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New cases of δ-aminolevulinic acid dehydratase deficiency: Functional insights into gene variants using an innovative mouse liver model

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Abstract. Di Pierro E, Solares I, Jericó D, Castelbón FJ, Solera JT, Riera-Mestre A, et al. New cases of δ-aminolevulinic acid dehydratase deficiency: Functional insights into gene variants using an innovative mouse liver model. *J Intern Med.* 2026;**299**:126–42.

Background. Dysfunction of δ-aminolevulinic acid dehydratase (ALAD), the second enzyme involved in heme biosynthesis, leads to two pathologies: genetic and acquired. The genetic form is an ultrarare, severe childhood-onset disease inherited in an autosomal recessive manner, whereas the acquired form usually affects adults due to enzyme inhibition by specific chemicals.

Aims and patient cohort. This study reports the molecular characterization of three pediatric patients with genetic ALAD deficiency porphyria (ADP), including two siblings, and five adults who exhibited features suggestive of heavy metal poisoning. Furthermore, using an innovative mouse liver model, we performed in vivo functional analysis of the pathogenic variants and lead susceptibility alleles identified in the *ALAD* gene.

Results. Siblings (one female) were found to carry the c440_441delinsTT (p.Arg147Leu) variant in homozygosis. However, the vector expression system confirmed a pathogenic role only for the c.440C > T substitution. The third patient

Elena Di Pierro, Bodo B. Beck, Montserrat Morales-Conejo, and Antonio Fontanellas jointly supervised this work.

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exhibited compound heterozygosity, with a c.839G > A (p.Gly280Glu) dominant variant and a hypomorphic c.724G > A (p.Val242Ile) allele. The rs1805313 and rs8177800 common intron variants were most prevalent in patients with acquired ADP. However, increased ALAD activity for the rs1139488 synonymous variant and a hexameric ALAD conformation for the rs1800435 missense variant have been established.

Conclusion. These findings underscore the molecular heterogeneity of the *ALAD* gene and present the first reported case of ADP in a female patient.

Keywords: delta-aminolevulinic acid dehydratase deficiency, heavy metal poisoning, porphyrinuria, ultrarare autosomal recessive disorder, unexplained recurrent abdominal pain

Abbreviations: Acq-ADP, acquired ADP; ADP, ALAD deficiency porphyria; AHPs, acute hepatic porphyrias; ALA, delta-aminolevulinic acid; ALAD, δ-aminolevulinic acid dehydratase; BLL, blood lead level; CKD, chronic kidney disease; CT, computed tomography; DTT, dithiothreitol; eALAD, erythrocyte ALAD activity; G6PD, glucose-6-phosphate dehydrogenase; HD, hydrodynamics-based procedure; MRI, magnetic resonance imaging; PBG, porphobilinogen; PEPT2, peptide transporter 2; PROTO, protoporphyrin; RBCs, red blood cells; SIADH, syndrome of inappropriate antidiuretic hormone secretion; WT, wild-type

Introduction

Delta-aminolevulinic acid (ALA) dehydratase deficiency porphyria (ALAD deficiency porphyria [ADP]; OMIM 612740) is an autosomal recessive disorder that results from a marked reduction in ALA dehydratase (δ-aminolevulinic acid dehydratase [ALAD], EC. 4.2.1.24), which is the second enzyme involved in heme biosynthesis [1]. This form of porphyria is classified as one of the four acute hepatic porphyrias (AHPs) because it is characterized by recurrent neurological attacks with neurovisceral and neuropsychiatric symptoms, complicated by progressive peripheral neuropathy [2]. ADP differs from dominant AHPs because it is not accompanied by elevated levels of porphobilinogen (PBG) in urine, a product of the ALAD enzyme, which can be easily detected using the Hoesch test [3]. Accordingly, these patients exhibit an almost complete loss of ALAD activity, which results in elevated levels of ALA in the plasma, ALA and coproporphyrin III in the urine, and zinc protoporphyrin (PROTO) within red blood cells (RBCs). ADP manifests as a clinically severe form of porphyria that primarily begins in childhood [4]. It is the rarest type of AHP, with only 10 documented cases reported worldwide, all in males [5–14].

ADP also exhibits high allelic heterogeneity, with 14 pathogenic variants identified in the *ALAD* gene (Institute of Medical Genetics in Cardiff. Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php, last entry October 2024). Here, we present the “in vivo” functional characterization of three *ALAD* variants identified in three young ADP patients, including two siblings, one of whom is a female.

In addition, ALAD enzyme deficiency can result from inhibition by chemicals, such as heavy metals (e.g., lead) [15] or succinylacetone [16], the primary biomarker in hereditary tyrosinemia type 1 (OMIM 276700). Lead easily absorbed by the body accumulates mainly in erythrocytes and affects the hematological system [17]. Several common *ALAD* gene variants have been associated with the accumulation and distribution of lead in the blood, bones, and internal organs, making individuals more susceptible to lead toxicity [18]. Although lead poisoning has declined due to preventive measures, exposure still occurs through various sources, including lead-containing pipes, cookware, dishes, cosmetics, retained bullets, illicit “moonshine” liquor, lead-adulterated opium, and certain herbal supplements. Among these, inhalation of lead-adulterated opium is associated with particularly high bioavailability compared with other exposure routes [19]. Ayurvedic remedies and online alternative medicines are also potential sources, with 20% of such products containing lead, arsenic, and mercury [20]. In the present study, the ALAD genetic backgrounds of five adults with clinical and biochemical symptoms of acquired ADP (Acq) were analyzed, and common *ALAD*-identified variants were tested for functionality.

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Patients and methods

Case description

Patient ADP1, a German boy born in 1992, presented at birth with muscular hypotonia and respiratory difficulties. Spinal muscular atrophy was excluded based on spontaneous recovery and the absence of a predominant molecular abnormality in the *SMN1* gene. Development remained normal until the age of 3 years, when deterioration led to a temporary loss of mobility and acute episodes with occasional lethargy. By the age 8 years, renal function had declined to eGFR of 48 mL/min/1.73 m^2. The patient subsequently developed persistent peripheral neuropathy, autonomic or vegetative symptoms, and tachycardia associated with elevated urinary ALA excretion. Notably, the patient carried a peptide transporter 2 (PEPT2) PEPT2*1/*2 protein variant, corresponding to exon 13CT/exon 15CT (Table 1), which was not associated with a poor renal prognosis [21]. Despite multiple courses of hemin therapy, ALA excretion remained elevated (Fig. S1).

By the age of 17 years, the patient's condition had significantly worsened; he became wheelchair bound and initiated hemodialysis due to chronic kidney disease (CKD, eGFR <10 mL/min/1.73 m^2). Hemodialysis was intensified to six sessions per week to reduce circulating ALA levels; however, this proved insufficient to prevent further neurological decline. The patient's condition progressed to tetraplegia with respiratory failure. In June 2010, at 18 years of age, the patient underwent a successful kidney transplantation, with the donor kidney positioned in the lower abdomen while retaining the native kidneys in situ. Subsequently, only sporadic minor flare-ups were observed. The patient was successfully weaned off mechanical ventilation 5 months after the transplant. Although he remained wheelchair bound, he was able to complete his high school education and pursue university studies. The patient remained clinically stable for more than 12 years posttransplantation until passing away in 2023 due to respiratory complications resulting from the COVID-19 infection.

