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AN ODD CASE OF HETEROALLELIC ACUTE INTERMITTENT PORPHYRIA IN THE ARGENTINEAN POPULATION

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Abstract

AIP is an acute liver disorder caused by a deficiency of porphobilinogen deaminase (PBGD) characterized by neuroabdominal symptoms. It is an autosomal dominant disease. However, homozygous dominant AIP (HD-AIP) have been described. In some cases erythrodontia was observed. CEP is an autosomal recessive disease produced by mutations in the uroporphyrinogen III synthase gene (UROS), characterized by severe cutaneous lesions and erythrodontia. The aim of the work was to establish the differential diagnosis of porphyria in a patient with abdominal pain, neurological attacks, skin symptoms and erythrodontia. The PBGD activity was reduced 50% and the genetic analysis indicated the presence of two genetic variants in the PBGD gene, p.G111R and p.E258G, a new genetic variant, revealing a case of heteroallelic HD-AIP. The patient, first diagnosed as a carrier of a dual porphyria: AIP / CEP based on the excretion profile of porphyrins, precursors and her clinical symptoms, would be an atypical case of human HD-AIP. These results would also suggest the presence of a phenocopy of the CEP, induced by an endogenous or exogenous factor. Our findings highlight the importance of genetic studies for a proper diagnosis of porphyria, prevention of its manifestation and its treatment.

Key words: Acute Intermittent Porphyria, Porphobilinogen deaminase, Hydroxymethylbilane synthase, Congenital Erythropoietic Porphyria, Uroporphyrinogen III synthase, Homozygous dominant AIP, Dual Porphyria.

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INTRODUCTION

The porphyrias are a heterogeneous group of metabolic disorders that result from the decreased activity of a specific enzyme of the heme pathway and are characterized by the overproduction and excretion of heme intermediates in urine and/or stool, and their accumulation in certain tissues (1). Except Porphyria Cutanea Tarda type I which is an acquired disorder, the porphyrias are inherited diseases. They can be classified in hepatic, erythropoietic or hepatoerythropoietic depending on the main site of expression of the specific enzymatic defect. Alternatively, they can be classified in cutaneous or acute depending on the clinical manifestations (1, 2).

Acute Intermittent Porphyria (AIP, OMIM 176000) is the commonest of the acute hepatic porphyrias. It is an autosomal dominant disorder caused by a deficient activity of porphobilinogen deaminase (PBGD, EC 4.3.1.8), also named hydroxymethylbilane synthase (HMBS), producing a markedly increase in the urinary excretion of δ-aminolevulinic acid (ALA) and porphobilinogen (PBG). The symptoms may appear at any time after puberty and are characterized by acute neurovisceral attacks that are frequently precipitated by different drugs, calories restriction or stress (3,4, 5).

Congenital Erythropoietic Porphyria (CEP, OMIM 263700) or Gunther's disease is a rare autosomal recessive cutaneous porphyria. CEP is caused by mutations in the gene encoding the enzyme uroporphyrinogen III synthase [UROS; hydroxymethylbilane hydrolyase (cyclizing), EC 4.2.1.75]. The significantly reduced activity of the enzyme results in the formation of the non-physiological and pathogenic isomers uroporphyrin I (URO I) and coproporphyrin I (COPRO I) which are accumulated in tissues and bones and are excreted by urine and faeces. Clinically, CEP is characterized by severe cutaneous lesions, massive hemolysis, hypersplenism, hypertrichosis, erythrodontia and osteolytic skeletal changes. Ultraviolet light activates the photosensitive URO I leading to tissues damage and formation of bullous lesions which rupture, often becoming infected, producing bone resorption and cutaneous deformity (5,6).

Patients with more than one heme enzyme deficiency are classified as having Dual Porphyria (DP). The clinical symptoms and biochemical parameters represent the superimposition of those found in each of the coexistent porphyrias.

However, a different and extremely rare form of porphyria, homozygous dominant AIP (HD-AIP), has been reported. Only four cases of heteroallelic and homoallelic HD-AIP have been described so far (7,8,9). A putative fifth case was published, although neither enzymatic nor molecular direct diagnosis were carried out, but the parents of the patient were heterozygous for a different mutation in the *PBGD* gene suggesting, alltogether that the patient was a HD-AIP (10,11). In most of those particular patients, they exhibited the same degree of mental retardation and/or neurological defects.

