

**A mutation in the iron-responsive element of ALAS2 is a modifier of disease severity in a patient suffering from CLPX associated erythropoietic protoporphyria**

Porphyrias are a group of eight genetically distinct disorders, each resulting from a partial deficiency or gain-of-function of a specific enzyme in the heme biosynthetic pathway.<sup>1</sup> Porphyrias are inherited as autosomal dominant, autosomal recessive or X-linked traits.<sup>2</sup> Erythropoietic protoporphyria (EPP) is a constitutive hematological disorder characterized by protoporphyrin IX (PPIX) accumulation in erythrocytes and other tissues resulting in acute skin photosensitivity, mild microcytic anemia, and rarely, severe liver disease. The majority of the patients with EPP present the autosomal EPP form (OMIM #177000) due to a partial deficiency of ferrochelatase (FECH), the last enzyme of the heme biosynthetic pathway.<sup>1,2</sup> In most EPP patients, the clinical expression requires the coinheritance of a *FECH* mutation, that abolishes or markedly reduces FECH activity, *in trans* to an hypomorphic *FECH* allele (rs2272783, NM\_000140.3; c.[315-48T>C]) carried by about 11% of Caucasians.<sup>3</sup> In Europe and the USA, 4-10% of EPP patients have been reported to harbor a gain-of-function mutation in the 11<sup>th</sup> exon of the erythroid δ-aminolevulinic synthase gene (*ALAS2*).<sup>4</sup> Rare cases of EPP have also been reported in few reference centers without any mutations in the *FECH* or *ALAS2* genes (personal communication from JC Deybach). EPP due to gain-of-function *ALAS2* mutations are inherited as an X-linked trait and result in a distinct biochemical feature not only with overproduction and accumulation of free PPIX but also zinc protoporphyrin (ZnPP) (XLPP) (OMIM #300751). In these patients, the FECH enzyme is functional and utilizes all available iron for heme production. The excess PPIX is used to make ZnPP in a reaction catalyzed by FECH.<sup>3,5</sup> Moreover, gain-of-function missense mutations altering the C-terminal part of *ALAS2* exacerbate congenital erythropoietic porphyria, suggesting that *ALAS2* is a gatekeeper of erythroid heme biosynthesis and may function as a modifier gene.<sup>6</sup>

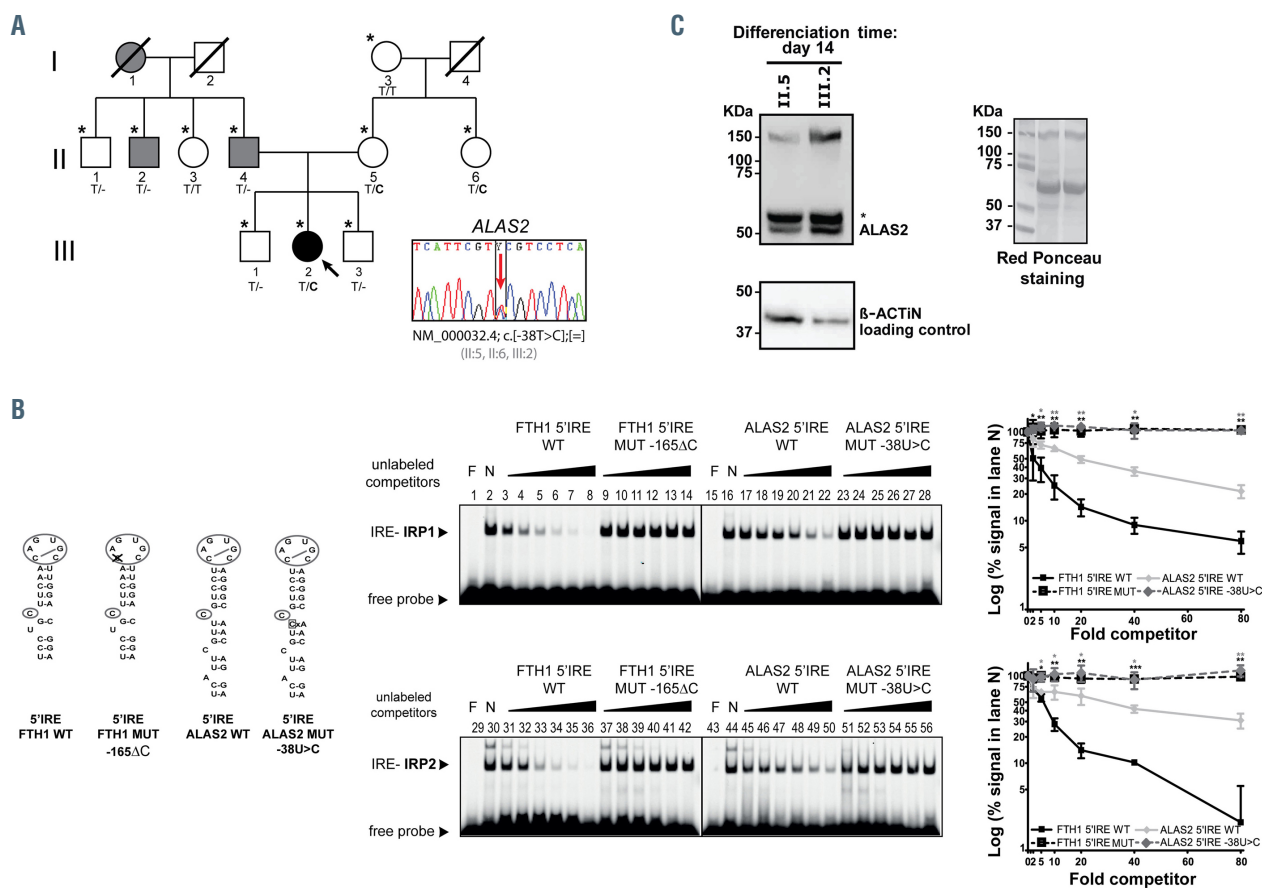
The 5' untranslated region (UTR) of *ALAS2* mRNA contains a cis-regulatory iron-responsive element (IRE) that confers iron-dependent posttranscriptional regulation by the iron regulatory proteins (IRP).<sup>7</sup> IRE mutations are known to cause human diseases. IRE mutations in ferritin L mRNA cause hereditary hyperferritinemia with cataract syndrome (HHCS) (OMIM #600886),<sup>8-9</sup> and a single point mutation in the IRE of ferritin H is responsible for an autosomal dominant iron overload phenotype (OMIM #615517).<sup>10</sup> These cases suggest a possible role for IRE mutations in the *ALAS2* gene in modifying the severity of hematologic diseases.

