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Homozygous Variegate Porphyria in South Africa: Genotypic Analysis in Two Cases[1]

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Variegate porphyria is an autosomal dominant disorder of heme metabolism which results from decreased activity of the enzyme protoporphyrinogen oxidase. Clinically, the disease manifests postpubertally and is characterized by photocutaneous sensitivity and/or acute neurovisceral crises. However, in homozygous variegate porphyria, onset of the disease usually occurs in infancy with severe skin manifestations. The molecular basis of variegate porphyria in two severely affected probands in two South African families is described. Mutation detection included combined SSCP-heteroduplex analysis followed by direct sequencing. The unrelated probands both had the common R59W mutation while the other lesion was Y348C or R138P (both novel mutations), causing homozygous variegate porphyria. © 2000 Academic Press

Key Words: **homozygous variegate porphyria; variegate porphyria; porphyria; mutations; protoporphyrinogen oxidase; heme biosynthesis.**

Variegate porphyria (VP) is an autosomal dominant disorder of heme metabolism which results from the reduced activity of protoporphyrinogen oxidase (PPO) (EC1.3.3.4, MIM No. 600923)—the penultimate enzyme in the heme biosynthetic pathway. The human PPO gene contains 13 exons and is located on chromosome 1q22–23 (1–3).

VP is encountered throughout the world and 79 mutations have been reported to date (4) including 4 from South Africa—R59W, R168C (5,6), H20P (6,7), and 537delAT (8). In the United Kingdom, it has been estimated to be a third as prevalent as acute intermittent porphyria, which has a prevalence of about 1–2 per 10,000 population (9). In South Africa, however, VP has an extraordinarily high prevalence. This has been attributed to a founder effect (10,11) and is supported by the finding that a single point mutation, R59W, is present in approximately 95% of South African cases of VP (5).

Typical symptoms of VP in heterozygotes include acute neurovisceral crises and photosensitive skin disease. Biochemically, the disease manifests with increased fecal excretion of protoporphyrin, coproporphyrin, and pentacarboxylic porphyrin. Elevated porphyrin concentrations in urine and plasma are frequently noted as well (7). Typically, neither clinical symptoms nor evidence of disturbed porphyrin excretion are present before puberty. "Silent" carriers, who carry mutations known to be associated with VP, but have no clinical or biochemical evidence of the disease, also have been described. The R59W gene frequency in South Africa is thought to be sufficiently high for homozygotes to be encountered (11), yet no R59W homozygote has been reported. Presumably, this suggests that homozygosity for the R59W mutation is lethal.

However, a small number of cases of apparently homozygous VP have been described around the world, including South Africa (12–19). These individuals are characterized by a severe reduction in the activity of PPO to <25% of normal (18,19), a severe phenotype marked by onset in infancy and the presence of atypical symptoms including growth retardation, brachydactyly, nystagmus, convulsions,

[1] The sequence data reported herein have been submitted to GenBank and assigned Accession Numbers Y348C-AF215864, R138P-AF215863.

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TABLE 1
Porphyrin Concentrations in Probands with HVP

	Proband 1	Proband 2	Reference range
Urine			
ALA	16	106	<45 μmol/10 mmol creatinine
PBG	8.1	106	<16 μmol/10 mmol creatinine
Uroporphyrin	11	1464	<20 nmol/10 mmol creatinine
7-COOH porphyrin	17.9	570	<1.5 nmol/10 mmol creatinine
6-COOH porphyrin	0	178	0
5-COOH porphyrin	6.1	1296	0
Coproporphyrin	177	4538	<240 nmol/10 mmol creatinine
Stool			
Uroporphyrin	2	33	<2 nmol/g dry mass
7-COOH porphyrin	0	74	0
6-COOH porphyrin	0	120	0
Pseudo 5-COOH porphyrin	23	376	0
5-COOH porphyrin	2	161	0
Isocoproporphyrin	0	114	0
Coproporphyrin	68.8	1212	<50 nmol/g dry mass
3-COOH porphyrin	0	634	0
Protoporphyrin	432	1804	<200 nmol/g dry mass

and mental retardation. Prior to the identification of the PPO gene, most of these cases were thought to represent VP homozygosity. Thus the syndrome was termed homozygous VP (HVP). No acute neurovisceral crises in these patients have been documented. Of the 11 cases of apparently homozygous VP reported to date, 7 have been investigated at the molecular level. Two of these patients were homoallelic (A433P/A433P, D349A/D349A) (19) while the remainder were heteroallelic for two different PPO mutations (R168C/R59W (5); G169E/G358R, G358R/SD (deletion of exon 7) (19,20); A219KANA/intron11 (19); I12T/P256R (21)). Thus approximately 70% of cases of HVP, including the R59W/R168C patient reported from South Africa, are in fact compound heterozygotes, rather than true homozygotes.