MATERIALS AND METHODS

Case report

The patient, a 43-years-old white woman, presented acute intermittent abdominal pains, erythrodontia, splenomegaly and dermatological manifestations (hyperpigmentation, scarring and photosensitivity). She also had elevated serum ferritin (1369 ng/ml, N.V: 12-150 ng/ml) and although she was a carrier of the p.H63D mutation in

the HFE gene associated with hereditary hemochromatosis (HH), the mutation was present in a heterozygous state, so excluding HH type I.

Informed consent was obtained from the patient following the standards of UNESCO Declarations-DD.HH Genome and Genetic Data (www.unesco.org/shs/ethics), Declaration of Helsinki and the study was approved by the Institutional Research Ethics Committee of the Research Center on Porphyrins and Porphyrias (CIPYP) - National Scientific and Technical Research Council (CONICET), University of Buenos Aires (UBA).

Biochemical studies

Determination of porphyrins in blood samples, urine, faeces and plasma

Total porphyrins in whole blood samples, urine, faeces and plasma (plasma porphyrin index) were measured by methods previously described (1).

Determination of PBGD activity

The PBGD activity from the patient and controls was determined in red blood cells (RBC) by the methodology of Batlle (1).

Determination of UROS activity

The activity of UROS was determined by a modification of the coupled enzyme assay (PBGD/UROS) described by Tsai (12). For preparation of the hemolysates (dilution 1:20) the erythrocytes were freezed-thawed 5 times and 50µl of the sample were diluted in 950µl of buffer (0.1M Tris-HCl pH 8, Ethylenediaminetetraacetic acid (EDTA) 1mM and 1% Tween).

A high-performance liquid chromatography (RP-HPLC) was used for the analysis of URO I and URO III according to a modification of Lim et al (13) method as follows. The isomers were resolved on a Merck LiChrospher 100 RP-18 column (125mm long, 4mm internal diameter and 5µm particle diameter), injection volume 20µl, eluted with 1M ammonium acetate buffer pH 5.16 10% in acetonitrile (solvent A) and methanol 10% in acetonitrile (solvent B) with a linear gradient from 10% B (v/v) to 90% B (v/v) in 13min; followed by a linear gradient from 90% B (v/v) to 10% B (v/v) for 1 min further, with a flow rate of 1ml/min. One unit of enzymatic activity was defined as the amount of enzyme that produced 1nmol of URO III per hour, per ml of RBC and per mg of protein.

Determination of the URO I and URO III isomers in urine

For the sample preparation 1ml of urine was mixed with 40µl of HCl (cc) and centrifuged at 11,000*g* (11,700 rpm) for 10min. Supernatant was used for separating URO I and URO III by the RP-HPLC method as described above.

Iron inhibition of the erythroid UROS enzyme

To assay the inhibition of erythroid UROS enzyme activity *in vitro*, both in patients and controls, we used the coupled assay protocol developed for measuring the enzyme activity with modifications described bellow, based on the procedure employed (14) to measure liver UROS in PCT patients.

We initially proceeded to the disruption of a RBC sample from a normal control by rapid freezing and thawing. Then a 1:20 dilution of the lysed red blood cells in buffer Tris-HCl 0.1M pH8, 1mM EDTA and 1% Tween was prepared; this sample was used as a source of UROS. Samples of 5µl of this lysate with exogenous PBGD, cysteine 0.804M, Tris-HCl 0.1M pH8 and iron as ammonium iron(ll) sulphate hexahydrate [$(NH_4)_2Fe(SO_4)_2 .6H_2O$] according to a final concentration from 0 to 1.2mM were preincubated in a water bath for 15 min at 37 ° C.

When preincubation time elapsed, PBG 3.4mM was added to each sample and incubated in a water bath at 37°C for 15 min. After this time, the reaction was finished adding TCA 10% to each tube. Finally, the samples were centrifuged and the supernatant was analyzed by RP-HPLC according to the protocol described above. Inhibition of the enzyme was expressed as percentage of conversion of hydroxymethylbilane (HMB) in URO III.

