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Pathogenic variants of *DNAJC12* and evaluation of the encoded co-chaperone as a genetic modifier of hyperphenylalaninemia

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ABSTRACT

Biallelic variants of the gene *DNAJC12*, which encodes a co-chaperone, were recently described in patients with hyperphenylalaninemia (HPA). This paper reports the retrospective genetic analysis of a cohort of unsolved cases of HPA. Biallelic variants of *DNAJC12* were identified in 20 patients (generally neurologically asymptomatic) previously diagnosed with phenylalanine hydroxylase (PAH) deficiency (phenylketonuria, PKU). Further, mutations of *DNAJC12* were identified in four carriers of a pathogenic variant of *PAH*. The genetic spectrum of *DNAJC12* in the present patients included four new variants,

two intronic changes c.298-2A>C and c.502+1G>C, presumably affecting the splicing process, and two exonic changes c.309G>T (p.Trp103Cys) and c.524G>A (p.Trp175Ter), classified as variants of unknown clinical significance (VUS). The variant p.Trp175Ter was detected in 83% of the mutant alleles, with 14 cases homozygous, and was present in 0.3% of a Spanish control population. Functional analysis indicated a significant reduction in PAH and its activity, reduced TH stability, but no effect on TPH2 stability, classifying the two VUS as pathogenic variants. Additionally, the effect of the overexpression of DNAJC12 on some destabilising PAH mutations was examined and a mutation-specific effect on stabilization was detected suggesting that the proteostasis network could be a genetic modifier of PAH deficiency and a potential target for developing mutation-specific treatments for phenylketonuria.

Keywords: *DNAJC12*, hyperphenylalaninemia, phenylketonuria, molecular chaperones, proteostasis network

INTRODUCTION

Hyperphenylalaninemia (HPA) is an inherited metabolic condition defined by increased blood phenylalanine (Phe) concentrations (>120 µmol/L). In 98% of cases, it results from a loss-of-function mutation in the *PAH* gene, which codes for the hepatic enzyme phenylalanine hydroxylase (PAH, EC1.14.16.1) responsible for the conversion of Phe to tyrosine (Tyr) in the presence of the cofactor tetrahydrobiopterin (BH4). PAH deficiency or phenylketonuria (PKU; MIM# 261600), one of the most common of all inborn errors of metabolism, affects approximately 1 in 10,000 newborns. Untreated (notably for blood Phe levels >600 µmol/L) it leads to progressive and irreversible intellectual disability, along with

motor deficits and other neurological problems (Blau, van Spronsen, & Levy, 2010). The remaining 2% of cases of HPA are caused by defects in either the synthesis or regeneration of BH4 and involve a more complex neurological phenotype; BH4 acts also as a cofactor for tyrosine hydroxylase (TH, EC: 1.14.16.2) as well as for tryptophan hydroxylase 1 and 2 (TPH, EC: 1.14.16.4), the rate-limiting enzymes in the synthesis of the neurotransmitters dopamine and serotonin respectively. Low concentrations of dopamine and the serotonin metabolites homovanillic acid (HVA) and 5–hydroxyindoleacetic acid (5–HIAA) in cerebrospinal fluid (CSF) may indicate a neurotransmitter deficit (Brennenstuhl, Jung-Klawitter, Assmann, & Opladen, 2019).

PKU treatment is focused on maintaining blood Phe below toxic levels and relies on life–long dietary restriction - although one subset of patients has shown a favourable genotype–dependent response to oral sapropterin (the commercial form of BH4) supplementation (Erlandsen et al., 2004). For deficiencies in BH4 metabolism, additional treatment with neurotransmitter precursors such as L–DOPA (for dopamine) plus carbidopa and 5–hydroxytryptophan (for serotonin) may be required (Blau, 2016).

The early diagnosis of HPA and the immediate start of treatment appropriate for the specific gene defect detected can prevent neurological symptoms and allow normal development. In many developed countries, HPA detection has been included in neonatal screening programmes for over 50 years. A positive result for HPA is followed by a differential biochemical diagnosis to identify a possible defect in BH4 synthesis or regeneration. Genetic confirmation is mandatory before prescribing a tailored therapy (Vockley et al., 2014).

