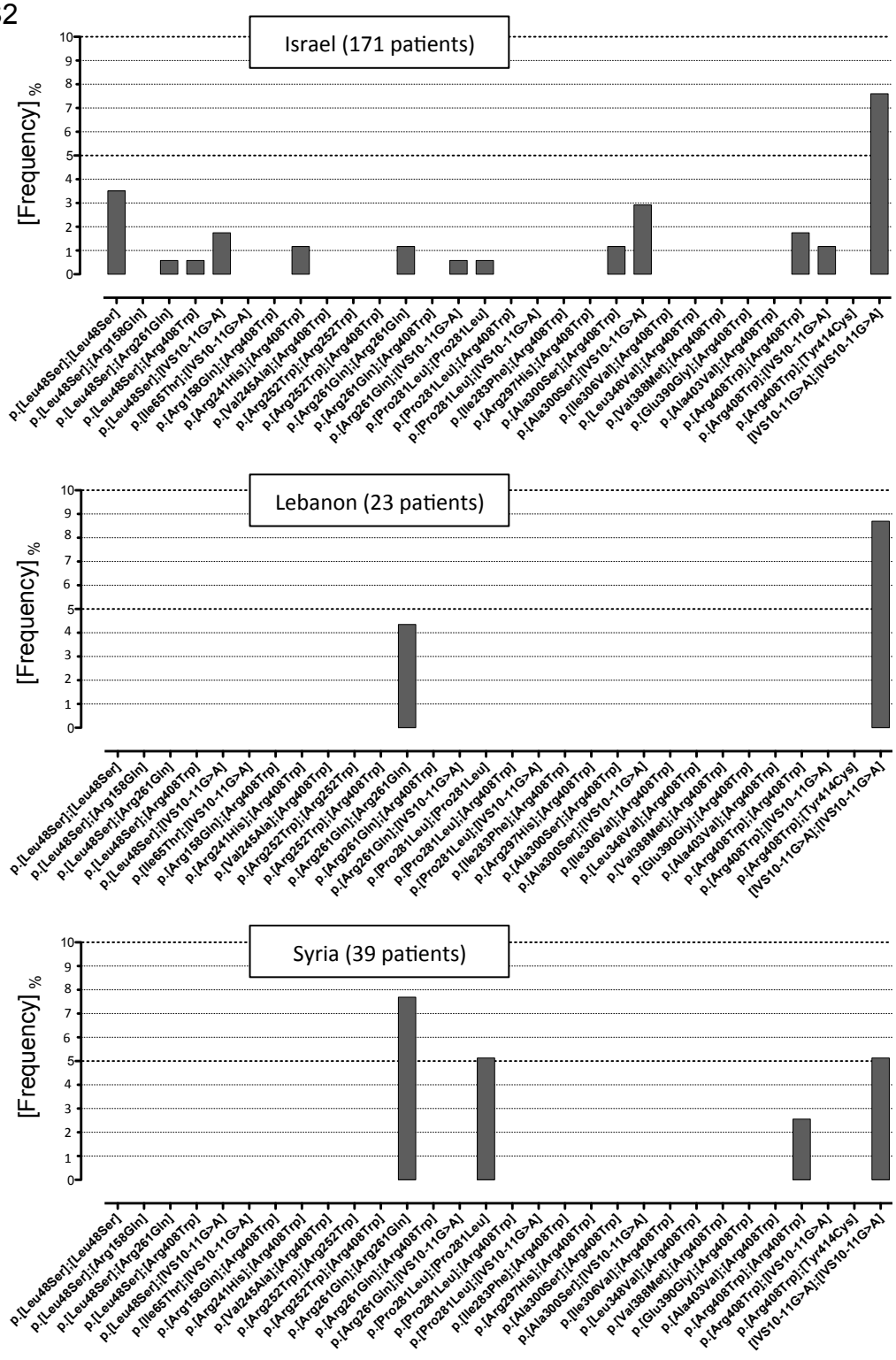
Israel (171 patients)
Lebanon (23 patients)
Syria (39 patients)
[Frequency] %
0
1
2
3
4
5
6
7
8
9
10
p.[Leu48Ser];[Leu48Ser]
p.[Leu48Ser];[Arg158Gln]
p.[Leu48Ser];[Arg261Gln]
p.[Leu48Ser];[Arg408Trp]
p.[Leu48Ser];[IVS10-11G>A]
p.[Ile65Thr];[IVS10-11G>A]
p.[Arg158Gln];[Arg408Trp]
p.[Arg241His];[Arg408Trp]
p.[Val245Ala];[Arg408Trp]
p.[Arg252Trp];[Arg252Trp]
p.[Arg252Trp];[Arg408Trp]
p.[Arg261Gln];[Arg261Gln]
p.[Arg261Gln];[Arg408Trp]
p.[Arg261Gln];[IVS10-11G>A]
p.[Pro281Leu];[Pro281Leu]
p.[Pro281Leu];[Arg408Trp]
p.[Pro281Leu];[IVS10-11G>A]
p.[Ile283Phe];[Arg408Trp]
p.[Arg297His];[Arg408Trp]
p.[Ala300Ser];[Arg408Trp]
p.[Ala300Ser];[IVS10-11G>A]
p.[Ile306Val];[Arg408Trp]
p.[Leu348Val];[Arg408Trp]
p.[Val388Met];[Arg408Trp]
p.[Glu390Gly];[Arg408Trp]
p.[Ala403Val];[Arg408Trp]
p.[Arg408Trp];[IVS10-11G>A]
p.[Arg408Trp];[Tyr414Cys]
[IVS10-11G>A];[IVS10-11G>A]

Figure S2

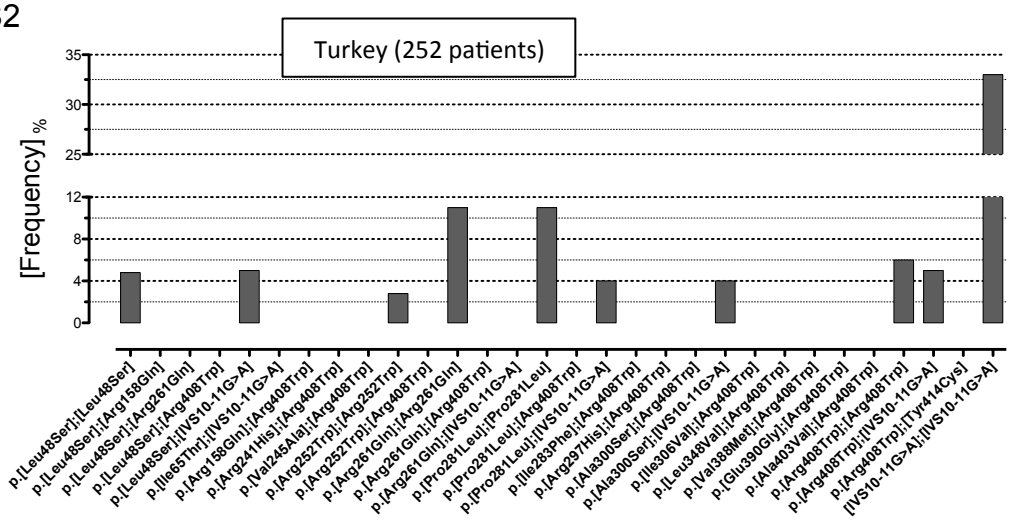

Figure S2 Region- and country-wise distribution of 30 most frequent *PAH* genotypes. 20 countries were classified into five geographical groups: Northern Europe (Lithuania, Sweden), Western Europe (Germany, France), Eastern Europe (Poland, Czech Republic, Slovakia), Southern Europe (Spain, Portugal, Italy, Croatia, Serbia, Slovenia) and Middle East (Armenia, Cyprus, Iran, Israel, Lebanon, Syria, Turkey). In each region, the overall frequency of individual genotype was calculated. Additionally, the frequency of each genotype was evaluated separately for each country belonging to the respective group. In case of mutation p.Gln355_Tyr356insGlyLeuGln, the trivial name (IVS10-11G>A) was used.

Figure S3

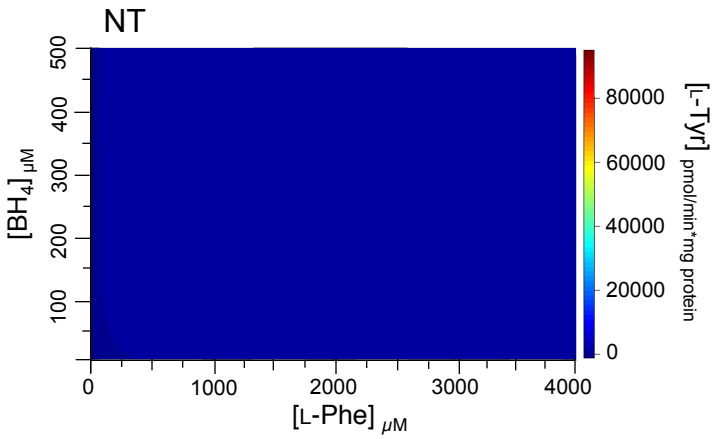

Figure S3 PAH activity landscape of not transfected (NT) COS-7 cells, used as a negative control.