Here, we describe an 18 year-old Caucasian female proband (III:2, Figure 1A) referred to the French Center of Porphyria because of early onset (9 months) acute photosensitivity characterized by painfully phototoxic reactions suggesting EPP. An incomplete genetic characterization of this case was previously reported to harbor a gain of function in the mitochondrial unfoldase gene, *CLPX*.<sup>11</sup> Written informed consent was obtained for all participants. This study was approved through the local ethical committees in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments (R162-16-7 and 145-15-4 French ethical agreement). In the proband, a high level of free erythrocyte PPIX and ZnPP confirmed the diagnosis of EPP (Table 1). At the age of diagnosis, the proband also presented with a microcytic iron deficiency anaemia (Table 1). FECH enzyme activity was normal (Table 1). No point mutation or large *FECH* gene deletion were identified, and chromosome 18, where *FECH* gene is located, was excluded by linkage and comparative genomic hybridization (CGH) array analyses. Moreover, the proband did not harbor the *FECH* low-expressed allele rs2272783, NM\_000140.3; c.[315-48T>C] (IVS3-48C). The father (II.4) and one uncle (II.2) of the proband presented with zinc- and free PP accumulation in erythrocytes that were associated with a mild photosensitivity, but without symptoms of EPP (Figure 1 and Table 1). Whole-exome sequencing (WES) analysis showed that proband (III.2), the father (II.4) and the uncle (II.2) carried a heterozygous single

**Table 1. Clinical and biochemical data in affected subjects of the erythropoietic protoporphyria proband's family.**

Pedigree	II:2	II:4	III:2	II:5	I:3	II:6	Normal values	
Sex	M	M	F	F	F	F		
Photosensitivity	mild	mild	Severe	no	no	no		
IRE <i>ALAS2</i> mutation	-	-	+	+	+	+		
Age at the visit (years)	56	46	18	20	43	78	51	
Total porphyrins in plasma (nmol/L)	54	52	936	780	22	19	20	< 20
Erythroid PPIX (µmol/L RBC):	30.4	26.7	140.9	89.5	2.1	1.9	2.0	<1.9
Free PPIX (%)	33	38	71	65	20	17	22	< 28
ZnPP (%)	67	62	29	35	80	83	78	> 72
FECH activity (nmol/mg prot. h)	4.4	3.9	3.6	3.9	4.3	4.2	4.2	>3.5
Hb (g/dL)	15.5	15.9	10.9	12.1	12.2	13.3	14.0	11.5 – 16.0
Serum iron (µmol/L)	25.0	20.0	4.0	11.0	21.0	14.0	20.0	12.0 - 26.0
Serum ferritin (µg/L)	196	171	5	11	12	137	85	15 – 250 ♂ 8 – 150 ♀
Transferrin saturation (%)	31	32	5	15	31	24	36	20 - 45
Soluble Transferrin receptor (mg/L)	1.24	1.41	3.95	1.75	1.15	1.26	1.19	0.76-1.76