In this study we report two further cases of homozygous VP in two unrelated South African VP families. Our findings suggest that all VP patients who appear to be severely afflicted should be investigated for HVP.

MATERIALS AND METHODS

Case Reports

Family 1. The proband and 10 other family members were investigated with respect to porphyria. The proband is a 7-year-old girl of Afrikaner (and therefore ultimately Dutch) descent, the daughter of nonconsanguinous parents, neither of whom are reported to have experienced clinical symptoms of VP. She first presented at the age of 10 months with severe skin disease and was found to have the typical urine and stool porphyrin excretion profile of VP (Table 1). Subsequently, when testing for the R59W mutation became available, she and her mother were shown to carry this mutation; her father was negative. Her current clinical status is typical of HVP. She has fragile skin with blisters, scars, and milia most marked in sun-exposed areas (Fig. 1A); although even unexposed skin is abnormal. She has brachydactyly (Fig. 1B), photo-onycholysis, myopia, nystagmus, a sensory neuropathy and problems with concentration. She has never had a typical acute attack.

Family 2. The proband is a 26-year-old female from the Cape mixed-race population, who are largely descended from the slave population imported by the Dutch to Cape Town in the 17th and 18th centuries, further modified by intermarriage with other indigenous and settler populations over the years. Their genetic inheritance is therefore largely Indian, Indonesian, European, African, and Khoi, although it is rarely possible to assess the contribution of each to a particular individual. She first presented at the age of 19 with symptoms suggestive of a porphyric acute crisis. A diagnosis of VP