In several patients with neither genetic defect in *PAH*, nor in any gene involved in BH4 metabolism, biallelic mutations in *DNAJC12* have been reported to cause HPA accompanied by heterogeneous neurological symptomatology or early–onset Parkinsonism (Anikster et al., 2017), (van Spronsen et al., 2017), (Straniero et al., 2017). DNAJC12 belongs to the DNAJ/Hsp40 family of co-chaperones that modulate the activity of molecular chaperone Hsp70 and mediate the productive delivery of its substrates (Choi, Djebbar, Fournier, & Labrie, 2014). Hsp70 is involved in a wide range of cellular processes, including the *de novo* folding of nascent chains and the refolding of aggregation-prone folding intermediates or misfolded proteins (Kampinga & Craig, 2010). DNAJC12 directly interacts with PAH, TH and TPH in human cells, and is therefore thought to play a critical role in the Hsp70–assisted folding of aromatic amino acid hydroxylases (AAAH) (Anikster et al., 2017). Moreover, a recent investigation has shown that DNAJC12 may play a role in the processing of misfolded ubiquitinated PAH, adding to the evidence that DNAJ proteins are important for the proper folding and degradation of their clients (Jung-Kc et al., 2019).

The complete structure of DNAJC12 is still to be determined, and little is known about the mechanism by which this co-chaperone exerts its function. Along with the rest of the Hsp40 family, DNAJC12 shares the conserved N-terminal JDP domain with the motif HPD that interacts with the molecular chaperone Hsp70, promoting its ATPase activity (Qiu, Shao, Miao, & Wang, 2006). The other major distinguishing feature of DNAJC12 is its highly conserved C–terminal heptapeptide KFRNYEI, which has been suggested involved in specific client binding (Hahn, Lee, Seong, Yoon, & Chung, 1999), (Lee, Hahn, Yun, Mita, & Chung, 2000).

The proteostasis network coordinates the balance between protein synthesis, folding and degradation. Molecular chaperones such as Hsp70 and their partners, constitute a complex machinery that guides the folding of nascent peptides and the refolding of stress–unfolded proteins, and/or facilitates their degradation when no recovery is possible (Kampinga & Craig, 2010). To avoid the accumulation of potentially toxic aggregates, the cell attempts to degrade meta-stable or aberrant polypeptides due to hypomorphic mutations (Dekker, Kampinga, & Bergink, 2015). Hsp70 recognizes the substrates fated for break-down and delivers them to downstream partners in the degradation pathway (Mayer & Gierasch, 2019). DNAJC12 might drive the specificity of this process.

PKU is a conformational disease, and about 80% of *PAH* mutations are missense variants that cause PAH destabilization and accelerated degradation, leading to the loss–of–function phenotype and the manifestation of disease (Gersting et al., 2008), (Scheller et al., 2019). However, some inconsistencies in genotype–phenotype correlation and BH4 responsiveness have been observed in certain patients with destabilizing mutations (Aldamiz-Echevarria et al., 2016), (Gamez, Perez, Ugarte, & Desviat, 2000). This lack of correlation may be explained by other genes acting as modifiers of disease severity (Citro et al., 2018). Genes involved in protein folding and stability, such as those coding for molecular chaperones and other proteostasis network components, may influence the association between genotype and phenotype in conformational disorders (Scriver, 2007).

Our laboratory is a reference centre for the genetic study of HPA in Spain. Of the 1407 HPA cases dealt with, biochemical differential diagnosis classified 98.5% under PAH deficiency. Biallelic mutations in *PAH* were found in 95% of

cases. The remainder cases had only a monoallelic variant or had no *PAH* mutation. The purpose of the present work was to identify possible defects in *DNAJC12* that might be the genetic cause of HPA in these latter cases, thus helping in the structuring of a tailored therapy and in the provision of appropriate genetic counselling. In addition, the potential of DNAJC12 overexpression as a means of ameliorating the destabilizing effects of PAH mutations was examined.

MATERIALS AND METHODS

Patient clinical features

This retrospective study examines a cohort of patients with HPA (n=50), all with a biochemical suspicion of PAH deficiency but without biallelic mutations in *PAH*. All had Phe levels of >120 µM (120–442 µM) at diagnosis, and had normal DHPR activity and urinary pterin levels. Sanger analysis was used to sequence the exonic region of *DNAJC12*. Nomenclature of the variants was verified using *VariantValidator* (*https://variantvalidator.org/*). All of the variations reported in this work were described with reference to *DNAJC12* transcript NM_021800.3 or *PAH* transcript NM_000277.3. Variants in *DNAJC12* were submitted to the LOVD (http://www.lovd.nl/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) databases. All these variants were classified according to ACMG guidelines, by the Varsome platform (Kopanos et al., 2019). The study protocol adhered to the Declaration of Helsinki and was approved by the Ethic Committee of Universidad Autónoma de Madrid.

Functional studies

Patient-derived fibroblasts carrying biallelic mutations in *DNAJC12* (taken with informed consent) were grown following standard conditions in minimal essential medium (MEM) supplemented with 1% glutamine, 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin).