The proband (III:2) was examined twice: first at the initial visit associated with iron deficiency and one year later after oral iron supplementation. PPIX: protoporphyrin IX; ZnPP: Zinc protoporphyrin; Hb: hemoglobin; FECH: ferrochelatase; RBC: red blood cells; m: male; f: female.



**Figure 1.** *ALAS2* iron-responsive element mutation as modifier for erythropoietic protoporphyria clinical severity. (A) Pedigree of the studied family. Filled black symbol indicates the patient with clinical overt erythropoietic protoporphyria (EPP). Grey symbols indicate relatives (II.2 and II.4) with protoporphyria IX (PPIX) over-production in red blood cells (RBC) associated with mild photosensitivity without EPP; the grandmother (I.1) presented with a suspected mild photosensitivity but no biochemical data were available; barred symbols indicate deceased subjects. Asterisks indicate subjects characterized at the molecular level. Partial chromatograms of *ALAS2* iron-responsive element (IRE) sequences where the -38T>C mutation is located. Reference sequence for *ALAS2*, NM\_000032.4. (B) Non-radioactive competitive electrophoretic mobility shift assays (EMSA) with wild-type (wt) and mutated (mut) *ALAS2* IRE. Graphic representation of the ferritin H (*FTH1*) IRE sequences (nucleotides 60-85 in NM\_002032.2) wt or mut -165 $\Delta$ C, *ALAS2* IRE sequence (nucleotides 93-125 in NM\_000032.4) wt or the -38U>C mut, used for the synthesis of RNA probes in competitive EMSA experiment. RNA Watson-Crick pairs are depicted as A-U; U-A; C-G or G-C; wobble pairings are shown as U-G and not possible pairings are depicted as C-A. Fluorescent-labeled *FTH1* IRE wt probe was incubated with increasing molar excess (2x, 5x, 10x, 20x, 40x and 80x) of unlabeled competitors corresponding to the *FTH1* IRE wt type sequence (lanes 3-8; lanes 31-26), or the *FTH1* IRE mutant -165 $\Delta$ C (lanes 9-14; lanes 37-42), *ALAS2* IRE wt sequence (lanes 17-22; lanes 37-42) or the *ALAS2* -38U>C mutation (lanes 23-28; lanes 51-56). Samples were incubated with recombinant IRP1 (upper panel) or IRP2 (bottom panel) and resolved on acrylamide gels. Quantification of the signals of the shifted bands was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences) and compared to the signal in lane N, set as 100%. Means  $\pm$  standard deviation of at least three independent experiments. Statistical analysis by Student's *t*-test (two-tailed) compares the signal given by the mutated IRE of *FTH1* or *ALAS2* sequences to the signal given by the corresponding wt IRE sequences, at each molar concentration \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. F: free probe; N: no competitor added. (C) Proteins from CD34<sup>+</sup> erythroid cells from proband's mother (II.5) and the proband (III.2) cultured and differentiated over 14 days were extracted. Twenty micrograms were separated (4-12% NuPAGE gel) with MES NuPAGE buffer. Proteins were transferred to nitrocellulose membrane. *ALAS2* was detected by chemiluminescence using specific antibody. The lower band is *ALAS2* whereas the upper band, indicated with an asterisk, is an unknown non-specific protein (Agiros Pharmaceutical). The membrane was then probed with an anti-human beta-actin antibody as a loading control.

nucleotide substitution (c.1102G>A; p.G298D) at the exon 7 of *CLPX* gene.<sup>11</sup>

The proband did not harbor mutations in the 11<sup>th</sup> exon of *ALAS2* gene demonstrating that she is affected by an unusual form of EPP. Sequencing of the rest of the *ALAS2* gene revealed an heterozygous T>C change at position -38 in the first exon, at the 5' UTR and located inside the *ALAS2* IRE motif (NM\_000032.4; c.[-38T>C];[=]) (Figure 1A and B). The mutation is absent in the 6,500 sequenced exomes in the Exome Variant Server database, excluding the possibility of being a neutral polymorphism. This variation was predicted to disturb the stability of the IRE, since the IRE position below the C8 bulge is critical to maintain closed the lower stem (IRE *ALAS2* wild-type [wt] predicted minimum free energy  $\Delta G = -7.00$  Kcal/mol vs. IRE *ALAS2* mutant predicted minimum free energy  $\Delta G = -5.60$

