



Short communication

Functional analysis of novel variants identified in *cis* in the *PCCB* gene in a patient with propionic acidemiaAinhoa Martínez-Pizarro^a, Nadège Calmels^b, Audrey Schalk^b, Camille Wicker^c, Eva Richard^a, Lourdes R. Desviat^{a,*}^a Centro de Biología Molecular Severo Ochoa UAM-CSIC, CIBERER, IdiPaz, IUBM, Universidad Autónoma de Madrid, Madrid, Spain^b Laboratoire de Diagnostic Génétique, Institut de Génétique Médicale d'Alsace, Nouvel Hôpital Civil, Hôpitaux Universitaires de Strasbourg, Strasbourg, France^c Centre de Compétence Maladies Héritaires du Métabolisme, filière G2M, Service de pédiatrie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

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ABSTRACT

Next-generation sequencing has improved the diagnosis of inborn errors of metabolism, allowing rapid confirmation of cases detected by clinical/biochemical studies or newborn screening. The challenge, however, remains for establishing the pathogenicity of the identified variants, especially for novel missense changes or small in-frame deletions. In this work we report a propionic acidemia patient exhibiting a severe neonatal form with coma and hyperammonaemia. Genetic analysis identified the previously described pathogenic *PCCB* variant p.R512C in the maternal allele and two novel *PCCB* variants in *cis* in the paternal allele, p.G246del and p.S322F. Expression analysis in a eukaryotic system confirmed the deleterious effect of the novel missense variant and of the one amino acid deletion, as they both exhibited reduced protein levels and reduced or null PCC activity compared to the wild-type construct. Accordingly, the double mutant resulted in no residual activity. This study increases the knowledge of the genotype-phenotype correlations in the rare disease propionic acidemia and highlights the necessity of functional analysis of novel variants to understand their contribution to disease severity and to accurately classify their pathogenic status. In conclusion, two novel *PCCB* pathogenic variants have been identified, expanding the current mutational spectrum of propionic acidemia.

1. Introduction

Propionic acidemia (PA; MIM#606054) is a rare and potentially lethal inherited metabolic disorder, caused by pathogenic variants in the propionyl-coenzyme A carboxylase (PCC, E.C.6.4.1.3) α (PCCA) or β (PCCB) subunits (encoded by the *PCCA* and *PCCB* genes, respectively), leading to PCC deficiency and subsequent accumulation of toxic metabolites (Richard et al., 2015; Shchelochkov et al., 1993). PA is characterized by recurrent life-threatening metabolic decompensation events and multisystemic complications. There are severe neonatal-onset cases and milder late-onset cases with different clinical manifestations. Currently, there are no effective therapies for PA that prevent the development of common neurologic, pancreatic or cardiac complications (Forny et al., 2021), although liver transplantation could limit the severity of the disease for some patients, and several genetic approaches targeting the underlying enzyme defects, such as mRNA

therapy or AAV-gene therapy, are in preclinical/early clinical development (Attarwala et al., 2023; Lomash et al., 2023; Jiang et al., 2020). Neonatal screening for PA by tandem mass screening followed by exome sequencing, which is available to date in many countries, allows timely medical and dietary interventions that may significantly improve the patients' condition and prognosis (Held et al., 2022; Martín-Rivada et al., 2022).

There are currently 207 variants reported for the *PCCA* gene and 190 for the *PCCB* gene (HGMD Professional release 2023.1). Missense variants are predominant, followed by small insertions and deletions, splicing variants and, in the case of the *PCCA* gene, by large genomic deletions (Shchelochkov et al., 1993). Several studies have investigated the effect of point mutations using different *in vitro* systems, classifying the variants as hetero-oligomeric assembly defects, catalytic defects, or folding defects (Chloupkova et al., 2002; Rivera-Barahona et al., 2018; Desviat et al., 2004; Kraus et al., 2012). Genetic diagnosis is used to

Abbreviations: ACMG/AMP, American College of Medical Genetics and Genomics and the Association for Molecular Pathology; ExAc, Exome Aggregation consortium; gnomAD, Genome aggregation consortium; PA, propionic acidemia; PCC, propionyl CoA carboxylase.