The ADP1 patient had no family history of porphyria. Both parents were asymptomatic with normal urinary porphyrin and ALA levels (data not shown). Erythrocyte ALAD activity (eALAD) was reduced to 16%, 25%, and 76% of normal levels in the proband, mother, and father, respectively. Enzyme activity was not restored by the in vitro addition of dithiothreitol (DTT) or zinc, suggesting a pathogenic variant of the ALAD gene (Table 1).

ADP2 (male) and ADP3 (female) patients exhibited refractory acute attacks. They were Italian siblings born to consanguineous parents. ADP2 was admitted to the intensive care to days after birth with respiratory failure and muscular hypotonia in the context of axonal polyneuropathy. By the age of 6 years, his neurological condition showed gradual improvement, and he regained autonomous motor function. However, by the age of 10 years, he began experiencing monthly acute attacks characterized by headache, nausea, abdominal and limb pain, and hypertensive attacks requiring hospitalization. At age of 13 years, ADP2 developed axonal polyneuropathy, persistent hypertension, mild cognitive impairment, sensorineural hearing loss requiring a prosthesis, and mood disturbances (Table 1).

ADP3 was diagnosed with intrauterine growth restriction, leading to delivery by cesarean section at 37 weeks. She remained in the neonatal care unit due to low birth weight and muscular hypotonia with limited spontaneous movement. Although she was not hospitalized again until the age of 4 years, she subsequently experienced progressive axonal neuropathy, reduced mobility, and loss of motor independence. By the age of 6 years, ADP3 presented with monthly acute attacks, axonal polyneuropathy, tetraparesis with tendon contractures, refractory hypertension, psychomotor retardation, and sensorineural hearing loss also requiring a prosthesis. She was undernourished and exhibited impaired growth (Table 1).

ADP2 and ADP3 required intensive care at various times to manage pain and blood pressure, whereas ADP3 required intubation following an episode of respiratory failure. Diagnosis of ADP was confirmed at ages of 14 and 7 years based on markedly elevated urinary ALA and porphyrin levels and reduced ALAD enzymatic activity (<6% of that observed in control samples) (Table 1). Notably, both siblings carried PEPT2*1/*1 variant 1, corresponding to exon 13CC/exon 15CC (Table 1), which is associated with a high affinity for ALA [21].

Despite treatment regimens including intravenous hemin (4 mg/kg/day), subcutaneous givosiran (2.5 mg/kg body weight/28 days), oral

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Table 1. *Biochemical characterization of patients with ADP and Acq.*

		Patients with ADP			Patients with unexplained severe abdominal pain and porphyrinuria				
Analyzed variables	Reference values	ADP1	ADP2	ADP3	Acq1	Acq2	Acq3	Acq4	Acq5
Urinary porphyrin excretion	<150 µg/g creat	1702	417	6610	1280	444.3	2753	NPDc	92
Urinary ALA excretion	<5 µmol/mmol creat	51.2	18	151	13.9	25.12	47.1	NPDc	43.2
Urinary PBG excretion	<1.5 µmol/mmol creat	0.25	0.74	2.8	5.3	0.32	1.1	NPDc	0.5
eALAD activity	100% vs. controls	16%	6%	3%	10%	25%	34%	15%	41% (NPDc)
Zinc + DTT eALAD activity (% vs controls)	100% vs. controls	30%	NPDc	NPDc	100%	115%	124%	124%	NPDc
Plasma porphyrin	5.72 ± 1.22 nmol/l	NPDc	NPDc	NPDc	49.8	NPDc	13.78	7.58	NPDc
Protoporphyrin levels in RBCs	<1.6 µmol/L	NPDc	22.7	11.0	2.9	NPDc	0.508	0.897	0.126 (NPDc)
Hemoglobin	14 ± 2 g/dL	7.4	10.3	9.8	10	8.6	9	13	11.5
ALT	12 ± 1.45 U/L	19	20	14	31.6	14	27	11.9	94
Triglycerides	<150 mg/dL	<150	87	184	84.6	145	126.5	161.9	50
Bilirubin	0.38 ± 0.098 mg/dL	NPDc	0.3	0.3	2.47	0.64	0.71	0.19	0.85
Blood lead levels	<100 µg/L	90	4	0.7	1380	732	877	487	7 (NPDc)
***PEPT2* genotype**		PEPT2 *1/*2	PEPT2 *1/*1	PEPT2 *1/*1	PEPT2 *1/*2	PEPT2 *1/3	PEPT2 *1/*2	PEPT2 *1/*2	PEPT2 *1/*2

Note: Elevated BLL is defined as greater than or equal to 10 µg/dL based on the CDC's Adult Blood Lead Epidemiology and Surveillance (ABLES) program [48]. Ancestry-informative markers are not available; thus, ethnicity was defined using a combination of self-reported information. Informed consent was obtained from all patients for participation in the study and access to their medical records.

Abbreviations: Acq, patients with acquired ADP; ADP, ALAD deficiency porphyria; ALA, δ-aminolevulinic acid; ALT, alanine aminotransferase; DTT, dithiothreitol; eALAD, erythrocyte δ-aminolevulinic acid dehydratase; NPDc, not performed during onset attack; PBG, porphobilinogen; PEPT2, peptide transporter 2; RBC´s, red blood cells; wt, wild type.

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hydroxyurea (10–20 mg/kg/day), initial trials of plasma or erythrocyte exchange, and opioid analgesia during acute exacerbation, prophylactic approaches failed to produce sustained clinical or biochemical improvement. ADP2's pain subsided for a year with near-complete neuromotor recovery but recurred with psychophysical stress, leading to severe motor deficits and dysphonia, making him wheelchair-dependent. ADP3 experienced more frequent and severe episodes of headache, nausea, and abdominal and limb pain than her brother, which were consistently accompanied by resistant hypertension. At 16 and 9 years of age, both siblings experienced severe neurovisceral attacks involving peripheral, central, and autonomic neurological dysfunctions, which significantly affected their quality of life.

Acq1 patient presented to the emergency care for abdominal pain. He indicated he was a nonsmoker, middle-aged Malian male bricklayer in Madrid, Spain. Despite normal findings on abdominal ultrasound and computed tomography (CT) angiography, he reported diffuse pain, nausea, weight loss, jaundice, and mild hypertension for 2 months. Blood analysis revealed anemia, hyperbilirubinemia (Table 1), normal haptoglobin levels (104 mg/dL), and slightly elevated reticulocyte levels (3%). Peripheral blood smear revealed acanthocytes, basophilic stippling, and spherocytes. Glucose-6-phosphate dehydrogenase (G6PD) deficiency was confirmed after negative autoimmunity studies and Coombs tests.

Elevated urinary levels of ALA and coproporphyrin III, normal urinary PBG excretion, and high blood lead levels (BLLs) suggested lead poisoning. In contrast to patients with ADP, the in vitro enzyme activity was fully restored by the addition of zinc and thiol reagents (DTT) to the incubation media (Table 1). These data are consistent with those of lead poisoning, which may be due to occupational exposure. Successful chelation therapy was performed using EDTA because contraindication of dimercaprol in persons with G6PD deficiency [22, 23].

