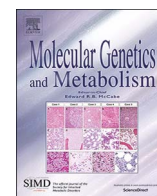




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Pyridoxine-5'-phosphate oxidase (Pnpo) deficiency: Clinical and biochemical alterations associated with the C.347g > A (P.Arg116gln) mutation

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ABSTRACT

Background: Pyridoxal-5'-phosphate oxidase (PNPO) deficiency presents as a severe neonatal encephalopathy responsive to pyridoxal-5'-phosphate (PLP) or pyridoxine. Recent studies widened the phenotype of this condition and detected genetic variants on PNPO gene whose pathogenic role and clinical expression remain to be established.

Objective: This paper aims to characterize the functional effects of the c.347G > A (p.Arg116Gln) mutation in the PNPO gene in order to define its pathogenicity and describe the clinical features of new patients with epilepsy carrying this mutation.

Methods: Arg116Gln protein variant was expressed as recombinant protein. The mutant protein was characterized with respect to structural and kinetic properties, thermal stability, binding constants of cofactor (FMN) and product (PLP). We also reviewed clinical data of 3 new patients carrying the mutation.

Results: The Arg116Gln mutation does not alter the overall enzyme structure and only slightly affects its catalytic efficiency; nevertheless, this mutation affects thermal stability of PNPO, reduces its affinity for FMN and impairs transfer of PLP to PLP-dependent enzymes.

Three boys with seizure onset between 8 months and 3 years of age, carrying the Arg116Gln mutation, are described. These three patients exhibited different seizure types associated with interictal EEG abnormalities and slow background activity. Mild/moderate intellectual disability was observed in 2/3 patients. A dramatic therapeutic response to pyridoxine was observed in the only patient who still had active seizures when starting treatment, while in all three patients interictal EEG discharges and background activity improved after pyridoxine treatment was initiated.

Conclusions: The reported data support a pathogenic role of the c.347G > A (p.Arg116Gln) mutation in PNPO deficiency. The later onset of symptoms and the milder epilepsy phenotype of these expand the disease phenotype.

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1. Introduction

Pyridoxine-5'-phosphate oxidase (PNPO) deficiency (OMIM#6032870) was originally reported as a severe life-threatening neonatal encephalopathy responsive to pyridoxal-5'-phosphate (PLP) but not pyridoxine (PN) [1–3]. Its phenotype included epileptic encephalopathy with suppression burst on electroencephalogram (EEG), drug-resistant neonatal seizures, prematurity, neonatal respiratory distress, dystonia, hepatomegaly, and metabolic acidosis [3]. Recent studies widened the age at clinical onset from 30 min to 5 months and detected phenotypic variants responding to pyridoxine [4–9].

To date, 18 pathogenic mutations of the PNPO gene and less than 40 patients have been reported in the literature [5,7,10]. In this context, a controversial role is still played by the c.347G > A (p.Arg116Gln) mutation. Its frequency varies in different populations (with a range of 4–10% depending on the subpopulation investigated) and it is higher in PNPO deficient patients if compared to the general population [5]. The c.347G > A (p.Arg116Gln) mutation was found in 1000 genomes (<http://www.ensembl.org>) with low frequency (A allele frequency 0.034; AA genotype frequency 0.004) and bioinformatic tools (SIFT, Polyphen-2, ConSeq v1.1, Mutation Taster, Meta SNP, ESE finder) provide discordant indications about its pathogenicity [5].

Here we describe the peculiar epilepsy phenotypes in three new patients carrying this mutation and the biochemical data supporting its pathogenic effects.

2. Materials and methods

We identified three patients with epilepsy carrying the c.347G > A (p.Arg116Gln) mutation. Data on clinical presentation, developmental milestones, EEG features, neuroimaging and treatment were collected from parents, medical records, and referring physicians.

The c.347G > A (p.Arg116Gln) mutation was identified through Sanger sequencing in Patient 1 and through a Next Generation Sequencing (NGS) panel including 95 genes associated with epilepsy in Patients 2 and 3 [11,12].

The Arg116Gln variant form of human PNPO was generated and expressed as recombinant protein. Wild type and mutant enzymes were purified through a modified version of a previously described protocol [13]. The mutant protein was characterized by size-exclusion chromatography, spectrophotometric and fluorimetric analyses in order to determine its quaternary structure, thermal stability, kinetics, dissociation constants for flavin mononucleotide (FMN) and PLP binding, and properties of PLP non-catalytic site. For all experiments, the mutant form was compared to freshly purified wild type human PNPO. Experimental procedures for the purification, biochemical characterization and molecular modeling of Arg116Gln PNPO mutant are described in Ref. [14].