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confirm the clinical/biochemical diagnosis of PA. However, for novel variants, especially missense changes, the main challenge is to establish their pathogenicity. The need for precise classification of novel variants, which are increasingly identified by next-generation sequencing approaches, has led to generating consensus recommendations from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP guidelines), that take into account the evidence from data of population frequencies, computational and predictive annotations, available functional assessments and other factors (Richards et al., 2015). The demonstration of the pathogenicity of a given variant can be confirmed using functional approaches in prokaryotic or eukaryotic systems.

Here we report a patient with PA in whom genetic diagnosis identified two novel *PCCB* variants in *cis* inherited from the father, and a previously reported *PCCB* pathogenic variant in the maternal allele. Expression analysis of the novel variants in an established eukaryotic system (Perez-Cerda et al., 2003) was performed to confirm which of the novel *PCCB* variants was responsible for the clinical phenotype.

2. Case report, materials and methods

2.1. Case report

The patient is the first child of unrelated parents, and he presented a neonatal form of the disease. Autistic traits are described in the mother's brother, but no other particular familial medical history was reported. At 33 weeks of amenorrhea, a premature delivery threat was observed, treated by corticotherapy to improve fetal maturation. Birth finally happened at 35 weeks and 3 days of amenorrhea. Adaptation to extra uterine life was initially good with normal Apgar score. After a few days of life with no particular symptoms, he presented progressive axial hypotonia with peripheral hypertonia, trembling, drowsiness and poor feeding. At day 9, blood tests found elevated ammoniemia at 286 $\mu\text{mol/L}$, normal pH and bicarbonates, and lactates at the upper limit at 2.78 mmol/L. Liver function, cranial ultrasound and electroencephalogram were normal. Food was stopped, and after transfer in an intensive care unit, a central venous line was inserted to infuse a poly-ionic 10 % carbohydrate solution to obtain a high glucose rate (10 mg/kg/min) in order to stop the catabolic condition. Ammonia scavengers were also administered: sodium benzoate (loading dose and then in continuous intravenous infusion) and carglumic acid, associated to carnitine. He didn't need haemodialysis since ammoniemia normalized in 18 h after the beginning of the treatment and he quickly became more reactive. Plasma amino acid chromatography showed low glutamine levels and slightly hyperglycinaemia. The diagnosis of PA was strongly suspected by the results of urinary chromatography of organic acids that showed elevated methylcitrate (5689 mmol per mmol of creatinine) and of the plasmatic acylcarnitine profile that showed a high level of propionylcarnitine (17,8 $\mu\text{mol/L}$), without elevated methylmalonyl-carnitine. After 24 h of improving his biological and clinical examinations, and despite the continuation of all appropriate treatments, his neurologic examination became again worse at day 11, with reappearance of metabolic acidosis with higher lactatemia (3.78 mmol/L), but normal ammoniemia. To rule out a potential meningitis, a lumbar puncture was performed. The analysis of cerebrospinal fluid showed high lactates at 4.6 mmol/L and low glutamine levels supporting the diagnosis of PA. The bacterial culture was finally negative. During the next days of treatment, he presented usual complications of the disease, as pancytopenia (by medullar toxicity) and mild renal tubulopathy with low plasmatic bicarbonates. A low protein diet was progressively introduced, and he finally completely recovered with a normal neurologic examination before hospital discharge, at 2 weeks of life. The evolution during the next years until now was quite good. With low protein diet, avoidance of fasting with nocturnal enteral feeding and chronic treatment by carnitine and carglumic acid, he didn't present any acute metabolic decompensation or hyperammoniemia. Staturo-ponderal

growth was difficult during the second year of life because of severe orality disorders and frequent nausea and vomiting. Feeding problems are still present today at the age of two and a half years, but the tolerance of the nutrition is much better since the last six months, allowing an improvement of growth. Psychomotor development is for the moment normal with regular kinesitherapy and speech therapies. The last cardiac assessment unfortunately found a cardiomyopathy with mild decreased left ventricular ejection fraction, for which a beta blocker treatment was introduced. Also, this patient is now waiting for a liver transplant.

