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1

2 **Five human phenylalanine hydroxylase proteins identified in mild hyperphenylalaninemia**
3 **patients are disease-causing variants**

4

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25 **Key words:** PKU, PKU mutations, PKU mutations functional analysis, expression studies of PKU
26 mutants, structural analysis.

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28

1 **ABSTRACT**

2 Hyperphenylalaninemia is a group of autosomal recessive disorders caused by a wide range of
3 phenylalanine hydroxylase (PAH) gene variants. To study the effects of mutations on PAH
4 activity, we have reproduced five mutations (p.N223Y, p.R297L, p.F382L, p.K398N and
5 p.Q419R) that we recently identified in a population of Southern Italy. Transient expression
6 of mutant full-length cDNAs in human HEK293 cells yielded PAH variants whose L-
7 phenylalanine hydroxylase activity was between 40% and 70% that of the wild-type enzyme.
8 Moreover, Western blot analysis revealed a 50-kD monomer in all mutants thereby indicating
9 normal synthesis of the mutant proteins. Because of the clinical mild nature of the phenotypes
10 we performed an *in vivo* BH4 loading test. This was positive in all tested patients, which
11 indicates that they are likely to respond to the coenzyme *in vivo*. We also analysed the
12 environment of each mutation site in the available crystal structures of PAH by using
13 molecular graphics tools. The structural alteration produced by each mutation was elucidated
14 and correlated to the mutated properties of the mutant enzymes. All the data obtained
15 demonstrate the disease-causing nature of the five novel variants.

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1 **1. INTRODUCTION**

2 Hyperphenylalaninemia (HPA; OMIM 261600) is the most frequent disorder of amino acid
3 metabolism and is mostly due to recessive mutations in the gene that encodes phenylalanine
4 hydroxylase (PAH: EC 1.14.16.1) [1-3]. This enzyme, which is mainly expressed by the liver,
5 converts L-phenylalanine to L-tyrosine in the presence of the essential cofactor
6 tetrahydrobiopterin (BH4) Few HPA cases are due to defects in the biosynthesis or
7 regeneration of BH4 [4].

8 HPA phenotypes are classified as follows: mild hyperphenylalaninemia (MHP or HPA
9 III) when plasma Phe levels of untreated subjects are between 120 and 600 μM ; mild
10 phenylketonuria (MPKU or HPA II) when untreated plasma Phe levels are between 600 and
11 1200 μM ; and classical phenylketonuria (PKU or HPA I) when untreated plasma Phe levels
12 are $>1200 \mu\text{M}$ (http://www.bh4.org/BH4_Deficiency_Nomenclature.asp). HPA patients with
13 a Phe plasma level $> 360 \mu\text{M}$ must adhere to a Phe-restricted diet throughout their lives to
14 prevent the neurotoxic effects of Phe and its metabolites. An increasing number of PAH
15 patients have been shown to benefit from BH4 supplementation, which in several cases can
16 substitute dietary restrictions [5-7]. Patients who may benefit from BH4 supplementation can
17 be identified with the BH4 loading test and by the presence of mutations found to be
18 associated with the BH4 responsive phenotype [2, 8-10].

19 To date, more than 500 gene variants have been identified in the PAH gene (PAHdb
20 Mutation Analysis Consortium database, at: www.pahdb.mcgill.ca) [11] that contains 13
21 exons spread over 80 kb. A number of PAH mutations are associated with a specific
22 metabolic phenotype. For example, some "mild" PAH mutations reduce the affinity of the
23 enzyme for BH4 and cause the BH4-responsive phenotype [12, 13]. Data regarding the
24 phenotypic impact of other mutations are either lacking or controversial
25 (<http://www.pahdb.mcgill.ca>). This is particularly the case of novel mutations, which are also
26 difficult to identify as disease-causing mutations because of the lack of functional analyses
27 [14].

28 PAH mutations are very heterogeneous in Italy. We recently identified 60 different gene
29 variants in 126 PAH patients from Southern Italy. We described ten novel mutations, four of
30 which are disease-causing (two nonsense, one splicing and one deletion). The others were
31 missense mutations and we suggested they may be disease-causing [15].

32 Other investigators have elucidated the 3D structure of the PAH enzyme in the bound
33 and unbound states [16-18]. Human PAH has a tetrameric architecture with each subunit
34 consisting of three domains: an N-terminal regulatory domain (residues 1-142) containing an
35 α/β sandwich, a large catalytic domain (residues 143-410 with an α/β fold), and a C-terminal

1 domain that is responsible for tetramerization (residues 411-452). The PAH tetramer is a
2 dimer of dimers, with the tetramer core formed by four α -helices (one from each subunit)
3 tightly packed in an antiparallel coiled-coil motif. Structural investigations of disease-causing
4 mutant enzymes have often revealed correlations between the 3D structure and the impaired
5 functioning of PAH in PKU [19-22].