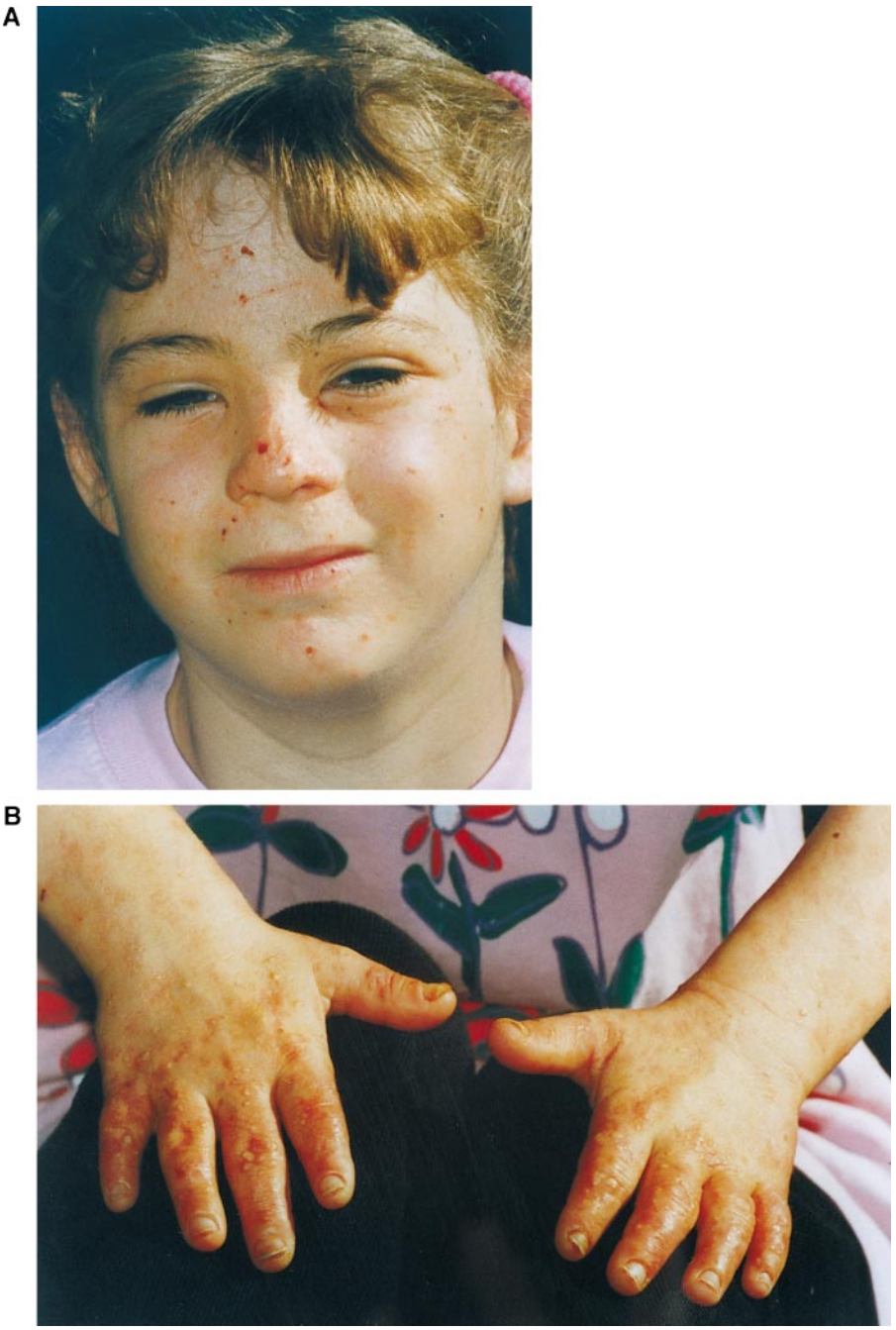

FIG. 1. Proband 1 showing typical features of severe HVP with marked erosions and scarring on the face (A) and severe skin changes and brachydactyly (B).

during the acute attack was made biochemically (Table 1). Subsequently she has experienced two milder acute attacks; however, she manifests an unusually severe form of skin disease with chronic blistering of the hands and face, hypertrichosis, and photomutilation of the hands with progressive shortening of the fingers and deformities of the joints of the fingers. Despite this, her general health has been excellent and she has two children.

Her sister, age 25, was subsequently found to be affected both clinically and biochemically with VP. She has a typical porphyrin excretion pattern and

typical skin disease. Unlike her sister, it is not regarded as unusually severe. Subsequent testing proved the presence of the R59W mutation in both siblings.

In the probands in both families, the presence of an abnormally severe phenotype led us to suspect the presence of more than one mutation, and hence they were investigated further.

Consent

All individuals gave informed consent prior to inclusion in the study which was formally approved by the Human Research Ethics Committee of the University of Cape Town Medical School (reference 051/98).

PCR Amplification and Mutation Analysis

Genomic DNA was extracted from whole blood (22). The 13 PPO exons were amplified by polymerase chain reaction (PCR) using primers as previously described (8).

Mutation Detection

We screened both families for the presence of the R59W defect using *Ava*1 restriction analysis as previously described (5).

Screening of all of the PCR products of the 13 PPO exons was performed by combined single-stranded conformational polymorphism (SSCP)-heteroduplex analysis as previously described (8). PCR products displaying abnormally migrating bands were directly sequenced using a Big Dye terminator cycle sequencing kit on an ABI Prism automatic sequencer. PCR conditions for exons 10 and 5, the exons of interest to this report, are given below.

Family 1. For exon 10, PCR was performed in a Robocycler (Stratagene) as follows: 95°C for 1 min; 35 cycles of (95°C for 30 s, 65°C for 30 s, 72°C for 30 s); 72°C for 7 min. This generated a 223-bp product which included exon 10.

Family 2. For exon 5, PCR was performed as follows: 95°C for 1 min; 10 cycles of (95°C for 30 s, 65°C for 45 s, 72°C for 30 s); 30 cycles of (95°C for 30 s, 60°C for 45 s, 72°C for 30 s); 72°C for 8 min. This generated a 247-bp product which included exon 5.

Restriction Analysis

Family 1. As the Y348C mutation creates an additional *Mae*III restriction site, restriction analysis was performed on the exon 10 PCR product by incubation at 55°C for 4 h. The digested fragments were separated by electrophoresis on a 6% acrylamide gel and visualized under UV light after ethidium bromide staining. Digestion of the wild-type product produces two fragments of 146 and 77 bp, whereas the mutant product has three fragments of 96, 50, and 77 bp.