Genetic analysis. After obtaining written consent of the family, peripheral blood was collected from the patient and his parents. Genomic DNA was extracted using QIAasympphony SP instrument (Qia-gen). Targeted sequencing of a panel of 392 genes involved in movement disorders was performed on the trio (adapted from Montaut et al., 2018). *PCCA* and *PCCB* were among the tested genes. Libraries prepared with SureSelect® enrichment probes (Agilent) were sequenced with Mid Output Kit v2.5 (300 cycles) on NextSeq550 instrument (Illumina). Data were analyzed using the homemade bioinformatics pipeline Stark. Variants were annotated and ranked according to VaRank software (Geoffroy et al., 2015). *PCCB* variants identified in the family were annotated according to the MANE transcript NM_000532.5. New *PCCB* variants described in this work have been submitted to ClinVar database.

Cell culture. *PCCB* deficient fibroblast cell line stably transformed with the T22 plasmid which contains SV40 DNA sequences was used for *in vitro* expression analysis (Perez-Cerda et al., 2003). Cells were cultivated according to standard procedures. Briefly, cells were maintained in Minimum Essential Medium supplemented with 1 % glutamine, 10 % foetal bovine serum (FBS) and antibiotics.

Expression analysis. *PCCB* variants were introduced by PCR mutagenesis (Quikchange Lightning Site-Directed Mutagenesis kit, Agilent Technologies) in the pRcCMVB52 vector coding for wild-type *PCCB* cDNA. Sequence analysis confirmed the identity of the mutant clones. The day before transfection, cells were seeded into 6-well cell culture plates (400,000 cells per well). Transfection was achieved by lipofection using Lipofectamine 2000 (Invitrogen), co-transfecting 2 μg of the wild-type or mutant *PCCB* vectors and 2 μg of the wild-type partner *PCCA* constructs to achieve maximal expression (Perez-Cerda et al., 2003). Cells were harvested 72 h after transfection.

PCC enzymatic assay. PCC activity was assayed by measuring the enzyme-dependent incorporation of radiolabelled bicarbonate into non-volatile products from the Krebs cycle as previously described (Suormala et al., 1985). Briefly, cells were resuspended in 20 mM Tris-HCl pH 8.0 buffer with 0.81 mM glutathione and lysed by freeze-thaw cycles. The enzymatic reaction was initiated with the addition of the homogenate, maintained at 30 °C during 20 min and stopped with trichloroacetic acid (TCA 30 %). The samples were centrifuged during 15 min at 16,000 \times g, the supernatant transferred to a scintillation vial and subjected to an evaporation process at room temperature for 24–48 h. The non-volatile products were measured in a Tri-Carb 2810 TR Liquid Scintillation Counter (PerkinElmer) after 48 h. PCC activity is expressed as pmol of incorporated (Perez-Cerda et al., 2003) C^{14} $\text{min}^{-1} \text{mg}^{-1}$ of total protein. Protein concentration in cellular extracts was determined by the Bradford method (Bio-Rad Laboratories).