6 In this study, we expressed five proteins, each containing one of the five novel
7 mutations (p.N223Y, p.R297L, p.F382L, p.K398N, p.Q419R) previously identified by our
8 group, to investigate the *in vitro* PAH activity of the mutant enzymes. We also studied the
9 BH4 response *in vivo* using the BH4 loading test in patients bearing these mutations. In
10 addition, we analyzed the structural perturbations induced by the mutations within the
11 framework of PAH crystal structures.

12

13 **2. MATERIALS AND METHODS**

14 **2.1 Mutagenesis**

15 PAH mutant constructs were derived from the wild-type PAH expression plasmid pCDNA3,
16 kindly provided by Prof. P. Knappskog (University of Bergen, Norway) and Prof. P. Waters
17 (McGill University-Montreal Children's Hospital Research Institute, Montreal, Canada). Each
18 mutation was introduced into the wild-type expression plasmid using mutagenic primers and
19 the Transformer II kit (Clontech, Palo Alto, CA). The resulting clones were sequenced to
20 verify the introduction of each single mutation.

21

22 **2.2 Expression studies**

23 Ten micrograms of wild-type and mutant cDNA expression vectors were introduced into $1.6 \times$
24 10^6 of human HEK293 cells using calcium phosphate (ProFection® Mammalian Transfection
25 System-Calcium Phosphate, Promega Italia, Milan, Italy). Forty-eight hours after transfection,
26 the cells were harvested by trypsin treatment, washed twice with 150 mM NaCl, resuspended
27 in the same buffer and frozen-thawed 6 times. All transfections were performed in triplicate.
28 Each triplicate was assayed for total protein content using a protein assay kit (Biorad,
29 Richmond, CA, USA). Parental vector pCDNA3, without insert, was transfected as negative
30 control. We co-transfected 10 μ g of a construct carrying a β -galactosidase reporter gene as a
31 control for transfection efficiency. Forty-eight hours after transfection, total RNA was isolated
32 using a standard protocol and RT-PCR analysis was performed using specific primers; the
33 resulting cDNAs were sequenced.

34

35 **2.3 Production of polyclonal antibody and Western blot analysis**

1 A synthetic peptide, based on the human PAH amino acid region (NH₂-Phe-Gly-Leu-Gly-
2 Arg-Lys-Leu-(Ser-Phospho)-Asp-Phe-Gly-Gln-Glu-Thr-Ser-Tyr-Ile-Glu-Asp-Asn-Cys-
3 COOH), was used to immunize rabbits. PAH antiserum was precipitated with ammonium
4 sulphate and the antibodies were purified on a protein A-Sepharose column. Immunoblotting
5 experiments were performed using 10 µg of protein extracts electrophoresed on a 10% SDS-
6 PAGE gel as previously reported [23].

7

8 **2.4 Enzyme analysis**

9 For each transfection, PAH activity was assayed on 50 µg of proteins, in duplicate, as
10 previously reported [24]. This test measures the amount of ¹⁴C radiolabeled Phe converted to
11 Tyr that were subsequently separated by thin-layer chromatography (TLC). Enzyme activity
12 of the wild-type and mutant PAH constructs was measured; the mean PAH activities was
13 calculated from the three sets of transfection data. The residual activities of mutant PAH
14 enzymes were expressed as a percentage of wild-type enzyme activity and normalized to
15 transfection efficiencies based on replicates β-galactosidase activities.

16

17 **2.5 BH4 loading test**

18 Patients (legal tutors for minors) provided informed consent to the BH4 loading test, which
19 was performed according to international guidelines [12]. Briefly, a watery solution of 20
20 mg/body kg of (6R)-5,6,7,8-Tetrahydro-L-Biopterin (Schircks Laboratories, Jona,
21 Switzerland) was given orally 30 min before feeding. Blood samples in EDTA were drawn 0,
22 2, 4, 6 and 8 hours later, and Phe serum concentration was measured. Patients ate as usual
23 during the test. A patient is considered “responder” if the Phe serum concentration decreases
24 more than 30% 8 hours after BH4 load, and “fast responder” if the decrease occurs after 2
25 hours.

26

27 **2.6 Structural analysis**

28 The effect of the mutations on the 3D-structure has been investigated by analysing the
29 structural environment of each substituted residue in the PAH crystal structures deposited in
30 the Protein Data Bank (PDB). Human PAH is a homotetramer with each subunit consisting of
31 three domains. A dimeric form also exists in equilibrium with the tetrameric form. Since no
32 crystal structure of any full-length tetrameric/dimeric PAH is available, different models have
33 been used to study the isolated catalytic domain (human PAH; PDB code 1kw0), the contact
34 interface between the catalytic and regulatory domain (rat PAH; PDB code 1phz) and the
35 oligomeric interfaces (human PAH; PDB code 2pah). A composite full-length monomeric

1 model was built by superimposing the respective catalytic domain regions (Figure 1A). The
2 notation for the secondary structure elements mentioned in the text refers to the assignment by
3 Erlandsen and Stevens [19].