Acq2 patient presented with recurrent lower abdominal pain with a medical history of dyslipidemia and hyperthyroidism. He had been a nonsmoker, 50-year-old Nepalese man residing in Murcia, Spain, for 15 years. Initial diagnostic evaluations, including gastroscopy, colonoscopy, CT, pelvic magnetic resonance imaging (MRI), and hepatitis serology, were unremarkable as well as autoimmunity tests. Blood tests revealed normochromic normocytic anemia with normal serum bilirubin levels. During hospitalization, the patient noticed reddish urine following a recent trip to Nepal, during which he had used a traditional herbal remedy for 2 weeks. At diagnosis, Acq2 had an extremely elevated BLL of 732 µg/dL (Table 1), with a follow-up of 722 µg/dL 15 days later. He was also diagnosed with hyperthyroidism (free T4: 2.70 ng/dL, normal range 0.8–1.8 ng/dL; TSH: 0.05 mIU/L, normal range 0.4–4.0 mIU/L), which was promptly treated. Hyperthyroidism can increase bone resorption in individuals with bone lead stores, releasing lead into the circulation and potentially exacerbating lead toxicity and porphyrin accumulation. Porphyria testing revealed elevated urinary ALA and porphyrin excretion, normal PBG levels, reduced eALAD activity (Table 1), and increased zinc-PROTO/free PROTO ratio (data not shown). These findings support a link between elevated BLL, the use of herbal remedies, and hyperthyroidism. Following cessation of exposure and therapy of hyperthyroidism (thiamazole/methimazole, 3 x 5 mg/day, Tirodril, Aldo-Union, Esplugues de Llobregat, Spain), BLL progressively decreased to 35.20 µg/dL within 5 months, falling below the threshold for chelation therapy.

Acq3 patient presented with intestinal pseudo-obstruction, arthralgia, and myalgia. He was a 45-year-old nonsmoker Indian male living in Barcelona, Spain. Abdominal CT scans were normal, but laboratory tests showed hyponatremia, normochromic normocytic anemia, and basophilic stippling on blood smears. Lead poisoning was also confirmed (Table 1). The patient received chelation therapy with intravenous calcium EDTA (500 mg twice daily for 5 days), followed by a succimer (DMSA) for 18 days. The protocol was followed until resolution of the condition was defined by the absence of symptoms and normalization of urinary ALA excretion levels. No sources of lead toxicity were identified. After ruling out occupational lead exposure, relatives were examined, which revealed elevated blood lead concentrations in his 32-year-old nonsmoking pregnant wife (Acq4). Although asymptomatic, with normal blood pressure and hemoglobin levels (Table 1), strict clinical monitoring was implemented, and scheduled delivery resulted in the birth of a healthy male. Pharmacological lactation inhibition was performed as a precautionary measure.

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Acq5 patient was admitted to the Internal Medicine Unit with diffuse abdominal pain, fever (38°C) and constipation. The patient was a 28-year-old Italian woman with an unremarkable medical history. Biochemical tests revealed a mild elevation in inflammatory markers (CRP, 6.5 mg/dL) and mild macrocytic anemia with vitamin B12 deficiency (Table 1). Further investigations, including abdominal ultrasonography, CT, MRI, celiac serology, and anti-parietal/intrinsic factor antibody, were negative, except for biliary sludge, for which ursodeoxycholic acid treatment (300 mg bid) was started. Gynecological evaluation using transvaginal ultrasonography revealed no significant findings. Elevated ALA levels and normal PBG and total urinary porphyrin levels suggested lead poisoning (Table 1). However, the BLLs (Table 1), zinc-PROTO/free PROTO ratio, and complete amino acid profile, including plasma succinyl acetone concentration (data not shown), were within the normal ranges. Fecal porphyrin and plasma porphyrin fluorescence tests were negative. Intravenous hydration with a 5% glucose solution and antibiotic therapy with amoxicillin–clavulanate were initiated, resulting in a progressive reduction in inflammatory markers and urinary ALA levels and symptom regression. Notably, eALAD activity remained reduced (41% of normal) 6 months after symptom resolution and normalization of inflammatory markers.

Four patients carried the PEPT2*1/*2 variant, corresponding to exon 13CT/exon 15CT (Table 1). Patient Acq2 carried PEPT2*3, corresponding to exon 13CC/exon 15TT, for which no data are currently available regarding affinity for ALA and renal prognosis [21].

In pediatric monogenic ADP cases, parents were continuously informed about the disease and its prognosis, and they provided written consent for publication of anonymized data. Adult Acq patients were also informed and gave consent for the analyses and tests performed during diagnostic work-up and treatment. They also consented to the potential anonymous publication of their results.

Genetic analyses

Total DNA was isolated from peripheral blood mononuclear cells using a standard phenol–chloroform procedure or an automatic Maxwell 16 instrument (Promega Corporation). Both erythroid-specific and housekeeping transcripts of the *ALAD* gene were analyzed using standard Sanger sequencing with previously reported primer sequences [24] on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) or via next-generation sequencing using SureSelect^{QXT} custom Target Enrichment (Agilent Technologies) on an Illumina MiSeq Sequencer. All variants were reported using the NM_000031.5. To analyze the rs1800435 polymorphism (Lys59Asn), a 916-bp sequence was amplified (forward primer: 5′-AGACAGACATTAGCTCAGTA-3′; reverse primer: 5′-GGCAAAGACCACGTCCATTC-3′) and treated with the MspI enzyme at 37°C for 2 h. Agarose gel electrophoresis revealed a C-to-G substitution resulting in 523 and 393 bp fragments. Exons 13 and 15 of the *PEPT2* (also known as *SLC15A2)* gene were amplified by PCR using previously described primers [21].

In vivo characterization of ALAD *variants*

The cDNA of human *ALAD* (*hALAD*; GenBank accession No. M13928.1, length cds: 990 bp) and the variant proteins were cloned into the pTRE2 vector (CLONTECH Laboratories, Inc.) under the control of a strong liver-specific promoter [25]. Using a hydrodynamics (HD)-based procedure [25], 50 µg of plasmids were transferred into hepatocytes via tail vein injection in 8-week-old C57BL/six mice. The mice were sacrificed 24 h post-HD. The procedure was approved by the Animal Care Committee of the University of Navarra (ethics committee approval #R-CP001-15GN) according to the European Council guidelines.

Successful transfection in all injected groups was confirmed through the detection of plasmid DNA copy numbers using iQ SYBR Green supermix and specific human *ALAD* primers (forward: 5′-GGGTTCCGCAGCTGACTCCGAGGA-3, reverse: 5′-CCACCTGACATCCTGCCTTGGCATACG-3′). Quantitative PCR (qPCR) was performed in an iQ5 real-time PCR detection system (Bio-Rad) under the following conditions: 35 cycles at 95°C for 15 s, 59°C for 30 s, and 72°C for 25 s, with the detection temperature set at 86°C. The melting curve confirmed amplification specificity. *ALAD* expression was measured using reverse transcription qPCR (RT-PCR). Total liver mRNA was extracted using TRIzol reagent and ALAD transcript levels were calculated as the fold change relative to

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(a)

siblings

	[ADP1] Germany (2013)$^{\text{ref. 7}}$	[ADP2] Italy	[ADP3] Italy
Sex / Age at first symptoms	male / birth	male / 8 yrs	Girl / 5 yrs
eALAD activity (% of control samples)	16 %	6 %	3 %
Allele 1	c.724G>A (p.Val242Ile)	c.440_441delinsTT (p.Arg147Leu)	c.440_441delinsTT (p.Arg147Leu)
Allele 2	c.839G>A (p.Gly280Glu)	c.440_441delinsTT (p.Arg147Leu)	c.440_441delinsTT (p.Arg147Leu)
In vivo expressed activity of alele 1	53.1 %	11.6 %	11.6 %
In vivo expressed activity of alele 2	9.4 %	11.6 %	11.6 %
Effect of IV heme on symptoms	Partial response	No response	No response

(b)

