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CLINICAL, BIOCHEMICAL AND GENETIC CHARACTERISTICS OF VARIEGATE PORPHYRIA IN ITALY

E. DI PIERRO[1]✉, P. VENTURA[2], V. BRANCALEONI[1], V. MORIONDO[2], S. MARCHINI[2], D. TAVAZZI[1], F. NASCIMBENI[2], M.C. FERRARI[2], E. ROCCHI[2] AND M.D. CAPPELLINI[1]

1 Dipartimento di Medicina Interna, Università degli Studi di Milano - Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena IRCCS Milano- Italy
2 Department of Medicines and Medical Specialties, University of Modena and Reggio Emilia; Italy
✉ Elena Di Pierro, PhD
Dipartimento di Medicina Interna - Fondazione Ospedale Maggiore Policlinico MA RE IRCCS
Via F.Sforza 35, 20122 Milano - Italy
Tel. 0039 02 55033363, Fax. 0039 02 50320296, E-mail: elena.dipierro@unimi.it

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Abstract – Variegate Porphyria (VP) is an autosomal dominant disorder found worldwide but is rare in Italy. In this study we provide an overview of clinical, biochemical and genetic background of 33 Italian VP patients diagnosed in the last fifteen years. About 70% of patients had experienced clinical symptoms: 43.4% had photosensivity, 8.7% acute attacks and 47.8% both. Among the 33 patients, 14 different mutations were identified. Of these only 6 defects have been previously described in other countries and 8 are unique having been identified for the first time in Italy. Two of these, the c.851G>T and the c.1013C>G, were found in two and four unrelated families respectively. No mutation has been found in homozygosis and no significant correlation has been observed between specific clinical and biochemical manifestations and the type of mutation. In contrast, normal faecal protoporphyrin excretion was high predictive of silent phenotype. Normal urinary excretion of PBG and ALA, predicted absence of neurovisceral symptoms. This paper represents the first compilation of data on genotype-phenotype relation in Italian patients with VP.

Key words: Porphyrias, Variegate Porphyria, Protoporhyrinogen oxidase, mutation, porphyrins, Heme

INTRODUCTION

Variegate porphyria (VP; MIM 176200) is a low-penetrance, autosomal dominant disorder resulting from the partial deficiency of protoporphyrinogen oxidase (PPOX; EC 1.3.3.4), the seventh enzyme of the heme biosynthetic pathway (4,27,31,43). PPOX is located on the intermembrane surface of the inner mitochondrial membrane and catalyzes the six-electron oxidation of protoporphyrinogen IX to protoporphyrin IX, in the penultimate step of heme biosynthesis (7).

Abbreviations: **VP**, Variegate Porphyria; **ALA**, δ-aminolevulinic acid; **PBG**, Porphobilinogen; **Copro**, Coproporhyrins; **Uro**, Uroporphyrins; **Proto**, Protoporhyrins; **PPOX**, Protoporphyrinogen Oxidase; **TotPo**r, Total Porhyrins; **PP**, Protoporhyrins.

Patients affected by VP are almost universally heterozygous for genetic defects in the PPOX gene (gene ID 5498) and usually display about 50% reduction in PPOX activity (4,14,48), nevertheless rare cases of true homozygotes or compound heterozygotes (characterized by profound reduction of enzyme activity and more severe clinical pictures), have been reported. (23,26,30,32,44). A PPOX deficiency results in an overproduction and increased urinary and biliary excretion of porphyrins and porphyrin precursors: in patients with VP, faecal excretions of copro- and protoporphyrin are usually elevated associated with urinary excretions of delta-aminolevulinic acid (ALA), porphobilinogen (PBG), uro- and coproporphyrins. Moreover, plasma of patients with overt VP shows a characteristic fluorescence spectrum with an emission peak at 626 nm (6,35,53).