4

5 **3. RESULTS**

6 **3.1 Mutation features and structural localization**

7 Table 1 shows the PAH genotype, clinical and biochemical classification of the five HPA
8 patients bearing on allele A2 the PAH missense mutations p.N223Y, p.R297L, p.F382L,
9 p.K398N, and p.Q419R. All patients were classified as HPA III; their serum Phe levels at
10 diagnosis ranged between 182 and 242 μ M. Table 2 shows the main features of the five
11 mutations. Some nucleotide changes involve a codon that contains other previously reported
12 gene variants (<http://www.pahdb.mcgill.ca>).

13 As shown in Figure 1A, four of the five mutations are located in the catalytic domain
14 (p.N223Y, Figure 1C; p.R297L, Figure 1D; p.F382L, Figure 1E; p.K398N, Figure 1F) and the
15 remaining one in the tetramerization domain (p.Q419R, Figure 1G). To evaluate the structural
16 implications of the missense mutations, we examined the 3D-localization of the substituted
17 residues in detail, taking into account the full tetramer (Figure 1B). The structural alterations
18 of the mutation sites are summarized in Table 2.

19 The c.667A>T nucleotide transversion changes the polar Asn residue at position 223 into
20 the polar, aromatic Tyr residue (p.N223Y). Asn223 is a moderately conserved amino acid.
21 Mutation p.R297L was identified in cis with mutation p.A403V (Table 1), which is widely
22 found in PAH patients [15]. The c.890G>T nucleotide transversion changes the charged Arg
23 residue at position 297 into the apolar, aliphatic Leu residue (p.R297L). Arg297 is a
24 moderately conserved amino acid. It is located in the α -helix C α 8 at the dimer interface
25 (Figure 1D).

26 The c.1146C>G nucleotide transversion changes the apolar, aromatic Phe residue at
27 position 382 into the aliphatic Leu residue (p.F382L). Phe382 is not strictly conserved. In
28 fact, it is frequently replaced by an aromatic Tyr residue. Phe382 is located at the end of the
29 loop 378-382, near the entrance to the active site. The c.1194A>C nucleotide transition
30 changes the polar, charged Lys residue at position 398 into the polar Asn residue (p.K398N).
31 Lys398 is a conserved amino acid. The c.1256A>C nucleotide transition in exon 12 changes
32 the polar Gln at position 419 into the polar, charged Arg residue (p.Q419R). Gln419 is a
33 poorly conserved amino acid.

34

35 **3.2 Functional analysis and expression studies by RT-PCR and Western blot**

1 We expressed in vitro five proteins containing the missense mutations p.N223Y,
2 p.R297L, p.F382L, p.K398N, p.Q419R. The p.R297L mutation, identified in cis together with
3 the well characterized p.A403V mutation, was reproduced in a recombinant protein as the sole
4 mutation (Table 1).

5 The RT-PCR (Figure 2A) showed a fragment, representing an 871-bp partial region of
6 PAH mRNA, in the wild-type and in all mutant constructs examined (lanes 2–7), but not in
7 the untransduced cells (lane 1) and in the RT-PCR negative control (water-lane 8). We
8 identified PAH mutant polypeptides in transfected HEK293 cell extracts by immunoblot
9 analysis using the anti-PAH serum (Figure 2B). The monomeric form of the enzyme appeared
10 as a 50-kD band, with different intensities in cells transfected with the wild-type enzyme (lane
11 2) as compared with mutants (lanes 3–7), but was absent in the untransduced cells (lane 1).

12 PAH enzyme assay was performed, in three independent experiments, on total protein
13 extracts harvested 48 hours after transfection. As shown in Figure 3, the residual enzyme
14 activity in cells expressing mutant cDNAs was 70% (p.N223Y), 42% (p.R297L), 56%
15 (p.F382L), 55% (p.K398N) and 71% (p.Q419R) that of the wild-type enzyme activity. There
16 was no enzyme activity in the untransduced cells.

17 18 **3.3 BH4 loading test**

19 Figure 4 shows the results of the BH4 loading test in patients bearing the genotypes
20 [p.N223Y]+[c.165delT] (magenta line and stars), [p.R297L+p.A403V]+[p.R243X] (cyan line
21 and diamonds), [p.K398N]+[p.442-5C>G] (red line and triangles) and [p.Q419R]+[p.S231P]
22 (black line and square). Patient [p.A403V]+[c.184delC] (blue line and circles) is a good
23 responder who served as a positive control in the test because c.184delC is a null mutation
24 and p.A403V is an in vivo responsive mutation [25]. The analysed patients proved to be
25 “responders” and even “fast responders” since the decrease in serum Phe concentration (from
26 61% to 87%) occurred already 2 hours after BH4 load. The patient bearing the p.F382L
27 mutation was not tested because his parents did not give consent.