For expression analyses, control and patient–derived fibroblasts were plated (2×10^6 cells) in 100 mm culture dishes, and on the following day transfected with 5 µg of the plasmids pRC-CMV-PAH, pCDNA3-TH (the kind gift of Dr. Angels García–Cazorla, Barcelona, Spain) or pCMV6-TPH2 (SinoBiological) using 25 µl of Lipofectamine 2000 reagent (ThermoFisher Scientific) according to the manufacturer's instructions.

For overexpression studies, COS–7 cells were plated in six–well plates at 3×10^5 cells per well and transfected with 1 µg of pRC-CMV-PAH expressing either wild–type PAH or one of several PAH mutants (p.Leu48Ser, p.Ile65Thr, p.Arg261Gln, p.Glu280Lys, p.Leu348Val or Val388Met). Alternatively, they were co–transfected with the pReceiver/*DNAJC12* plasmid (OriGene) using the jetPEI transfection reagent (Polyplus). In all cases, transfected cells were harvested with trypsin after 48 h.

For the functional analysis of *DNAJC12* variants, 250 ng of total RNA isolated from control and patient–derived fibroblasts were retrotranscribed using the NZY First Strand cDNA Synthesis Kit (NZYTech). *DNAJC12* was amplified from the obtained cDNA with the specific primers CAGTGAAGACGTCAATGCACT and GGTTGAAGCCAGCTCCTCT, and using the Perfecta SYBR Green Fast Mix Kit (Quanta Biosciences). *GAPDH* was

used as an endogenous control. qPCR reactions were performed in triplicate in a Light Cycler 480 II instrument (Roche Applied Bioscience). After determining the raw threshold cycle (Ct) values for the reference and target genes, the relative quantification (RQ) or fold–change was calculated according to the $2^{-\Delta\Delta Ct}$ method.

For protein analysis, total protein was extracted from fibroblasts or COS–7 cells using cOmplete™ Lysis-M buffer (Sigma-Aldrich) and protease inhibitor (Sigma-Aldrich) and its concentration determined via the Bradford assay (BioRad). Equal amounts of protein extract were prepared in NuPage®LDS 4x sample buffer (Invitrogen) and dithiothreitol (DTT) and subjected to electrophoresis in 4-12% NuPAGE Novex Bis–Tris precast polyacrylamide gel (Invitrogen). Proteins were transferred to a nitrocellulose membrane using the iBlot 2 Dry blotting system (Invitrogen). Membranes were blocked after 1 h with Tris-buffered saline (TBS) containing 5% non–fat dry milk and 0.1% Tween–20. Immunodetection was performed using commercially available antibodies against DNAJC12 (1:750, Abcam), PAH (1:1000, Santa Cruz Biotechnology), TH (1:1000, Sigma–Aldrich) and TPH2 (1:1000, Sigma–Aldrich). NPT-II (1:1000, Millipore) was used as a marker of plasmid transfection efficiency and β-actin (1:5000, Abcam) as a loading control to normalize the amount of protein. After incubation with secondary antibody (anti–rabbit 1:5000 or anti–mouse 1:2000 [Cell Signalling]), protein bands were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific).

PAH activity assays

The freeze–thaw method was used to lyse PAH–transfected fibroblasts and COS–7 cells. PAH activity was assayed at 25°C in a final volume of 50 µl. 10 µg of total

protein extract were mixed with Na–Hepes Buffer pH 7.0, 0.1 mg/ml catalase and 1 mM L–Phe and incubated for 5 min, adding 1 µM $Fe(NH_4)_2(SO_4)_2$ for the last minute. The reaction was started by the addition of 75 µM (6R)–tetrahydrobiopterin (BH4) and 5 mM DTT and incubated for 30 min; it was stopped with 12% $HClO_4$. The amount of tyrosine produced was measured by HPLC.

RESULTS

Genetic analysis

Of the 50 patients studied, 21 had variants in *DNAJC12*. Tables 1 and 2 summarize the patients' genotypes and phenotypes. The Phe levels at diagnosis ranged from 120 to 442 µM. All patients had normal DHPR activity and normal urinary pterin levels. CSF analysis for pterins and neurotransmitter metabolites HVA and 5-HIAA was performed for two patients (P13 and P17). P17 had normal levels for all of these, but P13 showed a slight reduction in HVA and 5-HIAA. Of the above 21 patients, 19 followed a normal diet. The Phe intake of P14 was limited during pregnancy, and P6 remains subject to mild Phe restriction. Only two patients (P7 and P13) have received treatment with sapropterin; P7 was treated with doses of 5-6 mg/kg (from 14 months to six years of age) and P13 with 10 mg/kg (from the 3 to 8 years of age). Some other patients showed an increase in Phe (400–500 µM) during febrile processes and were treated with sapropterin. Only four patients of the above 21 showed constant clinical symptoms, including psychomotor delay and seizures, autism symptoms or hyperactivity. No patient had Parkinsonism. Schooling was normal in all cases; only P10 and P16 needed some learning support. Among those with neurological symptoms, only P6 was

administered neurotransmitter precursors. The remaining patients are currently untreated.

