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Research paper

Sudden cardiac death triggered by minimal alcohol consumption in the context of novel PPA2 mutations in 2 unrelated families☆

Cristina Gómez González [a,b,c,d], Iván del Campo Cano [e,f], Ana Isabel Fernández-Avila [b,c,d,*], Maria Paz Suárez – Mier [g], María José Sagastizábal [h], Reyes Álvarez García-Rovés [b,c,i], Irene Méndez Fernández [a,b,c,d], Silvia Vilches [a,b,c,d], Miriam Centeno Jiménez [b,c,i], Ana Siles Sánchez –Manjavacas [e,f], Ana Usano Carrasco [e,f], Emiliano Gonzalez-Vioque [j], Juan Pablo Ochoa [k,l], Constancio Medrano [b,c,i], Esther González López [d,f,l], Pablo García-Pavía [d,f,l,m], Javier Bermejo [a,b,c,d], María Angeles Espinosa Castro [a,b,c,d]

[a] Department of Cardiology, Hospital General Universitario Gregorio Marañón, Madrid, Spain
[b] Facultad de Medicina, Universidad Complutense de Madrid, Spain
[c] Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain
[d] CIBERCV, Spain
[e] Department of Pediatrics, Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain
[f] IDIPHISA, Madrid, Spain
[g] Servicio de Histopatología. Instituto Nacional de Toxicología y Ciencias Forenses. Madrid. Spain
[h] Instituto de Medicina Legal y Ciencias Forenses de Madrid, Spain
[i] Pediatric Cardiology Department, Hospital General Universitario Gregorio Marañón, Madrid, Spain
[j] Department of Biochemistry, Hospital Universitario Puerta de Hierro, Madrid, Spain
[k] Health in Code. A Coruña. Spain
[l] Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain
[m] Department of Cardiology, Hospital Universitario Puerta de Hierro, Majadahonda, Madrid, Spain

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ABSTRACT

Biallelic variants in *PPA2* gene cause a rare but lethal mitochondrial disorder. We describe the first four cases reported in Spain of PPA2 disease in two unrelated families. We have conducted a revision of the clinical history, necropsies, and postmortem genetic testing from probands, and clinical evaluation, genetic testing and blood transcript analysis in family members. All the cases harbored biallelic *PPA2* variants in compound heterozygous status. Two brothers from family 1 suffered sudden death after a small first intake of alcohol in 2013 and 2022. The sister remains alive but affected with cardiomyopathy, extensive scar on cardiac imaging, and high sensitivity to alcohol intake. The three siblings carried *PPA2* c.290A > G (p.Glu97Gly) novel missense variant and *PPA2* c.513C > T (p.Cys171 =) altering splicing site variant, both probably leading to mRNA degradation based on in-silico and transcript analyses. A teenager from family 2 suffered sudden death after a small intake of alcohol in 2018 and carried *PPA2* c.683C > T (p.Pro228Leu) missense and *PPA2* c.980_983del (p.Gln327fs) novel frameshift variant, both probably leading to abnormal protein structure. All cases were asymptomatic until adolescence. Furthermore, the sister in family 1 has survived as an asymptomatic adult. *PPA2* disease can manifest as cardiac arrest in the young, especially after alcohol exposure. Our results show that *PPA2* deficiency can be related to different pathogenicity mechanisms such as abnormal protein structure but also mRNA decay

Abbreviations: PPA2, Pyrophosphatase Inorganic 2; PPi, inorganic pyrophosphates; SCD, sudden cardiac death; EKG, electrocardiogram; TTE, transthoracic echocardiogram; MRI, magnetic resonance imaging; NGS, Next Generation Sequencing; LGE, late gadolinium enhancement; ICD, implantable cardioverter defibrillator; WES, whole exome sequencing.

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* Corresponding author at: CIBERCV, Spain.
E-mail address: anaisabel.fernandez@cibercv.es (A. Isabel Fernández-Avila).

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caused by synonymous or missense variants. Strict avoidance of alcohol consumption and early defibrillator implantation might prevent lethal arrhythmias in patients at risk.

1. Introduction

PPA2 (Pyrophosphatase Inorganic 2) is a mitochondrial enzyme, encoded by nuclear DNA, responsible for the hydrolysis of inorganic pyrophosphates (PPi) into organic phosphate. PPi is generated in several cellular reactions which are necessary for DNA and RNA synthesis, and for protein and lipid formation.

Biallelic mutations in the *PPA2* gene have been recently described in a few dozen families with recurrent sudden cardiac death (SCD) in siblings (Guimier et al., 2016, Kennedy et al., 2016, Phoon et al., 2020, Guimier et al., 2021, Manzanilla-Romero et al., 2023). Some cases of acute heart failure with very rapid progression to multiorgan failure leading to death or cardiac transplant in children and young adults have also been reported (Bellaing et al., 2018, Vasilescu et al., 2018, Guimier et al., 2021). Clinical features of mitochondrial disease (myopathy, ataxia, and neuropathy) can be found in some patients along with acute cardiomyopathy presentation or in the few known survivors (Guimier et al., 2021). The first cases of PPA2-associated disease were reported in 2016 (Guimier et al., 2016, Kennedy et al., 2016). Subsequent published patient series and case reports confirm that PPA2 disease is a very lethal condition that manifests primarily in the form of death of cardiac origin in two age groups, infants before the age of 2 years old and SCD in adolescents, typically after small doses of alcohol exposure (Phoon et al., 2020, Guimier et al., 2021, Genthe et al., 2023, Graham et al., 2023, Manzanilla-Romero et al., 2023).