3. Results

3.1. Clinical phenotypes associated with the mutation c.347G > A (p.Arg116Gln)

3.1.1. Patient 1

This boy was admitted at the emergency department, due to focal clonic status epilepticus, at the age of 20 months. His previous neurological development was unremarkable. Seizures were interrupted by intravenous (IV) phenobarbital after three unsuccessful IV administrations of midazolam. In the following days the patient remained seizure-free on oral phenobarbital (5 mg/kg/day). Interictal sleep EEG recorded bilateral spikes, and slow waves in the fronto-temporal regions. Brain MRI was normal. PNPO gene sequencing was performed within a research project that aimed to study the possible role of its pathogenic mutations in epilepsies with onset before 24 months of life. After identifying the c.347G > A (p.Arg116Gln) mutation we started oral

pyridoxine (PLP is not commercially available in Italy) at 6.25 mg/kg/day.

In the following months, brief episodes of staring were observed, which promptly remitted after pyridoxine increase to 12.5 mg/kg/day. At age 24 months, phenobarbital was suspended. At age 28 months, psychomotor development was normal. At age 35 months, three prolonged focal clonic seizures with secondary generalization occurred. Pyridoxine was further increased up to 16.5 mg/kg/day and no further seizures occurred. At last evaluation, at 3 years 7 months, developmental milestones remained normal, while interictal sleep EEG showed diffuse spikes and polyspikes and wave discharges with posterior predominance.

3.1.2. Patient 2

This 12-year-old boy had normal developmental milestones before the onset of multiple per day myoclonic-astatic and generalized tonic-clonic seizures at the age of 3 years 2 months. Initial EEGs revealed slowing of background activity with generalized spike and wave discharges. In the following months, psychomotor stagnation was observed and at the age of 7 years cognitive testing revealed moderate intellectual disability (WISC-IV, Full scale IQ: 44), associated with attention deficit and hyperactive behavior. Brain MRI was normal. Since age 8 years multiple absences per day, and tonic or tonic-clonic seizures during sleep with a frequency of 1–2 seizures per week were observed, despite treatment with valproic acid and ethosuximide. Repeat EEG recordings showed slow background activity and multiple spike and wave discharges lasting up to 5–6 s (Fig. 1A,B). At age 11 years seizures were controlled by a polytherapy including valproic acid, ethosuximide and rufinamide. Moderate intellectual disability was confirmed at age 11 years (WISC-IV Full scale IQ: 40). At 12 years, after the PNPO gene alteration was detected by NGS, pyridoxine was added at 10 mg/Kg/day without further seizures and complications. At last evaluation, at 12 years 6 months, cognitive level remained stable (WISC-IV Full scale IQ: 44), but EEG was remarkably improved, with normal background and no discharges (Fig. 1C).

3.1.3. Patient 3

This 7 year old boy had an unremarkable family and perinatal history. Since age 8 months, sporadic seizures with generalized stiffening lasting up to one minute were observed, which subsequently appeared in clusters during a single febrile episode. Treatment with valproic acid was started. From the second through the seventh year of life an average of three seizures per year occurred, again as generalized stiffening, while falling asleep. Brain MRI was normal at age 5 years. Interictal EEG recordings, from epilepsy onset to the age of 6 years 6 months, revealed slowing of background activity and an association of focal and generalized spike and wave discharges (Fig. 2A). Language delay with phonological errors was evident since age 3 years, and mild intellectual disability was uncovered at 6 years (WISC IV Full scale IQ: 64). After detecting the PNPO gene mutation, pyridoxine was added at 10 mg/Kg/day. At last follow-up, at 7 years of age, EEG revealed normal background activity with no epileptiform abnormalities (Fig. 2B). Cognitive testing confirmed mild intellectual disability, with an improvement of the Verbal Comprehension Index (from 65 to 75).