Genetic characterization

DNA extraction

Genomic DNA was extracted from peripheral blood collected in EDTA using the commercial kit GE Healthcare Ilustra ™ blood genomic Prep Mini Spin Kit.

Mutational analysis

Mutational analysis was performed amplifying the promoters, all exons and the intron/exon boundaries of the *PBGD* and *UROS* genes by the polymerase chain reaction (PCR) using specific primers shown in Table 1 and Table 2. The amplified products were purified with the Bioneer Accuprep PCR Purification Kit and were automatically sequenced by Macrogen [Macrogen Inc, Gangseo-gu, Seoul, Korea (sequencer: ABI3730XL). The sequencing primers were the same as those for PCR amplification.

Prokaryotic expression of the novel PBGD mutation

The novel gene variant p.E258G was expressed in *Escherichia coli (E. coli)* strain BL21 using the pKK223-3 expression vector (Pharmacia Biotech Inc., Piscataway, NJ, USA) containing the cDNA encoding the housekeeping PBGD cloned into the Eco RI-Hind III site (pKK-PBGD-wt). The mutation was introduced in the vector pKK-PBGD-wt by site-directed mutagenesis (Fig. 1) using the following primers: FPBGD13 5´CCTTCCTGAGGCACCTGGGA 3´; RPBGD13 5´GCCAGCAACAAGTTGGCCAGGCTG 3´; FHMBS13 5´CGCATGGGCTGGCACAAC 3´; RHMBS13 5´ TCCCAGGTGCCTCAGGAAGG 3´ and the pKK-PBGD-wt plasmid as template. The PCR product and the plasmid pKK-PBGD-wt were digested separately with Nsi I (New England Biolabs, Beverly, MA, USA) and Apa I (Promega Corporation, Madison, WI, USA) restriction endonucleases. The purified Nsi I/Apa I fragment and the linear pKK-PBGD-wt were ligated using T4 DNA Ligase (Promega Corporation, Madison, WI, USA) and the resulting plasmid was transformed into *E. coli* BL21. The bacterial clones containing the expression construction pKK-PBGD-E258G were confirmed by automatic sequencing [Macrogen Inc, Gangseo-gu, Seoul, Korea (sequencer: ABI3730XL)].

Bacterial clones containing either the pKK-PBGD-wt and pKK-PBGD-E258G were grown to exponential phase and induced with 2mM isopropylthiogalactoside (IPTG) for 3h. Cells were centrifuged and the pellet was resuspended in 750µl of lysis buffer (0.1M Tris- HCl buffer, pH 7.5, 0.05%Triton X-100) and lysed by sonication. The supernatant was used to measure the PBGD enzyme activity

Table 1. Primers for amplification and sequencing the PBGD gene.

Primer	AMPLIFIED REGION	Sequence of the primer (5´→ 3´)
PR3	Promoter, Exon 1, Intron 1	TTGTGCTCCCACTTCAGTTACTTGTCTTTA
750D		GTCTGGTAACGGCAATGCGGCT
AD1		TCTGGACGAGCCGTGCAGCGATTG
PBGF3300		AGAGAAATGGGACCTCCG
PBGR3741		CCCAGATGTCTGGCTACA
PBGF3800	Promoter, Exon 2	GTCTACTCCATGTGGCAT
PBGR4260		CCTTGTCCAAGAACCAGA
LR3	Intron 2 to Exon 4	AAGGGACCAGCCTTGGAGTATTTCCCCACTC
PR4		GACGCCCATCTCTAAACCTAATCAGGACGGGAAG
EP71	Exon 4 to Exon 7	CCTAACCTGTGACAGTCT
EP48		AGACCTAGCATACTAGGG
EP77		CCTTTCCCTTTGGGGCCTGACCCT
AD76	Exon 7 to Exon 10	CCCTAGGCTCCACCACTGAAG
EP12		TTGTCTTTTTCCTTGGCTG
EP128		CGAGAGAGAATAGAGGTGAT
EP9310	Exon 10 to Exon12	GGGAAAGACAGACTCAGGCAGAG
EP15.1.12		GCAGGGAAGATTTCTTA
AD12.1	Exon 12 to Exon 15 n.c.	ATGCTTTGCGCCATTGGTTGG
EP1613		CAGTGATGTCCTCAGGTCTG
MV1030		TGGCTGCCCAGAACTTGGGCATC
LR4		GTTCCTATCTTCCCGCCAATTCCACACG

Table 2. Primers for amplification and sequencing the UROS gene.