Family 2. No suitable restriction enzyme was available. Thus screening of the controls and other family members was performed by SSCP-heteroduplex analysis.

Controls. For each family, DNA from 50 healthy individuals of similar racial descent was used as control DNA.

Biochemical Analysis

Quantitative thin-layer chromatographic fluoroscanning of urine and stool was performed (7). Additionally, plasma fluoroscanning was performed at a fixed excitation of 405 nm and emission scanning between 621 and 627 nm (Hitachi 650-10S) (23); the presence of a plasma porphyrin fluorescence spectrum with maximum emission at 625 nm is consistent with VP (24).

RESULTS

Family 1 (Fig. 2)

SSCP-heteroduplex analysis of the PCR products of the 13 exons revealed an abberant mobility shift in exon 10 in the proband (Fig. 3) and her father. In addition there was an abnormality in exon 3 in the proband and her mother which was confirmed by *Ava*I restriction analysis of the PCR product of exon 3 to represent the R59W mutation. Her sister also tested R59W positive by restriction analysis. All other exons appeared normal by SSCP-heteroduplex analysis. Direct sequencing in both a forward and a reverse direction of the exon 10 PCR product from the proband revealed an A to G transition in codon 348. This results in a tyrosine to cysteine substitution, Y348C; (GenBank Accession No. AF215864), numbering from the initiating methionine codon of the PPO cDNA sequence (25). *Mae*III restriction analysis confirmed this finding (Fig. 4). Subsequently we tested the 10 other family members for the Y348C mutation by *Mae*III restriction analysis. Results may be summarized as follows (Fig. 2).

The proband tested positive for both the R59W and the Y348C mutation. She is both clinically and

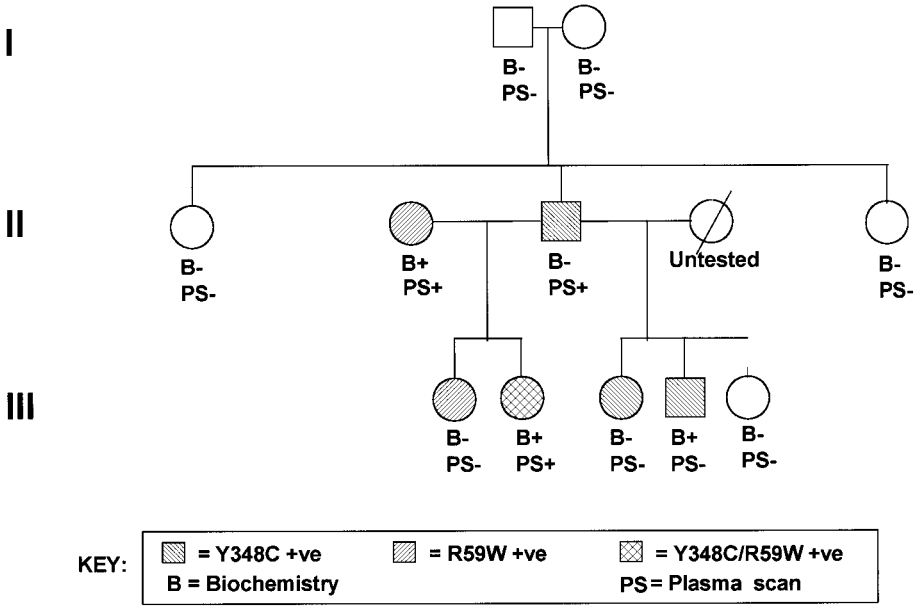

FIG. 2. Abridged pedigree illustrating three generations of family 1. *Mae*III was used to determine the presence or absence of the Y348C mutation and *Ava*I the presence or absence of the R59W mutation. Individuals labelled B+ showed a porphyrin excretion profile consistent with VP whereas B− demonstrated a normal excretion profile. Plasma scanning (PS) was performed at 625 nm.