3.2. Biochemical and molecular studies on the Arg116Gln PNPO protein variant

3.2.1. Arg116Gln mutation does not alter the overall structure of the enzyme and slightly affects the catalytic efficiency of human PNPO

The crystal structure of human PNPO in complex with cofactor FMN and product PLP [15] shows residue Arg116 to be located at the N-terminus end of an α -helix segment, whose positive dipole charge might stabilize the negative charge of the phosphate moiety of FMN cofactor inside the active site of the protein. Moreover, the positive guanidinium moiety of Arg116 is at fair close distance from the phosphate FMN

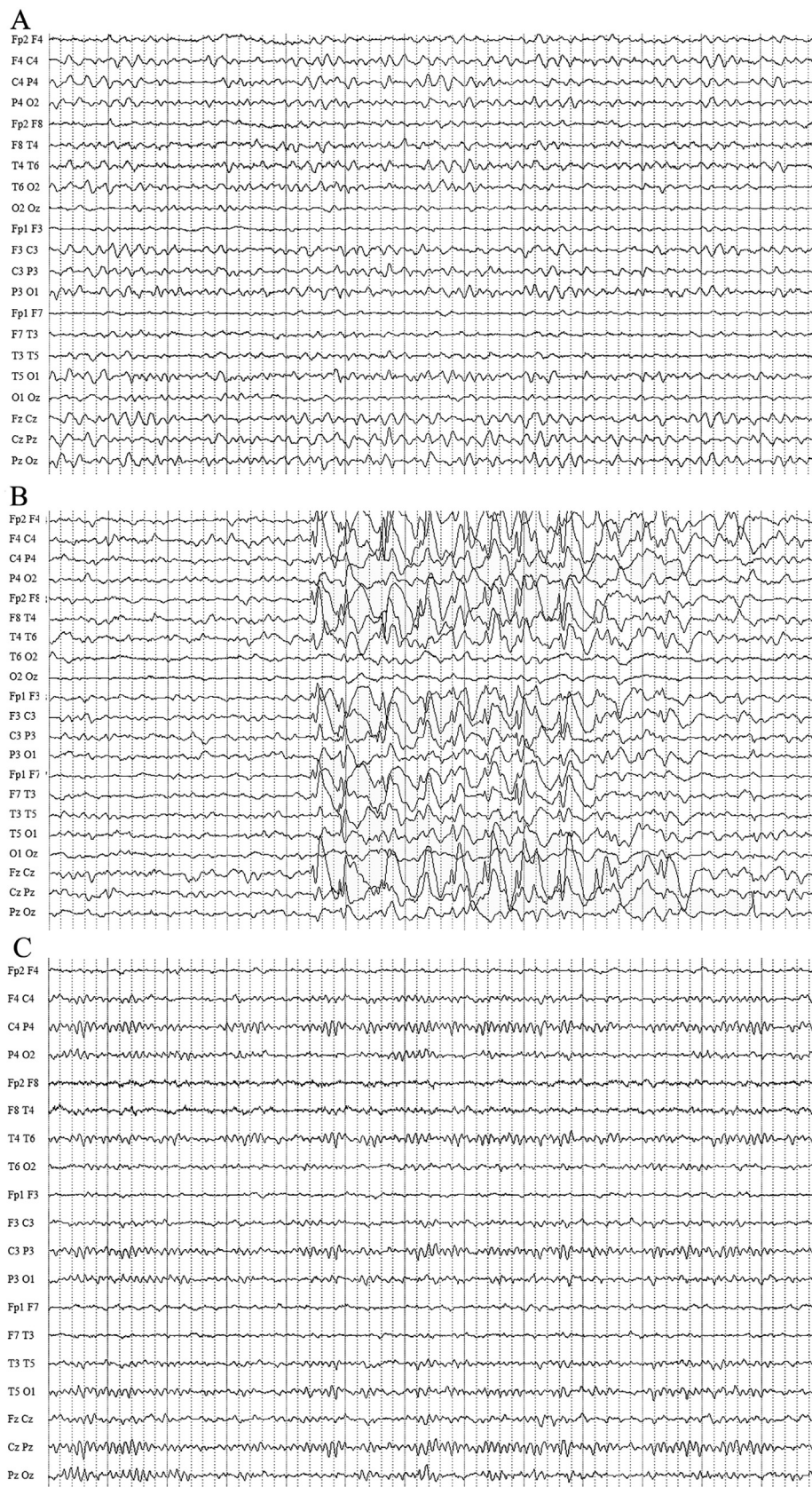


Fig. 1. EEG recording of Patient 2 performed at age of 8 years (A and B), shows A) slow and monomorphic background activity at 4–5 Hz; B) generalized spike and wave discharges. A follow-up recording performed at 12 years (C), shows normal background activity, with no discharges.