Primer	Amplified region	Sequence of the primer (5´→ 3´)
UROSFwPrE	Erythroid Promoter	ACGAGACCCAAAAGAATTCTAGTG
UROSRvPrE		TACACAAGAAGAAGGCAATCAAATG
PrEFw		CAGGATTAAAAAAGAAGACAAATGC
PrERv		TGGGTCTCGTTGGGTCATATTA
PrHFw	Housekeeping Promoter, Exon 1	TCAATGACCTCGTCCTCTTCA
PrHRv2		CTTTTAGGTCAAGAAATGCGCTC
UROSFw2-3	Exons 2, Exon 3, Intron 2	CCGTCCCCATCGGAAATT
UROSRv2-3		TGCCATGTTTTAAAGTTTGGGAA
UROSFw4	Exon 4	CTCCTTGGAAGATGTTGAA
UROSRv4		GTCTGTGACCTGATACCA
UROSFw5	Exon 5	CTAATAGTTTGCTTTGCTCACAG
UROSRv5		TCACTGCATTCTTATCAGTAGTA
UROSFw6	Exon 6	GCATACCTGTGTATTTGCACGTT
UROSRv6		ACCAAGAATGCACTGAGGAAA
UROSFw7	Exon 7	AACTGAGTCCTAGAAGCAGAG
UROSRv7		GCTCTCTGCAGGGCCACCCACTT
UROSFw8	Exon 8	AGCTTCCAAGCAGCTCGTT
UROSRv8		CCATTCCTAATTCTAGAATCCCAG
UROSFw9	Exon 9	ACCACTCCTGTGCTAAAACC
UROSRv9		TTCTAAGGCACCTGCTAGGC
UROSFw10	Exon 10	ACGTCACATGAGCAGTAACG
UROSRv10		AGGCTTGAGGCAGGAGTCT

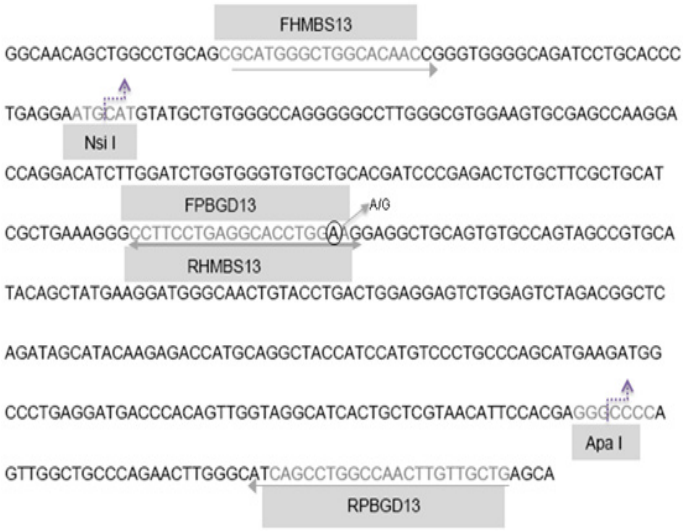

Figure 1. Site directed mutagenesis protocol**.** Expression of the novel mutation p.E258G in *E. coli* strain BL21 using the pKK223-3 expression vector.

as published (15) and the specific activity was expressed as nmol uroporphyrinogen/h/mg of protein. For the enzyme stability studies, samples from the bacterial lysates were incubated at 65°C for 150min. Aliquots were removed at intervals of 30 min and the PBGD activity was determined as already indicated.

RESULTS

The biochemical analysis (Table 3) showed increased urinary levels of ALA, PBG, URO and COPRO as well as increased levels of COPRO in stool, high values of porphyrins in blood and also a high plasmatic porphyrin index. Clinical symptoms and biochemical parameters therefore led to the diagnosis of a possible Dual Porphyria: AIP/CEP.