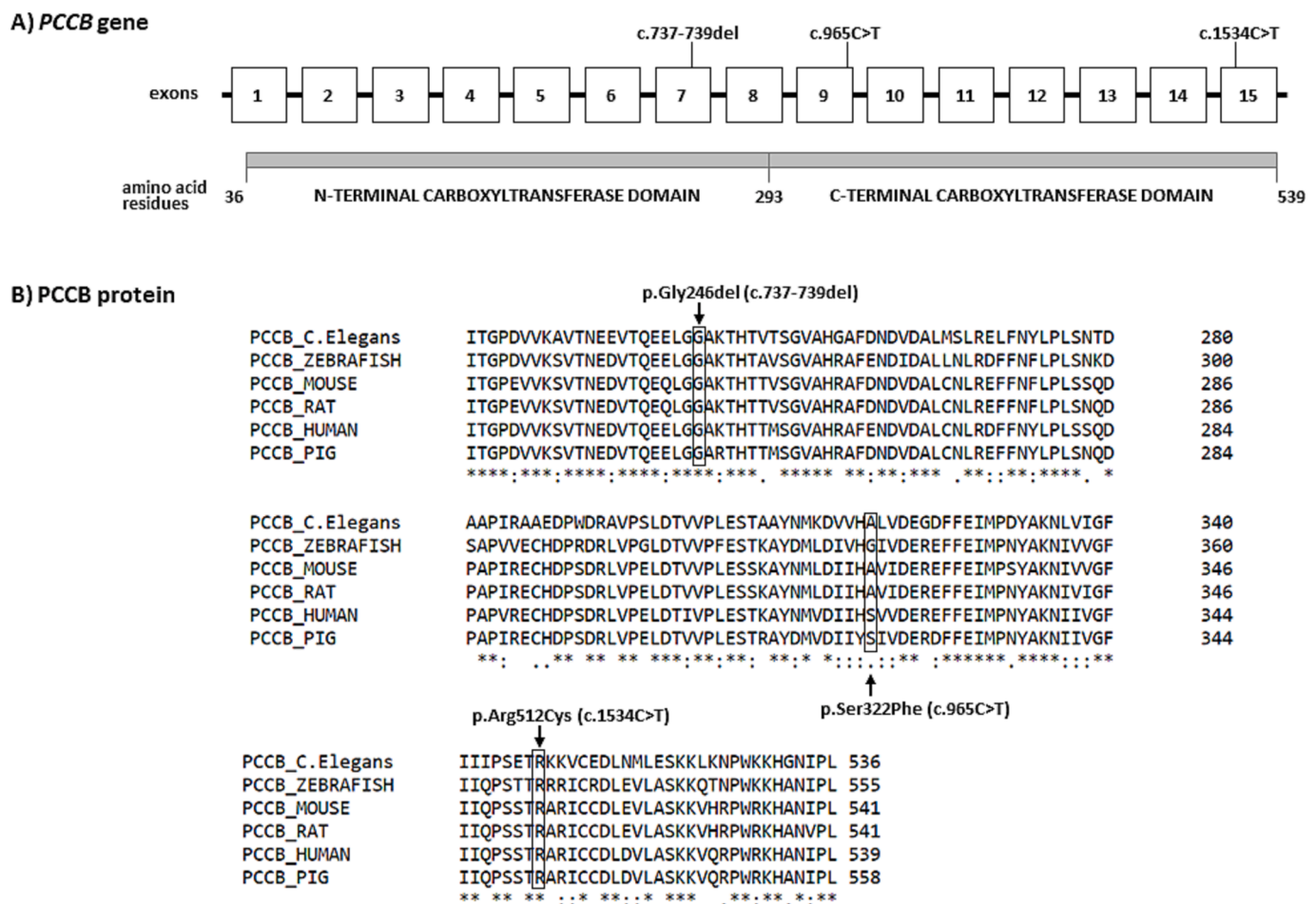
Western blot. Cells were disrupted by freeze-thawing in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % triton X-100 and 10 % glycerol) with protease inhibitors and centrifuged 10 min at 4 °C. The supernatant fraction was collected, and protein concentration was determined by the Bradford method (Bio-Rad Laboratories). Equal amounts of lysed extracts (50 μg protein) were loaded on a 10 % SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (iBlot 2 NC Mini Stacks) in an iBlot 2 Gel transfer device (Invitrogen). Immunodetection was carried out using commercially available antibody against *PCCB* (1:500, Santa Cruz Biotechnology, sc-393929). Secondary antibody used was anti-mouse IgG (1:2,000, Cell Signalling #7076S). For loading control,