biochemically severely affected as described above. Her mother and a sister age 12 were positive for R59W, but negative for Y348C. Her mother shows a fecal porphyrin excretion pattern typical of VP and positive plasma fluoroscanning but is clinically silent; the sister is negative both biochemically and clinically. Her father, a half-sister, and a half-brother are positive for the Y348C mutation by *Mae*III restriction analysis. Her father has a normal fecal porphyrin excretion profile but shows a small peak at 625 nm on plasma fluoroscanning. Her brother shows a typical VP fecal porphyrin excretion

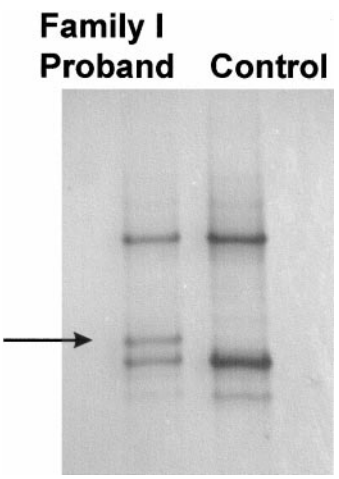

FIG. 3. SSCP of the PCR product of exon 10 on 1× MDE gel showing an additional band in the family 1 proband.

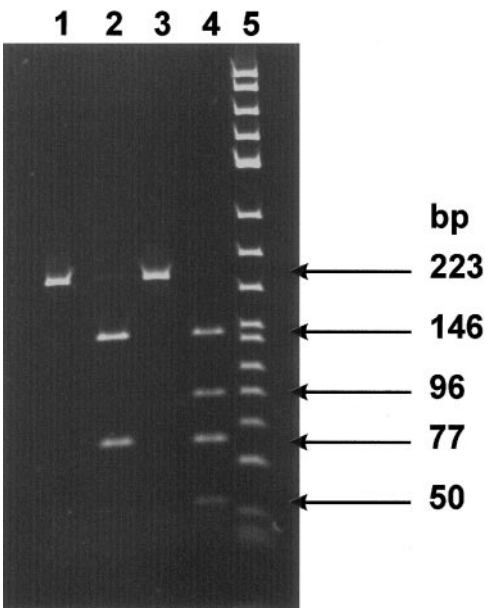

FIG. 4. *Mae*III restriction analysis on 6% acrylamide. PCR amplified DNA of exon 10 of the family 1 proband uncut (lane 3); cut (lane 4). Lanes 1 and 2 show DNA from a control individual pre- and postdigestion. Lane 5 is a DNA marker.

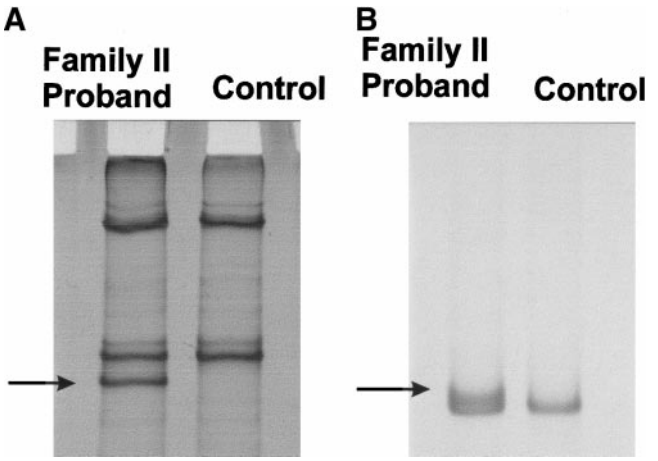

FIG. 5. (A) SSCP of the PCR product of exon 5 showing an additional band in the family 2 proband. (B) Heteroduplex analysis of the same. A heteroduplex is present in the proband.

pattern but has a normal plasma fluoroscan at 625 nm. There was no biochemical evidence of VP on fluoroscanning or fecal porphyrin analysis in her half-sister (age 12). Both of the probands' aunts were negative for both the R59W and the Y348C mutation. The proband's paternal grandparents are both negative for the Y348C mutation and have neither clinical nor biochemical evidence of VP. Genotype analysis using five randomly selected polymorphic microsatellite markers was consistent with their being the biological parents of the proband's father, confirming that the Y348C mutation has arisen *de novo* in her father. Additionally, the absence of Y348C in either of the aunts lends support to this notion. None of the 50 control individuals tested positive for the Y348C mutation.