28 29 30 **4. DISCUSSION**

31 Five new PAH mutations (p.N223Y, p.R297L, p.F382L, p.K398N, p.Q419R) were
32 previously identified by our group in a population of HPA patients from Southern Italy (Table
33 1) [15]. These mutations can be considered “mild”, because they were found in compound
34 heterozygote patients affected by a mild phenotype and bearing a “severe” mutation
35 (www.pahdb.mcgill.ca) on the other allele (allele A1 in Table 1) [25]. In particular, the

1 missense mutations p.R252W and p.S231P were previously described as severe mutations
2 [11]. The remaining ones are severe mutations leading to inactive PAH protein (c.165delT,
3 p.R243X, c.442-5C>G) [11].

4 On a molecular level, in our patients, the PAH tetramer may be formed from various
5 combinations of mutated alleles (Table 1). The two homotetramers (A1)₄ and (A2)₄, and the
6 heterotetramers (A1)₁(A2)₃, (A1)₂(A2)₂ and (A1)₃(A2)₁ can be formed at variable ratios,
7 depending on the effects generated by mutations (i.e. folding defects, reduced stability or low
8 amount of expression) [26]. In fact, co-expression of both mutants from two mutated alleles
9 resulted in activity values in between those of single mutants [26]. In our patients bearing a
10 severe mutation in allele A1, the functionality of the PAH tetrameric enzyme would mainly
11 result from the novel mutations in the allele A2. The resulting enzymes, being embodied in
12 homo- or heterotetrameric proteins, may influence the overall in vivo activity [26].

13
14 In this work, we reproduced in vitro the five mutant enzymes and measured the PAH
15 catalytic activity. The assays showed an activity decrease to between 42% and 71% that of the
16 wild-type protein. Western blot analysis showed that the five mutated constructs yielded a
17 relatively normal amount of regular-sized PAH polypeptides. This indicates that these
18 mutations do not induce changes that lead to increased degradation of the mutants nor to
19 alteration of transcription and translation processes. Therefore, the tested mutations are
20 disease-causing and the observed decreased enzymatic activity well correlates with the mild
21 patient phenotype.

22 Some residual PAH activity is a prerequisite for BH4 responsiveness [13] but BH4
23 responsiveness can only be established with an in vivo test. We therefore verified BH4
24 responsiveness for the studied HPA patients, some of them (ID 12, 74, 82, Table 1) may be
25 considered functionally hemizygous (coupled to a null mutation). In all cases, BH4
26 supplementation resulted in a relevant decrease (>30%) in serum Phe concentrations, which
27 remained low up to 8 hours after BH4 challenge. The BH4 response might be the result of a
28 correction by BH4 of the kinetics of the PAH mutant and/or of stabilization defects, because
29 the mutant activity is decreased and the mutations might affect the structural stability [18, 22].

30 HPA is responsive to BH4 supplementation in vivo in more than 70% HPA
31 phenotypes [2]. Infants undergoing BH4 treatment may be fed a less rigid PKU-restricted diet,
32 with consequent positive effects on weight gain and quality of life [5-7]. Moreover, this
33 treatment improves intellectual development especially in patients who have difficulty in
34 adhering to a semisynthetic Phe-restricted diet [6]. As the BH4 positive response requires
35 some residual PAH activity, BH4 is believed to activate the PAH enzyme [27]. Indeed,

1 classification of BH4-responsive patients, based on genotype and biochemical phenotype, and
2 elucidation of the molecular mechanism underlying BH4 responsiveness are crucial for PKU
3 treatment. Various mechanisms have been implicated in BH4 responsiveness [22, 28]:
4 increased enzyme activity [2, 12, 22, 27] correction or compensation of the BH4 decreased
5 affinity, protection towards catalytic inactivation and chaperone-like activity that, by
6 stabilizing the protein, protects it from proteolytic degradation [2, 3, 22]. In addition, BH4
7 supplementation may restore the optimal concentration of BH4 cofactor in hepatocytes [29,
8 30]. In all cases, BH4 responsiveness is dependent on the genotype, even though in some
9 cases the genotype is not a reliable predictor of phenotype [9]. The latter authors reported a
10 case of a patient bearing two null mutations on both alleles who was responsive to BH4
11 treatment. Generally, there is no single nor simple explanation for each patient who presents
12 an own complex phenotype that requires an ad hoc therapeutical protocol. However, new
13 therapeutic strategies that monitor the safety and the response to long-term treatment have
14 recently been reported [9, 10, 31].

