THEODORE WOODWARD AWARD

PATHOGENESIS OF BIOCHEMICAL ABNORMALITIES IN PROTOPORPHYRIA

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INTRODUCTION

The porphyrias are a group of metabolic disorders in which abnormalities in the heme biosynthetic pathway cause the excessive accumulation and excretion of porphyrins and porphyrin precursors (Fig. 1) (1). The first report of porphyria was in 1874, when Schultz described a patient with the clinical features of congenital erythropoietic porphyria (2). Fifteen years later, Stockvis reported the first case of acute hepatic porphyria in a drug addict who passed urine with the color of port wine after taking sulfonmethane (2). During the following century, the clinical and biochemical features of the individual porphyrias were described, each of which had a unique pattern of biochemical abnormalities that permitted the diagnosis to be made through measurements in blood, stool and urine (Table 1). However, the pathogenesis of the biochemical abnormalities remained unclear until 1970, when Strand and coworkers demonstrated a decrease in the hepatic conversion of porphobilinogen to porphyrins in patients with acute intermittent porphyria, implicating a deficiency of porphobilinogen deaminase activity as the fundamental defect in this disorder (3). Subsequently, a defect in an enzymatic step of the heme biosynthetic pathway has been defined for each of the porphyrias (Table 1).

The cloning and sequencing of cDNA and genomic DNA that encode

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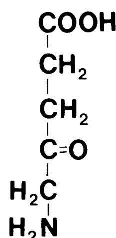
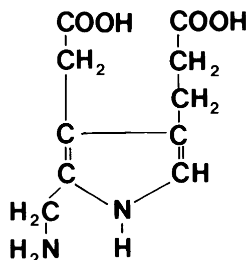
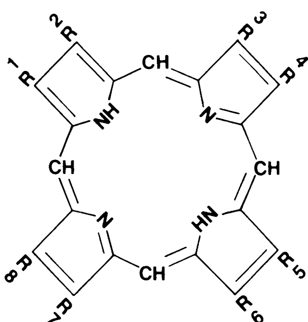
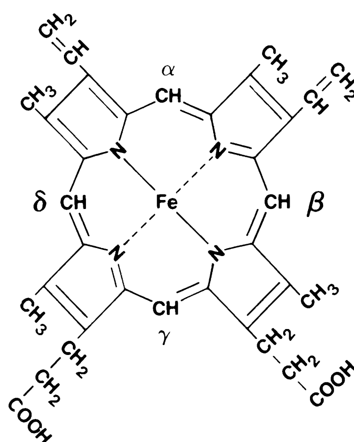
δ-AMINOLEVULINIC ACID (ALA)



PORPHOBILINOGEN (PBG)







PORPHYRIN **HEME**

FIG. 1. Porphyrins and porphyrin precursors (delta-aminolevulinic acid and porphobilinogen) are intermediates of the heme biosynthetic pathway. All porphyrins have the same tetrapyrrole ring structure, but they differ in the composition of side chains (R1-8) which are attached to the ring. Uroporphyrin has 8 carboxylic acid side chains, coproporphyrin has 4, and protoporphyrin has 2. This figure is reproduced with permission from Schiff's Diseases of the Liver, 8th edition, edited by ER Schiff, MF Sorrell, and WC Maddrey, Lippincott Raven Publishers, Philadelphia 1999, page 1152.

the enzymes of the heme biosynthetic pathway have made it possible to identify the gene mutations that cause the enzyme defects in the porphyrias. Acute intermittent porphyria has been the most intensively studied, and many different mutations have been described, establishing significant genetic heterogeneity (4). The mutations cause abnormal splicing of porphobilinogen deaminase mRNA, insertions or deletions in exons which cause premature termination of protein synthesis, nucleotide changes that produce stop codons which prevent complete translation of mRNA, and nucleotide changes that cause substitutions of amino acids which alter the catalytic activity of the

TABLE 1
Biochemical Abnormalities in the Porphyrias

Type of porphyria	Major biochemical features*	Enzyme defect	Chromosome Location of Enzyme DNA
Acute intermittent porphyria	ALA and PBG in urine	PBG deaminase	11q24.1 → q24.2
Variegate porphyria	ALA, PBG, and coproporphyrin in urine; protoporphyrin in feces	Protoporphyrinogen oxidase	14
Hereditary coproporphyria	ALA, PBG, and coproporphyrin in urine; coproporphyrin in feces	Coproporphyrinogen oxidase	9
ALA dehydrase deficiency	ALA in urine	ALA dehydrase	9q34
Porphyria cutanea tarda	Uroporphyrin in urine; isocoproporphyrin in feces	Uroporphyrinogen decarboxylase	1p34
Hepatoerythro-poietic porphyria	Zn-protoporphyrin in red cells; uroporphyrin in urine; isocoproprophyin in feces	Uroporphyrinogen decarboxylase	1p34
Congenital erythropoietic porphyria	Uroporphyrin in red cells and urine; coproporphyrin in feces	Uroporphyrinogen III synthase	10q25.3 → q26.3
Protoporphyria	Protoporphyrin in red cells and feces	Ferrochelatase	18q21.3