Kcal/mol).<sup>12</sup> In order to confirm the functional consequence of the -38U>C change in *ALAS2* IRE, we performed non-radioactive competitive electrophoretic mobility shift assays (EMSA) with recombinant IRP1 or IRP2<sup>13</sup> (Figure 1B). As expected, the ferritin H (*FTH1*) wt positive control showed efficient competition, while its corresponding negative control, the *FTH1* mutant -165 $\Delta$ C could not compete with the labeled *FTH1* wt probe (Figure 1B, compare lanes 3-8 to 9-14 in upper panel and 31-36 to 37-42 in bottom panel). In addition, the *ALAS2* -38U>C mutation totally abolished the ability of the *ALAS2* IRE to compete with *FTH1* wt probe for IRP1 or IRP2 binding, in contrast to the wt *ALAS2* IRE sequence (Figure 1B, compare lanes 17-22 to 23-28 in upper panel and 45-50 to 51-56 in bottom panel). Therefore, the -38U>C mutation in *ALAS2* IRE abolishes IRP1 and IRP2 binding affinity *in vitro*, which would

impair *ALAS2* mRNA regulation in response to iron levels. We hypothesized that under iron limiting conditions, IRP would not repress *ALAS2* mRNA translation, with consequent overproduction of *ALAS2* protein. This predicted increase in *ALAS2* protein levels was indeed observed by immunoblotting for *ALAS2* from cell lysates obtained from cultured and differentiated CD34<sup>+</sup> erythroid precursors from the proband and the mother (II.5) who only bears the *ALAS2* IRE mutation (Figure 1C). Therefore, our data supports the hypothesis that the 38U>C mutation in the *ALAS2* IRE increases *ALAS2* protein expression and modifies the severity of EPP caused by a confirmed *CLPX* mutation. In contrast, the *ALAS2* gain-of-function in XLPP is caused by increased enzymatic activity in the protein.<sup>14</sup>

Sequencing of the *ALAS2* gene in other family members showed that this mutation does not co-segregate with porphyrin accumulation (Figure 1A and data not shown). The proband inherited the -38U>C change from the maternal branch (II.5; II.6; I3, Figure 1A). Those relatives have higher than normal ZnPP levels and are asymptomatic (Table 1). Methylation analysis confirmed the absence of skewed X inactivation in relatives (II.5 and II.6) and the proband (III.2) who carried the IRE mutation.

Previously we and others described the important role of *CLPX* in heme biosynthesis through its regulation of *ALAS2* turnover and enzyme activity.<sup>11,15</sup> The G298D mutation in the *CLPX* gene, present in heterozygosity in this family (proband, father and paternal uncle), decreases the proteolytic activity of the *CLPX* protease, causing accumulation of *ALAS2*.<sup>11</sup> However, in comparison to the mild photosensitivity presented by the paternal family, the proband presents with a full and severe EPP phenotype due to the combination of the paternal *CLPX* G298D mutation and the maternal *ALAS2* -38T>C mutation present in the 5' IRE of *ALAS2* gene.

Collectively, our data suggests that the *ALAS2* IRE mutation is the modifier necessary for the clinical presentation of EPP in the proband. The IRE/IRP-dependent down regulation of *ALAS2* during iron deficiency is impaired and contributes to ALA overproduction and increases PPIX level in erythrocytes especially during iron deficiency. This postulate was supported clinically by the beneficial effect of the oral iron therapy when the patient was anemic (Table 1).

In summary, we have identified the first described mutation in the IRE of *ALAS2* gene that contributes to porphyrin accumulation in erythrocytes above a threshold leading to overt EPP. These data strongly support the role of *ALAS2* as an important modifier gene triggering the clinical manifestations in erythropoietic disorders and extend the involvement of its IRE/IRP system to human heme metabolism and erythropoietic porphyria.

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Contributions: MS, LG and HP developed the study concept and designed the research; KP, LG and HP recruited family members and collected patients' data; SD and HM performed patients DNA sequencing; Ckdid the X inactivation and CGH array experiments;

SL performed the IRE/IRP functional EMSA; HM did the erythroid progenitor cell culture; GN performed immunoblot experiments;

XF-C, YYY, HP and MS wrote the manuscript and all authors participated in data discussion, analyzed data, read and approved the manuscript.

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