The overproduction and the accumulation of porphyrins and porphyrin precursors occurs mainly in the liver, thus VP is classified as a hepatic porphyria and it resembles other acute porphyrias (18). As implied by its name, VP is characterized by a wide diversity of presenting clinical symptoms, ranging from none to severe skin photosensitivity, which, in turn, may occur alone or simultaneously with acute neurovisceral crises. Cutaneous photosensitivity is characterized by skin fragility, erosions, blisters and pigment changes in sun-exposed areas. These symptoms result from light-induced phototoxic reactions and tissue damage attributable to excess porphyrins that are present in plasma and/or skin. Except for solar radiation and mechanical trauma, no precipitating factors responsible for inducing skin symptoms of VP are known. Neurovisceral crises include intermittent attacks of abdominal pain, constipation, vomiting, hypertension, tachycardia and various central and peripheral nervous system manifestations (pain in the extremities or in the back, weakness that may progress to paresis). Acute attacks frequently result from exposure to porphyrinogenic drugs, alcohol ingestion, reduced calories intake due to fasting or dieting, infections or endogenous changes in sex-hormones balance. All of these factors are known to stimulate heme synthesis by induction of δ-aminolevulinic acid synthase (ALA-S), thereby increasing the production of the porphyrin precursors (ALA and PBG).

Skin symptoms usually arise in the second decade and in most cases are manifest continuously until the fifth decade at which time they may decline. Acute attacks are more frequent during the second and fourth decades and are rare before puberty. The disease is more often clinically manifested more often in women (18,28,29,31,41,46,55), although more than 50% of the carriers of a mutation in the PPOX gene remain symptom-free throughout their lives (41).
The human *PPOX* gene is located at chromosome 1q22-23; it consists of 13 exons, one of which is a non-coding exon, and 12 introns for a total of about 6kb. The *PPOX* gene expression is under control of a single promoter that lacks of the canonical TATA box, a property common to housekeeping promoters (9,13,49). A single 1.8kb mRNA transcript encodes a protein of 477 amino acids (8). The N-terminal 17 amino acid segment of human PPOX is responsible for initial targeting to the mitochondrion (10,40,54) but also internal targeting signals outside the N-terminal region, within residues 18-235, have also been identified (11). To date, more than 130 mutations in PPOX gene that are responsible for VP have been reported (50). These mutations are highly heterogeneous including insertions, deletions, splicing defects, missense and nonsense mutations. Moreover, no clear correlation appears to exist between type of mutation and clinical manifestations.(56). The disease has worldwide distribution, but has an exceptionally high frequency in South Africa (1:300), where the missense mutation R59W is largely predominant (37). In Europe the disease is rare with the highest frequency in Finland (1:50.000) (53).

Currently little is known about VP in Italy. The disease has been a diagnostic enigma due to its clinical and biochemical findings resembling those observed both in acute and chronic (cutaneous) porphyrias. The purpose of the present work was to assess clinical, biochemical and genetic aspects of VP in patients from 18 different Italian families, in order to provide more insights about this disorder.

MATERIALS AND METHODS

Patients

After informed consent, according to the good clinical practice, 18 Italian unrelated patients and their relatives (a total of 47 subjects) were studied. Approximately 10 ml of peripheral blood in Na_2-EDTA anticoagulant tubes were taken. Fluorimetric scanning of porphyrins and assays of hydroxyl-methyl-bilane synthase (HMBS) activity assay were performed for all patients using plasma or erythroid fraction respectively. Genomic DNA was purified from peripheral blood. Urine and fecal samples were obtained for ALA, PBG and porphyrin assessment. In all patients biochemical assessments were performed in remission phase of acute attack and never during overt cutaneous manifestations. For this study the VP diagnosis was made on the presence of a genetic defect in the PPOX gene.

Fluorometric scanning of plasma porphyrins

Using a ND 3300 NanoDrop Fluorometer (NanoDrop Technologies, Wilmington, DE USA) 2µl of freshly-drawn plasma were excited with UV led (max=365 nm). The fluorescence emission spectrum was analyzed by scanning from 400 to 750 nm. Using a Perkin Elmer LS-5 luminescence spectrometer (Perkin Elmer, Foster City, CA, USA) 100 microliters of plasma was isolated and diluted five-fold in a 0.25 M potassium phosphate buffer, pH 6.7. The fluorescence emission spectrum was recorded between 580 nm and 700 nm with excitation at 405 nm.