* Levels of the listed compounds are increased in urine, feces, or red cells as indicated.
ALA = delta-aminolevulinic acid; PBG = porphobilinogen

protein (4). Genetic heterogeneity has also been demonstrated in the other types of porphyria, even those which are very rare. Thus DNA analysis has not become a practical means for establishing the diagnosis except in geographical areas where a specific mutation has a high prevalence. However, it is the most definitive manner by which to examine other family members for the disease when a specific mutation has been identified in one family member.

Protoporphyria (erythropoietic protoporphyria, EPP) was described in 1961 by Magnus and co-workers when they reported a 35-year-old man who had lifelong swelling of his skin when exposed to sunlight and increased levels of protoporphyrin in his red cells and feces (5). The

major clinical feature of EPP is photosensitivity, which is caused by the photoreactive properties of protoporphyrin in skin. Some patients also develop hepatobiliary disease, due to the toxic effect of protoporphyrin on liver structure and function, that may cause progressive liver failure and necessitate liver transplantation (1). These patients typically have very high levels of protoporphyrin in red cells and serum.

Several studies have shown that the enzyme abnormality which underlies protoporphyrin accumulation in EPP is a deficiency of ferrochelatase (FC) activity (6–8). FC, which is the terminal enzyme of the heme biosynthetic pathway, is located on the matrix side of the inner mitochondrial membrane and catalyzes the insertion of ferrous iron into protoporphyrin to form heme (9). Its functional state in mitochondria appears to be that of a homodimer with a molecular size of approximately 80 kD (10). FC cDNA has been cloned and sequenced from several different species, including human. Human FC cDNA has an open reading frame of 1269 base pairs which encode a protein of 423 amino acid residues, 54 of which are the mitochondrial leader sequence (11). There is a single FC gene which contains 11 exons and maps to chromosome 18 at region q21.3 (12).

With this knowledge of the FC gene and FC cDNA, recent studies have begun to define the molecular causes of deficient FC activity in patients with EPP (13–32). In this report, we summarize the FC gene mutations which have been found by two laboratories in American patients with EPP. A particular focus has been on patients with advanced liver disease in order to determine if specific mutations in the FC gene are associated with this severe phenotype.

PATIENT POPULATION AND METHODS OF STUDIES

Patient Population

The patient population in this study consisted of 49 individuals from 38 separate families in whom a diagnosis of EPP was established on the basis of characteristic photosensitivity and an elevated red cell protoporphyrin (FEP) level (>65 mcg/dL). The age range of the patients was 5 years to 73 years, with 24 males and 25 females. Nine patients had advanced protoporphyric liver disease, and eight underwent liver transplantation. Liver specimens from these patients had the features typical of advanced protoporphyric liver disease. The livers were enlarged, firm in consistency and black in color. Histological examination revealed the presence of cirrhosis, cholestasis, and birefringent protoporphyrin pigment deposits in hepatocytes, Kupffer

cells, and biliary structures. The remaining 40 patients had no clinical evidence for liver disease, but three had mild abnormalities in liver chemistries, and their liver biopsy specimens showed mild portal inflammation.

Measurement of FC Activity

FC activity was measured in liver tissue and/or cells of 34 patients with EPP. This was done in liver tissue from 12 patients, cultured skin fibroblasts from 7 patients, EBV-transformed lymphoblasts from 5 patients, and peripheral blood leukocytes from 15 patients. Five patients had a measurement done in both liver tissue and skin fibroblasts or lymphoblasts.

Four different assays were used to measure FC activity. These were a radiochemical assay which measured the formation of radiolabeled heme (7), a pyridine hemochromogen method which measured the formation of deuteroheme (29), a fluorometric assay which measured the formation of zinc deuteroporphyrin (29), and a zinc chelatase assay in which protoporphyrin was used as the porphyrin substrate (27). In order to combine the results of these different studies, the level of FC activity measured was expressed as a percent of the mean normal value established for the particular method used.

Mutational Analysis

Mutational analysis of FC DNA was done in 38 patients with EPP and 24 of their asymptomatic relatives. This included 8 patients with advanced protoporphyric liver disease. Total RNA and genomic DNA were extracted from liver tissue, peripheral blood white cells, and EBV-transformed lymphoblasts using standard methods (27,29–31). Using extracted RNA, specific reverse transcriptase-polymerase chain reactions were used to amplify and sequence FC cDNA (27,29,31). The amplification and sequencing of individual exons and their flanking intron regions was done on genomic DNA by polymerase chain reactions, using oligonucleotides which were specific for the particular exon of interest (27,29–31). Haplotype analysis was done in select families using three microsatellite markers (31).