Bioinformatic and structural predictions. Bioinformatics tools used to predict the consequences of the missense mutations were MutationTaster (Steinhaus et al., 2021), PolyPhen2 (Adzhubei et al. 2010) and SIFT (Ng and Henikoff, 2001). PCC holoenzyme is a trimer of hetero-tetramers composed by two central PCCB-subunits and two peripheral PCCA-subunits. Structural prediction of the novel *PCCB* variants was performed in a previously reported (Gallego-Villar et al., 2013) homology model, using as template the homologous crystallized PCC holoenzyme from *Ruegeria pomeroyi* (PDB ID: 3n6r) (Huang et al., 2010), composed of PCCA and PCCB-subunits sharing 54 % and 65 % sequence identity to the human subunits, respectively. Structural modelling was performed as previously described (Rivera-Barahona et al., 2018), using the SWISS-MODEL server (<https://swissmodel.expasy.org/interactive>).

3.1. Genetic analysis identifies novel PCCB variants

glycine 246 (p.G246del) and c.965C > T, leading to the replacement of serine 322 by phenylalanine (p.S322F) (Fig. 1 and Supplementary Fig. 1). The p.R512C variant has already been described in PA patients (Perez-Cerda et al., 2003; Pérez-Cerdá et al., 2000) and is reported as Pathogenic in ClinVar (VCV000038879.25). Both p.G246del and p.S322F variants have never been described in PA patients nor in Genome Aggregation Data Base (gnomAD). The three base-pair deletion c.737_739del leads to the deletion of G246 in the carboxyltransferase domain of the enzyme. Consequences of the p.S322F missense variant are predicted as moderate by bioinformatics tools, with a CADD score of 25.30. No consequence on the splicing process is predicted even though the nucleotide change involves the penultimate base of exon 9. Both paternal rare variants are thus of unknown significance requiring further characterisation.

The predicted missense change (c.965C > T; p.S322F) and one amino acid deletion (c.737_739del; p.G246del) in the *PCCB* gene were analysed using an established eukaryotic expression system to confirm their pathogenicity (Rivera-Barahona et al., 2018; Perez-Cerda et al., 2003). The wild-type (WT) and mutant (with p.S322F, p.G246del or with both variants in *cis*) *PCCB* constructs were expressed in a *PCCB* deficient fibroblast cell line, according to previously described procedures (Perez-Cerda et al., 2003) and the resulting PCC activity and PCCB protein levels were quantified. The results are shown in Fig. 2. The mutant p. S322F construct resulted in greatly reduced PCCB protein levels (4 %),



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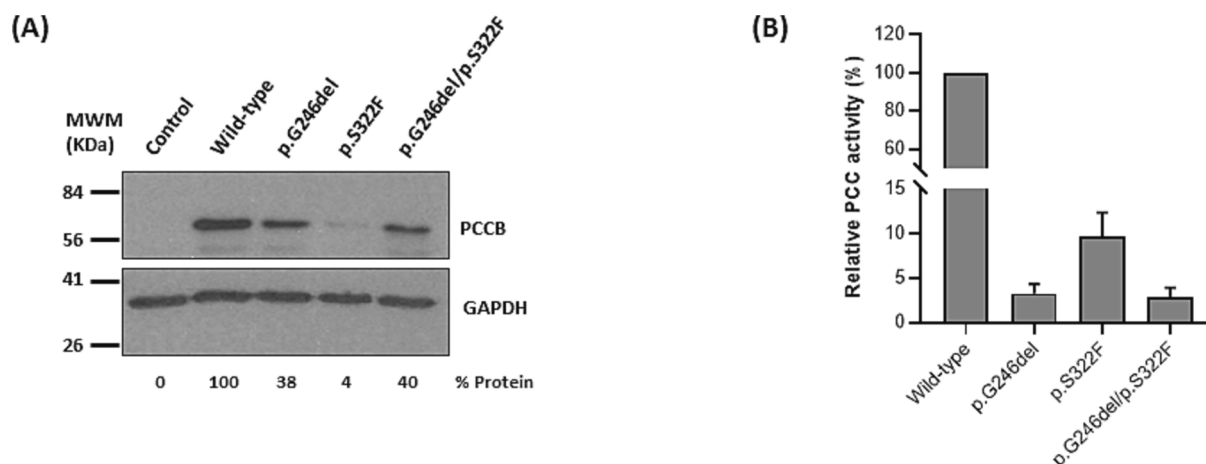