group (about 4.9 Å). On the other hand, Arg116 also makes an ion-pair interaction with the neighboring Glu143 residue (average distance about 3 Å), coming from the other chain of the dimeric structure (Fig. 1 in Ref. [14]). A previous study of enzyme efficiency with a HeLa cell

expression system showed the Arg116Gln mutation to result in a ~20% reduced level of PNPO catalytic activity [5]. To specifically understand how this mutation affects PNPO functionality, the Arg116Gln mutant was expressed and purified as recombinant protein. The enzyme is



Fig. 2. EEG recording of Patient 3 performed at age of 6 years A) before pyridoxine treatment shows slow background activity and generalized spike and wave discharge; A follow-up recording performed at 7 years B), shows normal background activity with no epileptiform abnormalities.



yellow, exhibiting absorption maxima typical of FMN-binding proteins (Fig. 2 in Ref. [14]). The $A_{278}:A_{448}$ ratio of the pure enzyme is 6.85, slightly higher than the one observed for wild type PNPO (5.65) [15], but still in the average range of other FMN-binding PNP oxidases [16], indicating that the enzyme active site is almost fully saturated with cofactor. The apo-form of the mutant enzyme, as for the wild type, is

colorless and shows no absorption above 300 nm (data not shown).

Size-exclusion chromatography showed mutant and wild type enzymes to exhibit similar elution profiles (Fig. 4A in Ref. [14]) with a single peak corresponding to an apparent molecular mass of 62 kDa. This matches to a dimeric quaternary structure of the enzyme. Far-UV CD spectra (Fig. 4B in Ref. [14]) revealed only minor differences in the

Table 1

Kinetic constants (K_m , k_{cat}) and dissociation constants (K_d) for FMN and PLP binding to wild type and Arg116Gln mutant human PNPO. Each experimental value is the estimate from three independent data collections on a same protein sample. Data were globally analyzed by non-least square fitting to the relevant equations, described in the [Material and Methods section](#). Mean and standard deviation values are those obtained from the fitting of experimental data. For all fittings, the R-square value was higher than 0.96.

Enzyme form	K_m (PNP) (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\cdot\text{mM}^{-1}$)	K_d (FMN) (nM)	K_d (PLP) (nM)	T_m ($^{\circ}\text{C}$)
Wild type	2.0 ± 0.1	3.5 ± 0.04	1.75	13.1 ± 1.7	136 ± 23	63.2 ± 0.2
Arg116Gln	3.1 ± 0.2	2.2 ± 0.03	0.71	251.3 ± 40.8	143 ± 11	57.0 ± 0.3

region between 225 and 235 nm, possibly indicating small structural alterations that might involve secondary structures, mainly α -helices. From these results, it can be concluded that the mutation did not affect the oligomerization state nor the overall structural integrity of the enzyme. Enzymatic activity measurements of wild type and Arg116Gln mutant were carried to determine K_m and k_{cat} values (Table 1 and Fig. 3 in Ref. [14]). The results show that the k_{cat} of Arg116Gln is 1.6-fold lower compared to wild type, while the K_m for PNP is about 1.5-fold higher with respect to wild type. Therefore, the catalytic efficiency (k_{cat}/K_m) of Arg116Gln enzyme is about 40% than that of wild type.

3.2.2. The Arg116Gln mutation reduces FMN affinity for PNPO

Concerning the potential involvement of Arg116 in the formation of a proper FMN phosphate binding site, we asked the question whether the replacement with Gln would lead to a decrease of FMN binding affinity that could in turn explain the observed minor loss of catalytic efficiency or lead to other effects on enzyme stability. We performed a fluorescence titration experiment with the apo-form of wild type and Arg116Gln enzymes. The dissociation constants for FMN and binding curves are shown, respectively, in Table 1 and Fig. 3A. Arg116Gln mutant binds the cofactor with a dissociation constant that is around 20-fold higher than wild type enzyme.