Informed consent for these studies was obtained in accordance with guidance and approval established by the local institutional review boards.

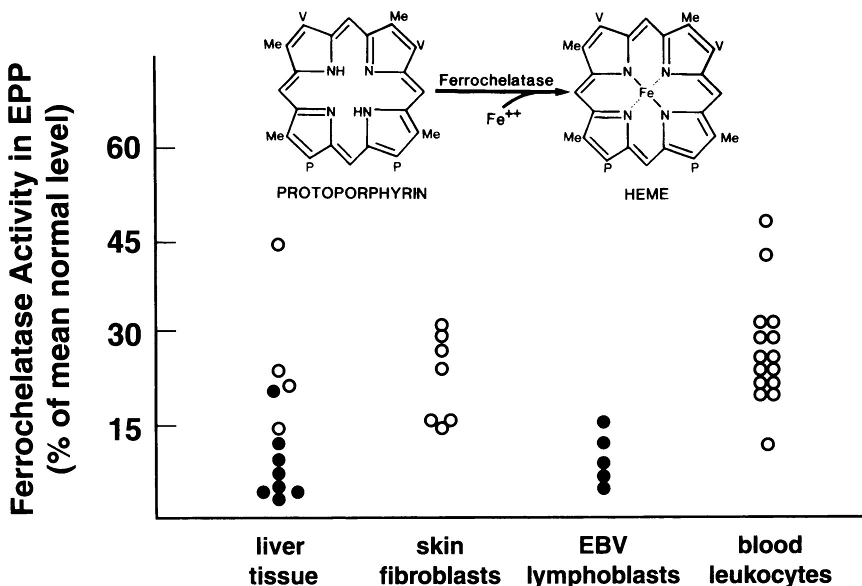


FIG. 2. Ferrochelatase, the last enzyme of the heme biosynthetic pathway, catalyzes the insertion of ferrous iron into protoporphyrin to form heme. Ferrochelatase activity in liver tissue and cells of patients with protoporphyria is significantly decreased compared to that in normal tissue. Each patient with protoporphyria had a level of FC activity which was less than 50% of the mean normal value, and 82% of patients had a level less than 30%. The lowest levels were seen in patients with advanced protoporphyric liver disease, shown by the closed circles.

RESULTS AND DISCUSSION

The level of FC activity in liver tissue and/or cells of each patient with EPP was less than 50% of the mean normal level (Fig. 2). In 82% of patients it was less than 30% of the mean normal level, which is striking for a disorder which is generally thought to be inherited as an autosomal dominant trait. This finding could be explained if the mutant protein exerts a dominant negative effect on the normal protein (14), since the functional state of FC in mitochondria may be that of a homodimer (10). In this situation, any dimer containing the mutant protein would be inactive, and the level of residual FC activity would be 25% of the normal level.

The lowest levels of FC activity were found in liver tissue from the patients with advanced protoporphyric liver disease (Fig. 2). They ranged from 4 to 20% (mean 8%) of the mean normal level, compared to 26% in the four patients whose liver biopsy specimens were either normal (2 patients) or showed only mild portal inflammation (2 patients). This profound decrease in FC activity could be due in part to

TABLE 2
Ferrochelatase (FC) Mutation Analysis in EPP Proband/Family Members

A. Patients with Advanced Cirrhosis

Subject gender/ age	FEP mcg/dL	Sequence of Genomic FC DNA	Sequence of FC cDNA	Predicted Effect on FC Protein	Ref
1. M/15*	6470	IVS2 + 11 A → G	exon 2 deletion	truncated protein 29 aa long	29
2. M/51*	5276	IVS3 + 2 T → G	exon 3 deletion	R65S and deletion of 40 aa	29
3. F/38*	5131	IVS3 + 2 T → G	exon 3 deletion	"	29
4. F/14*	2450	IVS3 + 6 A → C	exon 3 deletion	"	29
5. F/36*	8240	580 del 5	580 del 5	truncated protein 208 aa long	29
6. F/18*	6488	1135 A → T	exon 10 deletion	deletion of 20 aa or K379X	29
mother	33	"	"	"	
brother	25	"	"	"	
7. M/27*	6094	1135 AA → A	exon 10 deletion	deletion of 20 aa or L399X	29
8. M/13*	7679	none found	none found	—	29

* indicates patient had photosensitivity

the additional effect of the protoporphyric liver damage. The finding that EBV-transformed lymphoblasts from the patients with advanced protoporphyric liver disease also had a profound deficiency of FC activity (5 to 15% of the mean normal level) suggests that genetic factors may be important as well.