HMBS activity assay

The PPOX activity was not measured since the enzyme localization makes the assay very difficult to perform. Therefore HMBS was assayed according to the method of Meyer et al., with slight modifications (39). The packed red cells (RBC) were lysed in 10 volumes of 0.1 M Tris-HCl buffer, pH 8.0 containing 0.2% Triton X100. The

enzyme reaction was performed at 37°C on 25 µl of lysate in the presence of 0.2 ml of 0.1 mM Tris-HCl, pH 8.0 and 25 µl of 1 mM porphobilinogen (PBG). After incubation for 1 h, the reaction was stopped by addition of 1 ml of 10% trichloroacetic acid and the mixture centrifuged at 10,000 x g for 5 min. The supernatant's fluorescence emission (excitation at 405 nm, emission at 655 nm) was measured with a spectrofluorometer (Perkin Elmer, Foster City, CA, USA) and uroporphyrins were assessed using uroporphyrin I (Porphyrin Products Inc, Logan, UT, USA) as standard. The HMBS activity was expressed as pmoles of uroporphyrin formed per hour/mg haemoglobin (Hb) at 37°C. Haemoglobin concentration was determined by the cyanmethemoglobin method (16).

Urine and stool assessments

Urinary ALA and PBG were determined by ion exchange chromatography using a double column specific kit (BIORAD laboratories, Munich, Germany). Urine and feacal assessment of porphyrins were performed by HPLC techniques as previously described (17,33,34,47).

DNA analysis

Genomic DNA was isolated from blood with Puregene kit (QIAGEN). The promoter, coding region and splice junctions of the PPOX gene were amplified by polymerase chain reaction (PCR) in five fragments. (Primers are available on request from the authors). PCR reactions were carried out with 0.2mM dNTPs, 1.5mM $MgCl_2$, 67mM Tris-HCl at pH8.8, 16mM $(NH_4)_2SO_2$, 0.01% Tween 20 and 1U of DNA polymerase (Bioline, London, UK) in a final volume of 50µL. The PCR conditions were: an initial denaturation step at 94°C for 5 min followed by 30sec at 94°C, 30sec at 59-65°C and 1min at 72°C for 30 cycles. The PCR products were characterized by automated direct sequencing on both strands using ABI PRISM 310 (Applied BioSystems, Foster City, CA, USA). The complete sequence of the gene is available in GenBank (PPOX: NC_000001.9:159402141-159407724).

Statistical methods

Fischer's exact test was used for the comparison of categorical variables. Continuous variables were analysed using Mann–Whitney U-test, when two groups were compared, or Kruskal –Wallis test, when more than two groups were compared simultaneously. Simple logistic regression was employed to evaluate the association between dichotomous outcome variables (eg occurrence of skin symptoms or acute symptoms) and covariates (eg mutation group and biochemical tests). Statistical calculations were performed with SPSS version 17.0 (SPSS inc., Chicago, Illinois, USA). In statistical analysis a p value <0.05 was considered as significant.

RESULTS

Molecular results

A total of 47 subjects composed of eighteen unrelated Italian patients (probands) and their relatives were studied. Sequence analysis of the PPOX gene confirmed the molecular diagnosis of VP in 33 subjects. Among the 18 families, 14 different mutations were identified. Of these only 6 defects have been previously described in other countries and 8 are unique having been identified for the first time in Italy. Two of these, the c.851G>T and the c.1013C>G, were found in two and four unrelated families respectively. The new mutations have been submitted to the Human Genome Mutation Database (HGMD), a core collection of molecular defects described worldwide (50). **Table 1** summarizes both the mutations identified in this study and those identified in previous works on other nine Italian families affected by VP.

Clinical results

The study group consisted of 47 subjects. Of the 33 subjects with detected VP mutations (11 male, 22 female, age 21 – 68 years), 23 were symptomatic with 2 having experienced neurovisceral acute attacks, 10 having experienced photosensitivity and 11 experienced both symptoms. The 10 asymptomatic subjects had never experienced any VP symptoms. The overall frequency for skin symptoms (21/33) was 63.6 %, and for acute attacks (13/33) 39.3%, respectively. The remaining 14 subjects (8 female and 6 male, age 18-65 years) were relatives and they were not carrier of PPOX gene mutation. The prevalence in acute symptoms was significantly prominent among female patients with 12/13 (92.3%) of the patients experiencing neurovisceral crises being female. Similar results were found for cutaneous symptoms (19/21, 90.4% of cases). The median age of the patients at the onset of acute symptoms was 32 years (range 19– 51 years) and for skin symptoms 24 years (range 13 – 52 years), respectively. For subjects over the age of 40, only one patient experienced her first acute attack and only one patient experienced her first skin symptoms.