15
16 Previous studies on structure/function correlations showed that the disease-causing
17 mutations are spread throughout the protein structure, with a certain number localised at
18 dimer/tetramer interfaces. This finding highlights the relevance of interactions among
19 subunits for the correct functioning of PAH [16, 17]. The structural examination of the five
20 PAH mutants, which we describe, revealed that the substituted residues are not directly
21 involved in the active site or in the cofactor binding site. Mutations at positions 223 and 382
22 perturb the local structure of regions that are involved in substrate-induced molecular
23 motions; whereas mutations at position 297, 398 and 419 disturb the dimer/tetramer
24 interfaces. All the substitutions resulted in an enzyme with decreased residual catalytic
25 activity compared to the wild-type enzyme.

26 The p.N223Y substitution is found in a region that may be critical for substrate-
27 triggered molecular motions [17, 18, 32]. Indeed, it has been proposed that this residue is the
28 centre of an intra-domain hinge-bending region associated with the conformational transition
29 of PAH induced by L-Phe substrate binding [18, 32]. In all available PDB structures, the
30 Asn223 side-chain nitrogen is H-bonded to the main-chain Gly218 oxygen. This interaction
31 adds further stabilization to the β -turn (Table 2, Figure 1C). The change to Tyr would result in
32 the loss of this H-bond, thereby probably destabilizing the local structure. A further
33 perturbation could be caused by the exposure of the Tyr aromatic ring to the surface.

34 The p.F382L replacement might affect the accessibility of the substrate in the active
35 site. Indeed, Phe382 is located at the end of the loop region 378-382, which covers the

1 entrance to the active site. This loop is quite flexible in the absence of substrate and becomes
2 more ordered when bound to pterin cofactor and L-Phe substrate. In the latter case, Phe382
3 forms aromatic interactions (within 3.7 Å) with Tyr277 thereby protecting the substrate from
4 the solvent. In both the substrate-free and the substrate-bound structure, Phe382 forms
5 aromatic interactions with Tyr356, whereas Tyr277 forms van der Waals interactions with
6 His146. Upon substrate binding, the relative approaching of Phe382 and Tyr277 results in a
7 network of hydrophobic interactions (Figure 1E). Substitution of the aromatic Phe382 with a
8 less bulky and aliphatic Leu residue would impair the efficiency and stabilization of the side-
9 chain packing upon substrate binding.

10 The mutations (p.R297L, p.K398N and p.Q419R) perturb the dimer/tetramer
11 architecture and hence may destabilize the enzyme. In the p.R297L enzyme, replacement of
12 the Arg side chain by an apolar group would disrupt the dimer-stabilizing electrostatic and
13 polar interactions. The Arg side chain is indeed close to acidic residues from the other subunit
14 of the dimer. It can also engage a H-bond with a main-chain oxygen from residue 71 in the
15 regulatory domain (Table 2). It is noteworthy that in our patients p.R297L was identified in
16 cis with the p.A403V substitution on the same allele (Table 1). The p.R297L and p.A403V
17 mutation sites are far apart (about 19 Å). Ala403 is located at the end of α -helix C α 12 (Figure
18 1A), close to other apolar residues, namely Ala306 and Ala309 belonging to C α 8. The
19 p.A403V substitution might affect the close packing of C α 8 and C α 12 helices by introducing
20 a larger, β -branched, Val residue at the helix-helix interface. This results in a further reduction
21 of dimer stability. Two other mutations at position 297 are reported in the PAH database,
22 p.R297H and p.R297C. The database also includes a unique case of a patient with the
23 [p.R297C]+[p.Y356X] genotype and a moderate phenotype (HPA II). Because mutation
24 p.Y356X is a null mutation, it is conceivable that mutation p.R297C induces a moderate
25 reduction of enzyme activity, consistent with dimer destabilization.

26 In the p.K398N enzyme, substitution of Lys with a smaller and uncharged Asn would
27 perturb the charge distribution at the tetramer interface. Indeed Lys398 protrudes toward a
28 dimer hydrated interface in a negatively-charged surrounding, being the acidic residues
29 Asp394 and Glu390 within 6 Å (Figure 1F). Lys398 belongs to the C α 12 helix (392-403) and
30 contributes to helix stabilization through ionic interactions with residues Glu390 and Asp394.
31 It is noteworthy that the C α 12 helix is a key secondary structure element that undergoes
32 significant motion upon substrate binding [17, 32]. Furthermore, in the tetramer, the C α 12
33 helix is directly connected to Ser439 of the Ta1 helix (430-449) of another subunit, via a H-
34 bond formed by Glu397, the residue preceding K398 (Table 2, Figure 1F). The substitution

1 may therefore also interfere with correct positioning of the C α 12 helix, which is involved in
2 interactions with the tetramerization domain, and in molecular motions [14].

3 In the p.Q419R enzyme, the replacement of Gln with a charged Arg would increase the
4 charge repulsion between basic residues of the same subunit (Table 2). In the homotetramer
5 (A2)₄, the replacement p.Q419R would also result in a close localization of two corresponding
6 Arg419 residues on different subunits of the dimer (Figure 1G). The replacement of Gln with
7 a charged Arg residue may hamper oligomerization.