Sanger sequencing revealed 20 patients with biallelic variants in *DNAJC12*, leading to the identification of four unreported nucleotide changes: c.298-2A>C, c.309G>T (p.Trp103Cys), c.502+1G>C and c.524G>A (p.Trp175Ter). Three patients homozygous for the p.Trp175Ter variant and one heterozygous also presented the following monoallelic variants in *PAH*: c.194T>C (p.Ile65Thr), c.143T>C (p.Leu48Ser), c.912+1G>A (p.?) and c.441+5G>T (p.?) (P18–P21).

The changes c.298-2A>C, c.502+1G>C and p.Trp103Cys in *DNAJC12* were not detected in the gnomAD worldwide population database, nor in the Spanish database. The splicing variants were predicted to be pathogenic according to the rules of the American College of Medical Genetics (ACMG) (Richards et al., 2008), by the Varsome platform (Kopanos et al., 2019), while the variants p.Trp103Cys and p.Trp175Ter were classified as VUS. The nonsense variant p.Trp175Ter, which causes the introduction of a premature termination codon in the last exon of *DNAJC12*, was detected in 80% of the variant alleles. This change is recorded in the gnomAD database and in a Spanish consortium database with frequencies of under 0.016% and 0.3%, respectively. It would appear to be particularly common in the Latino population, although no homozygous control individuals were found in the population databases consulted.

Given that *in silico* analysis failed to determine the pathogenicity of the p.Trp175Ter and p.Trp103Cys variants, functional assays were needed to offer a reliable diagnosis with clinical significance.

Functional effect of *DNAJC12* variants

Primary skin fibroblasts derived from three cases with DNAJC12 deficiency (P14, P16 and P20) were used to assess the functional effect of the p.Trp175Ter and p.Trp103Cys variants on *DNAJC12* expression at the transcriptional and protein levels. Both P14 and P20 were homozygous for the p.Trp175Ter variant, and P16 presented p.Trp175Ter in compound heterozygosity with the p.Trp103Cys variant. P20 also carried a mutated *PAH* allele. Quantitative real time PCR (qPCR) analysis showed a reduction in *DNAJC12* mRNA levels in all three patients' fibroblasts compared to three healthy, unrelated controls (Fig. 1A). Immunoreactive DNAJC12 p.Trp175Ter protein was undetectable by Western blot in the patients' fibroblasts. The introduction of a premature termination codon in the last exon of *DNAJC12* led to a protein lacking the last 23 amino acids from the C–terminus, preventing its recognition by commercially available antibodies. In the case of the compound heterozygous P16 (p.Trp103Cys/p.Trp175Ter), protein levels were slightly diminished (Fig. 1B).

DNAJC12 has been proposed to play a key role in the proper folding of PAH, TH and TPH (Anikster et al., 2017). To gain further insight into the pathomechanisms related to the DNAJC12 deficiency of the present patients, the steady–state levels of the three aromatic amino acid hydroxylases (AAAH) in the presence of the p.Trp175Ter and p.Trp103Cys variants were determined. Given that these hydroxylases are not expressed in primary skin fibroblasts, control and patient–derived fibroblast lines were transfected with plasmids coding for PAH, TH and the neurological isoform of the TPH protein (TPH2). Western blot analysis revealed a pronounced reduction in PAH and TH protein levels in the presence of the *DNAJC12* variants (Fig. 2A and C). Correlating with these results, PAH

enzymatic activity was markedly reduced (Fig. 2B). Nevertheless, no difference in the TPH2 expression profile was seen between the control and patients' cells under the present study conditions (Fig. 2D), suggesting that the p.Trp175Ter and p.Trp103Cys variants may have a greater effect on PAH and TH stability than that of TPH. Moreover, DNAJC12 levels were intriguingly reduced after the transfection of hydroxylase cDNA, probably pointing to some intracellular co-aggregation. Taken together, these data support the pathogenic nature of these *DNAJC12* variants, explaining the HPA observed in the present cases.