Biochemical results

No difference has been recorded in the fluorometric scanning of plasma porphyrins using the two different methods for the two different fluorimeters.

As expected, a high rate of symptomatic patients showed the typical plasma fluorescence peak at 626nm (19). Only 1 patients (4.3%) did not show the peak. The plasma fluorescence test results were negative for all 10 (100%) VP mutation carrier with a negative history for symptoms and in 1 patient (1/2, 50%) who experienced only neurovisceral symptoms. In patients with only cutaneous symptoms and in patients who experienced both kind of symptoms, the plasma fluorescence test results were positive in 100% of the cases.

Table 1: Reported mutations in the Variegate Porphyria in Italy

Mutation	Exon	Protein or RNA defect	Cases	First report	Ref.
In this study					
c.121delG	3	p.Gly41SerfsX27	2 (4.3%)	Italy	(45)
c.218 T>C	3	p.Leu73Pro	2 (4.3%)	UK	(56)
c.306_307insC	4	p.Cys103ArgfsX41	3 (6.4%)	Italy	(36)
c.416 G>A	5	p.Gly139Asp	2 (4.3%)	Italy	(2)
c.419_420 delAA	5	p.Lys140ArgfsX3	1 (2.1%)	Italy	(5)
c.503 G>A	6	p.Arg168His	2 (4.3%)	Germany	(21)
c.532 G>C	6	p.Leu178Val	2 (4.3%)	Argentina	(12)
c.694 G>C	7	p.Gly232Arg	1(2.1%)	France	(15)
c.745_746insC	7	p.Val249AlafsX32	1 (2.1%)	France	(56)
c.851 G>T	8	p.Ser284Ile	7 (14.9%)	Italy	(36)
c.1013 C>G	10	p.Ser338X	7 (14.%)	Italy	(36)
c.1082_1083insC	10	p.Gly362TrpfsX19	1 (2.1%)	UK	(56)
c.1190C>A	11	p.Ala397Asp	1 (2.1%)	Italy	(3)
c.1353T>G	13	p.Tyr451X	1 (2.1%)	Italy	(1)
In previous study					
c.384G>A	5	p.Trp128X	1	Italy	(5)
c.419_420 delAA	5	p.Lys140ArgfsX3	1	Italy	(5)
c.1098+2 T>G	Ivs10	r.spl?	1	Italy	(5)
c.1291+1G>C	Ivs12	r.spl?	1	USA	(22)
c.694 G>C	7	p.Gly232Arg	2	France	(15)
c.745_746insC	7	p.Val249AlafsX32	1	France	(56)
c.795delA	7	p.Glu265GlufsX8	1	Italy	(5)
c.848T>A	8	p.Ile283Asn	1	Italy	(5)

HMBS activity was significantly lower in carriers of VP mutations (n= 33) than in relatives who were not carriers of mutations (n=14) (87.51 ± 21.2 % vs. 100.8±11.1 %, respectively, p=.008). Within VP mutation carriers, no significant difference was observed among symptomatic patients (n=23) and their asymptomatic relatives (n=10) (82.93±18.22 % vs. 98.2±24.5%, respectively, p=.054). Within the symptomatic group, no significant difference was observed between patients with cutaneous signs only (n=10) with respect to patients with neurovisceral crises (n=13) (87.27±19.13 vs. 78.76±14.13, respectively, p=.249). **Table 2** presents the biochemical and clinical features of the subjects. Values are means of at least three different determinations that were all made during the remission phase (absence of neurovisceral and/or cutaneous active lesions).