8 In conclusion, all the data reported herein support the pathologic nature of the five
9 studied variants. In fact, the enzyme activity exerted by the recombinant protein variants was
10 from 40% to 70% that of the wild-type enzyme, which well correlates with the mild
11 hyperphenylalaninemia found in our patients. Accordingly, the analysis carried out in silico
12 shows that the structural alterations do not directly affect the binding site of either the
13 substrate or the coenzyme — rather they interfere with favourable interactions at the
14 molecular surface or at its quaternary structure, and hence may destabilize structural integrity.
15 Finally, the beneficial effect of BH4 supplementation in patients indicates that altered
16 activity/stability could be partially reversed, thus supporting the not severe nature of HPA in
17 patients bearing the variant proteins studied in this report.

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32

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1 **Figure legends**

2

3 Figure 1. Schematic representation of PAH structure.

4 A: The localization of the five mutations is represented by red spheres in the composite
5 monomeric model (see text); the position of residue 403 is also shown (mutation p.R297L is
6 in cis with p.A403V). The catalytic domain, the regulatory domain, and the tetramerization
7 domain are shown in blue, magenta, and green, respectively. BH₄ cofactor is drawn in yellow,
8 thienylalanine in cyan, Fe ion as a grey sphere.

9 B: The tetrameric model of human PAH containing the catalytic and tetramerization domains
10 (PDB code 2pah). Dimers are coloured blue/cyan and red/orange. C: Asn223 site. Segment
11 217-224 is drawn in ball-and-stick form. The stabilizing H-bonds displayed by Asn223
12 (magenta) in the β -turn 220-223 are represented by dotted lines. D: Arg297 site. The
13 negatively-charged side-chains (Glu422 and Asp415) close to Arg297 (magenta) are shown.
14 The Arg71 residue is also shown, with its main-chain oxygen within H-bond distance of
15 Arg297. E: Phe382 site. Phe382 (magenta) and nearby residues (Tyr356, Tyr277, His146) are
16 highlighted in the substrate-bound structure (PDB code 1kw0). The substrate analogue,
17 thienylalanine (cyan), and the BH₄ cofactor (yellow) are also shown. F: Lys398 site. The
18 negatively-charged side-chains close to Lys398 (magenta) are shown. In the dimer, the H-
19 bond between Glu397 (C α 12) and Ser439 (T α 1) is also drawn. G: Gln419 site. Two Gln419
20 (magenta) residues at the dimer interface are shown with the positively-charged Arg241
21 residues nearby.

22

23 Figure 2. RT-PCR (panel A) and Western blot analysis (panel B) in transfected human
24 HEK293 cells. A: Forty-eight hours after transfection, total RNA was extracted for RT-
25 PCR analysis; an 871-bp region of PAH cDNA was present in all samples (2 to 7)
26 examined but not in untransduced cells (lane 1), or in an RT-PCR negative control (water-

1 lane 8). B: a 50-kD band was detected on immunoblots of cells transduced with wild-type
2 PAH (lane 2) but not in untransduced cells (lane 1). Lane 1: Untransduced cells; 2: Wild
3 type; 3: p.F382L; 4: p.K398N; 5: p.R297L; 6: p.N223Y; 7: p.Q419R; 8: RT-PCR negative
4 control (water).

5

6 Figure 3. Residual enzymatic activity of PAH mutants expressed as percent of wild-type PAH
7 activity. The columns show the mean value and the standard deviation derived from three
8 different transfections, for each construct.

9

10 Figure 4. Temporal decrease of serum Phe concentrations after 20 mg/kg BH4 loading test.

Table 1. Biochemical phenotype and PAH genotype of HPA III patients.

| Patient ID | Serum Phe (μmol/L) | Allele A1 | Allele A2* |
|------------|--------------------|------------|-----------------|
| 12 | 242 | c.165delT | p.N223Y |
| 74 | 242 | p.R243X | p.R297L+p.A403V |
| 96 | 182 | p.R252W | p.F382L |
| 82 | 242 | c.442-5C>G | p.K398N |
| 135 | 205 | p.S231P | p.Q419R |

*Mutations studied

Table 2. Features of the studied PAH mutations.