Role of DNAJC12 in the presence of PAH mutants

As described above, protein misfolding is the main pathomechanism in PKU, with most of the missense mutations affecting PAH folding and stability (Gersting et al., 2008). Boosting DNAJC12 expression might offer a therapeutic option for PKU. To this end, wild–type DNAJC12 was overexpressed in COS–7 cells transfected with wild–type or different PAH mutants previously characterized by our group: p.Leu48Ser, p.Ile65Thr, p.Arg261Gln, p.Glu280Lys, p.Leu348Val and Val388Met (Gamez et al., 2000). These mutations have been associated with discrepancies between *in vitro* residual activity and patient clinical phenotype, as well as with some inconsistencies in the response to sapropterin (Aldamiz-Echevarria et al., 2016). The resulting effect on PAH was examined via Western blotting and enzyme activity assays. Unexpectedly, DNAJC12 overexpression triggered an increase in protein levels for the p.Leu48Ser, p.Ile65Thr and p.Arg261Gln PAH mutants, correlating with enhancements of enzyme activity of 23, 5.2, 1.4 and 2.8-fold respectively. In the case of the remaining mutants, PAH levels were reduced after DNAJC12 co–transfection (Fig. 3). This mutation-dependent result might reflect a selective DNAJC12 function in the presence of

different PAH mutants, guiding the Hsp70 machinery towards the specific folding or degradation of the client protein.

DISCUSSION

The present work reports the detection of 20 patients with HPA carrying new biallelic variants of *DNAJC12*, and one with a pathogenic variant in that gene. Four patients also bore a pathogenic variant in *PAH*. These last four patients had been previously classified as having PAH deficiency and had been treated in accordance with that misdiagnosis. All had plasma Phe levels corresponding to a benign HPA phenotype, and all presented biochemical hallmarks similar to those of PAH deficiency (Blau, Martinez, Hoffmann, & Thony, 2018). This shows it is important to conduct retrospective genetic analysis for all unsolved cases of HPA, regardless of the presence of neurological symptoms, since in our cohort only four subjects had clinical symptoms. The remaining unsolved 29 of the 50 cases might bear pathogenic variants in regulatory or intronic sequences of *PAH* or *DNAJC12*, or in other genes which phenocopy *PAH* or *DNAJC12* deficiencies. Genomic or transcriptional analysis should be performed so that tailored therapy can be prescribed.

Two loss–of–function variants were identified - c.298-2A>C and c.502+1G>C - affecting the 3' and 5' conserved splice sites of exon 4 respectively (they were hence considered pathogenic variants). In addition, two exonic nucleotide changes were identified - p.Trp103Cys and p.Trp175Ter - classified as VUS according to ACMG rules (Richards et al., 2008). Taking into account this classification, and the high frequency of p.Thr175Ter in the general Spanish population, functional genomic analysis was deemed necessary. The results

revealed both VUS variants to be pathogenic. After transfection with PAH cDNA, the reduction in the amount of PAH and PAH activity observed in fibroblasts derived from two patients homozygous for the p.Trp175Ter variant, and in those of one patient compound heterozygous for p.Trp103Cys/p.Trp175Ter, confirmed the inability of mutant DNAJC12 to properly contribute to the PAH folding process.

It is remarkable that steady–state TH levels were reduced in the presence of DNAJC12 variants in patient–derived fibroblasts, while TPH levels did not seem to be affected. Unfortunately, it was not possible to rule out the absence of the p.Thr175Ter DNAJC12 mutant protein in the patient-derived fibroblasts due to the lack of recognition by the antibody, and if present it may have interacted with TPH, assisting with folding. The disappearance of the specific heptapeptide owed to the premature stop codon introduced by the p.Trp175Ter variant might have affected the correct recognition of PAH and TH by DNAJC12, but not that of the TPH2 protein.

It was initially reported that patients with HPA suffered progressive dystonia and intellectual disability (Anikster et al., 2017). Later reports broadened the clinical spectrum of DNAJC12 deficiency to range from a very mild neurological phenotype to early-onset dopa–responsive Parkinsonism (van Spronsen et al., 2017), (Straniero et al., 2017). In the light of the present results, the more important involvement of the DNAJC12 C–terminal domain in the stability of PAH than in other AAAH seems plausible, and might explain the practical absence of neurological manifestations in the present patients. These results suggest that the phenotypic characteristics expressed depend on the variants involved and/or other genetic modifiers. Further, all patients ever reported in the literature have presented with reduced CSF HVA and 5-HIAA concentrations,

revealing a central deficit of dopamine and serotonin respectively. Since neurotransmitters levels in the CSF were measured only in two patients, it cannot be ruled out that they might develop future neurological complications. It might be advisable to perform a CSF analysis of neurotransmitter–derived metabolites to rule out this possibility, although this test is controversial since most cases in our series are currently asymptomatic.