3.2.3. Arg116Gln mutation affects thermal stability of PNPO

In order to verify if the Arg116Gln mutation affects thermal stability of the enzyme, we performed two different experiments. First, we checked the structural stability of the enzyme through thermal denaturation. Mutant Arg116Gln showed a melting temperature (T_m) about 6° lower than wild type enzyme (Fig. 3B, Table 1). This difference led us to further investigate the stability of enzymatic activity when incubating the proteins at different temperatures for a prolonged period of time. Beside the physiological body temperature of 37°C we also chose the “altered” temperature of 42°C to mimic fever status often related to seizures. The results (Fig. 4) show a significant difference between wild type and Arg116Gln mutant. After 22 h of incubation at 37°C , the wild type retained about 70% of the initial enzymatic activity, whereas Arg116Gln retained only half of its activity (Fig. 4A). When the mutant form was incubated at 42°C , it lost about 80% of its initial activity after 22 h, whereas the wild type enzyme only lost about 40% (Fig. 4B). The first few hours of incubation seem to be critical, with the mutant enzyme losing up to 60% of its activity within 3 h at 42°C . Addition of an over-saturating amount of FMN stabilized enzyme activity (Fig. 4C). This was true for both enzymatic forms and at both incubation temperatures (data not shown for the experiments at 37°C), although the more drastic differences were revealed for the mutant enzyme when incubated at 42°C . In particular, the protection elicited by FMN was most significant within the first 4 h of incubation (about 30% protection), and the enzyme retained 15% more activity over a 24-hour incubation time.

3.2.4. Arg116Gln mutation only slightly reduces PLP affinity for PNPO but impairs its transfer to PLP-dependent enzymes

Previous studies with human and *E. coli* PNPO demonstrated that both holo- and apo-enzyme bind tightly one molecule of PLP on each subunit. In the crystal structure of *E. coli* PNPO, this secondary PLP binding site (also defined as tight binding, non-catalytic binding site)

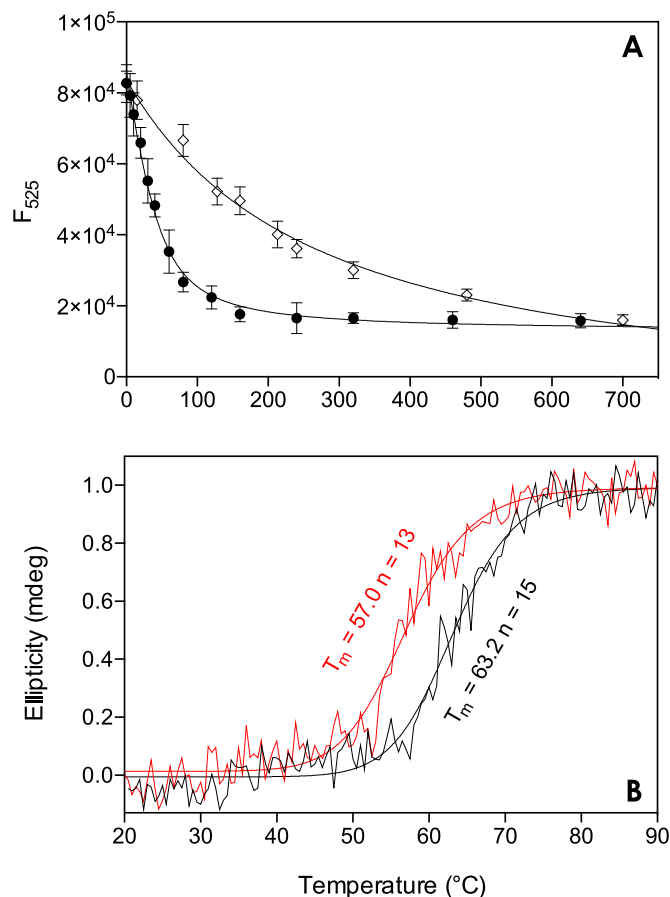


Fig. 3. A) Fluorescence emission of FMN (excitation wavelength of 450 nm, emission 525 nm) upon binding to apo-forms of PNPO: wild type (closed circles, ●), Arg116Gln (open squares, ◇). All experimental points are the average \pm standard deviation of at least three independent measurements. The continuous lines through the experimental points were obtained by non-least square fitting of experimental data to Eq. (2) in Ref. [14]; B) Thermal denaturation profiles for wild type (black) and Arg116Gln (red) PNPO. On each curve, the values corresponding to T_m and n (reflecting the steepness of the sigmoid transition) are shown. The continuous black (wild type) and red (Arg116Gln mutant) lines through the experimental points were obtained by non-least square fitting of experimental data to Eq. 3 in Ref. [14]. Arg116Gln mutant unfolds at lower T_m and with a less steep transition than wild type enzyme. Such behavior suggests that Arg116Gln has a quite different stability curve with respect to wild type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

has been identified [17]. Binding of PLP to the non-catalytic site of PNPO might be one way for cells to regulate free PLP concentration in vivo and it was suggested that PNPO might serve as a significant reservoir of PLP in the cell [18,19]. According to this hypothesis, PLP tight binding site on PNPO would function as a stable storage site, preventing the cofactor from reacting with the many nucleophiles present in the cell. The tightly bound PLP must rapidly transferred to the plethora of newly synthesized apo-enzymes that require it for activity. Residue Arg116 in human PNPO lies at close distance from the PLP present at the putative tight binding site (Fig. 3, lower panel, in