In symptomatic patients urinary ALA and PBG showed a high prevalence (7/9, 78%) of very significant increase (from two to three fold higher than normal, and with PBG increase greater than ALA increase) where measured during an acute neurovisceral attack; ALA and PBG remained mildly elevated (slightly higher than reference values) during remission phase only in about 80% of patients. Patients with cutaneous symptoms exhibited a great variability in ALA and PBG urinary excretion, even in the presence of overt cutaneous lesions (data not showed), and only 25% had elevated ALA and PBG (at least one fold more than the reference value) levels during acute exacerbation of

Table 2 Biochemical features of considered patients

	VP Mutation Carriers			Controls (n=14)		
	All (n=33)	Symptomatic (n=23)	Asymptomatic (n=10)		p^1	p^2
Urinary ALA mg/g creatinine (r.v. < 5)	6.85±2.83	7.76±2.64	4.75±2.11	2.44±0.99	.000	.002
Urinary PBG mg/g creatinine (r.v. < 2)	5.77±3.63	7.03±3.57	2.9±1.61	1.25±0.55	.000	.000
Urinary total Porphyrins mg/g creatinine (r.v . < 100)	702±672	893±727	262±91.5	53.7±12.8	.000	.000
Urinary Coproporhyrins mg/g creatinine (r.v . < 100)	354±373	437±421	181±59.8	52.1±20.2	.000	.001
Urinary Uroporhyrins mg/g creatinine (r.v . < 100)	116±85.7	139±92.9	62.8±20.9	14.2±6.21	.000	.005
Fecal total Porphyrins μg/g of dry weight (r.v . < 100)	707±610	864±674	347±79.8	63.8±18.5	.000	.001
Fecal Coproporphyrins μg/g of dry weight (r.v.< 50μg/g)	178±207	212±240	100±51.2	21.8±19.1	.000	Ns
Faecal Protoporphyrins μg/g of dry weight (r.v.< 30 μg/g)	205±345	274±395	45±28	17.8±5.54	.000	.011

Values are mean of at least three different assessment, all performed during remission phase of disease. p^1= Controls vs. Carriers (All); p^2= Symptomatic vs. Asymptomatic

Table 3 Correlation between genetic, biochemical and clinical parameters

Mutation	Urinary	Fecal	Symptoms
	ALA/PBG/PorTot/Uro/Copro	**PorTot/Copro/Proto**	**C / NV / B / A**
c.121delG (n=2)	2 / 2 / 1 / 1 / 2	2 / 2 / 2	2 / 0 / 0 / 0
c.218 T>C (n=2)	1 / 2 / 2 / 2 / 2	1 / 1 / 1	0 / 0 / 1 / 1
c.306_307insC (n=3)	3 / 3 / 3 / 3 / 3	3 / 3 / 3	0 / 0 / 2 / 1
c.416 G>A (n=2)	2 / 2 / 2 / 2 / 2	2 / 1 / 2	0 / 1 / 0 / 1
c.419_420 delAA (=1)	1 / 1 / 1 / 1 / 0	1 / 0 / 1	1 / 0 / 0 / 0
c.503 G>A (n=2)	2 / 2 / 2 / 2 / 2	2 / 2 / 2	1 / 0 / 1 / 0
c.532 G>C (n=2)	2 / 2 / 2 / 2 / 2	2 / 2 / 2	1 / 0 / 1 / 0
c.694 G>C (n=1)	1 / 1 / 1 / 1 / 1	1 / 1 / 1	0 / 0 / 1 / 0
c.745_746insC (n=1)	1 / 1 / 1 / 1 / 1	1 / 1 / 1	0 / 1 / 0 / 0
c.851 G>T (n=7)	3 / 4 / 7 / 7 / 7	7 / 7 / 5	2 / 0 / 1 / 4
c.1013 C>G (n=7)	4 / 6 / 7 / 7 / 6	7 / 7 / 7	3 / 0 / 1 / 3
c.1082_1083insC (n=1)	1 / 1 / 1 / 1 / 1	1 / 1 / 1	0 / 0 / 1 / 0
c.1190C>A (n=1)	1 / 1 / 1 / 1 / 1	1 / 1 / 1	0 / 0 / 1 / 0
c.1353T>G (n=1)	1 / 1 / 1 / 1 / 1	1 / 1 / 1	0 / 0 / 1 / 0

The numbers indicate, for each correspondent mutation of PPOX gene, the number of patients with altered (i.e. higher than reference value, see table 2) urinary or faecal biochemical parameter (ALA= δ-aminolevulinic acid; PBG=Porphobilinogen; PorTot=Total Porphyrins; Copro=Coproporhyrins; Uro=Uroporphyrins; Proto=Protoporhyrins) or the number of patients with clinical symptoms (C=only cutaneous; NV= neurovisceral; B=both (NV+C); A=asymptomatic). In bold 8 mutation observed only in Italy are indicated.

cutaneous symptoms. Nevertheless, even within this group (cutaneous symptoms only) we found a high prevalence of patients with urinary ALA and PBG levels elevated significantly above reference values. This was even true for subjects during the remission phase of diseases (more than 60%).