	Germany (1979)$^{\text{ref.11}}$	Germany (1979)$^{\text{ref. 11}}$	Sweden (1987)$^{\text{ref. 9}}$	Belgium (1989)$^{\text{ref. 10}}$	Germany (2004)$^{\text{ref. 8}}$	USA (2006)$^{\text{ref 5}}$	Netherlands (2019)$^{\text{ref. 6}}$	USA (2024)$^{\text{ref. 14}}$	Cape Verde (2024)$^{\text{ref. 13}}$
Sex / Age at first symptoms	Male / 15 yrs	Male / 15 yrs	Male / birth	Male / 63 yrs	Male / 15 yrs	Male / 12 yrs	Male / birth	Male / neonate	Male / 3 yrs
eALAD activity (% of control samples)	<1 %	<1 %	<2 %	1 %	10 %	<8 %	11 %	n/a	< 2 %$^{\#}$
Allele 1	c.718C>T (p.Arg240Trp)	c.457G>A, (p.Val153Met)	c.397G>A (p.Gly133Arg)	c.177G>C (p.Lys59Asn) & c.397G>A, p.Gly133Arg	c.114-11C>A	c.265G>A (p.Glu89Lys)	c.114-11C>T	c.397G>A (p.Gly133Arg)	n/a
Allele 2	c.820G>A (p.Ala274Thr)	c.818_819delTC	c.823G>A (p.Val275Met)	WT	c.114-11C>T	c.394T>C (p.Cys132Arg)	c.164+2T>A	c.415G>A (p.Gly139Arg)	n/a
In vitro expressed activity of alele 1	3.5 %	41 %	8.1 %	8.1 %	n/a	10 %	n/a	n/a	n/a
In vitro expressed activity of alele 2	19.3 %	0 %	1.2 %	100 %	n/a	4 %	n/a	n/a	n/a
Effect of IV heme on symptoms	Partial response	Partial response	No response	No response to heme (partial response to iv glucose)	Partial response	Inital good response	Partial response	n/a	Partial response

Fig. 1 ***Features of ADP cases documented through DNA studies***. *(a) Patients in the present study and (b) previously reported cases were identified by their location and the first published report. ALAD activity in erythrocytes and expressed in in vitro or our in vivo model for advanced molecular studies and response to heme administration is included. ADP, ALAD deficiency porphyria; ALAD, δ-aminolevulinic acid dehydratase; eALAD, erythrocyte ALAD; IV, intravenous administration. # personal communication.*

the internal control gene, *actin* (forward primer: 5′-CGCGTCCACCCGCGAG-3′, reverse primer: 5′-CCTGGTGCCTAGGGCG-3′, detection temperature of 88°C). The results were expressed according to the formula $2^{-(Ct\ \text{Actin}-Ct\ \text{gene})}$, where *Ct* represents the point at which the fluorescence rises appreciably above the background fluorescence. Finally, ALAD expression in the liver was analyzed using enzymatic activity and immunoblotting. To validate the new expression system, previously reported *ALAD* variants (F12L and C132R) showed reduced ALAD activity (5.6% and 8.9% of wild-type [WT] hALAD activity) when expressed in the livers of WT mice (Fig. 1a).

Biochemical assessments

ALAD activity was tested by incubating the ALA substrate with proband RBCs or liver homogenates from mice expressing human WT or *ALAD* variants. PBG production over 1 h was determined colorimetrically using Ehrlich's reagent as previously reported [26]. Restored enzymatic activity was measured after the addition of zinc and DTT to the incubation media [26]. To assess the thermal stability of the *ALAD* variants, they were preincubated at 48°C without substrate for up to 60 min, cooled on ice, and the ALAD activity was measured as described previously. Erythroid ALAD activity was expressed as a percentage of the

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activity in healthy controls used in each assay. For *ALAD* variants expressed in the mouse liver, the endogenous activity from sham-operated animals was subtracted from the results. To account for the variability in transfection efficiency between livers, enzymatic activity was normalized to plasmid abundance, as quantified by qPCR. A value of 1 corresponded to the plasmid amount in control animals.

Immunoblot analysis

Immunoblotting was performed using either a polyclonal rabbit IgG anti-ALAD antibody (HPA022124; Atlas Antibodies) or a polyclonal goat anti-ALAD antibody (sc-50779, Santa Cruz Biotechnology, Inc.). Liver tissue was homogenized in RIPA buffer, and protein concentration was measured after centrifugation. Extracts were mixed with sample buffer (30% glycerol, 6% SDS, 62.5 mM Tris HCl, pH 6.8, 0.01% Bromophenol Blue and 0.3 M DTT), boiled, and run on SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane, probed with antibodies, and detected using a Western Lightning Plus ECL kit. Band intensity was quantified using ImageJ software.

Native polyacrylamide gel electrophoresis (PAGE)

Liver tissue (50 mg) was lysed in buffer (50 mM Tris pH 6.8, 150 mM NaCl, 75 µL NP-40), homogenized, and incubated on ice for 20 min. Samples were centrifuged at 13,000 × *g* for 45 min at 4°C, and the supernatant was collected. The protein concentration was measured using the Bradford method. A 50 µg protein sample was mixed with loading buffer (1 M Tris pH 6.8, 40% glycerol, 2% bromophenol blue) and run on a 6% acrylamide native gel without SDS. Protein detection was performed as previously described.

Results

Molecular characterization of genetic ADP patients

Molecular analysis identified two base changes in patient ADP1: the c.839G > A (ALAD: p.Gly280Glu or p.G280E) variant in exon 10 inherited from the mother and the c.724G > A (ALAD: p.Val242Ile or p.V242I) variant in exon 11, which is present in the paternal genome (Fig. 1a and Fig. S2). Expression of the novel *ALAD* variants in the mouse liver showed that the p.V242I mutation retained 53% of the WT hALAD activity, whereas p.G280E showed a residual activity of approximately 9.4% (Figs. 1a and 2a). Immunoblot analysis of native-polyacrylamide gel electrophoresis (PAGE) showed that p.G280E ALAD predominantly migrated as hexamers, whereas the WT and p.V242I ALAD forms adopted an octamer conformation (Fig. 2b). Notably, the co-expression of p.V242I and p.G280E at a 50% ratio resulted in octameric (55%) and hexameric (45%) bands, whereas the co-expression of p.V242I at 80% predominantly yielded an octameric migration pattern (87%, Fig. 2b). However, the observed enzyme activity was lower than expected in both co-expression groups. For the 50% co-expression, the expected activity was 29.8 nmol PBG/mg prot/h (U), but only 11.4 ± 3.8 U were measured. Similarly, for the 80% p.V242I and 20% p.G280E co-expression, an activity of 16.05 ± 7.7 U was obtained, compared to the expected 42.2 U (Fig. 2c). These data suggest that the p.G280E monomer interferes with the p.V242I subunit during hetero-octamer formation. Finally, thermal stability analysis revealed that p.V242I was more stable than p.G280E. However, the heteroconformation likely present in the patient exhibited reduced stability, comparable to the p.G280E homomer (Fig. 2c).

Targeted sequencing of *ALAD* gene from relatives of Italian siblings (ADP2 and ADP3) showed two adjacent nucleotide substitutions at the same codon: the c.440G > T substitution causing a mutated p.Arg147Leu (R147L) protein and the c.441C > T substitution without affecting the p.Arg147Arg protein (Fig. 1a and Fig. S3). The simultaneous presence of both substitutions (c.440_441delinsTT) resulted in a mutated p.Arg147Leu protein.