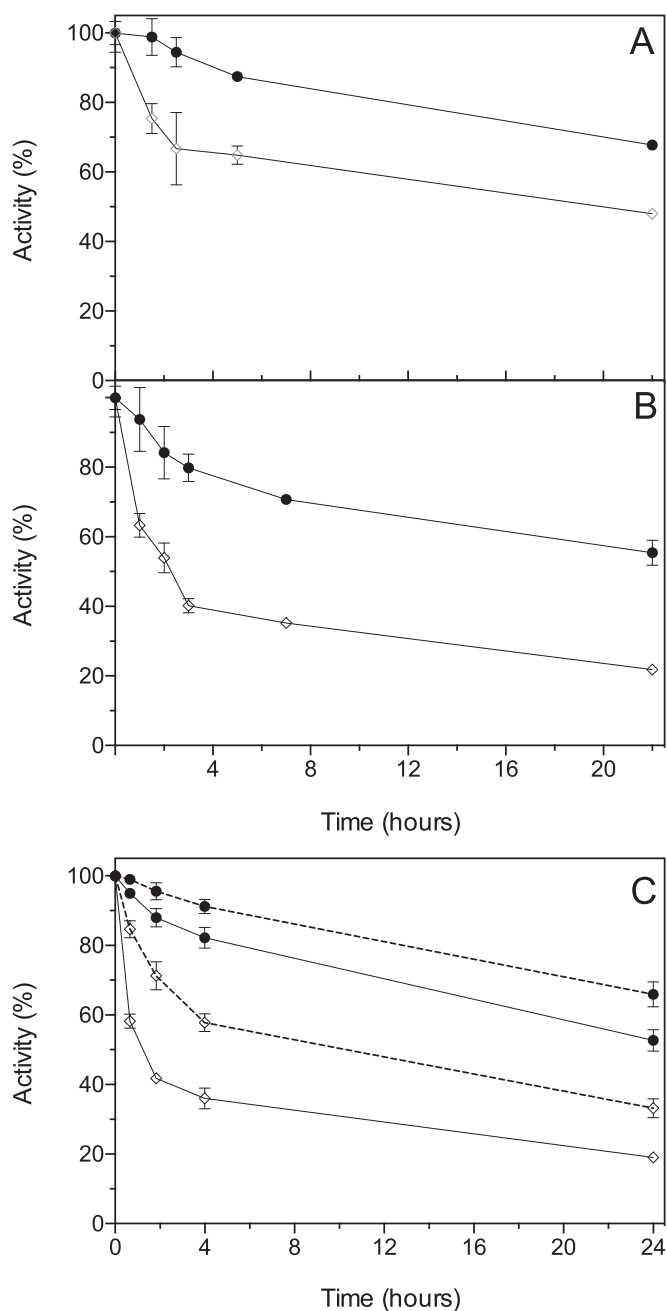


Fig. 4. Residual enzymatic activity vs. incubation time for wild type (closed circles, ●) and Arg116Gln PNPO mutant form (open squares, ◇). Panel A, incubation temperature 37 °C; Panel B, incubation temperature 42 °C; Panel C, incubation temperature 42 °C with (dashed line) or without (continuous line) addition of 5 μM FMN. All experimental points are the average \pm standard deviation of at least three independent measurements.

Ref. [14]). Mutation of Arg116 could locally perturb protein structure and influence the ability of PNPO to tightly bind PLP and transfer the cofactor to PLP-dependent apo-enzymes.