Family 2

Both the proband and her sister tested positive for the R59W mutation by *Ava*I restriction analysis. SSCP-heteroduplex analysis of the PCR products of exon 5 from the proband (Fig. 5A) and her sister revealed an aberrant mobility shift and a heteroduplex (Fig. 5B). All other exons, apart from exon 3 (due to the R59W), appeared normal. Direct sequencing of the PCR product of the proband showed a G to C transversion resulting in an arginine to proline substitution (R138P) (GenBank Accession No. AF215863).

Both patients were clinically affected as described above. The proband had an abnormally severe phenotype; her sister was moderately affected, but not unusually severe. The fecal biochemical profile of the proband and her sister, assessed by thin-layer chromatography and fluoroscanning, was typical of VP and demonstrated extremely high porphyrin concentrations. Plasma fluoroscanning in both cases showed a peak at 625 nm confirming these results.

None of the 50 control individuals screened by SSCP-heteroduplex analysis demonstrated any aberrant mobility shift of the PCR product of exon 5.

DISCUSSION

To date, only 11 cases of HVP have been reported, 1 of whom is South African. Here we identify 2 further cases of HVP in the South African VP population. Both are heteroallelic for the R59W mutation and for a second mutation, Y348C or R138P, respectively. One would expect that, for survival, at least one of the mutations in a patient with HVP should be relatively mild; this would translate at the enzyme level to appreciable residual activity, as we showed in our original patient who was heteroallelic for R59W/R168C. In that case we were able to show that the R59W protein expressed essentially no kinetic activity, whereas the R168C protein had approximately 20% residual activity.

The Y348C mutation results in a tyrosine to a cysteine substitution, i.e., from an aromatic amino acid to a sulfur-containing amino acid. This tyrosine is conserved in 31% of the PPO species listed using Jpred (www.expasy.ch) (man (25); mouse (26), *Solanum tuberosum* (27), *Chlamydomonas reinhardtii* (28), and *Arabidopsis thalianum* (29)). This mutation may alter the secondary structure of the protein, and it is reasonable to infer that this leads to some reduction in activity.

The R138P mutation results in an arginine to proline substitution, a change from a basic to a nonpolar amino acid with an aliphatic hydrocarbon R group. A high incidence of substitutions to proline has been noted in the PPO gene (4). The R138P represents another such mutation. As in the case of Y348C, this represents a significant replacement. This arginine is conserved in 25% of the species listed in Jpred (man (25), mouse (26), *soybean* (Accession No. AB025102), and *Aquifex aeolicus* (30)).

The Y348C/R59W genotype exhibited severe porphyric skin symptomatology consistent with the HVP phenotype, suggesting that the Y348C mutation negatively complements the effect of the R59W mutation. In contrast, both R138P/R59W individuals did not manifest symptoms in early childhood, suggesting that the R138P mutation may have more

residual PPO activity than the Y348C mutation. Nevertheless the R138P/R59W proband is clearly more severely affected than the R59W heterozygotes with whom our clinic is familiar, as exemplified by the photomutiliation and skeletal abnormalities of the hands. It was surprising that her sister also was heteroallelic, yet in comparison with many symptomatic R59W heterozygotes, her symptoms were unremarkable.

From our experience, we suggest that: (1) the homozygous state for severe mutations such as R59W which effectively abolish PPO activity is probably lethal; (2) although HVP may have a severe phenotype, at least one of the mutations is likely to be associated with a benign phenotype in heterozygotes, such that family members heterozygous for this mutation are likely to be clinically and biochemically silent; (3) HVP patients will in most cases have a severe phenotype in terms of photocutaneous symptoms; thus patients with biochemical evidence of VP who are unusually and severely affected should be investigated for a second mutation; and (4) even in HVP, as exemplified by our proband 2's sister, there may be considerable phenotypic variability.

When we reviewed eight cases of HVP in 1993 (18), we remarked on the intriguing observation that, with one exception (a patient whose biochemistry was not in keeping with VP and whom we suspected might have been incorrectly classified), no patient had shown elevated ALA or PBG levels, the biochemical markers of the acute neurovisceral crisis. Furthermore, no patient had experienced such a crisis clinically, despite their very low enzyme activity. This observation remains unexplained. However, proband 2 has indeed suffered an acute attack. This may indicate that such attacks can occur in HVP. It would be interesting to know whether if any of the previously reported HVP patients developed acute attacks after puberty.

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