Expression of the c.440G > T variant in the liver of mice was 9% of the WT enzyme activity (Figs. 1a and 2a). However, proteins with both c.440G > T and c.441C > T substitutions, which did not lead to any further reduction in activity. Finally, expression of the c.441C > T variant alone had no effect on the enzymatic activity (Fig. 3a), protein conformation (Fig. 3b), or thermostability (Fig. 3c), thereby confirming the pathogenic role of the c.440G > T substitution. Native-PAGE analysis of p.R147L suggested an intermediate migration pattern between the WT octamer (296 kDa) and the hexameric form of the p.G280E mutant protein (222 kDa), likely resulting from the octameric conformation of ALAD monomers containing a 28 amino acid deletion (Fig. 2d).

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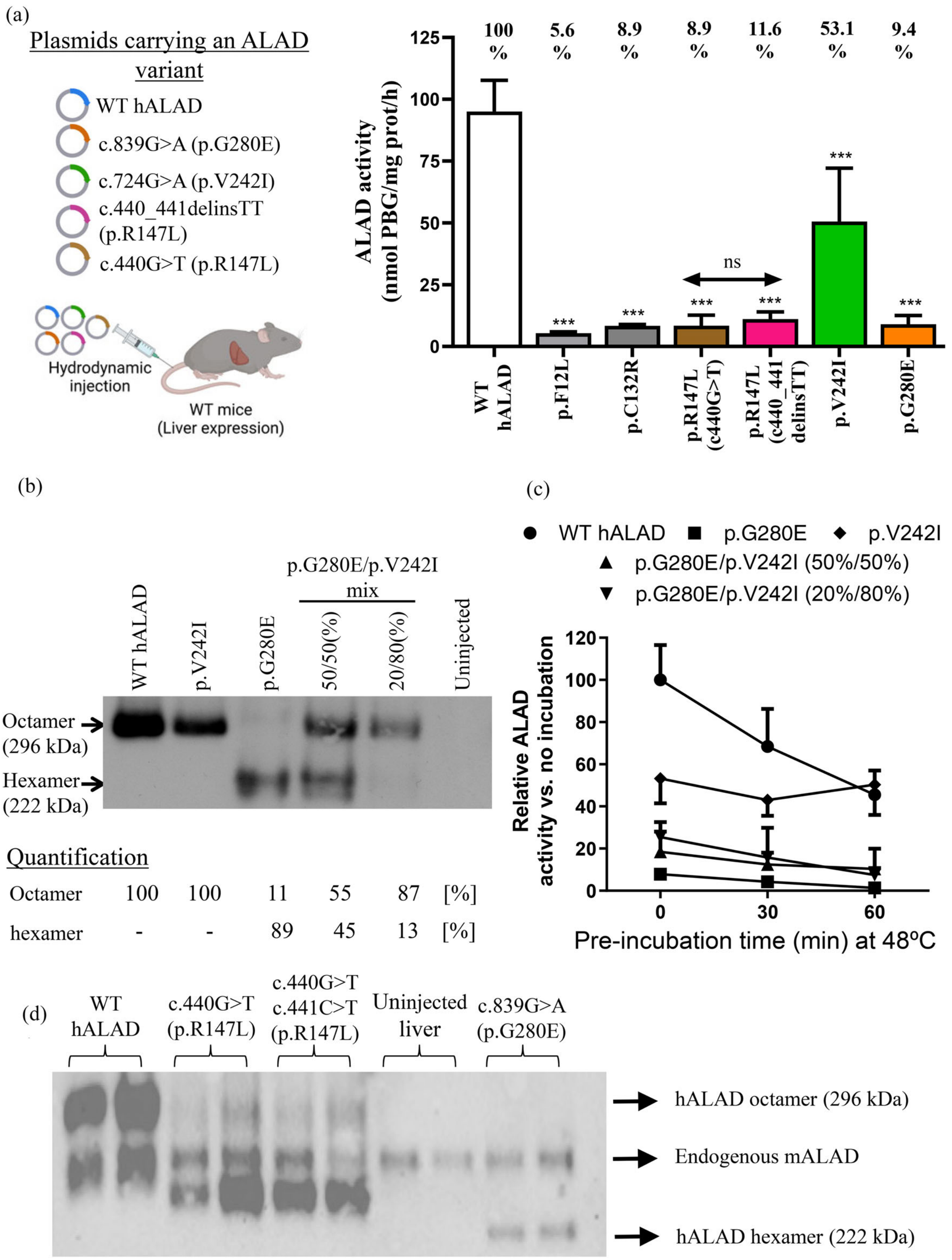

Fig. 2 ***Molecular characterization of novel ALAD variants expressed in mouse liver***. *(a) Study design of the hydrodynamic procedure (left). Mice were injected with plasmids expressing either the human ALAD protein or its variants. Specific ALAD activity was quantified as the relative increase over endogenous activity in the mouse liver (right). Additionally, plas-*

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Molecular characterization of the acquired ADP cases

Four symptomatic patients with acquired ADP shared the common c.931 + 66 T > C variant (rs1805313) in homozygous status, except for Acq5, which inherited this polymorphism in heterozygosity; three of these patients also carried additional common polymorphisms associated with heavy metal poisoning (c.165 − 196G > A, rs8177800; c.168T > C, rs1139488; c.398 − 34G > C, rs1805312). These variants, which are mostly located in introns or near the splicing sites, are likely to influence *ALAD* mRNA processing. In contrast, Acq4, which was asymptomatic despite a high BLL, exhibited heterozygosity for the rs1800435 polymorphism alone (c.177G > C), which resulted in the pLys59Asn ALAD variant being reported to have increased lead affinity (Table 2).

Functional characterization of the selected ALAD SNPs was performed in a mouse liver model. The c.168T > C (p.Tyr56 =) variant exhibited increased enzymatic activity compared to WT hALAD expressed in the mouse liver (Fig. 3a). The c.177 G > C (p.Lys59Asn) variant displayed activity comparable to that of the WT protein (Fig. 3a) but showed greater thermostability (Fig. 3b). Notably, in non-denaturing electrophoresis, the variant displayed an altered migration pattern compared to WT hALAD, along with a faint band with a migration profile similar to that of the p.G208E variant, which predominantly forms a hexameric quaternary structure (Fig. 3c). Both the c.414C > T (p.Asn138 =) and c.441C > T (p.Arg147 =) variants demonstrated activities and structural profiles similar to those of WT hALAD (Fig. 3).

Discussion

Our findings demonstrated the pathogenic role of the three novel variants found in patients with ADP, confirming the high molecular heterogeneity of the *ALAD* gene (Fig. 1a,b). Indeed, the “in vivo” expression confirmed a marked reduction in ALAD activity for the c.839G > A (p.Gly280Glu) pathogenic variant. Human ALAD has a TIM barrel fold with eight active sites on its surface [27]. Although this variant did not affect the catalytic, zinc-binding, or Lys263 (which forms a Schiff base linkage with the substrate) sites, substitution of the aliphatic amino acid glycine with a negatively charged glutamine side chain likely caused structural changes, simultaneously disrupting the conformation and activity of the octamer. Conversely, the c.724G > A variant, causing a substitution between two nonpolar aliphatic amino acids (from valine to isoleucine), resulted in high residual activity, high thermostability, and preservation of the octamer conformation. However, the reduced activity levels compared to the WT, along with the high residual activity of eALAD in the father (73%), strongly suggest that this variant acts as a hypomorphic allele. Notably, the co-expression findings confirmed that the G280E monomer exerted a dominant effect on the V242I monomer in the hetero-octamer structure, resulting in lower activity than expected.