The patient´s erythrocyte PBGD activity was 37.73U/ml RBC, value 50% lower than those for normal subjects (normal range 81.51±11.96 in women) confirming the diagnosis of AIP. However, in our patient the enzymatic activity of UROS in RBC, which is always significantly reduced in CEP patients, was unexpectedly higher (5.16U/mg protein/ml RBC) than values obtained in normal subjects (3.65U/mg protein/ml RBC).

The RP-HPLC analysis of the urinary isomers UROI and UROIII revealed the predominantly excretion of UROI, as expected for an AIP/CEP patient (Fig 2).

The genetic study revealed no mutations in the *UROS* gene. However, in the *PBGD* gene two genetic variants were detected, one in exon 7, producing the transition of a G to an A at position c.331, changing a glycine (G) for an arginine (R) at position 111 of the protein (p.G111R) (Fig 3 I) and a novel gene variant in exon 13, the transition of an A to a G at position c.773 leading to the change of a glutamic acid (E) for a glycine (G) at position 258 of the protein (p.E258G) (Fig 3 II). Moreover, the genetic study of the *PBGD* gene of her asymptomatic mother revealed that she was heterozygous for the p.E258G mutation.

Prokaryotic expression of the new *PBGD* gene variant showed that the mutated protein has a residual enzyme activity of about 80% (pkk-PBGDwt: 71.37 ± 0.87 nmol

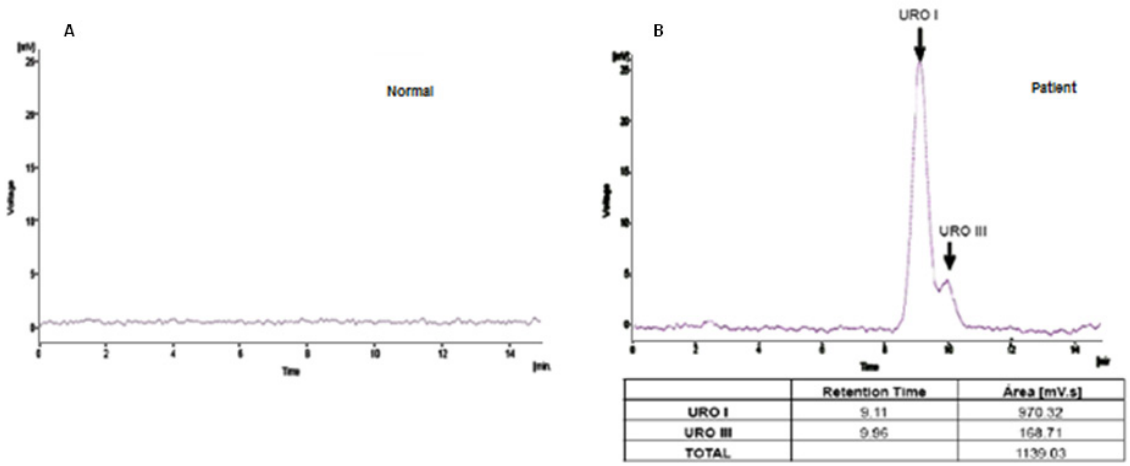

Figure 2. RP-HPLC analysis of the urinary isomers UROI and UROIII**.** A**)** Normal sample B**)** Patient ´s sample.

Table 3. Biochemical values in urine, faeces, blood and plasma.

	URINE							
	TP	**ALA**	**PBG**	**%C**	**%P**	**%Hx**	**%H**	**%U**
PATIENT	13.512	18.00	57.70	19	6	1	4	70
REFERENCE VALUES	20-250	< 4	< 2	100	0	0	0	0

	FAECES								BLOOD	PLASMA
	TP	**%Pr**	**%I**	**%C**	**%P**	**%Hx**	**%H**	**%U**	**TBP**	**PPI**
PATIENT	655	5	5	80	1	1	3	5	427	5.30
REFERENCE VALUES	30-130	35	25	35	2	1	1	1	150 ±40	<1.30

Urine TP: Total porphyrins (µg /24h); ALA: δ- aminolevulinic acid (mg/24h); PBG: porphobilinogen (mg/24h); Fecal TP: Total porphyrins (µg/dw; C: coproporphyrin; P: pentaporphyrin; Hx: hexaporphyrin; H:heptaporphyrin; U: uroporphyrin. Pr: Protoporphyrin; I: Isocoproporphyrin; TBP: Total Blood Porphyrins (µg /100 ml RBC); PPI: Plasma Porphyrin Index (λ= 619nm).

uroporphyrin/h/mg.prot.; pkk-PBGD-E258G: 56.20 ± 1.41 nmol uroporphyrin/h/mg.prot.). Other studies revealed no significant difference in the thermal stability between the wild type and the mutated protein at 65°C (Fig 4).