| Exon | Nucleotide | Amino acid | Other mutations described for the codon | Enzyme domain/ Secondary structure position* | Amino acid conservation score§ | Structural contacts in WT / Consequent alterations in mutated enzyme |
|------|------------|------------|---|--|--------------------------------|---|
| 6 | 667A>T | N223Y | None | Catalytic/ β -turn (220-223) | 5 | N223 is located in a solvent-exposed β -turn (residues 220-223) connecting α -helices C α 4 and C α 5 (Fig. 1C). N223 H-bonds to G218 oxygen / Loss of a H-bond; Tyr exposure |
| 7 | 890G>T | R297L | R297C and R297H | Catalytic/ α -helix C α 8 (293-310) | 5 | R297 points toward (~ 4 Å) negatively-charged residues (E422 and D415) of the other subunit of the dimer (Fig. 1D). In the structure (PDB code 1phz), R297 forms a salt bridge with E422 and a H-bond with R71 oxygen in the regulatory domain of the other subunit of the dimer / Loss of polar interactions at the dimer interface |
| 11 | 1146C>G | F382L | None | Catalytic / Long loop preceding C α 12 (392-403) | 4 | F382 forms aromatic interactions (~ 3.7 Å) with Y356. In the substrate-bound state, it is close to Y277 that in turn displays contacts with H146 (Fig. 1E) / Loss of aromatic interactions |
| 11 | 1194A>C | K398N | K398K (c.1194A>G) | Catalytic/ α -helix C α 12 (392-403) | 6 | K398 points toward a hydrated interface in a negatively-charged surrounding and makes ionic interactions with D394 and E390 within 6 Å (Fig. 1F). In the tetramer, the C α 12 helix is connected to the Ta 1 helix (430-449) of another subunit via a H-bond (E397 \cdots S439) / Perturbation of charge distribution at the subunit interface and possible interference with the correct positioning of the C α 12 helix |
| 12 | 1256A>C | Q419R | None | Tetramerization / Loop (415-420) connecting two β -strands | 1 | Q419 lies in a rather hydrated region on the dimer interface, close to R241 on the same subunit (Fig. 1G). In the substrate-bound structure, it is close to Q419 on the other subunit of the dimer / Unfavourable ionic interactions at the dimer interface |

* According to the nomenclature of the composite model [17]

§ Deduced from data deposited in the PDBsum database (ID: 2pah) in the range from 1 (variable) to 9 (conserved)

Figure 1

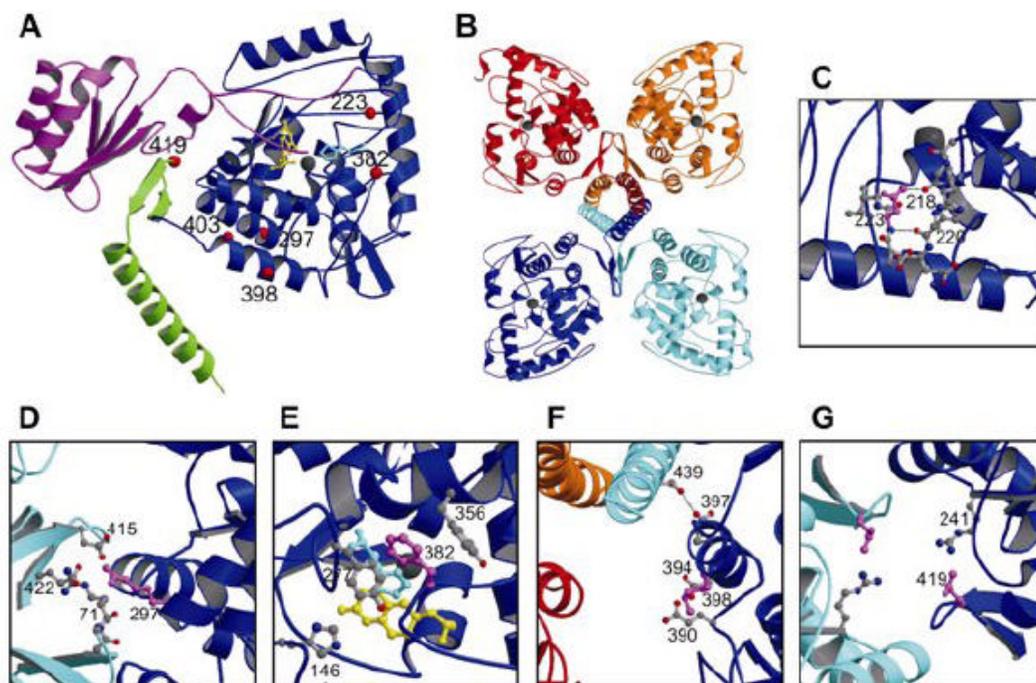
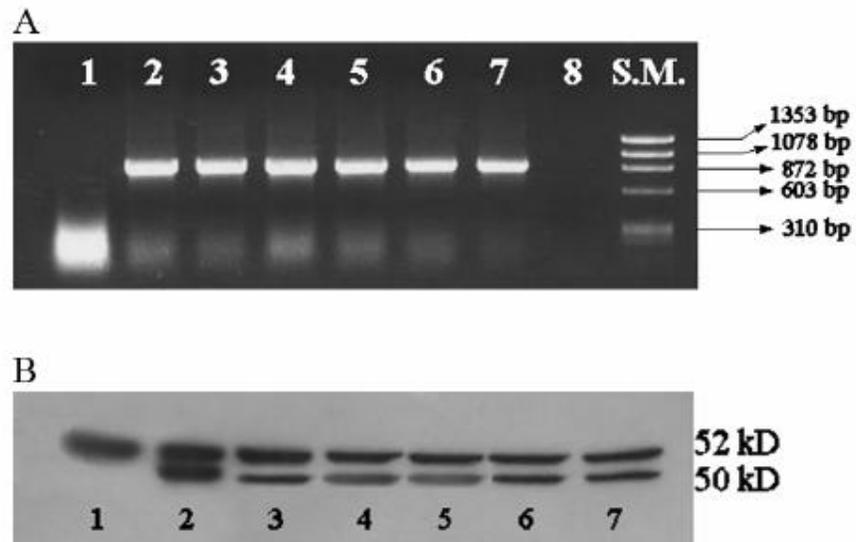
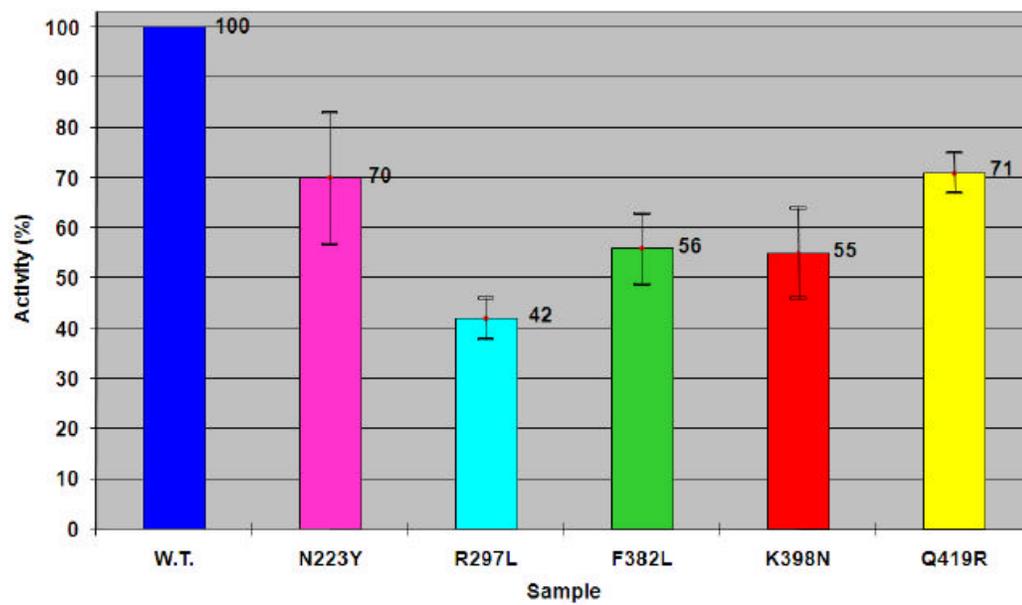