Figure S4

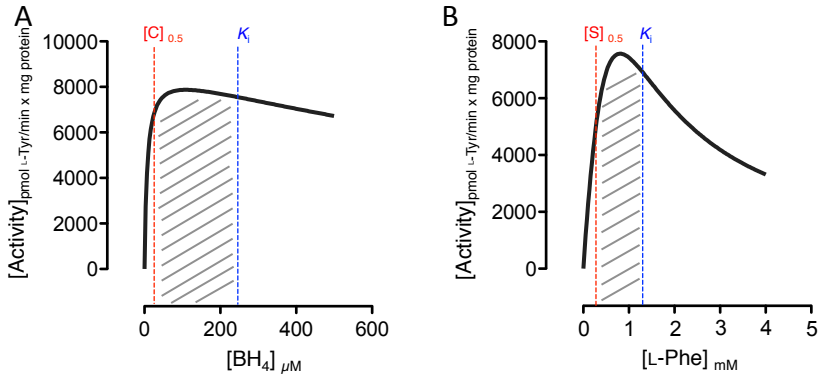

Figure S4 Determination of optimal working ranges of PAH activity with respect to substrate and cofactor. The optimal working range of PAH activity assessed for p.[Arg261Gln];[Arg261Gln] is given as an example (peak activity: 762 μM L-Phe and 94 μM BH_4). A) Optimal working range for BH_4 is depicted as boundaries set by $[C]_{0.5}$, which reflects the concentration of the cofactor at half maximal enzyme activity and K_i, which is the concentration of the cofactor needed to inhibit the enzyme by 50 %. Similarily, B) the optimal working range for L-phenylalanine is described by $[S]_{0.5}$ and K_i values where $[S]_{0.5}$ is the concentration of the substrate needed for the enzyme to achieve 50 % of enzyme's maximal activity and K_i is the substrate concentration at which the activity of the enzyme is decreased by 50 %.

Figure S5

Group 1

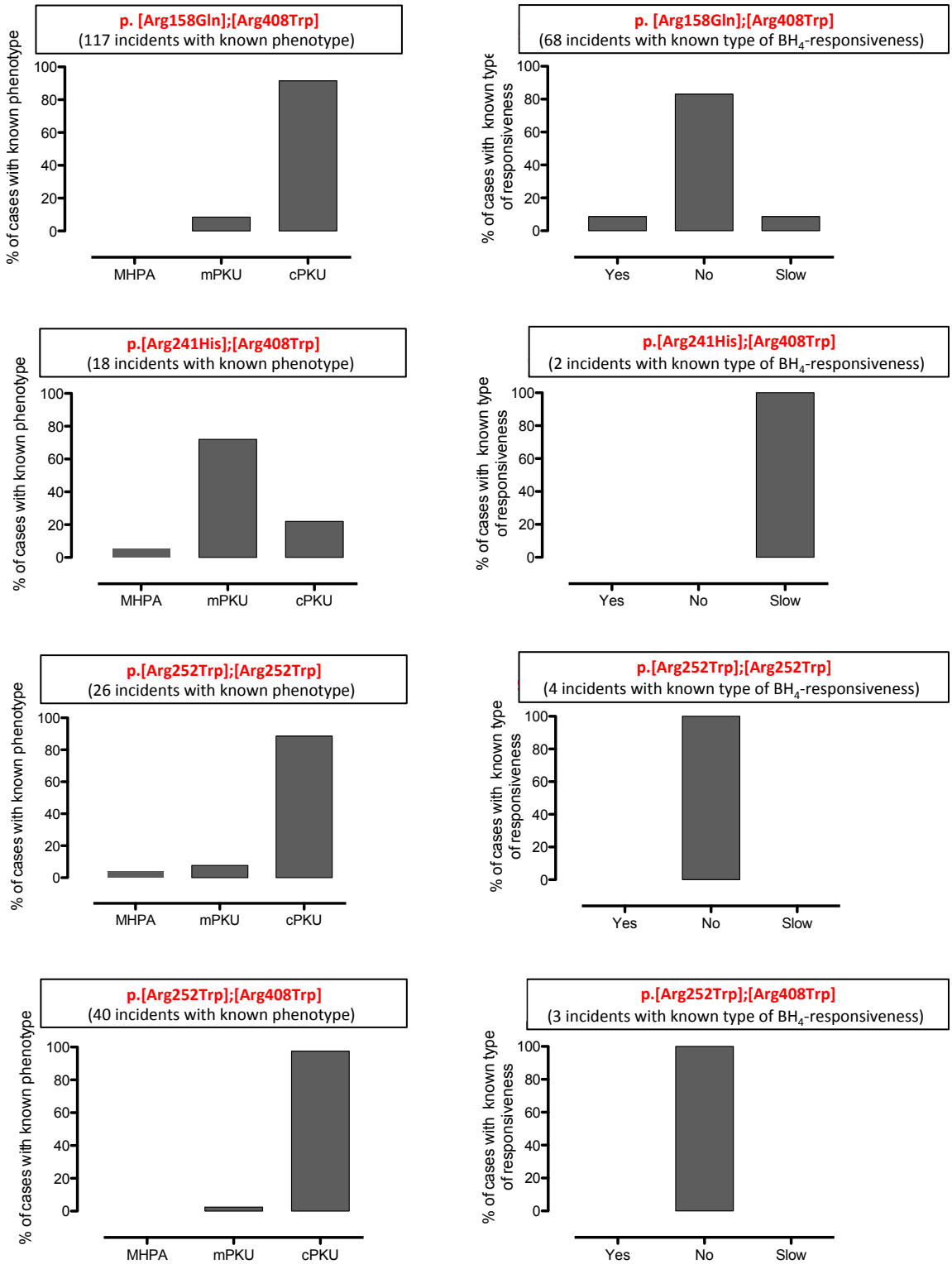

Figure S5

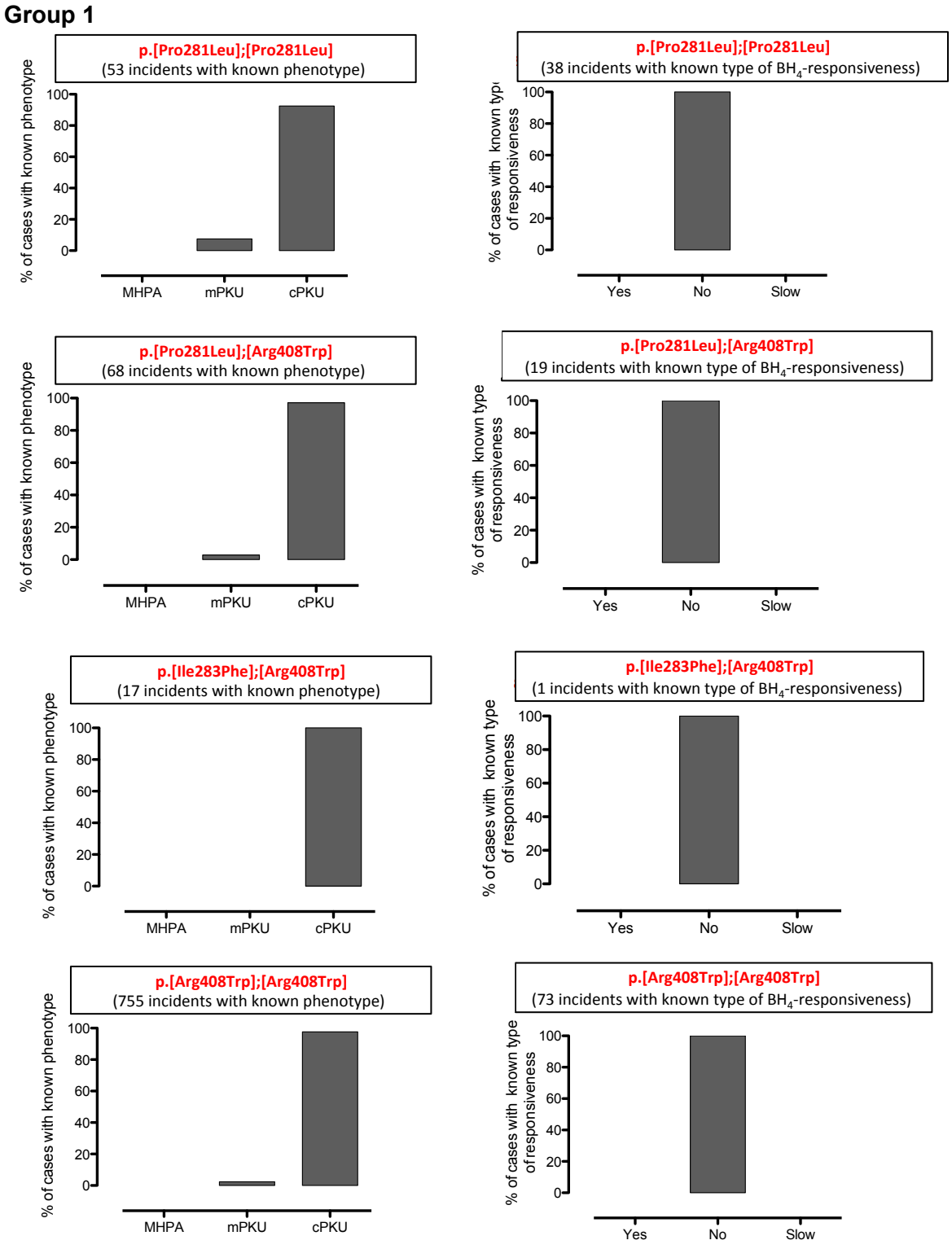
Group 1
p.[Pro281Leu];[Pro281Leu]
(53 incidents with known phenotype)
% of cases with known phenotype
100
80
60
40
20
0
MHPA
mPKU
cPKU
p.[Pro281Leu];[Pro281Leu]
(38 incidents with known type of BH4-responsiveness)
% of cases with known type of responsiveness
Yes
No
Slow
p.[Pro281Leu];[Arg408Trp]
(68 incidents with known phenotype)
p.[Pro281Leu];[Arg408Trp]
(19 incidents with known type of BH4-responsiveness)
p.[Ile283Phe];[Arg408Trp]
(17 incidents with known phenotype)
p.[Ile283Phe];[Arg408Trp]
(1 incidents with known type of BH4-responsiveness)
p.[Arg408Trp];[Arg408Trp]
(755 incidents with known phenotype)
p.[Arg408Trp];[Arg408Trp]
(73 incidents with known type of BH4-responsiveness)