We tested whether the mutant Arg116Gln was able to bind PLP tightly, as defined by PLP not dissociating during size exclusion chromatography. In our experimental conditions, both wild type and Arg116Gln showed about 1 molecule of PLP bound per subunit. This value refers to the enzymes right after elution from the HisTrap affinity column, following the fast purification procedure described herein. When samples were subjected to dialysis and concentration through centrifugal filters, the amount of bound PLP was about 0.15 mol per subunit, as previously found for wild type human PNPO subjected to laborious purification procedures and extensive dialysis [15]. When our

dialyzed enzyme samples were incubated with a threefold excess of PLP and chromatographed on a size exclusion column, we found again about one molecule of PLP bound per subunit of enzyme. The dissociation constant of tightly bound PLP was determined by fluorescence titration and showed no significant difference between wild type and Arg116Gln mutant (Table 1 and Fig. 5 in Ref. [14]).

In order to determine if wild type and Arg116Gln were able to transfer to the same extent their tightly bound PLP molecule to apo-PLP-dependent enzymes, we measured the kinetics of transfer of tightly bound PLP from PNPO-PLP complexes to apo-human cytosolic serine hydroxymethyltransferase (hcSHMT). Fig. 4 shows that Arg116Gln is undoubtedly able to transfer its tightly bound PLP to hcSHMT, although with a slightly slower kinetics compared to wild type. Moreover, with the mutant PNPO-PLP form, reactivation of hcSHMT does not seem to go to completion, reaching only 70% of the activity of fully saturated holo-hcSHMT within the experimental time frame.

4. Discussion

A recent genome-wide association analysis has suggested the involvement of 17q21.32 locus, including the PNPO gene, in the seizure susceptibility for genetic generalized epilepsy and the idea that the phenotype of PNPO deficiency could be wider than previously reported [4,9]. The present study further expands the epilepsy phenotype of patients with PNPO deficiency related to the so far poorly characterized c.347G > A (p.Arg116Gln) variant. Up to date, the isolated homozygous c.347G > A mutation was only reported in 2 patients with PNPO deficiency and severe epilepsy: a 5-month-old boy with West syndrome and a girl with severe neonatal seizures [5,7]. Phenotypes in our series are milder in terms of age at seizure onset, seizure severity and developmental outcome. Patients 1 and 2 are the first reported individuals with PNPO deficiency and seizure onset after the first year of life. Patient 3, too, had a later onset than the previously described older patient diagnosed post-natally (8 months versus 5 months of age) [5]. Patient 1 had normal cognitive development and unusually mild focal epilepsy, easily controlled with pyridoxine. Rare generalized seizures were reported in patient 3. Patient 2 is the first reported in whom PNPO deficiency is associated with myoclonic astatic epilepsy.

Our *in vitro* experimental studies demonstrate that the c.347G > A (p.Arg116Gln) mutation causes different functional alterations in PNPO. Although the catalytic properties of the enzyme are not drastically affected, the catalytic efficiency (k_{cat}/K_m) is only about 40% of wild type (Table 1). This is in agreement with previous measurements in a eukaryotic cell system, though the experimental conditions were quite different [5]. The affinity for FMN is reduced in the mutant variant by 20 fold (Fig. 3A). The mutant enzyme is more susceptible to thermal denaturation if compared to wild type, showing a T_m of about 6 °C lower (Fig. 3B); thus, the stability of the mutant enzyme may be impaired, especially when moving from body temperature to febrile status. In addition, the combination of high temperature and low FMN concentration seems to have deleterious effects on the stability of the mutant enzyme activity (Fig. 4). Altered stability of PNPO mutant enzyme with temperature could be linked to the increased susceptibility to febrile seizures observed in Patient 3 (noteworthy, the same susceptibility was reported in children with PNPO deficiency carrying other mutations) [7,20].

Another interesting feature of the Arg116Gln PNPO mutant is its diminished ability to transfer tightly bound PLP to PLP-dependent enzymes (Fig. 5). Such impairment has already been reported with the Arg95Cys PNPO mutation [21] and might represent a critical feature considering the importance of PNPO in PLP cellular homeostasis [19]. The observed difficulty to transfer PLP by the above-described PNPO mutants may result in a reduced bioavailability of this cofactor. This might have a drastic effect on the biosynthesis of neurotransmitters such as dopamine, serotonin, glycine, epinephrine, norepinephrine, D-serine, L-glutamic acid, gamma-aminobutyric acid (GABA) and