We present the first four cases diagnosed in Spain of PPA2-related sudden cardiac death after consuming minimal amounts of alcohol in two unrelated families. All affected patients harbored two mutations in *PPA2* in compound heterozygosis. We additionally describe three of these variants as novel likely pathogenic variants, including a "synonymous" change previously described as likely benign.

2. Methods

2.1. Families

After the SCD of two teenagers, their respective families, in accordance with approved protocols, were referred by the Histopathology Service of the National Institute of Toxicology and Forensic Science to two referral centers for genetic heart conditions for assessment.

Family 1 had four members, mother and three teenage children, including the proband who suffered SCD in 2013 and his brother that died suddenly in 2022. Family 2 had four members, mother, father and two teenage daughters, one of whom suffered SCD in 2018. We underwent a comprehensive review of the clinical records, family history and results of the post-mortem examination and post-mortem genetic testing. In addition, we performed clinical evaluation and genetic testing to all family members.

2.2. Postmortem investigation

In the three sudden death cases, we performed an exhaustive post-mortem investigation with toxicological analyses and histopathologic examinations according to guidelines for autopsy investigation of sudden cardiac death (Basso et al., 2017). Blood samples of the deceased were frozen for genetic testing.

2.3. Clinical evaluation

The clinical screening conducted on the relatives included physical examination, electrocardiogram (EKG), transthoracic echocardiogram (TTE), Holter monitoring, exercise test and cardiac magnetic resonance imaging (MRI).

2.4. Genetic evaluation

We conducted genetic tests using a whole exome sequencing (Twist Bioscience) approach in family 1. Sequencing was conducted on Illumina NextSeq platform. All samples achieved a mean depth $>$ 50X with over 99.3 % of targeted regions. In family 2, we analysed 90 genes related to arrhythmias without structural heart disease with Next Generation Sequencing (NGS) technology. We analysed and interpreted the sequencing data on the platform SOPHiA DDM using ClinVar, dbNSFP, ESP, ExAC, G1000, GnomAD and OMIM public databases. We employed SIFT, Polyphem2, MutationTaster tools for amino acid replacement impact prediction, and Human Splice Finder and Splice AI Lookup tools for splicing impact predictions. In addition, we used RNAfold to predict the secondary mRNA structure and usDSM for predicting deleterious synonymous mutations (Tang et al., 2021). We classified the genetic variants according to standard guidelines (Richards et al., 2015). We performed validation and segregation analyses by conventional Sanger sequencing method, using the primer pairs described in Table1.

2.5. Transcript analysis

We isolated RNA samples from whole blood collected in EDTA tubes using NucleoSpin RNA Blood kit (Macherey-Nagel, Germany), and synthetized cDNA using iScript Advantage cDNA synthesis kit (BioRad, Spain). We conducted PPA2 transcript PCR amplification from exons 2 to 7 (NM_176869/2 reference mRNA sequence) using the primer pair described in Table1, and *GAPDH* as the reference gene. After PCR, we run amplicons in an agarose gel.

3. Results

3.1. Clinical and histology findings

3.1.1. Family 1 (Fig. 1A)

The proband (Individual II-1) was a healthy teenager who presented SCD in 2013, at the age of 16 years old. He reported having drunk two alcoholic beverages, the first time he had drunk alcohol, feeling nausea and vomiting shortly after and suffering a cardiac arrest in the following minutes. He could not be resuscitated.

Autopsy revealed an undetectable blood alcohol level and abnormal cardiac histology with scattered myocyte necrosis with inflammatory infiltrates and large areas of myocytolisis (myocyte dropout) with contraction bands in the myocardial tissue, which at first, was considered probably secondary to acute myocarditis (Fig. 2A).

First-degree family members were referred for cardiological evaluation. No family history of SCD or cardiac disease was found: the mother was a healthy 45 year-old woman, the father had died several years earlier, due to severe traumatic injuries, with no history of cardiac disease.

The sister (Individual II-2) was first evaluated in 2013 at the age of 13 years-old.

The initial study was normal including EKG, TTE, 24 h Holter monitoring and an exercise test. Regular follow-up was recommended. In 2017, at the age of 17 years old, a cardiac MRI was performed to complete cardiac evaluation. Biventricular function was entirely normal and there were no regional wall motion abnormalities but extensive mid-wall ring-like late gadolinium enhancement (LGE) on the left ventricle was present (Fig. 3B).

The younger brother's first evaluation (Individual II-3) was carried out at the age of 9 years old (2013), including EKG, TTE, 24 h Holter monitoring and stress test, all of them were normal. The first cardiac MRI in 2017 (at the age of 13 years old) showed mid-wall LGE limited to the interventricular septum. (Fig. 3A).

Considering siblings' MRI findings along with features of myocarditis in the proband's histologic analyses, we suspected an arrhythmogenic cardiomyopathy in family 1 with atypical findings though. Genetic testing (cardiomyopathy and channelopathy NGS panel of 199 genes) was performed in II-2, the relative with the more severe phenotype, in 2017 but no pathogenic or likely pathogenic variants were found.