Figure S5

Group 1

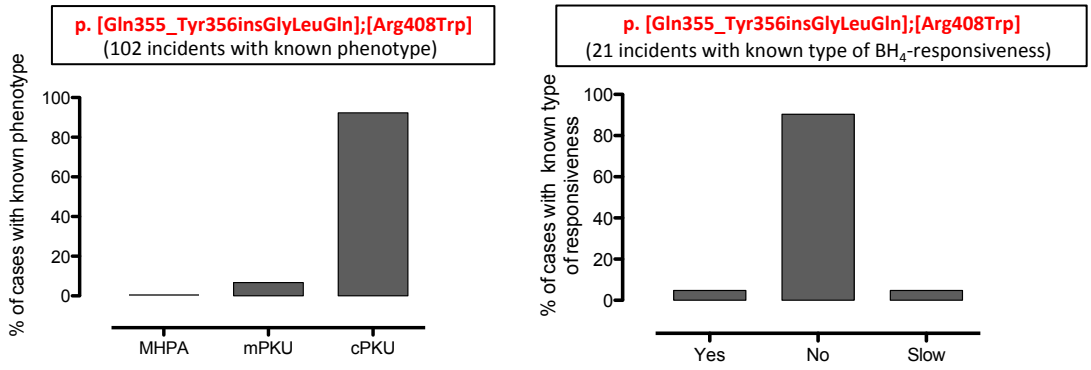

Figure S5

Group 2

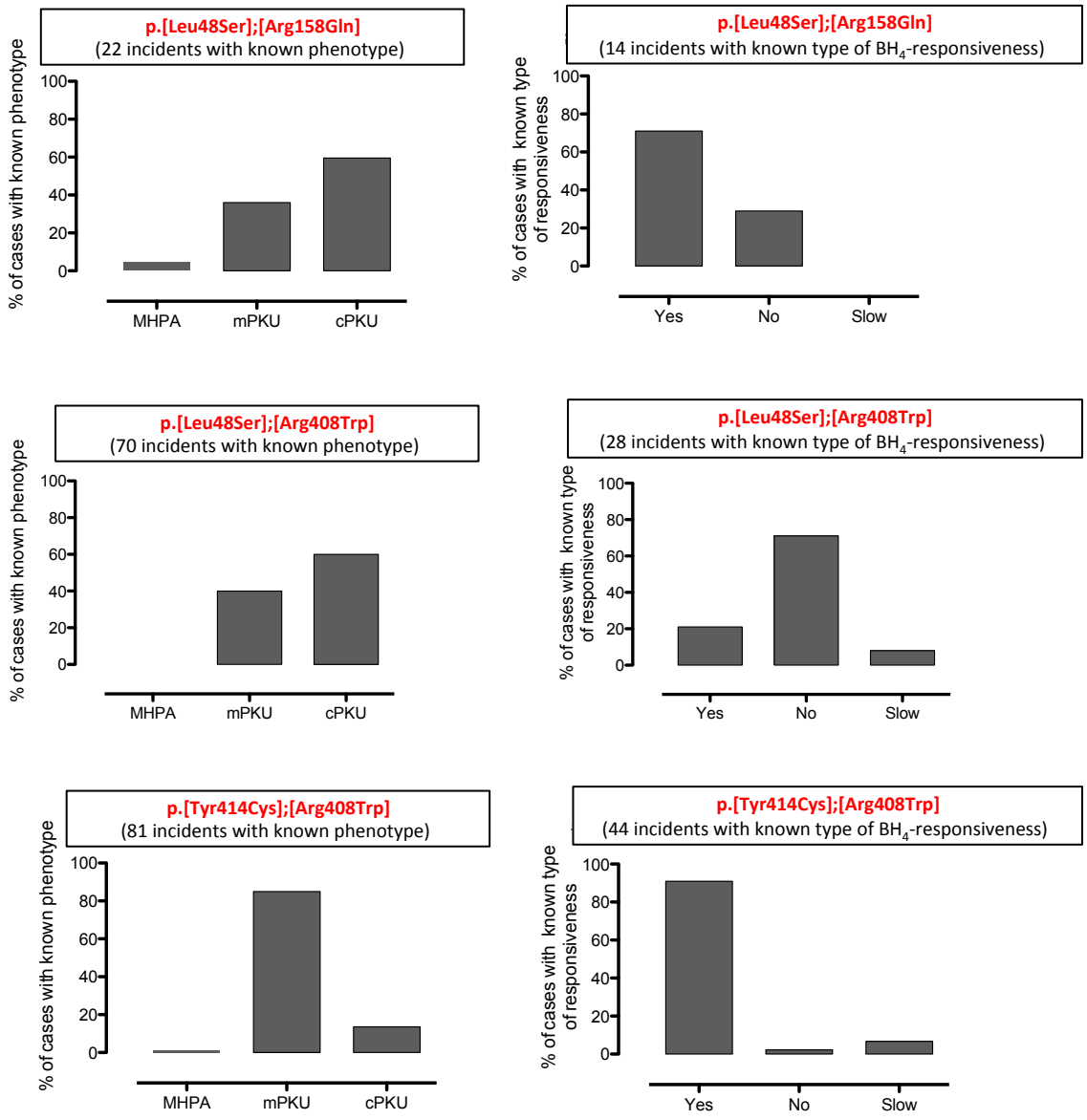

Figure S5

Group 3

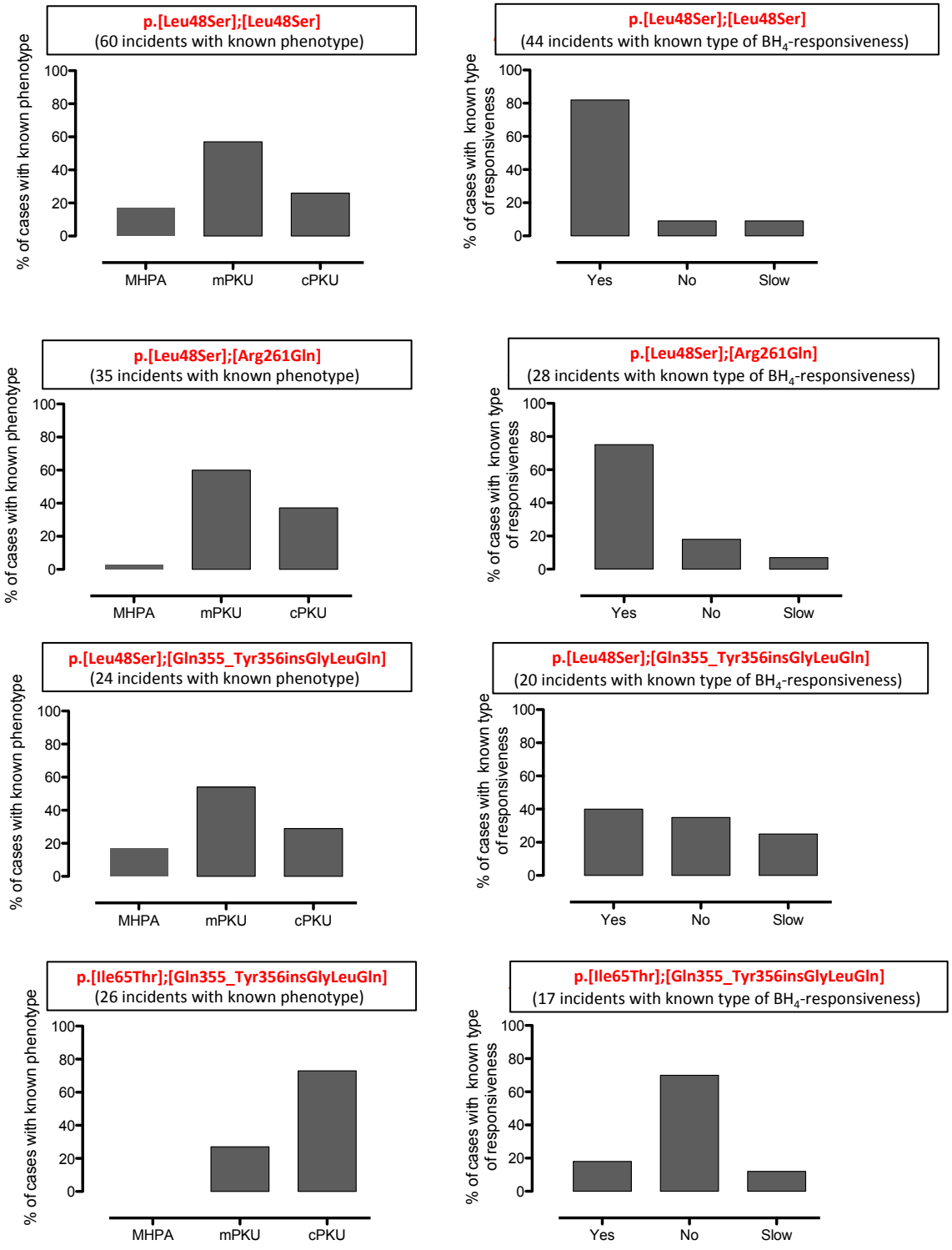

Figure S5

Group 3

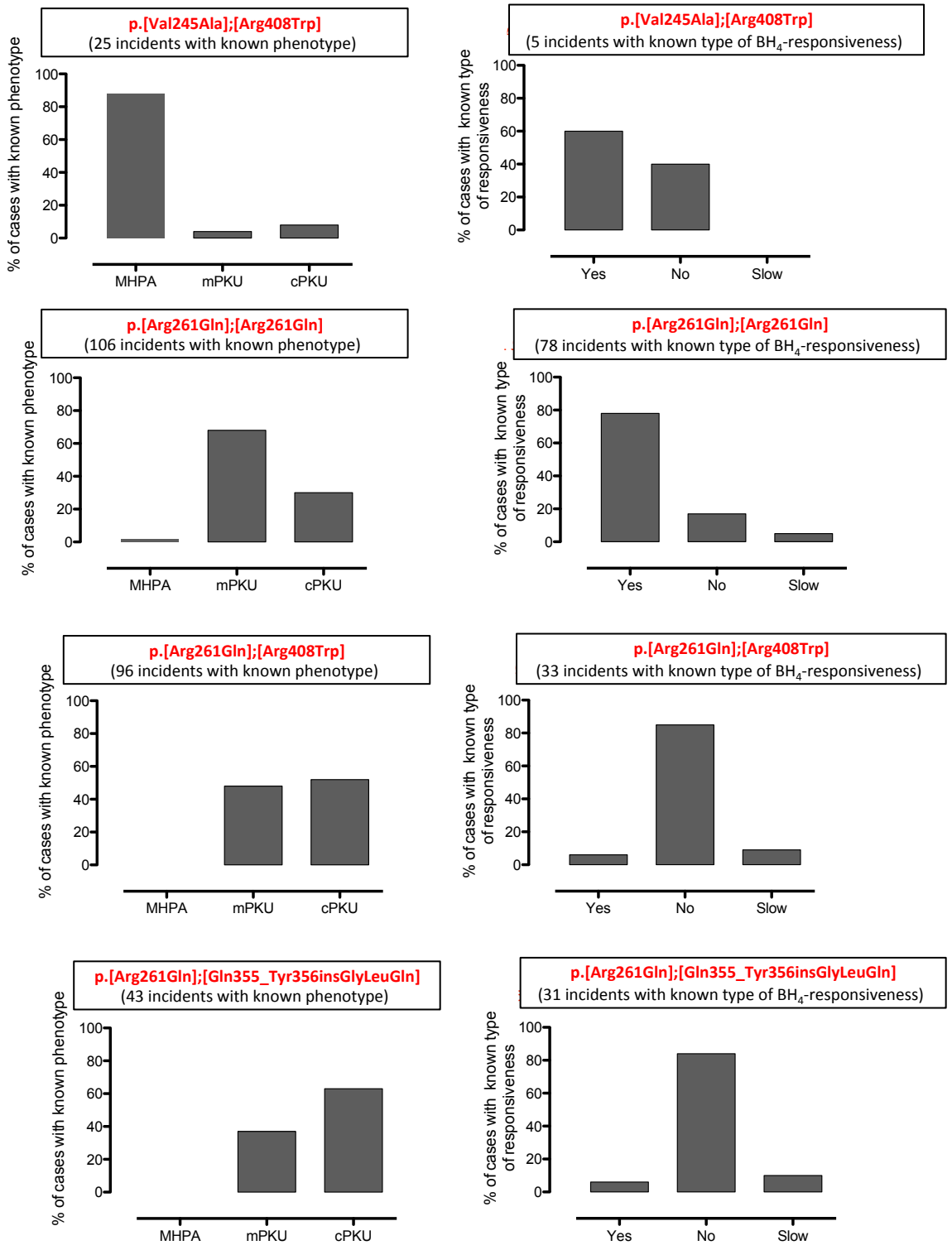

Figure S5

Group 3

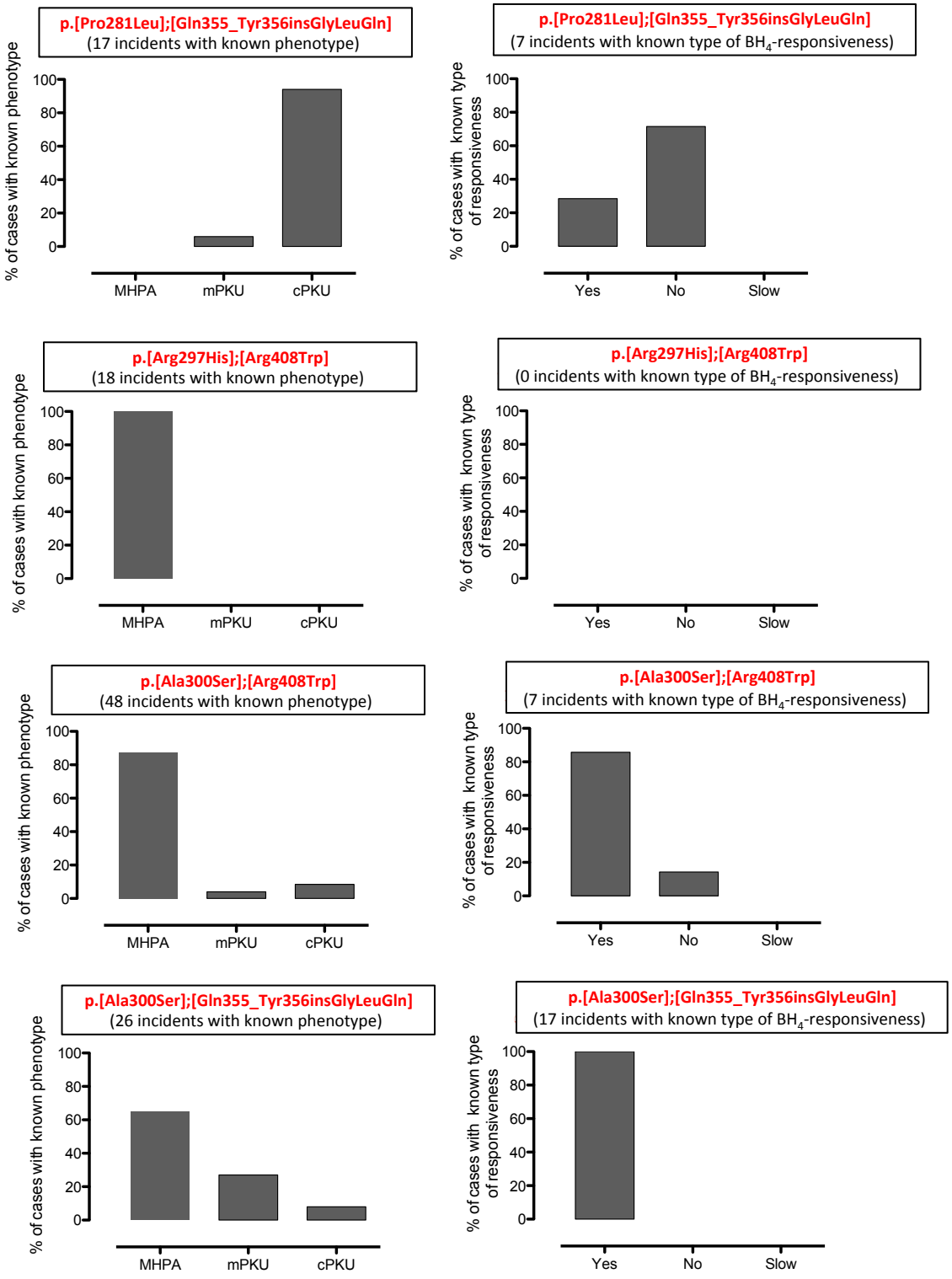

Figure S5

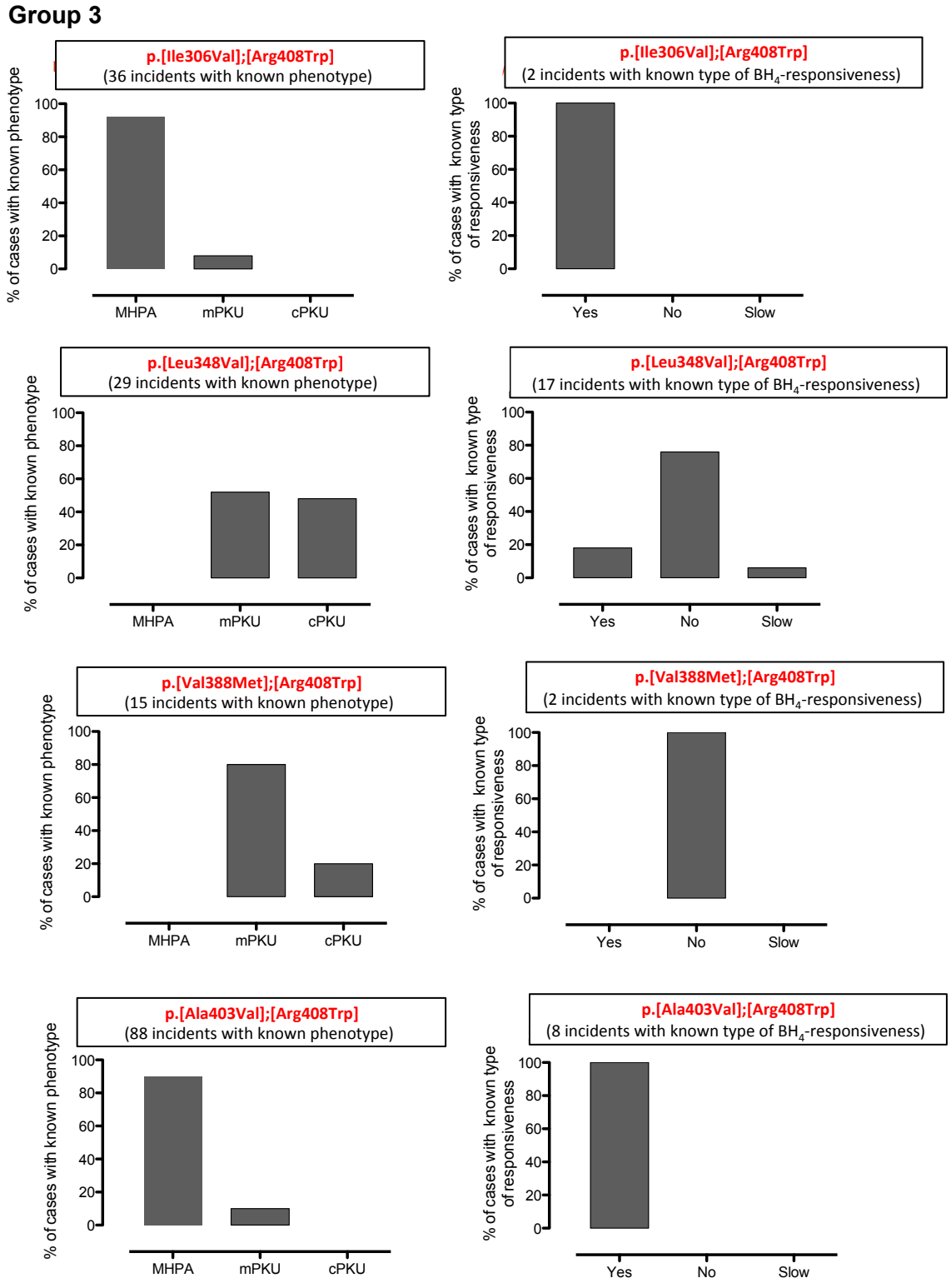
Group 3
p.[Ile306Val];[Arg408Trp]
(36 incidents with known phenotype)