Figure 5 summarize the results obtained for the inhibition of the UROS enzyme by iron. These results showed, *in vitro*, an inverse relationship between the activity of erythroid UROS and iron concentration so, increasing concentrations of iron inhibited the enzyme.

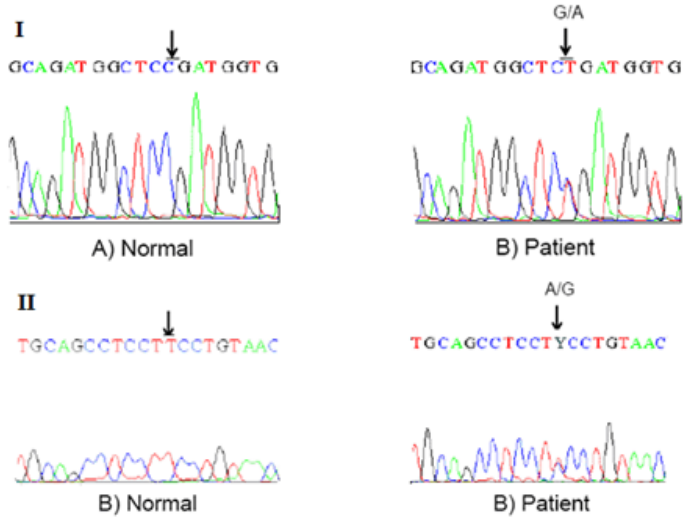

Figure 3. I) Mutations in the PBGD gene. I) c.331 G > A, p.G111R; II) c.773 A > G, p.E773G and their corresponding normal sequences.

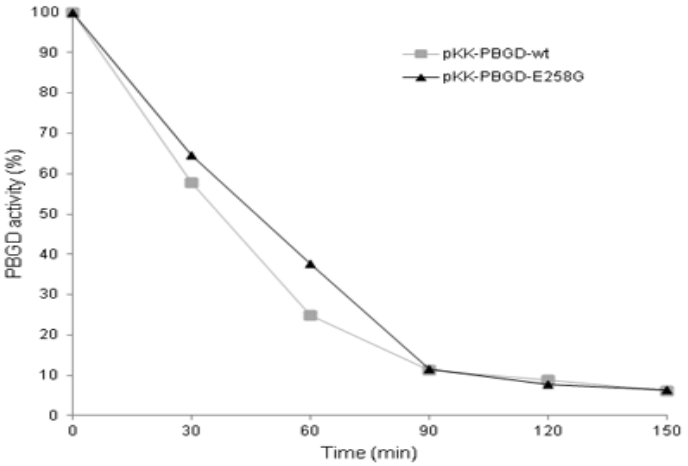

Figure 4. Thermostability at 65ºC of wild type and p.E258G proteins**.** PBGD activity was expressed as the percentage of the initial activity.

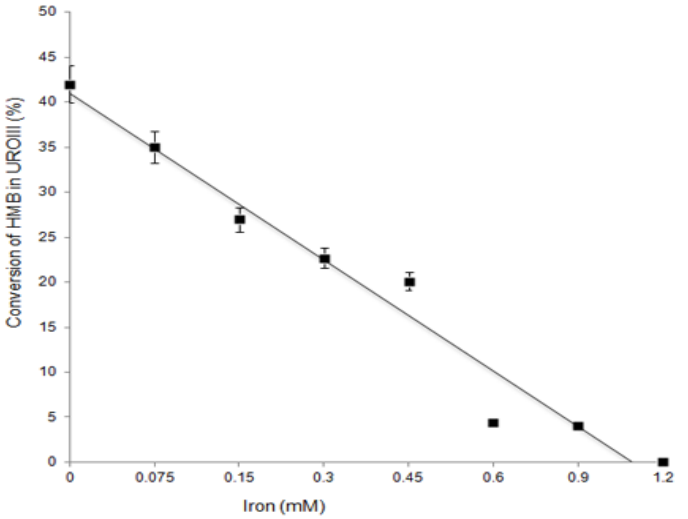

Figure 5. Iron inhibition of the erythroid UROS enzyme**.** Inhibition of the enzyme was expressed as percentage of conversion of HMB in UROIII with respect to the control value without iron.