DNAJC12 overexpression ameliorated the effects of the p.Leu48Ser, p.Ile65Thr or p.Arg261Gln variants on PAH stability, but worsened those of the p.Glu280Lys, p.Leu348Val and Val388Met variants. Thus, PAH missense mutations lead to different degrees of conformational destabilization (Gersting et al., 2008). The overexpression of DNAJC12 leads the mutant protein towards the stabilization pathway or to its degradation via the ubiquitin–proteasome system (Scheller et al., 2019). The different effect of DNAJC12 might depend on the different pathogenic mechanism of each variant; thus, those mutants with a tendency to aggregate might stabilize after DNAJC12 overexpression while mutants with tendency to degrade would not. Further assays are needed to confirm this hypothesis. This highlights the major role of DNAJC12 in targeting misfolded PAH for degradation (Jung-Kc et al., 2019). These results open up the possible use of pharmacological treatments designed to upregulate levels of molecular chaperones or co-chaperones.

DNAJC12 would appear to be a modifier of PAH deficiency, with different variants stabilizing/degrading the protein to different degrees. This could explain some of the inconsistencies in the genotype–phenotype correlation described in patients with PKU, as well as the highly variable response to sapropterin therapy reported for people with certain mutant alleles, such as p.Val388Met or Leu348Val

(Sarkissian et al., 2012). Further investigations are needed to understand the relationship between DNAJC12 and its clients, which would help reveal the role of *DNAJC12* as a modifier gene in PKU and its involvement in the development of neurological disorders.

In summary, the present work emphasizes the need to perform periodic re-evaluations of unsolved cases of genetic diseases based on new scientific findings in order to confirm the gene affected and prescribe adequate therapy. The identification of patients with DNAJC12 deficiency informs on the specific biochemical tests (e.g., neurotransmitter analyses) required to assess possible clinical complications. Given the high frequency of carriers of the p.Trp175Ter pathogenic variant in the Spanish population, there may be many people with DNAJC12 deficiency (1/500,000) who were not identified by neonatal mass screening, and who might benefit from L-DOPA treatment (Straniero et al., 2017).

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Data Availability Statement

The variants identified in this study are openly available at http://www.lovd.nl/ with reference numbers 0000644164, 0000645396, 0000644166 and 0000405673.

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Titles and legends to figures

Figure 1. Functional characterization of DNAJC12 patient–derived fibroblasts. A) Relative expression of *DNAJC12* in control–derived fibroblasts (C) and fibroblasts from DNAJC12 patient 14 (p.Trp175Ter/p.Trp175Ter, P14), patient 16 (p.Trp103Cys/p.Trp175Ter, P16) and patient 20 (p.Trp175Ter/p.Trp175Ter, P20) measured by qPCR. Each bar represents the mean ± SD of three independent experiments. *DNAJC12* mRNA levels were normalized using *GAPDH* as an endogenous control. **B)** Representative Western blot of DNAJC12 protein from control (C) and patient–derived fibroblasts (P14, P20 and P16). β-actin was used as a loading control.

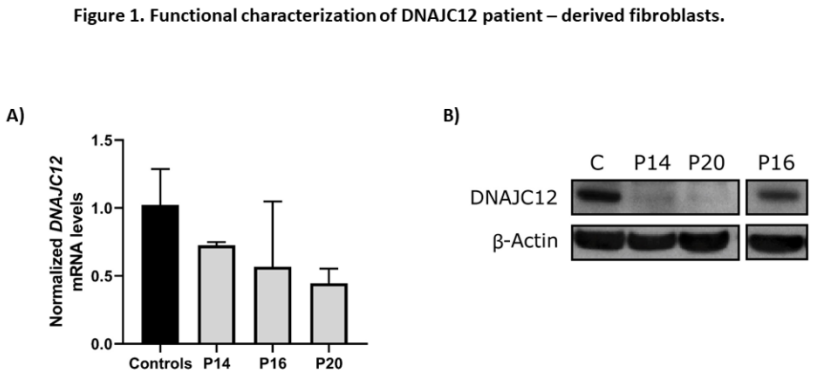

Figure 2. Functional effect of *DNAJC12* variants on amino acid hydroxylases. A, C, and D) Western blot assays of PAH (A), TH (C) or TPH2 (D) and DNAJC12 proteins in fibroblasts from a control individual (C lane) and DNAJC12 patients 14 (p.Trp175Ter/p.Trp175Ter, P14), patient 16 (p.Trp103Cys/p.Trp175Ter, P16) and 20 (p.Trp175Ter/p.Trp175Ter, P20) after transfection with 5 µg of the corresponding plasmid (+ lanes), and the negative control (- lanes). β-actin was used as a loading control. **B)** PAH activity in control and patient–derived fibroblasts transfected with 5 µg of plasmid pRC–CMV–PAH, expressed as the amount of tyrosine produced, as measured by HPLC.