Fig. 2. Functional analysis of novel *PCCB* variants. A) Representative blot of *PCCB* protein from lysates of *PCCB*-deficient fibroblast cell lines co-transfected with the mutant *PCCB* and wild-type *PCCA* constructs. At least two experiments were performed for each mutant construct. Control lane corresponds to non-transfected cells. GAPDH was used as loading control. MWM: molecular weight marker. B) Percentage of PCC activity for each variant relative to wild-type levels, which was set to 100 %. Data represent the mean \pm standard deviation of 2 experiments.

while p.G246del and the double mutant resulted in approximately 40 % levels of protein compared to the WT protein. *PCCB* variant p.S322F retained low residual activity (9.7 %), while p.Gly246del and the double mutant exhibited < 5 % PCC activity relative to the WT construct.

3.3. Structural predictions of *PCCB* variants

The G246 residue is well conserved in different species and a missense variant affecting this residue (p.G246V) has been previously reported (Perez et al., 2003), thus p.G246del can be classified as probably pathogenic. On the contrary, S322 is not conserved, and p.S322F is classified as a variant of unknown significance (VUS). The structural effect of the variants was carried out using a homology model of the human PCC holoenzyme (Rivera-Barahona et al., 2018; Gallego-Villar et al., 2013). The $\alpha\beta\alpha$ heterotetrametric model was visualized with Pymol and the affected *PCCB* changes were *in silico* mapped (Fig. 3). The variants were predicted to disturb and destabilize protein structure. The p.G246del change predictably disturbs the conformation of an α -helix at the interface between *PCCB* subunits (Fig. 3A), thus altering their interaction, while p.S322F results in the loss of an H-bond with I319 (Fig. 3B,C). Both affected residues (G246 and S322) are far apart in the structure, therefore no additional alterations are predicted in the double mutant.

4. Discussion

In this work, we discuss a case with a severe form of PA presenting with coma and hyperammonaemia at 9 days of life. Genetic analysis identified variants in the *PCCB* gene, a pathogenic variant in the maternal allele (c.1534C > T) and two newly found variants in *cis* (c.965C > T and c.737_739del) in the paternal allele. Thanks to initiatives such as the Exome Aggregation consortium (ExAC) project (Lek et al., 2016), its successor gnomAD consortium (Karczewski et al., 2020), and the published ACMG/AMP guidelines (Richards et al., 2015), it is now possible to provide an interpretation of sequence variations identified in the genomes of patients with genetic diseases. Variants are classified into 1 of 5 categories (pathogenic, likely pathogenic, likely benign, benign, uncertain significance) (Richards et al., 2015). However, for a correct genetic diagnosis, experimental evidence of the pathogenicity of the identified variants is necessary.

The c.1534C > T (p.R512C) variant has been reported earlier and *in vitro* studies have shown it results in an unstable *PCCB* protein that renders the PCC enzyme devoid of activity, correlating with a severe phenotype in homozygous/functionally hemizygous patients (Perez-Cerda et al., 2003; Perez-Cerda et al., 2000). The c.965C > T (p.S322F) and c.737_739del (p.G246del) variants have not been described before and are classified as VUS (c.965C > T) or probably pathogenic