DISCUSSION

Dual Porphyrias show the coexistence of clinical symptoms and biochemical excretion profiles associated to each individual porphyria. In our case, the patient presented acute intermittent abdominal pains and an increased urinary excretion of ALA and PBG characteristics of AIP. She also had cutaneous symptoms, splenomegaly and erythrodontia; urinary porphyrins were mainly URO I and COPRO I and the faecal porphyrins were mostly COPRO, all typical signs of CEP. Taken together both the biochemical and clinical signs, the patient appeared to be a case of a Dual Porphyria: AIP/CEP.

However, the UROS activity was higher than control values and the sequencing of the gene encoding this enzyme rules out the presence of mutations in exons and promoters, being not consistent with a CEP case. On the other hand, the determination of PBGD activity showed that it was approximately 50% of control value and sequencing of the PBGD gene revealed the presence of p.G111R mutation and p.E258G gene variant. The p.G111R mutation is located on the surface of the protein and although the molecular effect of the change of G amino acid by R is not exactly known, it was reported that the abnormal cDNA was unable to synthesize an active protein in the bacterial expression system, as already described (16). This mutation has a high prevalence in AIP families genetically studied in Argentina (17,18). The p.E258G gene variant had not been previously described worldwide, and its prokaryotic expression did not cause a dramatic decrease either in the activity or in the stability of the PBGD.

In all cases of homozygous dominant AIP reported, erythrocyte PBGD activity was lower than 15%, unlike this case with an activity of about 50%, similar to expected values for an individual with heterozygous AIP. To our knowledge our patient is the first HD-AIP case described in adults and the absence of severe neurological manifestations may be explained by the high activity of PBGD as compared to other HD-AIP patients. Moreover it is interesting to recall that four unrelated lines of AIP cats which presented phenotypically CEP, except cutaneous photosensitivity have been recently reported (19). In three lines of cats the porphyria was present as an autosomal dominant trait, whereas the fourth line was a homozygous AIP case (19). These cases can not be associated directly with the expression of the porphyria in humans since in wild cats PBGD activity was markedly low.

In our case, the patient presented high values of serum ferritin, not associated with any of the porphyrias diagnosed as AIP or CEP, suggesting the presence of some other pathology not related with porphyrias. It was reported that hepatic UROS and uroporphyrinogen decarboxylase (UROD) were inhibited by iron and that the inactivation of UROS produces a marked synthesis of URO I (14). We have also shown *in vitro*, that erythroid UROS was inhibited by increasing concentrations of iron (Table 8 and Fig 6). Iron inhibition of erythroid UROS would explain the increased accumulation and excretion of URO I and COPRO I in our patient. High levels of URO I, would be responsible for the cutaneous manifestations observed. The erythrocyte porphyrins were markedly enhanced and

can be accumulated in tissues and bones, corresponding to the CEP phenotype, as well as erythrodontia, splenomegaly and skin symptoms. In fact when a splenectomy was performed, her serum ferritin levels normalized and her cutaneous symptomatology improved.

We can not rule out however, the possibility of the presence of a gain function mutation in δ-aminolevulinic acid synthase 2 (ALAS2), the regulatory enzyme of heme biosynthesis in erythrocytes, as recently reported (20) .

In conclusion, the patient first diagnosed as a carrier of a dual porphyria: AIP / CEP based on the excretion profile of porphyrins, precursors and her clinical symptoms, would be an atypical case of human HD-AIP. The results would also suggest the presence of a phenocopy of CEP induced by an endogenous or exogenous factor, very likely iron overloading. Our findings highlight the importance of genetic studies for the correct diagnosis of porphyria, the prevention of its manifestation and treatment.

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