Patients with ADP typically exhibit eALAD activity levels below 10% of the normal values. However, in the patient with ADP1, we observed a relatively high eALAD activity (16% of normal), comparable to that of his asymptomatic mother (25% of normal). Activity measurements were conducted 3 years after kidney transplantation when the reticulocyte count was within the normal range. Therefore, elevated eALAD activity is unlikely to be attributable to recent blood transfusions or increased enzyme levels from reticulocytosis. Despite this residual activity, the patient developed severe polyneuropathy and tetraplegia requiring mechanical ventilation alongside the onset of renal failure.

CKD was one of the most common long-term complications in AHPs [28]. The prevailing pathophysiologic model proposes that during AIP attacks,

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*mid abundance was quantified by qPCR to normalize enzymatic activity based on the level of plasmid transfection in each liver. (b) Immunoblot analysis of ALAD proteins (up) and quantification of octamer and hexamer forms (down) for the mutations identified in the German patient with ADP. (c) Thermal stability kinetics of ALAD proteins overexpressed in mouse livers. (d) Immunoblot analysis of ALAD proteins for mutations identified in the Italian patients with ADP. Statistical analyses were performed using GraphPad Prism (v6.01). To normalize variances, data were log-transformed using the formula log(1+X). Two-way ANOVA was then performed, followed by pairwise comparisons using Bonferroni’s Multiple Comparison Tests. Significance levels: ***p < 0.001 versus WT hALAD activity; *p < 0.05 and **p < 0.01 versus ALAD activity without preincubation time at 48°C. ADP, ALAD deficiency porphyria; ALAD, δ-aminolevulinic acid dehydratase; PBG, porphobilinogen; qPCR, quantitative PCR; RBC’s, red blood cells; WT hALAD, wild-type human housekeeping ALAD.*

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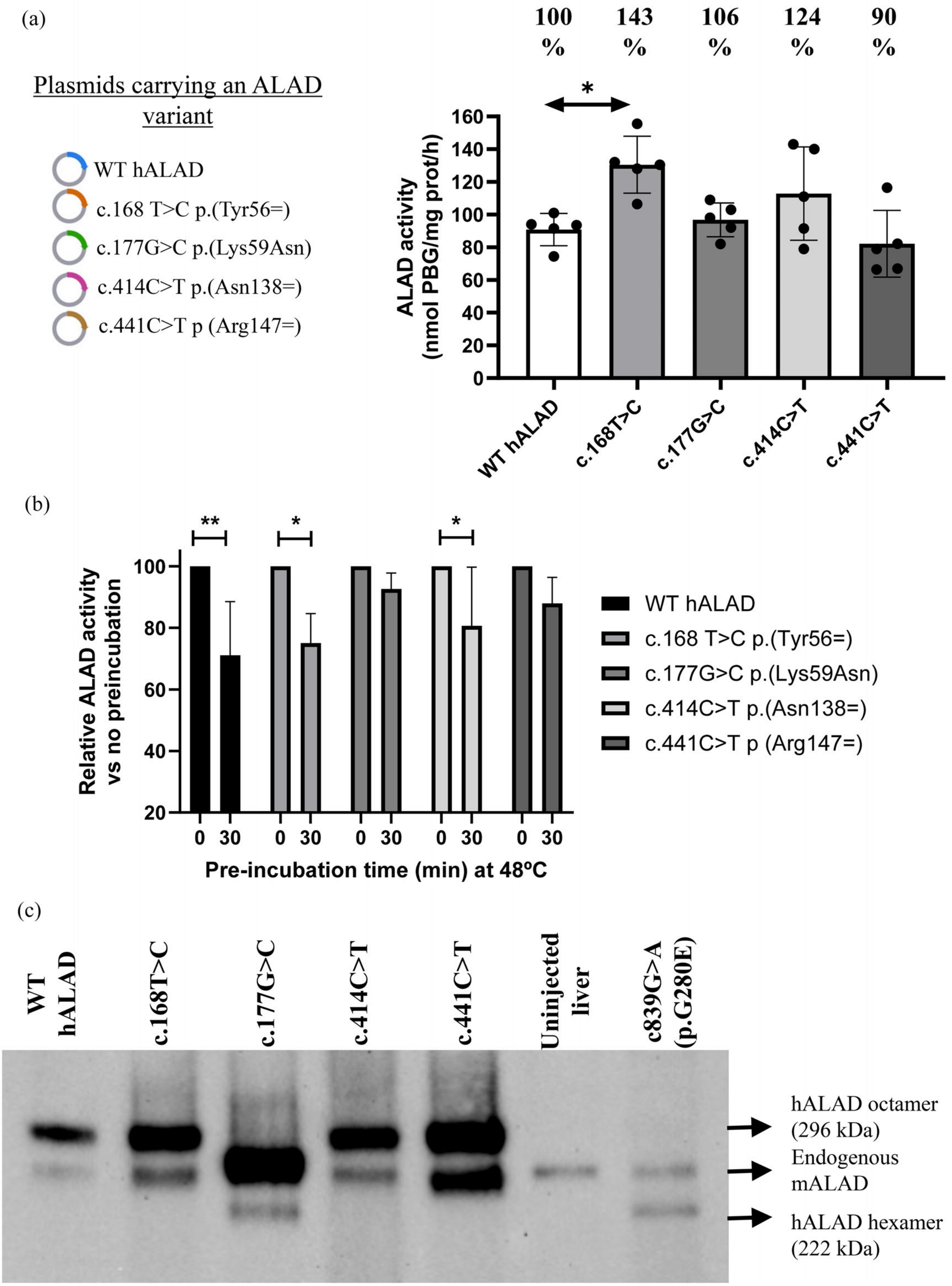

Fig. 3 ***Molecular characterization of ALAD SNPs expressed in mouse liver***. *(a) ALAD activity quantified as the relative increase over endogenous liver activity. (b) Thermal stability profiles of the SNP variants after preincubation at 48°C for 30 min. (c) Immunoblot analysis of ALAD proteins to assess octameric and hexameric forms. Statistical analyses were*

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excessive urinary excretion of ALA and PBG leads to tubular toxicity, typically manifesting later in life (≥55 years), with an annual decline in eGFR of >1 mL/min/1.73 m^2 [21]. Notably, carriers of the high-affinity *PEPT2*1/*1* variant have poorer renal outcomes than those with lower-affinity alleles [21]. However, patients with ADP1 carry the *PEPT2*1/*2* genotype, for which no data are currently available regarding renal prognosis. Interestingly, the patient developed CKD during adolescence, suggesting that the renal failure in this case was unlikely to be secondary to ALA-mediated tubular toxicity. Instead, CKD may impair ALA excretion, thereby exacerbating porphyria symptoms due to serum ALA accumulation. This aligns with evidence from dominant AHPs, in which renal impairment can trigger the recurrence of acute attacks and necessitate combined liver and kidney transplantation [29, 30].

In an AHP mouse model, renal insufficiency worsened porphyria attacks by increasing hepatic ALAS1 expression and reducing ALAD activity [26]. Similarly, decreased eALAD activity has been reported in experimental renal failure models [31] and patients with chronic renal failure [30]. These findings suggest that impaired renal function may have contributed to the severe clinical course in the ADP1 patient, despite eALAD activity exceeding 10%, a level below which ADP is typically diagnosed.

This hypothesis is further supported by the patient's increasing frequency of acute attacks and hospitalizations as CKD progresses as well as by the clinical improvement following initial hemodialysis and subsequent kidney transplantation, but not with hemin, which primarily targets hepatic heme synthesis. Notably, acute porphyria attacks have been successfully managed with hemodialysis in patients with syndrome of inappropriate antidiuretic hormone secretion [32] and in patients with AHPs from countries where hemin is not readily available [33].