After internal discussion, regular follow-up was recommended in II-3 and a subcutaneous implantable cardioverter defibrillator (ICD) was considered in II-2. She has remained with no cardiac symptoms and no ICD shocks, though she reported nausea, vomiting and severe myalgias after her first exposure to a small amount of alcohol (2–2.5 g). Since then, she has not consumed alcohol again.

Individual II-3 also remained asymptomatic in the follow up but in 2022 he suffered SCD being 17 y.o. after having one alcoholic drink, reported as the first time he had drunk alcohol. He also experienced nausea and vomiting before the cardiac arrest. The first rhythm registered was ventricular fibrillation (VF) and he could not be resuscitated even though he was promptly assisted by the emergency services. Refractory VF persisted after numerous defibrillation shocks and 50 min of advanced cardiopulmonary resuscitation.

The autopsy revealed a low alcohol level in vitreous humor (0.20 g/L), undetectable in blood, and no relevant macroscopic findings. Cardiac histology was compared side by side with the proband's (individual II-1) samples. No myocardial inflammatory infiltrate was noted in II-3 subject but otherwise almost identical findings were present in the brothers: large areas of myocytolisis and contraction bands (Fig. 2A and 2B).

The mother was initially studied after the death of individual II-1 with TTE and 24 h Holter monitoring that were normal. She is currently 55 years old and reported occasional alcohol consumption during the past 35 years (from small alcohol amounts to exceptional higher intake), with no symptoms of intolerance.

3.1.2. Family 2 (Fig. 1B)

The proband (individual II-1) was a 14-year-old female, with a previous history of suspected epilepsy in infancy that required treatment with an antiepileptic drug until the age of 10. She suffered SCD in 2018, at a party after having a small-dose alcohol intake. She had no cardiac history and no previous symptoms, except for one syncopal episode classified as vasovagal related to pain at the age of 8 years old. The autopsy revealed very low alcohol levels in vitreous humor (0.19 g/L) and blood (0.12 g/L) and no macroscopic or microscopic relevant findings. Specifically, cardiac histology was normal (Fig. 2C).

Her parents were healthy, occasional alcohol consumers since young adulthood and denied any symptoms related to alcohol intake, and she also had a younger sister, that was 11 years old at that time. They were all studied with EKG, TTE, 24 h Holter monitoring and stress test that were normal. Additionally, a cardiac MRI was performed in both parents, showing no abnormalities in both cases.

3.2. Genetic results

We conducted a whole exome sequencing (WES) study in individuals II-2 and II-3 from family 1 after the death of subject II-2. Both patients shared in heterozygosis two variants in *PPA2*: NM_176869.3:c.290A > G (NP_789845.1:p.(Glu97Gly)) and NM_176869.3:c.513C > T (NP_789845.1:p.(Cys171 =)). In family 2, the proband case II-1, showed other two *PPA2* variants: NM_176869.3:c.683C > T (NP_789845.1:p.(Pro228Leu)) and NM_176869.3:c.980_983del (NP_789845.1:p.(Gln327ArgfsTer9)), in heterozygosity. No other pathogenic or likely pathogenic variants were found in any of these two families.

Sanger sequencing was conducted on the mother in family 1 and in both parents and sister in family 2 to determine variant segregation (Supplementary Image). Additionally, a blood sample intended for microbiological studies from proband II-1 of family 1 was available for *PPA2* variant analysis by Sanger sequencing. The mother in family 1 carried the *PPA2* c.513C > T (p.Cys171 =) variant in heterozygosity. Individual II-1 carried both variants: *PPA2* c.290A > G (p.Glu97Gly) and c.513C > T (p.Cys171 =). The father in family 2 harbored the *PPA2* c.683C > T (p.Pro228Leu) variant in heterozygosity while the mother and sister were carriers of the *PPA2* c.980_983del (p.Gln327fs) in heterozygosity (Supplementary Image). These results support the *PPA2* variants status in compound heterozygosis (trans) in the affected patients II-1, II-2 and II-3 in family 1 and II-1 in family 2.

Detailed information on patient demographic data, survival status and gene variants are summarized in Table 2.

3.2.1. PPA2 variants interpretation and ACMG classification

The NM_176869.3:c.683C > T variant, (NC_000004.12: g.105399137G > A) is found at a frequency of 0.02 % in population databases (GnomAD). This change replaces proline, which contains an imino group, cyclic and non-polar, with leucine, which is aliphatic and non-polar, at codon 228 of the PPA2 protein (Fig. 4A). Five of five in-silico tools predict a damaging effect of the variant on protein function. This variant has been described in the literature in several individuals from non-related families with PPA2-related disease (Kennedy et al., 2016, Phoon et al., 2020). Most affected patients were also carriers of other pathogenic/likely pathogenic variant in *PPA2* in compound heterozygosis state. *PPA2* c.683C > T (p.Pro228Leu) variant has also been described in patients in homozygous state (Manzanilla-Romero et al., 2023). Functional studies in vitro show that this variant reduces PPA2 enzymatic activity, around 25 % residual activity compared to wild type PPA2 (Kennedy et al., 2016). This variant, located in exon 8, is therefore classified as pathogenic according to ACMG criteria (PS3-SUP, PM2-MOD, PM3-MOD, PP1-STR, PP3-SUP).