Genotype-phenotype relationship

Table 3 contains the summary of the biochemical (as number of patient with increase in urinary porphyrins and/or porphyrin precursors) and clinical data (history of cutaneous, neuro-visceral, or both) with respect to the underlying identified PPOX gene mutation. **Table 4** presents data showing the association between individual biochemical parameters (presence of a positive test) and the symptomatic state (cutaneous, neurovisceral, or both). Due to the small sample it was not possible to perform a similar test for each kind of subgroup of symptomatic disease (neurovisceral/cutaneous only), nor a multivariate analysis. Normal fecal protoporhyrin excretion (< 50 μg/g dry weight) was significantly associated to a symptom free status (OR=3.44, 95% CI 2.5÷84.2, p=.002); normal ALA and PBG urinary excretion (together) were significantly associated with the absence of neurovisceral crisis history (OR= 4.56, 95% C.I. 1.23÷78.5, p=.014).

Table 4. Association between different positive tests and presence of symptomatic disease

	OR (95% CI)	p
Urinary ALA	15.7 (2.31÷107)	.005
Urinary PBG	9.42 (.841÷105)	.069
Urinary Coproporhyrins	2.44 (1.37÷43.7)	.025
Urinary Uroporhyrins	3.21 (1.53÷121.7)	.015
Fecal Coproporphyrins	4.52 (1.12÷56.8)	.012
Faecal Protoporphyrins	5.50 (1.41÷78.1)	.003

Due to the small sample size it was not possible to perform a similar test for each kind of subgroup of symptomatic disease (neurovisceral / cutaneous only).

DISCUSSION

Herein we summarized the results of clinical, biochemical and molecular assessments from 18 Italian families affected by Variegate Porphyria. While previous studies have focused more specifically upon genetic issues (5) the current work represents the first compilation of data relating VP genotype to phenotype in Italy. In this study among 18 families, 14 different mutations were identified. Although most of mutations are family-specific, only 2 defects (c.851G>T and the c.1013C>G) were found in two and four unrelated families respectively. These data, while limited, strongly suggest the absence of any predominating VP mutation in Italy. This heterogeneity of genetic defects is typical for what has been observed in other countries with the notable exceptions of variegate porphyria in South Africa (37) and acute intermittent porphyria in northern Sweden (20), where particular mutation have been transmitted over generations from single founders. The genetic heterogeneity is reflected in the sorts involves also the kind of mutations found. In fact, missense, nonsense, small deletions-insertions and splicing junction mutations have been identified without a prevalence of any single type. The data presented herein also did not reveal any evidence of specific hot spots. The majority of exons have been involved without distinction with a slight exception for exons 5 and 7. Out of a total of 19 reported mutations in Italy (summarized in Table 1), 12 have been identified for the first time in our country, thus they could be restricted to the Italian population, while 7 have been found in other countries.