Moreover, of the c.440G > T and c.441C > T adjacent nucleotide substitutions inherited from homozygous Italian siblings (ADP2 and ADP3), only the former resulted in a marked reduction in ALAD activity. The substitution of a basic amino acid, arginine, for the nonpolar hydrophobic amino acid, leucine, could result in structural changes in the ALAD protein, resulting in an intermediate migration pattern between the WT octamer and the hexameric form. However, severe protein dysfunction due to the activation of an alternative splice site, causing a 28-aa deletion, can also be hypothesized. Indeed, unexpected splicing of mRNAs expressed in human cDNAs, resulting in protein isoforms, has already been reported in transgenic plants and transiently transfected cells [34]. Moreover, sequence analysis using the human splicing finder (HSF) tool showed that both substitutions may activate an alternative donor splice site or cause a significant alteration of auxiliary sequences, potentially leading to the exclusion of an 84 bp exon (28 aa) while maintaining the reading frame of the protein.

Furthermore, the *PEPT2*1/*1* haplotype in patients with ADP2 and ADP3 may contribute to the phenotypic expression of ALA neurotoxicity. This high-affinity haplotype facilitates efficient ALA efflux across the blood–brain barrier, potentially correlating with less severe neurological impairment, as previously proposed [35].

Interventions targeting the hepatic heme biosynthesis pathway, such as hemin in ADP1 and hemin or givosiran in ADP2 and ADP3, result in only a limited reduction in ALA levels and minimal clinical improvement. These therapies downregulate hepatic ALAS1 and reduce urinary ALA excretion in dominant AHPs. The persistence of elevated ALA levels suggests that hemin does not repress erythroid ALAS2 and that any potential erythroid effect of givosiran is limited by its predominant hepatic tropism. Similar findings were observed in a patient with ADP who underwent a 3-day therapeutic challenge with heme arginate [13]. Although urinary ALA levels decreased by 35% after the first injection, ALA concentrations in the CSF and RBC remained unaffected. Likewise, a previously reported case of ADP showed persistent

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*performed using GraphPad Prism (v6.01). To normalize variances, data were log-transformed using the formula log(1+X). Two-way ANOVA was then performed, followed by pairwise comparisons using Bonferroni's Multiple Comparison Tests. Significance levels: *$p < 0.05$ versus WT hALAD activity; *$p < 0.05$ and **$p < 0.01$ versus ALAD activity without preincubation time at 48°C. ALAD, δ-aminolevulinic acid dehydratase; mALAD, murine ALAD; PBG, porphobilinogen; WT hALAD, wild-type human housekeeping ALAD.*

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Table 2. *Molecular characterization of the ALAD gene in lead poisoning patients.*

Localization of nucleotide change	Exon	ALAD Polymorphism	Minor allele frequency in control population	Acq1	Acq2	Acq3	Acq4	Acq5
c. 165–196G > A p. (?)		rs8177800	0.06	wt/wt	Heterozygous	Homozygous	wt/wt	wt/wt
c.177G > C p.(Lys59Asn)	Exon 4	rs1800435	0.08	wt/wt	Heterozygous	wt/wt	Heterozygous	wt/wt
c.931 + 66 T > C p.(?)		rs1805313	0.35	Homozygous	Homozygous	Homozygous	wt/wt	Heterozygous
c.414C > T p.(Asn138=)	Exon 6	rs2228083	0.11	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
c.168 T > C p.(Tyr56=)	Exon 4	rs1139488	0.36	wt/wt	wt/wt	wt/wt	wt/wt	Heterozygous
c.398 − 34 G > C p.(?)		rs1805312	0.08	wt/wt	wt/wt	wt/wt	wt/wt	Heterozygous

Abbreviations: Acq, patients with acquired ADP; ADP, ALAD deficiency porphyria; ALAD, δ-aminolevulinic acid dehydratase; Wt, wild type.

excretion of ALA and coproporphyrin, even after liver transplantation [36]. One month postoperatively, the child experienced recurrent attacks followed by progressive neuromuscular deterioration and generalized paralysis, including involvement of the respiratory muscles, underscoring the partial role of the liver in the pathogenesis of the disease.

A separate case of late-onset ADP has been reported in a 63-year-old man with myeloproliferative syndrome [37]. Genotype analysis revealed that the patient carried two variants in one allele, whereas the other allele was normal. The onset of ADP symptoms occurred later in life, presenting as “acquired ADP,” presumably driven by clonal expansion of erythroid cells harboring the mutant allele [38]. As suggested by previous studies, the pathophysiology of ADP involves a significant erythropoietic contribution, and combined therapies targeting both the erythroid and hepatic heme pathways may offer therapeutic benefits. Notably, partial suppression of erythroid heme synthesis through blood transfusion and hydroxycarbamide in combination with hemin therapy resulted in symptomatic improvement in a middle-aged man [6]. However, in ADP2 and ADP3, oral hydroxyurea aimed at suppressing erythroid proliferation and initial trials of plasma or erythrocyte exchange to reduce circulating ALA precursors failed to improve the disease. Taken together, these data suggest a complex and case-to-case variable pathophysiology for this ultrarare porphyria, which is associated with profound enzyme deficiency in all cells of the body [36, 39].

Here, we identified SNP variations potentially linked to an increased lead poisoning risk in five adult patients presenting with Acq-ADP. In four of these patients, erythrocyte ALAD activity was fully restored by the addition of zinc and thiols (DTT) to the incubation medium, highlighting the dependence of the enzyme on zinc and its sensitivity to—SH group-blocking agents, such as lead or heavy metals. Elevated BLLs typically result from chronic exposure [15, 17, 18, 40, 41], with approximately 99% of the lead bound to ALAD in erythrocytes [42]. Symptoms correlate with BLLs; most patients are asymptomatic below 30 µg/dL, whereas levels over 100 µg/dL can cause headaches and abdominal pain [43]. Lead also diffuses into soft tissues (kidneys, brain, and liver) before accumulating in bones, teeth, and hair. Common laboratory findings include normocytic or microcytic anemia, basophilic stippling, Burton’s lines on the

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gums [44], and progressive kidney or liver dysfunction. Genetic predisposition may play a clinically significant role in heavy metal toxicity. Epidemiological studies of a cohort of lead-exposed workers have identified *ALAD* polymorphisms that may influence individual susceptibility to lead toxicity. Among the SNPs detected, intronic rs1805313 [45] was the most prevalent in our cohort; three individuals (Acq1–Acq3) carried it in a homozygous form, and one (Acq5) carried it in a heterozygous form. Located at position c.931 + 66 T > C, this SNP contains a non-conserved nucleotide (phyloP: −0.27 [−19.0, 10.9]). The allele frequency was lower in the African population (52.14%) than in the European population (66.24%), with no data available for the South Asian population. In terms of its clinical significance, the rs1805313 variant was classified as benign using ClinVar (ref: 1180338). Additionally, the HSF tool predicted no significant impact on the splicing signals affecting exon 11/12.

Another intronic polymorphism, rs8177800, which is commonly found in patients with lead poisoning [45], was identified in both Acq2 and Acq3. It was classified as benign using ClinVar (Ref: 1242974). Predictive tools, including SSF, MaxEntScan, NNSplice, and GeneSplicer, indicated that this variant does not create a novel splice site that is stronger than the natural splice site; however, the HSF tool predicts the activation of a cryptic acceptor site affecting exon 3/4 splicing.