The NM_176869.3:c.290A > G variant change (NC_000004.12: g.105449381 T > C) replaces glutamic acid, which is acidic and polar, with glycine, which is aliphatic and nonpolar, at codon 97 of the PPA2 protein. This variant is not present in population databases (GnomAD, ExAC). Computational prediction tools and conservation analyses support that this missense variant has a deleterious effect on protein structure and function. Besides, secondary mRNA structure alteration prediction, conducted with RNAfold tool (Fig. 4A), indicates that this missense variant could produce an important change in mRNA structure that makes it susceptible to mRNA mediated decay. The variant, located

Table 1
Primer pairs used for *PPA2* Sanger validation and segregation, and transcript PCR amplification (Reference Gene sequence NG_053007.1. Reference mRNA sequence NM_176869/3).

Analysis	Forward: 5́-3́	Reverse: 5́-3́	Location	Size (pb)
PPA2c.290A > G	CGGGGGAAAACATCTAAGGT	ATGCCAAACAACTTGCAACA	Intron 3–4 to intron 4–5	227
PPA2c.513C > T	AAAACTTTGTGATTATGTGGAAAGA	GGCATGAATAAACCAAAACTCA	Intron 5–6 to intron 6–7	224
PPA2c.683C > T	TTGTGCTTCTGGTTAGACTGGG	TTTTTCAGCCTTGTTGATTCACTT	Intron 7–8 to intron 8–9	446
PPA2c.980_983del	ATGAGAGAACTTGGGACGAAAG	GTGCCTGGCCAAAATCTTCTTTTT	Intron 11–12 to exon 12	426
PPA2 transcript	TCCCCCTTTCATGATATTCCTCTG	TTTCCAATCTGTTTCACCTTCATCA	Exon 2 to exon 7	429

in exon 4, is present in the three affected siblings of family 1. We classify this variant as variant of unknown significance according to ACMG criteria (PP4-SUP,PP3-STR,PM2-MOD, PP1-MOD). However, since other variants in nearby positions have previously been described as likely pathogenic and pathogenic: *PPA2* c.280A > G; (p.Met94Val), c.318G > T (p.Met106Ile), we propose that the variant is likely a pathogenic mutation.

The NM_176869.3:c.980_983del variant (NC_000004.12: g.105369747_105369750del) has not previously described in the literature in affected individuals with cardiomyopathy or sudden cardiac death. It is present in population databases (0.004 % GnomAD). This variant produces a frameshift change generating a premature stop codon and loss of the last seven amino acids of the protein (Fig. 4A). Loss of function variants (nonsense and splice site) have been previously classified as pathogenic or likely pathogenic in the literature. Moreover, there is another frameshift variant in a relatively close position (p. Ser313*) that has previously been classified as pathogenic as it was identified in 2 affected individuals.(Guimier et al., 2021) Thus, we classify this variant, located in exon 12, as pathogenic (PVS1-VSTR, PM2-MOD,PM3-MOD,PP4-SUP).

The NM_176869.3:c.513C > T variant (NC_000004.12: g.105437965G > A) results from a C to T substitution in coding exon 6 of the PPA2 gene. It is a synonymous variant presents in the general population at a frequency of 0.2 % (GnomAD database), and therefore it is classified as likely benign in ClinVar. However, only 2 homozygous have been identified, suggesting that this variant may be causative for a recessive condition. Moreover, in silico predictors indicate that although *PPA2* c.513C > T is a synonymous variant, it could have a deleterious impact (usDSM tool) and alter splicing sites (Human Splice Finder and Splice AI Lookup tools) (Fig. 4A), which could result in aberrant splicing that could trigger mRNA decay. We therefore reclassified this variant, located in exon 6, as variant of unknown significance (VUS) (PP1-MOD, PP4-SUP, BP6-SUP).

3.3. Transcript analysis

The analysis of the transcripts in family 1 (Fig. 4B) revealed that for the mother carrier of *PPA2* c.513C > T (p.Cys171 =) variant, an amplicon with the expected canonical transcript size (429 bp) was detected although with lower expression than control sample. While for subjects II-2 and II-3, carriers of both *PPA2* c.513C > T (p.Cys171 =) and c.290A > G (p.Glu97Gly) variants, the expected transcript was undetectable. These results, together with the conducted predictions and the presence of the two variants in the 3 affected siblings support that both *PPA2* c.513C > T (p.Cys171 =) and c.290A > G (p.Glu97Gly) may impact on mRNA decay and potentially on PPA2 protein expression. We therefore suggest that these PPA2 variants are likely contributing to the disease. The abnormal splicing transcript due to *PPA2* c.513C > T (p.Cys171 =) was not detected in this procedure as it probably results in a too long or short RNA fragment (due to exon skipping or intron retentions).

The analysis of the transcripts in family 2, mother and father, carriers in heterozygosity of *PPA2* c.683C > T (p.Pro228Leu) and *PPA2* c.980_983del (p.Gln327fs), respectively, revealed no alterations (Fig. 4B).

4. Discussion

In this study, we report the first cases of PPA2- cardiac disease diagnosed in Spain, three PPA2- related sudden cardiac deaths and a patient with cardiomyopathy. All these patients harbored biallelic *PPA2* mutations in compound heterozygosis. We also describe two novel relevant genetic variants (*PPA2* c.290 A > G and *PPA2* c.980_983del) and reclassify *PPA2* c.513C > T that was previously considered benign because it is synonymous. Our findings in family 1 supports previous studies that suggest that some variants in *PPA2* may induce mRNA mediated protein decay instead of creating an abnormal protein with altered function.