% of cases with known phenotype
100
80
60
40
20
0
MHPA
mPKU
cPKU
p.[Ile306Val];[Arg408Trp]
(2 incidents with known type of BH4-responsiveness)
% of cases with known type of responsiveness
Yes
No
Slow
p.[Leu348Val];[Arg408Trp]
(29 incidents with known phenotype)
p.[Leu348Val];[Arg408Trp]
(17 incidents with known type of BH4-responsiveness)
p.[Val388Met];[Arg408Trp]
(15 incidents with known phenotype)
p.[Val388Met];[Arg408Trp]
(2 incidents with known type of BH4-responsiveness)
p.[Ala403Val];[Arg408Trp]
(88 incidents with known phenotype)
p.[Ala403Val];[Arg408Trp]
(8 incidents with known type of BH4-responsiveness)

Figure S5

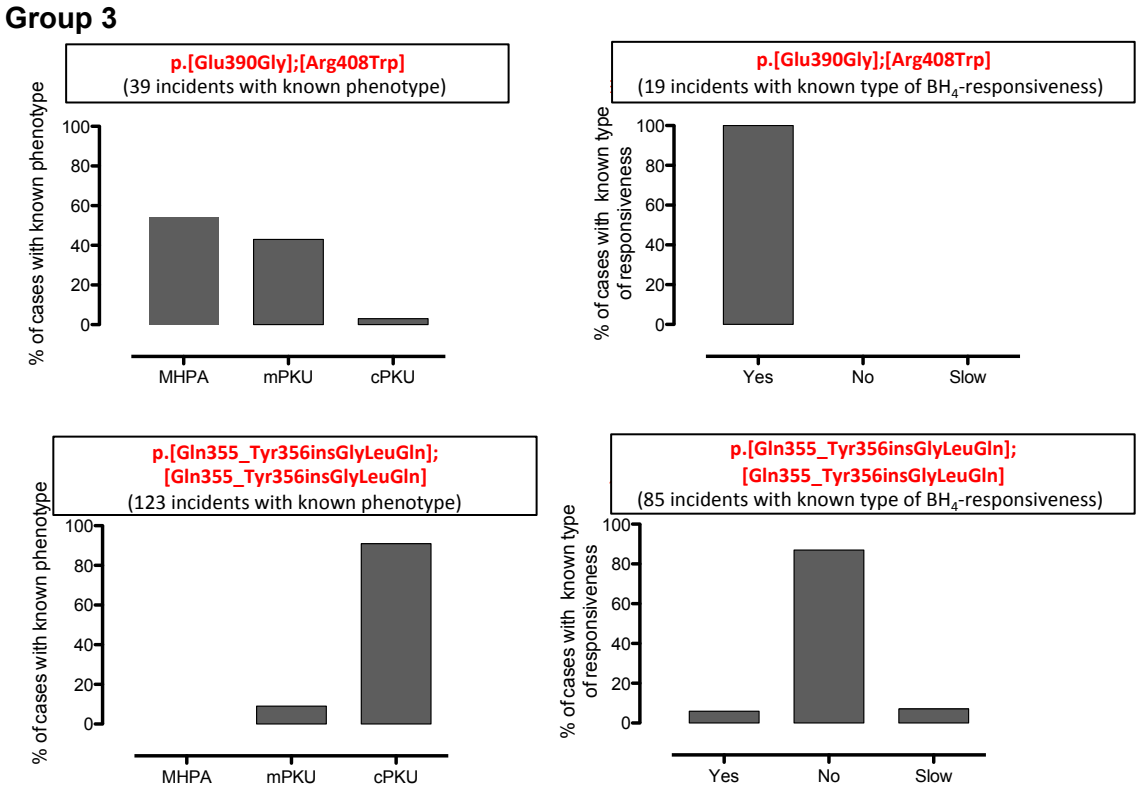

Figure S5 Clinical phenotypes associated with analyzed *PAH* genotypes. The data on clinical phenotypes and BH_4-responsiveness were obtained from BIOPKU (www.biopku.org).