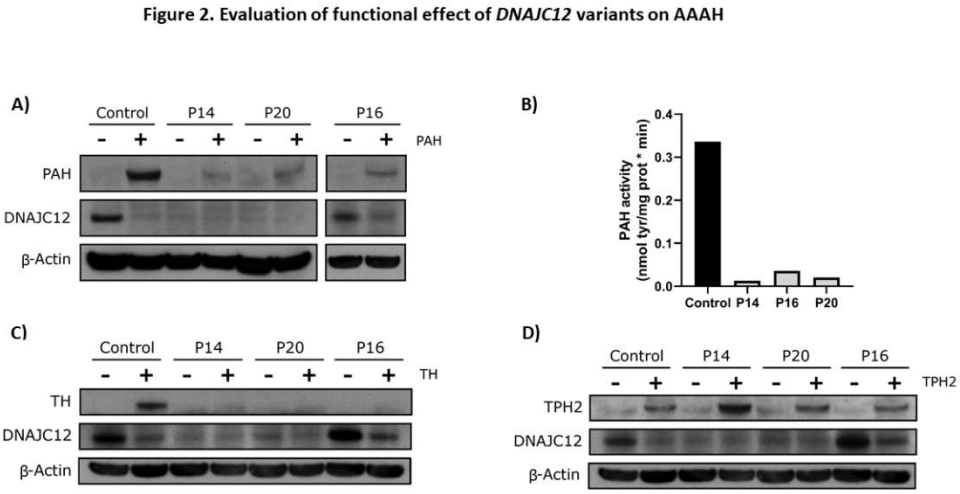

Figure 3. Role of DNAJC12 in the presence of different *PAH* mutations. A) Representative Western blot of wild–type and different mutant PAH proteins overexpressed in COS-7 cells alone (- lanes) or in combination with DNAJC12 (+ lanes). 1 µg of each expression plasmid was used for transfection. Neomycin phosphotransferase II (NPT-II) was used as a marker of plasmid transfection efficiency. β-actin was used as a loading control. **B)** Quantification of relative PAH protein levels after DNAJC12 plasmid co–transfection. The basal PAH level without DNAJC12 overexpression was considered as '1'. Each bar represents the mean ± SD of at least three independent experiments. **C)** Relative PAH activity after DNAJC12 plasmid co–transfection, expressed as fold change. *p<0.05, **p<0.01, ***p<0.001