PPA2 disease is a relatively new described pathology. Including our two families, 65 affected patients have been reported worldwide to date, 85 % of them presented with SCD or acute heart failure leading to death; most cases were infants under 2 years but 21 % were healthy teenagers who suffered cardiac arrest after minimal alcohol exposure (Guimier et al., 2016, Kennedy et al., 2016, Vasilescu et al., 2018, Phoon et al., 2020, Guimier et al., 2021, Genthe et al., 2023, Graham et al., 2023, Manzanilla-Romero et al., 2023). Abnormal PPA2 enzyme activity was found to be disruptive for mitochondria survival and function due to their inability to maintain an electric transmembrane action potential caused by deficiencies in respiratory chain function and abnormal adenine exchange through the membrane(Guimier et al., 2016). According to previous cases reported and the two new families we describe, PPA2 enzyme seems to have a major role in cardiomyocytes in comparison to other cells with high energetic demands and typically affected in mitochondrial disorders, as the initial symptoms are mainly cardiological and very severe. Hence, both adult and pediatric cardiologists should be aware of this rare but lethal condition and *PPA2* gene should

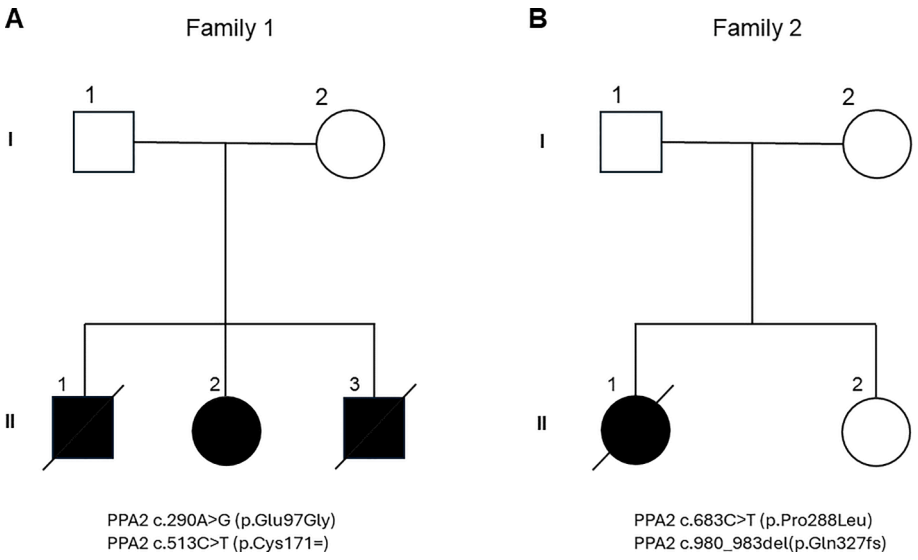

Fig. 1. Pedigree of Family 1 and Family 2.

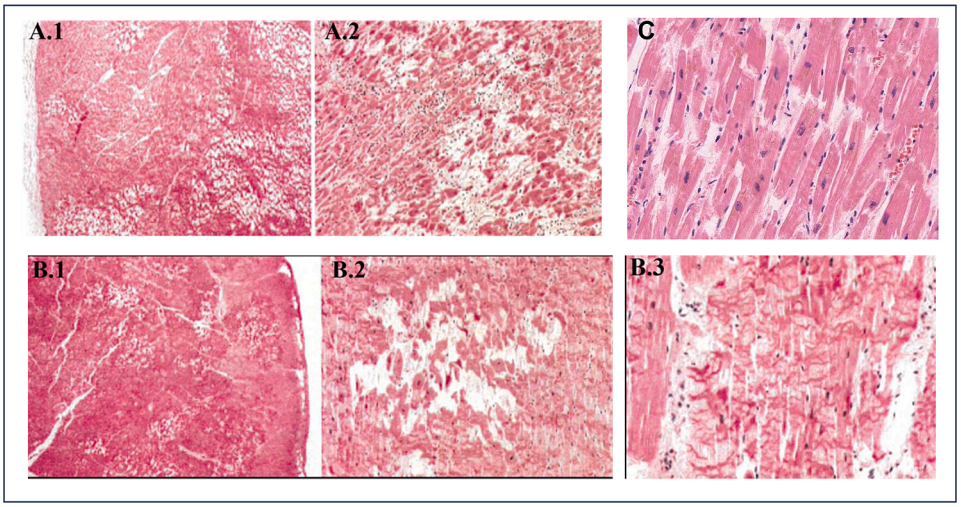

Fig. 2. Cardiac histology of the deceased individuals from family 1 and 2. A) II-1 patient of family 1. scattered myocyte necrosis with inflammatory infiltrates and large areas of myocytolisis (A.1) with contraction bands in the myocardial tissue (A.2). B) II-3 patient of family 1. Large areas of myocytolisis (B.1-B.2) and contraction bands (B.3). C) II-1 patient of family 2. Normal myocardial tissue.

be included in genetic panels for the assessment of cardiomyopathy and sudden cardiac death.