Figure S6

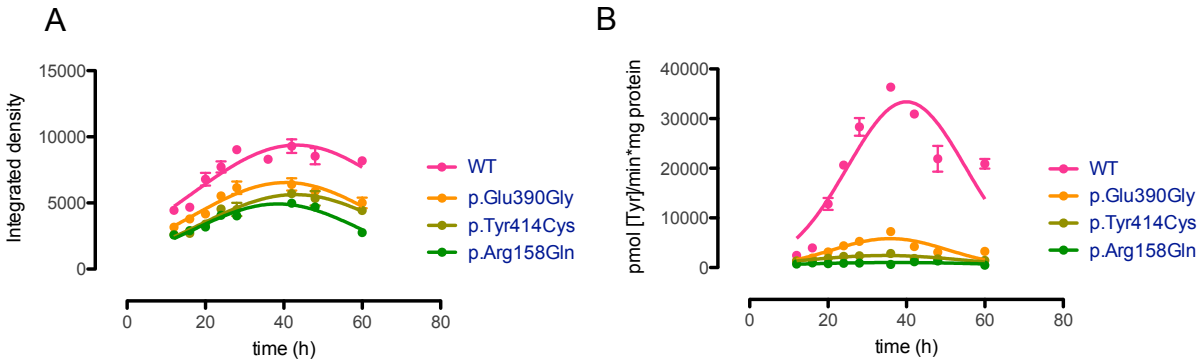

Figure S6 Assessment of the PAH protein production over time. COS-7 cells were transiently transfected with PAH plasmids harboring the WT, p.Glu390Gly, p.Tyr414Cys and p.Arg158Gln genes respectively. Next, the cells were harvested at different time points (12 – 60 h after transfection). The production of PAH protein was analyzed by the dot blot accompanied by the PAH activity assay. This allowed for the determination of an optimal time point (~ 40 h) where the protein production is in balance with the protein degradation. A) The PAH protein production analyzed by dot blots and evaluated with the Dot Blot Analyzer for ImageJ toolset (image.bio.methods.free.fr/dotblot.html). B) Analysis of the PAH activity in transfected cells harvested at different time points post-transfection.

Figure S7

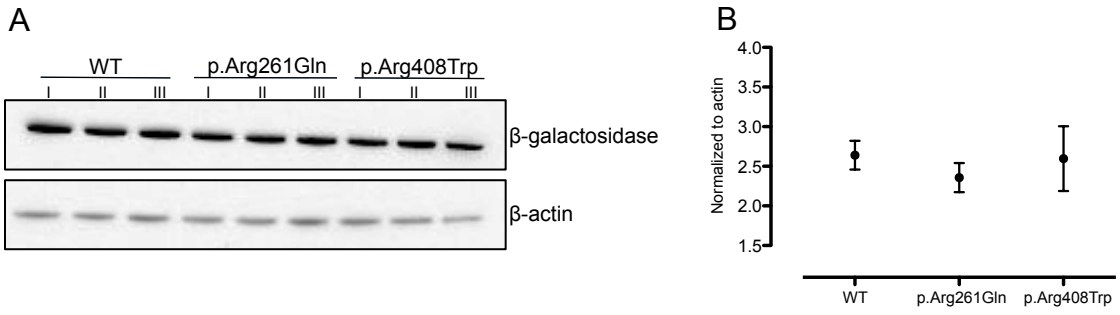

Figure S7 Assessment of transfection efficiency in co-transfection experiments. The positive control plasmid pEF/GW-51/*lac*Z, coding for β-galactosidase, was co-transfected together with plasmids coding for wild-type PAH (WT), p.Arg261Gln or p.Arg408Trp. The molar ratio of control plasmid to plasmids coding for PAH was 1:5. On the protein level, the production of the control protein β-galactosidase did not change in the presence of wild-type or variant PAH proteins. A) Western blot analysis of transient expression of β-galactosidase protein in the presence of WT PAH, p.Arg261Gln or p.Arg408Trp of three independent transfections (I-III). β-galactosidase was detected using primary anti-V5 antibody (life technologies) and secondary anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology). For the detection of β-actin, the anti-β-actin (C4) HRP-conjugated antibody was used (Santa Cruz Biotechnology). The resulting protein bands were visualized with Pierce ECL Western Blotting Substrate (ThermoScientific). B) ImageJ analysis of β-galactosidase protein production (http://imagej.nih.gov/ij/download.html).

Figure S8

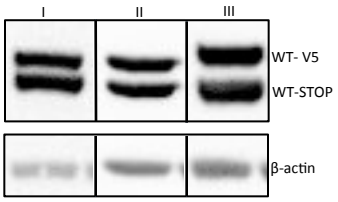

Figure S8 Western blot analysis verifying a 1:1 molar ratio of wild-type PAH derived from co-transfection of two different wild-type PAH expression plasmids (pEF-DEST51-V5 and pEF-DEST-STOP) in three independent experiments (I-III). PAH protein was detected using mouse monoclonal anti-PAH antibody (PH8; Millipore) and anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology) wherease for the detection of β-actin the anti-β-actin (C4) HRP-conjugated antibody was used (Santa Cruz Biotechnology). The resulting protein bands were visualized with Pierce ECL Western Blotting Substrate (ThermoScientific).

Table S1. Sources for *PAH* genotypes from different countries included in the study.

Country	References	No. of patients
Armenia	Konstandyan et al. 2011[1]	34
Croatia	Karačić et al. 2009[2]	58
Cyprus	Georgiou et al. 2012[3]	12
Czech Republic	Réblová et al. 2013[4]	46
France	own data	97
Germany	own data Gramer et al. 2009[5]	217
Iran	Zare-Karizi et al. 2011[6]	122
Israel	Bercovich et al. 2008[7]	171
Italy	Fiori et al. 2005[8] Daniele et al. 2009[9] Guzetta et al. 1997[10]	154
Lebanon	Karam et al. 2013[11]	23
Lithuania	Kasnauskiene et al. 2003[12]	92
Poland	Bik-Multanowski et al. 2013[13]	1286
Portugal	Rivera et al. 2011[14]	67
Serbia	Stojiljkovic et al. 2006[15] Djordjevic et al. 2012[16]	92
Slovakia	Polak et al. 2013[17]	207
Slovenia	Tansek et al. 2012[18]	36
Spain	Couce et al. 2013[19] Desviat et al. 2004[20] Bueno et al. 2013[21]	175
Sweden	Svensson et al. 1993[22]	50
Syria	Murad et al. 2013[23]	39
Turkey	Dobrowolski et al. 2011[24]	252
	Total number of patients	**3230**
	Number of patients with full genotypes	**3066**

Resources for *PAH* genotypes. On the basis of a PubMed literature search, 24 publications were selected. In addition, data from Medical Centers in Munich and Nancy were included.

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Table S2. Frequent *PAH* mutations in the study population.