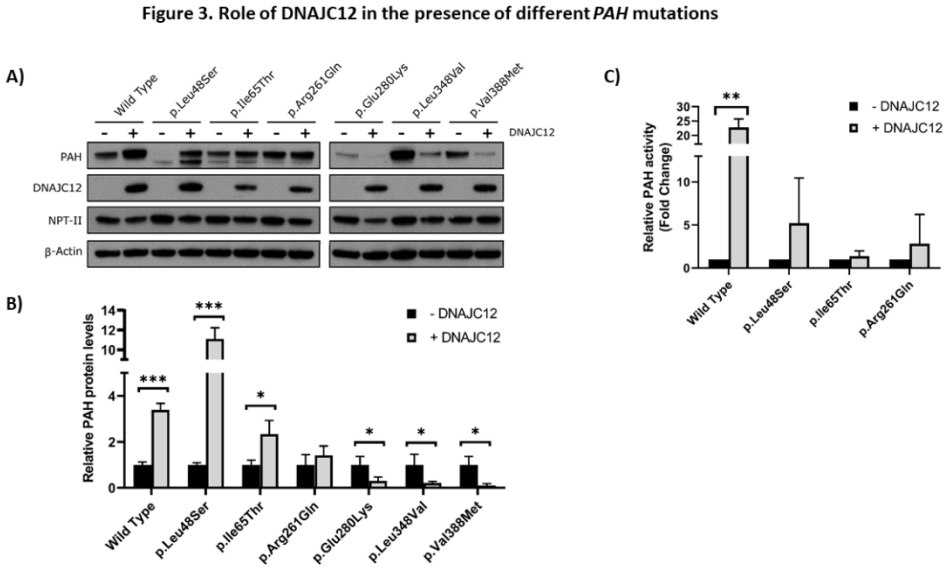

Table 1. Genotype and phenotype of *DNAJC12* cases

Patient	Allele 1	Allele 2	Age of Diagnosis	Current age	Phe at diagnosis[a] (µM)	Prolactin[b] (ng/ml)	Melatonin[c] (nmol/mmol creat)	Dietary treatment (Age)	Neurological Symptoms	Others	Treatment
1	p.? (c.298-2A>C)	p.Trp175Ter (c.524G>A)	23 days	6 years 6 months	158	11,7		-	-	-	-
2	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	1 month 17 days	9 years 4 months	120	9.0		-	-	Migraines	-
3	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	1 month 13 days	7 years 10 months	139	25,5		-	-	-	-
4	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	6 months 15 days	13 years 3 months	127	-		-	-	-	-
5	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	27 days	6 years 10 months	224	-		-	-	-	-
6	p.? (c.502+1G>C)	p.Trp175Ter (c.524G>A)	21 months	6 years 6 months	442	-		Mild Phe restriction (21 months)	Speech delay, occulogyric crisis	West Syndrome, myoclonic epilepsia	Started treatment with L-DOPA
7	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	Newborn screening	8 years 9 months	131	6,44		-	-	Elevations of Phe in febrile episodes. Hyperpigmented spots on the trunk	Sapropterin 5-6 mg/kg (14 months - 6 years)
8	p.Trp175	p.?	-		-	-		-	-	-	-

	Ter (c.524G >A)	(c.297+5 G>T)									
9	p.Trp175 Ter (c.524G >A)	p.Trp17 5Ter (c.524G >A)	Newb orn screen ing	30 years 4 mont hs	184	9.35		-	-	-	-
10	p.Trp103 Cys (c.309G >T)	p.Trp17 5Ter (c.524G >A)	9y	16 years 3 mont hs	266	-	14.7	-	Intellect ual disabilit y (limit), attentio n difficult ies, speech delay, dystonia , limb hyperto nia	Behaviour problems, anxiety, agresivity	-
11	p.Trp175 Ter (c.524G >A)	p.Trp17 5Ter (c.524G >A)	12 month s	14 years	218	6,3	12.4	-	-	-	-
12	p.Trp175 Ter (c.524G >A)	p.Trp17 5Ter (c.524G >A)	38 days	10 years 4 mont hs	240	-		-	-	-	-
13	p.? (c.502+1 G>C)	p.Trp17 5Ter (c.524G >A)	23 days	8 years 2 mont hs	324	-		-	-	-	Sapropterin 10 mg/kg (3-8 years old)
14	p.Trp175 Ter (c.524G >A)	p.Trp17 5Ter (c.524G >A)	34 years	40 years	236	-		Limite d during pregn ancy	-	-	-
15	p.Trp175 Ter (c.524G >A)	p.Trp17 5Ter (c.524G >A)	1 month 4 days	5 years	122-421 (fever)	-		-	-	-	-
16	p.Trp103 Cys (c.309G >T)	p.Trp17 5Ter (c.524G >A)	2 month s	7 years 4 mont hs	204	-	18.80	-	Borderli ne Intelect ual disabilli	Macrocep haly	-

									ty, Attention difficulties, Speech delay		
17	p.Trp175Ter (c.524G>A)	p.Trp175Ter (c.524G>A)	7 days	7 years 3 months	97	-	-	-	Mild (CI per WPPSI-IV of 86, intelligence normal-low range), attention difficulties, Autistic features	Mild autistic spectrum disorder, with mild psychosocial disability and Asperger-like traits	-

DNAJC12 transcript NM_021800.3

[a]Normal value >120 µM

[b] Normal values: 4,8-23 ng/ml

[c] Normal values: 11.9-66 nmol/mmol creatinine

Table 2. Genotype and phenotype of *DNAJC12* deficient patients with monoallelic pathogenic variant in *PAH*

Patient	***DNAJC12***		***PAH***	**Age of Diagnosis**	**Current age**	**Phe at diagnosis[a] (µM)**	**Prolactin[b] (ng/ml)**
	Allele 1	**Allele 2**					
18	(p.Trp175Ter) c.524G>A	(p.Trp175Ter) c.524G>A	(p.Ile65Thr) c.194T>C	1 month 10 days	24 years 6 months	<363	-
19	(p.Trp175Ter) c.524G>A	(p.Trp175Ter) c.524G>A	(p.Leu48Ser) c.143T>C	6 days	9 years 6 months	151	31.2
20	(p.Trp175Ter) c.524G>A	(p.Trp175Ter) c.524G>A	(p.?) c.912+1G>A	1 month 29 days	6 years	163	-
21	(p.Trp175Ter) c.524G>A	?	(p.?) c.441+5G>T	-	18 years	>151 (max. 236)	-

DNAJC12 transcript NM_021800.3. *PAH* transcript NM_000277.3

[a]Normal value >120 µM

[b] Normal values: 4,8-23 ng/ml