It has been hypothesized that enzyme deficiency with secondary progressive accumulation of PPi and subclinical reduction in ATP availability produces chronic damage to the cardiomyocytes and leads to fibrosis (Kennedy et al., 2016). Moreover, myocardial fibrosis is a common finding either in histology or CMR imaging. Inflammatory infiltrate, as shown in cardiac histology from patient II-1 of family 1, is also common (Guimier et al., 2016, Kennedy et al., 2016, Manzanilla-Romero et al., 2023) so in some cases autopsy may be misdiagnosed as myocarditis. Other findings include areas of myocyte necrosis (Guimier et al., 2016, Kennedy et al., 2016), contraction bands in cardiac muscle fibers (Phoon et al., 2020) or reduced amount of myofibrils (Kennedy et al., 2016). These histological changes were present in deceased siblings from family 1. Additionally, an autopsy with no pathological findings ("negative autopsy") was described in one case (Phoon et al., 2020) and also in our patient II-2 from family 2.

Cardiac fibrosis in PPA2 patients can be present with left ventricle dilation or reduced LVEF but also with normal ventricular volumes and function as we show in family 1. Mid-wall LGE in the septum and/or mid-wall or subepicardial inferolateral LGE has been predominantly described (Kennedy et al., 2016, Guimier et al., 2021, Genthe et al., 2023, Manzanilla-Romero et al., 2023), similar to arrhythmogenic cardiomyopathy. Notably, II-3 of family 1 subject had mid-wall fibrosis at an early age, (13y.o.) suggesting that, even if some patients remain asymptomatic in infancy, there are already signs of structural disease that can be misdiagnosed with only TTE imaging. However, whether myocardial fibrosis is the substrate of the fatal arrythmia like other cardiomyopathies remains unclarified. Some patients show extensive myocardial scar (patient II-2 of family 1) with no arrythmias while whereas others have suffered SCD with normal cardiac histology (individual II-1 of family 2). Moreover, acute mitochondrial and cardiomyocyte disfunction with subsequent cellular death has been proposed as the main mechanism for ventricular arrhythmias in PPA2 disease. Hence, when the concentration of PPi rises beyond a certain threshold (generally after a trigger as alcohol consumption, fever, or viral infections) it results in a competitive inhibition of mitochondrial translocases of ADP/ATP. This precludes energy generation in the cardiomyocyte leading to an acute myocardial dysfunction and acute heart failure or VF. This could also explain why patients sometimes do not respond to defibrillation attempts (Kennedy et al., 2016).

A total of 25 variants have been described in patients with PPA2 associated disease in the literature in previous reports, causing disease only when presenting as biallelic mutations: homozygosis or compound heterozygosis (Guimier et al., 2016, Kennedy et al., 2016, Bellaing et al., 2018, Vasilescu et al., 2018, Phoon et al., 2020, Guimier et al., 2021, Genthe et al., 2023, Graham et al., 2023, Manzanilla-Romero et al., 2023). The majority of these are missense (20) resulting in an abnormal protein with altered function, although there are also nonsense variants (4) and one splice-site which are expected to result in loss of function by premature protein truncation or nonsense-mediated mRNA decay. A reduction in the PPA2 enzymatic activity, in the amount of protein and in the protein stability are pathogenic mechanisms previously described in PPA2 variant analyses (Phoon et al., 2020). It should be noted that different consequences are predicted depending on tissue or cell types and even in carriers for the same variants (Phoon et al., 2020). This supports a variety of mechanisms of PPA2 deficiency, probably involving different protein-coding isoforms.

In the current study, we found different potential pathogenicity mechanisms. We detected normal transcripts in blood for individuals

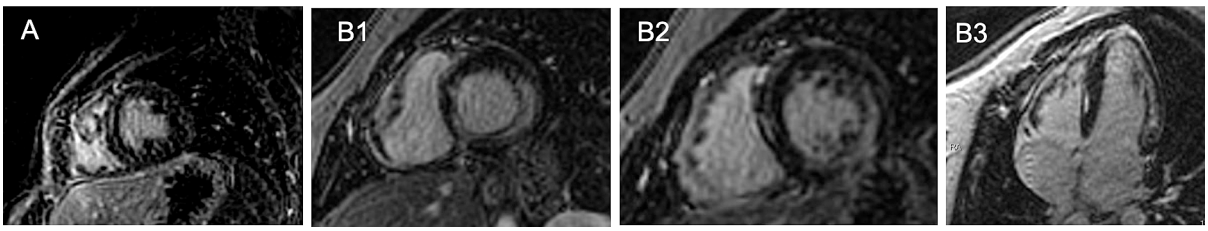

Fig. 3. Cardiac Magnetic Resonance Imaginf (MRI) of individuals II-3 and II-2 from family 1. A) Patient II-3. Short axis (SA) LGE at papillary muscles (PM) level showing mid wall fibrosis in the septum. B) Patient II-2. B1 and B2: basal and PM SA view showing mid-wall ring-like LGE at inferior, inferolateral and lateral walls. B3: 4 chamber view with extensive mid LGE at lateral wall and in the septum.