In this study the sequence analysis of the PPOX gene confirmed the molecular diagnosis of VP in 33 of 47 analyzed subjects. Our series includes both carriers of PPOX mutation with a history positive for VP-related symptoms (69.7%) (patients) and subject carriers of PPOX mutation without a history of VP-related symptoms (carriers with normal phenotype) (30.3%). The proportion of patients with acute attacks (39.4%) is similar to that observed in some extensive family studies, where up to 38% of patients had experienced acute attacks. The frequency of photosensitivity (63.6%) is also quite similar to that reported previously in South Africa and France (about 65-70%) (17,56). Since 47.8 % of symptomatic patients suffered both photosensitivity and acute attacks, they represented the majority of our patients, being those with each of these manifestations 8.7% (neurovisceral only) and 43.4% (cutaneous only). This is in contrast with previous studies where 79 and 77% of symptomatic patients experienced either photosensitivity or acute attacks, but not both (17,56). We have observed that the occurrence of acute attacks has decreased markedly during the last fifteen years, as well (even in a less extent) for skin symptoms. The decrease has been most prominent in males. This suggests that different pathogenetic factors, including hormonal factors, are important in the development of skin symptoms and acute attacks. In our data symptomatic patients had a high incidence of elevated plasma fluorescence. The specificity of these tests in symptom-free individuals was also very high (about 100%). This results support the theory that the severity of symptoms (especially cutaneous symptoms) is likely to depend on the permanent circulating levels of porphyrins (41) and they confirm this simple and inexpensive screening method as a specific marker for symptomatic VP patients. Our fluorometric screening results, however, result from a relatively small sample size and included no children carrying the genetic defect (6,25,35).

The responsible enzyme activity (PPOX) was not measured since the enzyme localization makes the assay very difficult to perform. Therefore HMBS was assayed according to the method of Meyer et al. As anticipated, HMBS activity was significantly lower in carriers of VP mutations than in tested relatives who were not carriers of any VP mutation. Interestingly, within VP mutation carriers, no significant difference was observed among symptomatic patients and their asymptomatic relatives. This enzymatic reduction could explain the high level of ALA and PBG precursors in patients compared to normal subjects (Table2).

In the current study we attempted to find a genotype-phenotype correlation by evaluating the biochemical parameters of patients not experiencing the acute phases of the disease in an effort to identify possible significant predictors of disease severity and of disease type (cutaneous, neurovisceral or both). Unfortunately, according to disease's name, even our data showed "variegate" results.

No mutation has been found in homozygosis and no significant correlation has been observed between specific clinical manifestations and the type of mutation. In four patients carrying the same c.1013C>G mutation, 1 experienced only

photosensitivity, 1 only neurological symptoms and the 2 both clinical manifestations.

Urinary and faecal excretions of porphyrins in remission phases differed significantly between symptomatic and asymptomatic group, being significantly lower in the latter group (Table 3). This is in distinction from others (55) no significant difference between values from different mutations (data not shown). These data result may be explained by the fact that in many cases the sample size was insufficient to carry out a significant comparison; moreover, within the more represented mutation (c.851 G>T and c.1013 C>G, see Table 1 and 2), about half of patients were asymptomatic. On the other hand, it is well known that environmental influences and of other metabolic gene(s) (at presently not all completely identified) play a role in modifying the porphyrin excretion in general. Moreover several polymorphisms in the cytochrome P450 enzymes exist that make these genes good candidates as modifiers of modifying porphyrin metabolism and VP phenotype (24,42,51,52).

In the pathogenesis of acute attacks, the induction of ALA-synthase (ALAS), the rate-limiting enzyme of the heme synthetic pathway, is believed to play a key role. Moreover, if liver has an increase of heme requirement, ALAS is induced resulting in the accumulation of porphyrins and porphyrin precursors in those patients (38). In our studies, the history of symptoms was related to a significant increase in urinary copro- and uroporphyrin and fecal copro- and protoporhyrin excretion (Table 4). In contrast, normal fecal protoporphyrin excretion as well as negative plasma fluorescence was highly predictive for the absence of skin symptoms, while a normal excretion of protoporphyrin in feces was predictive of freedom from both skin symptoms and acute attacks for patients. Normal urinary excretion PBG and ALA, predicted freedom from neurovisceral symptoms. Other authors have shown that high urinary coproporphyrin excretion (but only in case of significant increased excretion, such as fivefold the reference value) was independently associated with an increased risk of both skin symptoms and acute attacks and virtually all patients with theses values had experienced either skin symptoms, acute attacks, or both (41).

Our paper represents the first attempt to collect data on genotype-phenotype relation on Italian patients with VP. While the sample size was small, these preliminary data show many results similar to those reported in other studies of genotype-phenotype relationship in VP (41) in term of phenotype prediction. Additional studies will needed to reach sample sizes sufficient to satisfy all statistical assumptions and hence to obtain more complete results whose inference should be able to build prognostic models for clinical aspects of this disease based on the association of different variables.

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