Figure 2



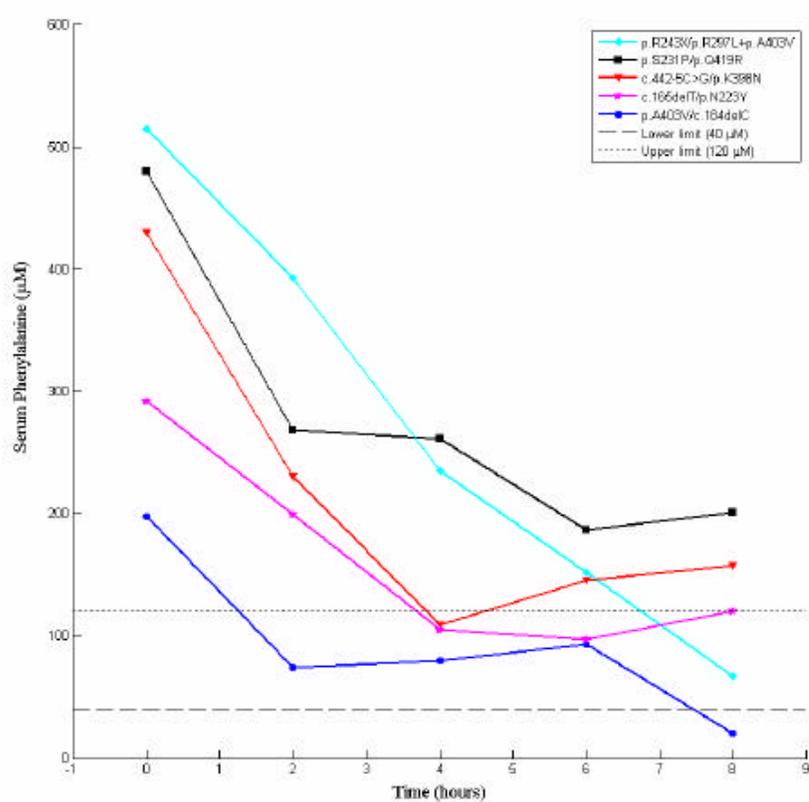
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Figure 3



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Figure 4



AC