Table 2
Summary of *PPA2* genetic variants, demographic data and survical status of the members of the two families

Gene	Nucleotide change	Protein change	Chr	Position	Carriers	Zygosity	Sex	Age	Status	Cardiovascular death
PPA2	c.290A > G	p.(Glu97Gly)	4	105,449,381	Family 1 I-1	*Heterozygous**	M	47	Deceased	No (traumatic brain injury)
					Family 1 II-1	Compound heterozygous (c.513C > T in trans position)	M	16	Deceased	Yes (cardiac arrest)
					Family 1 II-2	Compound heterozygous (c.513C > T in trans position)	F	23	Alive	–
					Family 1 II-3	Compound heterozygous (c.513C > T in trans position)	M	17	Deceased	Yes (cardiac arrest)
PPA2	c.513C > T	p.(Cys171 =)	4	105,437,965	Family 1 I-2	Heterozygous status	M	55	Alive	–
					Family 1 II-1	Compound heterozygous (c.290A > G in trans position)	M	16	Deceased	Yes (cardiac arrest)
					Family 1 II-2	Compound heterozygous (c.290A > G in trans position)	F	23	Alive	–
					Family 1 II-3	Compound heterozygous (c.290A > G in trans position)	M	17	Deceased	Yes (cardiac arrest)
PPA2	c.683C > T	p.(Pro228Leu)	4	105,399,137	Family 2 I-1	Heterozygous	M	42	Alive	–
					Family 2 II-1	Compound heterozygous (c.980_983del in trans position)	F	14	Deceased	Yes (cardiac arrest)
PPA2	c.980_983del	p.(Gln327fs)	4	105,369,747	Family 2 I-2	Heterozygous	F	41	Alive	–
					Family 2 II-1	Compound heterozygous (c.683C > T in trans position)	F	14	Deceased	Yes (cardiac arrest)
					Family 2 II-2	Heterozygous	F	16	Alive	–

Chr: Chromosome; M: Male; F: Female; *: No tested, obligated carrier.

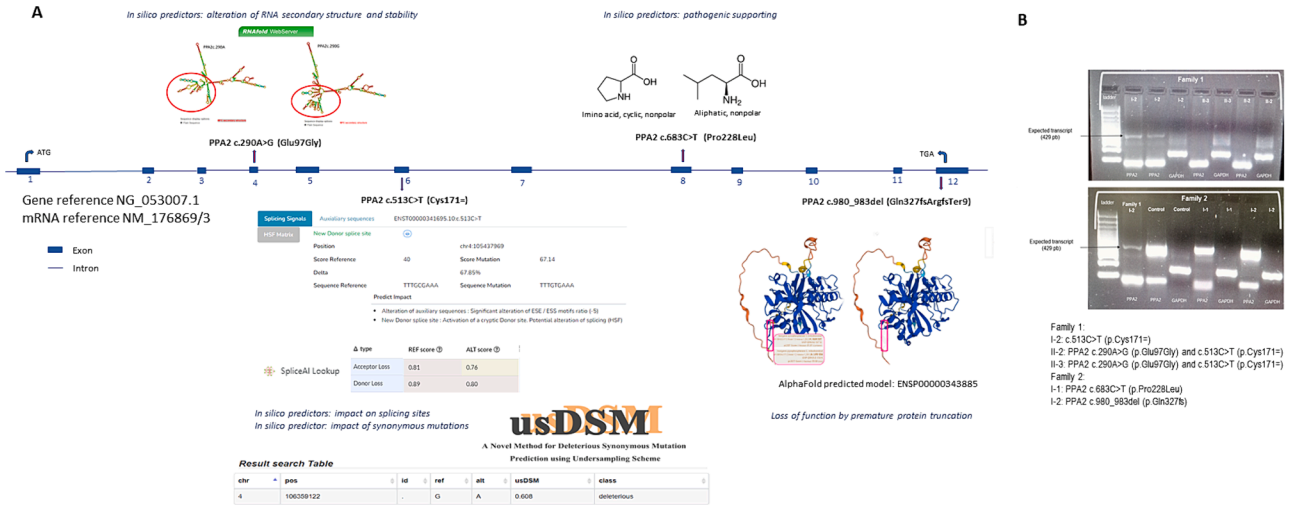

Fig. 4. *PPA2* impact variant predictions and transcript analysis in family 1 and family 2. A) Variant localization and impact by in silico prediction tools. B). Transcript amplification.

harboring *PPA2* c.683C > T (p.Pro228Leu) missense or *PPA2* c.980_983del (p.Gln327fs) frameshift variants segregating in family 2. These are expected to result in an abnormal or truncated protein with altered function, the most common mechanism causing PPA2 disease. In fact, *PPA2* c.683C > T (p.Pro228Leu) has been previously described in both compound heterozygous in trans position with another pathogenic or likely pathogenic variant, and in homozygosis state in one family (Kennedy et al., 2016, Vasilescu et al., 2018, Phoon et al., 2020, Guimier et al., 2021). Furthermore, previous studies have shown that, although PPA2 is present, its function is altered with only around 25 % residual activity compared to wild type PPA2 (Kennedy et al., 2016). However, we detected absence of normal transcript in blood for individuals harboring both *PPA2* c.290A > G (p.Glu97Gly) and *PPA2* c.513C > T (p.Cys171 =) variants in family 1, probably mediated by abnormal mRNA degradation. Although these results should be taken with caution, as blood is not the target tissue, and the RNA degradation cannot be assured, previous studies support that some missense *PPA2* variants such as c.280A > G (p.Met94Val) significantly decrease PPA2 protein expression, maybe due to mRNA instability, at least in fibroblast of affected individuals (Guimier et al., 2016, Kennedy et al., 2016, Vasilescu et al., 2018). Moreover, it is well recognized that synonymous variants may manifest changes at DNA, RNA and/or protein levels, most frequently on RNA level altering splicing site or splicing regulatory motifs leading to mRNA degradation (Vihinen, 2021), as predicted for *PPA2* c.513C > T (p.Cys171 =) in family 1. Additional functional evidence such as protein quantification, tissue-specific expression or a cellular model would unequivocally support the pathogenicity of both *PPA2* c.290A > G and *PPA2* c.513C > T variants but that is currently beyond our scope. However, in agreement with our available information, the disease presentation which is very specific of PPA2-related