Additionally, the intronic polymorphism rs1805312 was identified in the Acq5. This variant involves a non-conserved nucleotide (phyloP score: −0.87 [range: −19.0 to 10.9]). The allele frequency was lower in the European population (8.04%) than that in the global population (10.91%). The functional significance of rs1805312 remains unclear [46], although it is classified as benign, consistent with ClinVar (Ref: 1178261) annotations, and no effect on splicing was predicted.

In addition to the benign intronic variants rs1805313 and rs1805312, patient Acq5 carried the rs1139488 polymorphism in exon 4. This variant involves a non-conserved nucleotide (phyloP: −0.15 [−19.0, 10.9]). The c.168 T > C substitution is synonymous, resulting in p.(Tyr56 =), and is found in approximately 36% of the global population. Codon usage biases can influence both the transcription and translation efficiencies. Notably, the frequency of the variant TAC codon (coding for Tyr) was higher than that of the reference TAT codon in both human (15.6 vs. 12, respectively) and mouse (16.5 vs. 12.2, respectively) genomes. Consistent with this, experimental data showed that liver expression of the c.168 T > C variant in mice yielded higher ALAD activity per unit of the transferred DNA plasmid.

Although rs1139488 is classified as benign in ClinVar (Ref: 364653), computational predictions suggested the activation of a cryptic donor splice site at exon 4 (c169), with MaxEnt and GeneSplicer indicating increases of +148.7% and +244%, respectively. However, as *ALAD* cDNA was used for expression, the potential impact of this base change on splicing could not be assessed in our model. Further studies are warranted to explore whether rs1139488 influences splicing and alters enzyme activity.

Notably, elevated ALT and bilirubin levels in Acq5 suggest increased susceptibility to factors, such as infections or chronic inflammation, potentially mimicking a mild acute attack of dominant porphyria. Targeted sequencing of the *ALAD* promoter in this patient revealed the c.−9C > G (rs1771220) variant in heterozygosity, which, according to GTEx data, significantly reduces ALAD expression (slope ≈ −12.99) and function as eQTL in whole blood. This promoter variant, alone or in combination with the rs1139488 polymorphism, may reduce ALAD activity and increase the susceptibility to triggering factors that exacerbate acquired ADP symptoms.

Acq2 and Acq4 are heterozygous carriers of the rs1800435 variant, which is the only SNP that results in an amino acid substitution (pLys59Asn) [18]. This variant, also known as ALAD2, involves a weakly conserved nucleotide (phyloP: 1.93 [−19.0, 10.9]). The allele frequency was higher in the South Asian population (14.587%) than in the global population (8.27%). Some authors have suggested that the ALAD2 isoform has a higher lead affinity than the more common ALAD1 allele. Consequently, individuals with ALAD 1–2 or 2–2 genotypes may experience a protective effect, as the enzyme can sequester lead and mitigate oxidative organ damage [47]. Regarding clinical significance, it is classified as benign or likely benign in ClinVar (ref: 16864), but both Benign and Pathogenic classifications appear in the dbSNP (rs1800435). The AAG codon (Lys) was more frequent than the AAC (Asn) in both human (32.9 vs. 19.5, respectively) and mouse (33.7 vs. 20.7, respectively)

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livers. However, in our experimental model, the enzyme activity was comparable to that of the WT protein but showed greater thermostability. The high lead affinity of ALAD2 isoform could explain the asymptomatic presentation in Acq4, despite exhibiting elevated BLL.

Conclusions

Using an innovative mouse liver model, we functionally characterized three novel *ALAD* pathogenic variants identified in three patients with genetic ADP, including the first reported female patient. In addition, we found that the common intronic variant rs1805313 was the most prevalent among patients with acquired ADP (four of five patients). Computational analyses predicted mRNA splicing alterations for the intronic variant rs8177800 and the synonymous variant rs1139488, both of which were present in three of the five patients. Furthermore, two patients carried the common variant rs1800435, which involves both a nucleotide change and an alteration in the quaternary protein structure that may increase susceptibility to environmental or physiological triggers, exacerbating acquired ADP symptoms. Taken together, these findings highlight the molecular heterogeneity of the *ALAD* gene and underscore the need for further studies investigating potentially abnormal *ALAD* isoforms.

Author contributions

Daniel Jericó, Ana Sampedro, Francesco Urigo, and Antonio Fontanellas designed and performed the experiments and processed animal samples. Isabel Solares, Francisco J. Castelbón, Javier Tomás Solera, Antoni Riera-Mestre, María Barreda-Sánchez, Carlo Poci, Annamaria Nicolli, Rafael Enríquez de Salamanca, Matteo Marcacci, Pauline Harper, Marta G. Fanlo-Maresma, Encarna Guillén-Navarro, Giovanna Graziadei, Bodo B. Beck, Paolo Ventura, and Montserrat Morales-Conejo managed patients with ADP and lead poisoning. Elena Di Pierro, Daniel Jericó, Ana Sampedro, Francesco Urigo, Bodo B. Beck, Andrea Wenzel, and Antonio Fontanellas determined *ALAD* and WB in patient samples and performed genetic analysis. Isabel Solares, Francisco J. Castelbón, Rafael Enríquez de Salamanca, Marta G. Fanlo-Maresma, Encarna Guillén-Navarro, Bodo B. Beck, Montserrat Morales-Conejo, and Antonio Fontanellas created and designed the original manuscript. Elena Di Pierro, Rafael Enríquez de Salamanca, Pauline Harper, and Montserrat Morales-Conejo wrote the manuscript, assisted by Daniel Jericó and Francesco Urigo for the Figs. and tables. All authors critically revised the manuscript for important intellectual content and approved the final version.

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Conflict of interest statement

The authors declare no conflicts of interest.

Data availability statement

Data supporting the findings of this study are available from the corresponding author upon request. Additional Supporting Information can be found in the online version of this article.

Prior presentation

Presented at the International Congress of Porphyrins and Porphyrias, September 21–25, 2024, Pamplona, Spain.

Patient consent statement

Informed consent for the use of these data was obtained from the patients, or their parents in the case of children.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Urinary ALA and PBG excretion over time in ADP1. Urinary ALA and PBG excretion during progressive peripheral neuropathy and renal failure. Hemodialysis frequency was increased to six sessions per week in an effort to reduce circulating ALA levels. However, this intervention was insufficient to prevent neurological deterioration. By December 2009, the ADP1 patient required mechanical ventilation and developed flaccid tetraplegia. Following a successful kidney transplant in June 2010, the proband experienced only minor flares, none requiring hospitalization.

Figure S2: Erythrocyte ALAD (eALAD) activity and genetic analysis in the family of the ADP1 patient. (A) Analysis of eALAD activity in the proband, mother, and father. (B) DNA sequence chromatograms illustrating compound heterozygous mutations in the ALAD gene of the proband. The c.839G > A (p.Gly280Glu, G280E) variant in exon 10 was inherited from the mother, whereas the c.724G > A (p.Val242Ile or V242I) variant in exon 11 was inherited from the father. Wild-type sequence chromatograms are shown in black, with mutation position highlighted in red below the WT sequence. The corresponding amino acid sequence is displayed above the DNA sequence.

Figure S3: NGS analysis in the ADP2 and ADP3 cases. The variant calling shows the reference sequence and the variant alleles in red. Both patients present two adjacent nucleotide substitutions (c.440G > T and c.441C > T) in homozygosis. The two variants are inherited in cis from both consanguineous parents. The resulting genotype in the family is also presented. ■

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