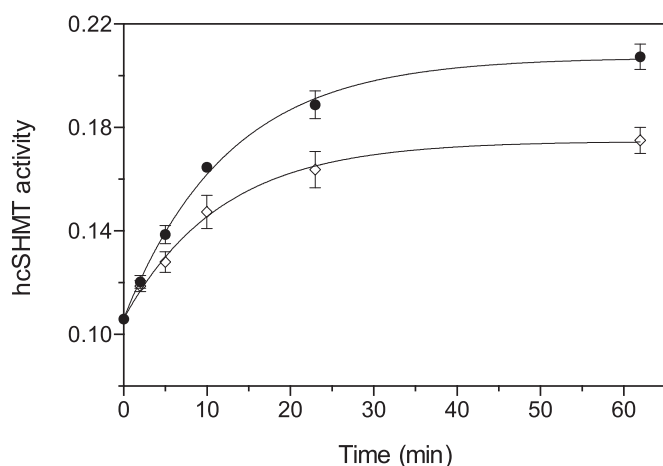


Fig. 5. Kinetics of apo-hcSHMT reactivation upon delivery of PLP from wild type (closed circles, ●) and Arg116Gln (open squares, ◇) PNPO-PLP complexes. All experimental points are the average \pm standard deviation of at least three independent measurements. The continuous lines through the experimental data were fitted to an exponential process.

histamine because the several PLP-enzymes responsible for the biosynthesis of these compounds might be in competition for PLP availability [22]. The reduced PLP availability for PLP-dependent enzymes, including glutamic acid decarboxylase and GABA transaminase, results in an impaired glutamatergic and gabaergic neurotransmission and in subsequent epileptogenic processes.

All these considerations suggest that Arg116Gln mutant enzyme is endowed with a residual functionality that could account for the relatively mild epilepsy phenotype of our patients carrying c.347G > A mutation and their heterogeneous response to treatment: patient 1 in our series and patient 8 of Mills' responded to pyridoxine, patient 7 by Mills' responded to PLP, our patients 2 and 3 were seizure-free before introducing pyridoxine. In patient 2 no cognitive improvement could be demonstrated after pyridoxine was started at 12 years, probably due to the delayed initiation. In Patient 3, both paroxysmal abnormalities and slow background EEG activity disappeared after pyridoxine was started and a mild trend towards improved Language skills also emerged. A longer follow-up would be necessary to properly assess the overall clinical effects of treatment on [5]. In basal conditions, the mutant enzyme may provide enough PLP to sustain cellular requirements but in particular physiological situations (e.g., local low concentration of PLP, FMN, high temperature) and/or in specifically localized environments in selected tissues the enzyme performance may be drastically reduced. On the other hand, when exogenous pyridoxine as precursor of PNP or PLP are supplemented through therapy, the enzyme may be saturated with substrate and increase its activity back to nearly normal levels, so preventing the emergence of seizures. An alternative to PLP or pyridoxine could be represented by pyridoxal, another B₆ vitamin diffusible across the blood-brain barrier, which can be re-phosphorylated by pyridoxal kinase to PLP [22]. Also supplementation with riboflavin could be a useful therapeutic strategy to improve functionality and stability of PNPO (in view of the fact that the mutant Arg116Gln binds FMN with lower affinity) even if no specific studies are available to date.

5. Conclusions

Our data suggest that clinicians should consider PNPO deficiency in children with epilepsy onset beyond the first months of life, irrespective from the seizure type and severity of the associated neurological status. The dissemination of this message among pediatric neurologists and experts of metabolic disorders might be relevant if we consider that PNPO deficiency is a potentially treatable disorder and that an earlier intervention is more likely to be more beneficial. Since there are no

peripheral biomarkers in plasma or urine for PNPO deficiency, a diagnostic and therapeutic trial with pyridoxine (or PLP where available) could be considered in any form of epilepsy with onset in infancy. Dosages and durations of these trials remains an open question that requires specific studies.

The demonstration that the Arg116Gln PNPO mutant has an altered functionality proves that molecular and biochemical studies might favor tailored treatments via a better comprehension of the pathogenic mechanisms of PNPO deficiency.

Conflict of interest

None

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Compliance with ethical standards

Informed consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from parents of all patients for being included in the study.

Animal rights

This article does not contain any studies with animal subjects performed by any of the authors.

Details of the contributions of individual authors

MLDS and MMA planned the whole structure of the study. MLDS and RC planned the biochemical experiments; IN, AT, and MLDS performed the experiments. AT, MLDS, and RC analyzed the biochemical experimental data. AP performed molecular modeling. MMA, MMo, RG and VL were responsible for clinical evaluation and follow-up of the patients. MT and DM performed the molecular genetic studies of the patients. MLDS, MMA, RC, and VL wrote the paper. RC, RG and VL revised the paper.

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