disease, the lack of other pathogenic variants for a disorder that, based on the family pedigree, is clearly suggestive of an autosomal recessive inheritance, the identified *trans*-variants in a recessive inheritance condition, the correlation disease-genotype in the 3 affected individuals and the predicted impact on mRNA and transcript analysis, support that the reported variants are likely contributing to the disease.

How the PPA2 deficiency leads to cardiomyopathy or lethal arrhythmias is not fully understood. It has been hypothesized that some *PPA2* variants are relatively more "benign" than others, producing a defective protein with a higher residual activity allowing some patients to survive and even being asymptomatic until adolescence or early adulthood. However, this genotype-phenotype correlation has not been clearly supported. Guimier et al. (Guimier et al., 2016) described high residual activity (40 %- 50 %) for the variant *PPA2* c.476C > T (p. Thr159Met) in three individuals who survived the first 2 years of life, two surviving until adolescence. However, the *PPA2* c.686G > T (p. Gly229Val) predicted to have also relatively high residual activity, was identified in homozygous state in a patient with heart failure as a neonate that died at 3 months old. In our families, patients survived and were asymptomatic until adolescence. Furthermore, our II-2 patient from family 1 is now 23 years old and fully asymptomatic except for intolerance to alcohol while her two brothers suffered SCD.

Minimal alcohol consumption triggers PPA2 disease. However, the single PPA2 adult carriers evaluated in the current study are occasional alcohol consumers, but they have never experienced any symptoms, supporting that single carriers have enough integral protein to ensure adequate residual PPA2 activity and normal mitochondrial function.

Data regarding the natural history of *PPA2* surviving patients are scarce. To our knowledge, only 10 patients (including individual I-2 from family 1) remain alive. A wide spectrum of phenotypes has been described, including asymptomatic status, alcohol intolerance, cardiomyopathy with significant myocardial fibrosis, rapidly progressive heart failure and acute myocarditis with or without signs and symptoms of mitochondrial disease (Kennedy et al., 2016, Guimier et al., 2021, Genthe et al., 2023). Thus, we believe a multidisciplinary approach is warranted for all at risk individuals with *PPA2* disease. Patients require regular neurological assessment. Avoidance of alcohol and vinegar consumption and aggressive treatment of fever are crucial to prevent lethal arrhythmias. Cardiac MRI imaging can be useful to detect subclinical disease. Finally, though there are still limited data, early ICD implantation should be considered in all individuals who harbor biallelic mutations in *PPA2*.

5. Conclusions

PPA2 mitochondrial disease is a rare but highly lethal condition that should be suspected in cases of recurrent SCD in siblings or cardiac arrest in the young, particularly after small alcohol intake. Cardiac MRI can detect early subclinical disease. PPA2 deficiency can be related to different pathogenicity mechanisms such as abnormal protein structure but also mRNA decay caused by synonymous or missense variants. Patients should be warned regarding alcohol consumption and ICD implantation should be considered early in all at risk individuals who harbour biallelic variants in PPA2.

Ethics approval and consent to participate

All patients signed an informed consent document authorizing the use of their genetic data for research purposes. The study was approved by the Ethics Committee of the Hospital General Universitario Gregorio Marañón (CEIm 39/18) and Puerta de Hierro and performed in compliance with the Declaration of Helsinki.

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CRediT authorship contribution statement

Cristina Gómez González: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Iván del Campo Cano:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Ana Isabel Fernández-Avila:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition. **Maria Paz Suárez – Mier:** Resources, Methodology, Investigation, Conceptualization, Supervision, Writing – review & editing. **María José Sagastizábal:** Writing – review & editing, Methodology, Investigation. **Reyes Álvarez García-Rovés:** Writing – review & editing, Methodology, Investigation, Data curation. **Irene Méndez Fernández:** Writing – review & editing, Methodology, Investigation, Data curation. **Silvia Vilches:** Writing – review & editing, Methodology, Investigation. **Miriam Centeno Jiménez:** Writing – review & editing, Methodology, Investigation. **Ana Siles Sánchez –Manjavacas:** Writing – review & editing, Methodology, Investigation. **Ana Usano Carrasco:** Investigation, Methodology, Writing – review & editing. **Emiliano Gonzalez-Vioque:** Methodology, Validation. **Juan Pablo Ochoa:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Constancio Medrano:** Resources, Supervision, Writing – review & editing. **Esther González López:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Pablo García-Pavía:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Javier Bermejo:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **María Angeles Espinosa Castro:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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

No data was used for the research described in the article.

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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.2024.148437.

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