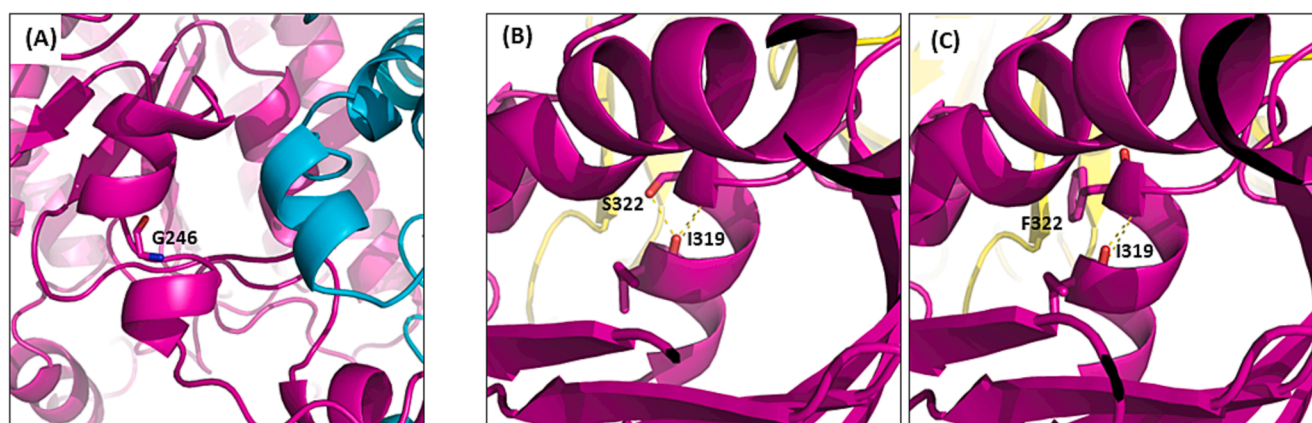


Fig. 3. Location of disease-causing mutations in the *PCCB* subunit. Cartoon representation using Pymol software of *PCCB* subunits (magenta and blue) interface and *PCCA* subunit (yellow). Protein residues affected by mutations and key interacting residues are shown in sticks with the following atom colors: nitrogen, blue; oxygen, red, and hydrogen bonds, yellow dashed line. *PCCB* affected residues A) p.G246, B) WT structure and C) p.S322 variant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(c.737_739del). Accurate classification of novel variants is relevant for adequate genetic counselling and may guide the correct prognosis and management of the PA patient. In addition, knowing the exact effect of each variant leads to a more profound understanding of the structure–function relationship of the PCC enzyme and reveals potential genotype–phenotype correlations (Pérez-Cerdá et al., 2000).

The results of the functional analysis of the novel variants identified *in cis* in the *PCCB* gene show that, individually, both have a clear damaging effect on protein function and can thus be considered disease-causing. The p.S322F change clearly results in a folding defect, as the mutant protein is highly unstable, although partially functional. This is the case for other described *PCCB* variants classified as destabilizing variants retaining some protein and residual activity, which can be partially recovered when expressed at a folding-permissive temperature (Rivera-Barahona et al., 2018), indicating that chaperones or proteostasis regulators improving PCC folding may be of therapeutic value for patients harbouring such folding defects (Chloupkova et al., 2002; Gamez et al., 2018; Kelson et al., 1996).

Variant p.G246del results in a functionally null mutant protein, although up to 38 % of protein levels are detectable in the expression studies performed, so it can be classified as having both a folding and a catalytic defect, as described for other *PCCB* variants (Rivera-Barahona et al., 2018). The combination of both variants renders the resulting mutant *PCCB* protein devoid of activity, although some residual protein can be detected. The presence of two pathogenic variants in the same allele is an unusual event, and to our knowledge, has not been reported in PA patients.

The clinical application of our study is the confirmation of the pathogenicity of the novel variants identified, thus validating a precise genetic diagnosis and enabling accurate genetic counselling in the family, while providing a prognostic view of the probable course of the disease. Although the genotype–phenotype correlations in PA are not always straightforward (Pérez-Cerdá et al., 2000), the results obtained in this work indicate that the patient's genotype, with two mutant *PCCB* alleles resulting in nearly null enzyme activity *in vitro*, is clearly in agreement with the severe neonatal phenotype exhibited. However, the functional studies have some limitations, such as the overexpression of the mutant proteins under the control of a strong viral promoter and the lack of co-expression of both the maternal and paternal *PCCB* mutant alleles contributing to the assembly of the PCC dodecamer. Therefore, the final phenotypic outcome in PA may not always be easily predicted, especially for private genotypes identified in individual patients as is the case reported here.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2023.147902>.

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