	Mutation		**% of mutant alleles**
	Protein	Nucleotide	
1	**# p.Arg408Trp**	**c.1222C>T**	**36.3**
2	# p.Gln355_Tyr356insGlyLeuGln	c.1066-11G>A (IVS10-11G>A)	10.2
3	**# p.Arg261Gln**	**c.782G>A**	**5.4**
4	**# p.Pro281Leu**	**c.842C>T**	**3.8**
5	**# p.Arg158Gln**	**c.473G>A**	**3.4**
6	**# p.Leu48Ser**	**c.143T>C**	**3.1**
7	# p.?	c.1315+1G>A (IVS12+1G>A)	2.9
8	**# p.Ala403Val**	**c.1208C>T**	**2.8**
9	**# p.Ala300Ser**	**c.898G>T**	**2.2**
10	**# p.Tyr414Cys**	**c.1241A>G**	**2.2**
11	**# p.Arg252Trp**	**c.754C>T**	**1.4**
12	**# p.Glu390Gly**	**c.1169A>G**	**1.2**
13	**p.Ile306Val**	**c.916A>G**	**1.1**
14	p.?	c.441+5G>T (IVS4+5G>T)	1.1
15	**# p.Ile65Thr**	**c.194T>C**	**1.0**
16	# p.Arg243*	c.727C>T	0.9
17	**# p.Val388Met**	**c.1162G>A**	**0.9**
18	p.Phe55Leufs*6	c.165delT	0.9
19	**p.Val245Ala**	**c.734T>C**	**0.7**
20	# p.Arg261*	c.781C>T	0.7
21	# p.Ser349Pro	c.1045T>C	0.6
22	p.?	c.168+5G>C (IVS2+5G>C)	0.6
23	p.Arg243Gln	c.728G>A	0.5
24	**# p.Leu348Val**	**c.1042C>G**	**0.5**
25	**p.Arg297His**	**c.890G>A**	**0.4**
26	**p.Arg241His**	**c.722G>A**	**0.4**
27	# p.Gly272*	c.814G>T	0.4
28	# p.Glu280Lys	c.838G>A	0.4
29	p.Asp415Asn	c.1243G>A	0.4
30	p.Arg270Lys	c.809G>A	0.4

The 30 most frequent mutations in the *PAH* gene based on the analysis of 6273 mutant alleles identified in 3230 PKU patients from Europe and the Middle East.
PAH mutations previously identified as frequent in the European population (Zschocke 2003). Mutations contributing to genotypes analyzed in this study are given in bold. The mutation c.847A>T (p.Ile283Phe), additionally analyzed in this study, contributes to the 30 most frequent genotypes but was not among the 30 most frequent mutations.

Table S3. PAH enzyme activity determined at standard conditions

Genotype	PAH enzyme activity [pmol L-tyrosine/min x mg protein] ± SEM	Residual activity (% of WT)
WT	52 981 ± 10 769	100
NT	664 ± 142	1.2
p.[Leu48Ser];[Leu48Ser]	3501 ± 258	6.6
p.[Leu48Ser];[Arg158Gln]	1818 ± 354	3.4
p.[Leu48Ser];[Arg261Gln]	5948 ± 67	11
p.[Leu48Ser];[Arg408Trp]	1981 ± 99	3.7
p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln]	4882 ± 418	9.2
p.[Ile65Thr];[Gln355_Tyr356insGlyLeuGln]	4808 ± 195	9
p.[Arg158Gln];[Arg408Trp]	815 ± 212	1.5
p.[Arg241His];[Arg408Trp]	1246 ± 67	2.3
p.[Val245Ala];[Arg408Trp]	5632 ± 238	10.6
p.[Arg252Trp];[Arg252Trp]	953 ± 14	1.8
p.[Arg252Trp];[Arg408Trp]	937 ± 44	1.8
p.[Arg261Gln];[Arg261Gln]	7678 ± 775	14.5
p.[Arg261Gln];[Arg408Trp]	4445 ± 329	8.4
p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln]	5690 ± 919	10.7
p.[Pro281Leu];[Pro281Leu]	1081 ± 106	2
p.[Pro281Leu];[Arg408Trp]	1307 ± 134	2.5
p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln]	2877 ± 324	5.4
p.[Ile283Phe];[Arg408Trp]	1288 ± 121	2.4
p.[Arg297His];[Arg408Trp]	7655 ± 135	14.4
p.[Ala300Ser];[Arg408Trp]	2909 ± 245	5.5
p.[Ala300Ser];[Gln355_Tyr356insGlyLeuGln]	4654 ± 480	8.8
p.[Ile306Val];[Arg408Trp]	2143 ± 228	4
p.[Leu348Val];[Arg408Trp]	4682 ± 697	8.8
p.[Val388Met];[Arg408Trp]	3842 ± 120	7.2
p.[Glu390Gly];[Arg408Trp]	5616 ± 345	10.6
p.[Ala403Val];[Arg408Trp]	3210 ± 245	6
p.[Arg408Trp];[Arg408Trp]	609 ± 66	1.1
p.[Arg408Trp];[Gln355_Tyr356insGlyLeuGln]	1736 ± 116	3.3
p.[Tyr414Cys];[Arg408Trp]	2120 ± 88	4
p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln]	3192 ± 473	6

PAH enzyme activities associated with frequent PAH genotypes as determined at standard conditions (1 mM L-phenylalanine and 75 µM BH_4). Data were extracted from the 96 well PAH activity matrix.
WT refers to wild-type PAH, NT refers to the negative control of not transfected COS-7 cells.

Table S4. PAH enzyme activity of PAH homozygotes determined at standard conditions

Genotype	PAH enzyme activity [pmol L-tyrosine/min x mg protein] ± SEM	Residual activity (% of WT)
NT	907 ± 139	1.5
pEF/GW-51/*lac*Z control vector	636 ± 18	1.1
WT	60 853 ± 4 505	100
p.[Leu48Ser];[Leu48Ser]	4 395 ± 561	7.2
p.[Ile65Thr];[Ile65Thr]	6 474 ± 610	10.6
p.[Arg158Trp];[Arg158Trp]	1 101 ± 163	1.8
p.[Arg241His];[Arg241His]	3 639 ± 416	6
p.[Val245Ala];[Val245Ala]	37 713 ± 2 845	62
p.[Arg252Trp];[Arg252Trp]	543 ± 22	0.9
p.[Arg261Gln];[Arg261Gln]	9 443 ± 336	15.5
p.[Pro281Leu];[Pro281Leu]	569 ± 25	0.9
p.[Ile283Phe];[Ile283Phe]	1 762 ± 70	2.9
p.[Arg297His];[Arg297His]	58 412 ± 1 777	96
p.[Ala300Ser];[Ala300Ser]	10 615 ± 1 864	17.4
p.[Ile306Val];[Ile306Val]	4 266 ± 65	7
p.[Leu348Val];[Leu348Val]	14 497 ± 852	24
p.[Val388Met];[Val388Met]	7 173 ± 1 203	12
p.[Glu390Gly];[Glu390Gly]	33 504 ± 2 177	55
p.[Ala403Val];[Ala403Val]	7 498 ± 1 264	12
p.[Arg408Trp];[Arg408Trp]	772 ± 48	1.3
p.[Tyr414Cys];[Tyr414Cys]	5 865 ± 839	10
p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln]	3 863 ± 135	6.3

PAH enzyme activities associated with homozygous *PAH* genotypes as determined at standard conditions (1 mM L-phenylalanine and 75 μM BH_4).

Table S5. Frequency of null genotypes and active genotypes in different countries

Country	Null genotypes [%]	Active genotypes [%]
Lithuania	89	11
Iran	75	25
Poland	76	24
Slovakia	76	24
Armenia	75	25
Turkey	71	29
Croatia	71	29
Lebanon	67	33
Syria	62	38
Spain	61	39
Portugal	54	46
Israel	52	48
Germany	54	46
Sweden	54	46
Slovenia	50	50
Serbia	50	50
Czech Republic	45	55
Italy	41	59
France	29	71

Percentages of patients carrying genotypes with residual activity below 5 % (null genotypes) or above 5 % (active genotypes) in different countries. Data are sorted by decreasing percentages for null genotypes.
The calculation only applies to the 30 most frequent *PAH* genotypes selected for experimental work in this study (Table 1).
Null genotypes: p.[Arg408Trp];[Arg408Trp], p.[Gln355_Tyr356insGlyLeuGln];[Gln355_Tyr356insGlyLeuGln], p.[Arg408Trp];[Arg158Gln], p.[Arg408Trp];[Gln355_Tyr356insGlyLeuGln], p.[Pro281Leu];[Pro281Leu], p.[Arg408Trp];[Pro281Leu], p.[Arg408Trp];[Arg252Trp], p.[Leu48Ser];[Arg408Trp], p.[Arg252Trp];[Arg252Trp], p.[Pro281Leu];[Gln355_Tyr356insGlyLeuGln], p.[Leu48Ser];[Arg158Gln], p.[Arg408Trp];[Ile283Phe], p.[Arg241His];[Arg408Trp].
Active genotypes: p.[Arg261Gln];[Arg261Gln], p.[Leu48Ser];[Leu48Ser], p.[Ala403Val];[Arg408Trp], p.[Arg261Gln];[Arg408Trp], p.[Tyr414Cys];[Arg408Trp], p.[Ala300Ser];[Arg408Trp], p.[Ile306Val];[Arg408Trp], p.[Arg261Gln];[Gln355_Tyr356insGlyLeuGln], p.[Glu390Gln];[Arg408Trp], p.[Arg297His];[Arg408Trp], p.[Ala300Ser];[Gln355_Tyr356insGlyLeuGln], p.[Leu48Ser];[Gln355_Tyr356insGlyLeuGln], p.[Leu48Ser];[Arg261Gln], p.[Val245Ala];[Arg408Trp], p.[Val388Met];[Arg408Trp], p.[Ile65Thr];[Gln355_Tyr356insGlyLeuGln], p.[Leu348Val];[Arg408Trp].

Table S6. PAH enzyme activity determined at standard conditions without and with co-transfection of control plasmid

Genotype	**PAH enzyme activity [pmol L-tyrosine/min x mg protein] ± SEM**	**Residual activity (% of WT)**
WT	60 853 ± 4 505	100
WT + pEF/GW-51/*lac*Z	62 340 ± 2 691	100
p.[Arg261Gln];[Arg261Gln]	9 443 ± 336	15.5
p.[Arg261Gln];[Arg261Gln] + pEF/GW-51/*lac*Z	11 546 ± 435	18.5
p.[Arg408Trp];[Arg408Trp]	772 ± 48	1.3
p.[Arg408Trp];[Arg408Trp] + pEF/GW-51/*lac*Z	624 ± 121	1.0

PAH enzyme activities determined at standard conditions (1 mM L-phenylalanine and 75 µM BH_4) with and without co-transfection of the positive control plasmid pEF/GW-51/*lac*Z. Residual activities of p.[Arg261Gln];[Arg261Gln] and p.[Arg408Trp];[Arg408Trp] refer to WT, residual activities of p.[Arg261Gln];[Arg261Gln] + pEF/GW-51/*lac*Z and p.[Arg408Trp];[Arg408Trp] + pEF/GW-51/*lac*Z refer to WT + pEF/GW-51/*lac*Z.

SUPPLEMENTARY MATERIALS AND METHODS

Data sources for *PAH* genotypes and phenotypes

A PubMed literature search using the terms <phenylketonuria> and <genotypes> resulted in 687 hits covering publications from 1967 to present (access date 17 March 2014). Only papers describing full genotypes of PKU patients due to PAH deficiency were selected. Studies with pre-selection constraints that focus on specific subgroups such as mild phenotypes or the phenotype of BH_4-responsiveness were excluded. Twenty-four publications reporting *PAH* genotypes from 19 countries in Europe and Middle East published between 1993 and 2013 were selected. Additional unpublished data on *PAH* genotypes was included from German and French medical centers. For comparison of types and frequency of mutations, data were accessed from the Human Gene Mutation Database (www.hgmd.org; access date 17 March 2014), the *PAH* locus knowledgebase *PAH*db (www.pahdb.mcgill.ca; access date 19 April 2014) and the BIOPKU database (www.biopku.org; access date 23 April 2014). Data on PKU clinical phenotypes and BH_4-responsiveness associated with *PAH* genotypes were obtained from BIOPKU.

Mutations

For functional analyses, 18 *PAH* missense mutations, c.143T>C (p.Leu48Ser), c.194T>C (p.Ile65Thr), c.473G>A (p.Arg158Gln), c.722G>A (p.Arg241His), c.734T>C (p.Val245Ala), c.754C>T (p.Arg252Trp), c.782G>A (p.Arg261Gln), c.842C>T (p.Pro281Leu), c.847A>T (p.Ile283Phe), c.890G>A (p.Arg297His), c.898G>T (p.Ala300Ser), c.916A>G (p.Ile306Val), c.1042C>G (p.Leu348Val), c.1162G>A (p.Val388Met), c.1169A>G (p.Glu390Gly), c.1208C>T (Ala403Val), c.1222C>T (p.Arg408Trp), and c.1241A>G (p.Tyr414Cys), were generated by site-directed mutagenesis (KAPAHiFi Hot Start PCR Kit; peqlab). In addition, a construct based on the intronic mutation c.1066-11G>A (IVS10-11G>A), which results in the insertion of three amino acids (p.Gln355_Tyr356insGlyLeuGln)[1] was generated. All mutant *PAH* constructs were cloned into the eukaryotic expression vector pEF-DEST51 (life

technologies). Authenticity of constructs was verified by DNA sequencing. Other *PAH* mutations appearing in tables and figures were not expressed in cells; c.1315+1G>A (IVS12+1G>A), c.441+5G>T (IVS4+5G>T), c.165delT (p.Phe55fs), c.727C>T (p.Arg243*), c.781C>T (p.Arg261*), c.168+5G>C (IVS2+5G>C), c.728G>A (p.Arg243Gln), c.809G>A (p.Arg270Lys), c.814G>T (p.Gln272*), c.838G>A (p.Glu280Lys), and c.1243G>A (p.Asp415Asn). See online supplementary table S2.

Transient expression of PAH in COS-7

COS-7 cells were maintained in basic RPMI 1640 medium with stable glutamine supplemented with 10 % fetal bovine serum and 1 % antibiotic-antimycotic (gibco by life technologies). In order to assess the time point of PAH expression at the equilibrium of synthesis and degradation, cells transfected with plasmids coding for wild-type PAH or the variants p.Arg158Gln, p.Glu390Gly, and p.Tyr414Cys were harvested at different time points (12 to 60 h) followed by quantitative dot blot analysis (ImageJ, http://image.bio.methods.free.fr/dotblot.html) and analysis of PAH enzyme activity (figure S6). Based on these data, landscape experiments were performed 36 h to 40 h post-transfection. In order to rule out potential variation in transfection efficiency in co-expression experiments, the positive control plasmid pEF/GW-51/lacZ (life technologies) was co-transfected with wild-type PAH, p.Arg261Gln or p.Arg408Trp in a 1:5 ratio (control:PAH). On the protein level, the production of the control protein β-galactosidase did not change in the presence of wild-type or variant PAH proteins (figure S7). Accordingly, wild-type or variant PAH enzyme activity did not vary in the presence or absence of the control vector (table S6). A total of 6 μg DNA per 6 million cells was used in single transfections (homozygotes) or in co-transfections (1:1 molar ratio of two different PAH expression plasmids) accounting for compound heterozygosity. Western blot analysis verified a 1:1 molar ratio of wild-type PAH derived from transfection of two different wild-type PAH expression plasmids (figure S8). Cells were harvested and lysed by three freeze-thaw cycles in a lysis buffer containing 20 mM HEPES (Sigma-Aldrich), 200 mM NaCl (Millipore), pH 7.0 and protease inhibitors

(cOmplete Protease Inhibitor Cocktail Tablets, Roche) followed by 20 min centrifugation at 20 000 g, 4°C. Recovered supernatants were subsequently used for PAH activity landscapes and the total protein amount in the lysate was determined by Bradford assay (BioRad). All transfections were performed using single cuvette Amaxa electroporation system (Lonza).

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PERSONAL DATA

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WORKSHOPS

04/2015	EMBO Laboratory Management Course
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CONFERENCES

09/2014	Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Innsbruck, Austria **Poster:** "Phenylalanine hydroxylase genotypes in Europe and the Middle East"
09/2013	12th International congress of inborn errors of metabolism Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Barcelona, Spain **Talk:** "Mechanisms underlying interallelic complementation: lesson learnt from phenylketonuria, glutaric aciduria type I and Alzheimer disease"
03/2013	5th European Phenylketonuria Group (EPG) Sypmosium 'Advances and Challenges in PKU **Talk:** "Structure-function relationship of the pharmacological chaperone tetrahydrobiopterin stabilising phenylalanine hydroxylase"
03/2013	International Conference on Tetrahydrobiopterin, Phenylketonuria and Nitric Oxide Synthase, St. Moritz, Switzerland **Talk:** "Insights into the mechanisms underlying the interallelic complementation in recessively and dominantly inherited disorders: PKU, GA-1 and Alzheimer disease"
06/2012	44th EMG Conference "Protein Misfolding", Munich, Germany
08-09/2011	Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Geneva, Switzerland

08-09/2010	Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Istanbul, Turkey **Talk:** "New insights into interallelic complementation of phenylalanine hydroxylase in phenylketonuria"
03/2008	International Conference on Tetrahydrobiopterin, Phenylketonuria and Nitric Oxide Synthase, St. Moritz, Switzerland
11/2007	International Symposium: "PKU and other hyperphenylalaninemias: where are we now?", Fulda, Germany
10/2005	Cell Dynamics: from Molecular Structure to Cellular Motility; SFB413, Munich, Germany
09/2005	IX Cell Biology Conference, Lódz, Poland **Poster** 'Lovastatin-induced changes in human skin fibroblasts'

AWARDS

03/2013	‚Award of the Best Oral Presentation in Basic Research' assigned by the Scientific Committee of the Serono Symposia International Foundation during the 5th European Phenylketonuria Group (EPG) Symposium ‚Advances and Challenges in PKU'

LIST OF PUBLICATIONS

Mapping the functional landscape of frequent phenylalanine hydroxylase genotypes promotes personalised medicine in phenylketonuria.
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