We thus anticipated that mutational analysis of the FC gene might provide important information regarding the severity of disease expression in EPP, in particular the possibility that specific mutations or types of mutations are associated with development of protoporphyric liver disease. Instead, several different mutations were found (Table 2A), indicating that genetic heterogeneity exists even in the most severe phenotype of EPP. Notably, however, mutations causing a deletion of exon 3 in FC mRNA were found in three patients. A mutation which produces a deletion of exon 3 has been reported in only one other patient, an 11-year-old female with severe photosensitivity whose FC activity was only 11.7% of normal (23). Careful observation of this patient for development of liver disease will thus be important.

Sarkany et al previously described two siblings who were compound heterozygotes for an exon 10 deletion and who developed liver failure in childhood (22). They proposed that recessive inheritance could be the factor which predisposes patients with EPP to liver disease. However, neither the patient with liver disease reported by Nakahashi et al (16), in whom the mutation resulted in a deletion of exon 9, nor by Schneider-Yin et al (33), in whom the mutation caused a stop codon, had evidence for recessive inheritance (34). Rüfenacht et al recently

TABLE 2 Continued

B. Patients without Liver Disease

Subject gender/age	FEP mcg/dL	Sequence of Genomic FC DNA	Sequence of FC cDNA	Predicted Effect on FC Protein	Ref
1. M/33*	1694	1135 A → T	exon 10 deletion	deletion of 20 aa or K379X	31
mother	31	"	"	"	
sister*	900	"	"	"	
son	37	"	"	"	
2. M/30*	1886	1135 A → T	exon 10 deletion	"	31
mother	62	"	"	"	
brother*	926	"	"	"	
3. F/71*	681	1135 A → T	exon 10 deletion	"	17, 31
sister*	990	"	"	"	"
4. F/29*	2890	IVS10 + 3 A → G	exon 10 deletion	deletion of 20 aa	24
father	61	"	"	"	
5. F/10*	1830	463G → C	exon 4 deletion	truncated protein 124 aa long or A155P	25
6. F/33*	621	IVS4 + 1 G → T	exon 4 deletion	truncated protein 124 aa long	31
7. M/33*	1600	IVS7 + 5G → A	exon 7 deletion	deletion of 33 aa	20
father	77	"	"	"	
8. M/15*	1449	IVS6 + 1G → A	exon 6 deletion	deletion of 35 aa and frame shift	31
mother	110	"	"	"	
sister	114	"	"	"	
m gmother	—	"	"	"	
9. M/12*	1945	IVS9 + 2 T → A	exon 9 deletion	deletion of 55 aa	31
father	150	"	"		
p gmother	76	"	"		
p aunt*	68	"	"		
10. F/30*	554	912 G → T	exon 9 deletion	deletion of 55 aa	24
father	82	"	"	"	
11. F/55*	1387	175 C → T	175 C → T	Q 59 X	
mother	77	"	"		—
12. M/38*	996	343 C → T	343 C → T	R 115X	—
13. F/61*	1687	343 C → T	343 C → T	R 115X	—
14. M/47*	1527	678 del G	678 del G	frame shift	27
sister*	383	"	"	"	
15. F/64*	917	205 ins A	205 ins A	frame shift	27
daughter*	426	"	"	"	
16. F/36*	1471	400 del A	400 del A	frame shift	27
mother	88	"	"	"	
17. M/13*	694	215 ins T	215 ins T	frame shift	27
18. M/10*	—	40 del G	40 del G	frame shift	—
19. M/18*	517	966 G → A	not sequenced	uncertain	31
father	62	"	"	"	
brother*	110	"	"	"	
p gmother	76	"	"	"	
20. F/37*	1319	IVS1 + 5G → A	not sequenced	uncertain	31
mother	158	"	"	"	
21. F/49*	342	"	"	"	31
22. M/11*	1612	"	"	"	31
father	83	"	"	"	
23. F/47*	1658	IVS1 + 5 G → A; also 185 C → G	not sequenced	uncertain P62R	31, 32
brother	125	IVS1 + 5 G → A	"	uncertain	
sister	—	"	"	"	
son	—	"	"	"	
daughter	—	185 C → G	"	P62R	

* Indicates patient had photosensitivity

reported an additional three patients with liver complications who were heterozygous for mutations in the FC gene (28). Similarly, none of the eight patients reported here (Table 2A) had more than one mutation and thus appear to be heterozygotes. Recessive inheritance thus accounts for only a small number of the cases of EPP who develop advanced liver disease.

Gouya et al presented evidence that low expression of an apparently normal FC allele modulated the phenotype in a family with dominant inheritance of a mutation that caused exon 10 deletion (26). Haplotype analysis in one of our families, in whom an 18 year old female developed liver disease and had transplantation, demonstrated that she and her asymptomatic brother inherited the FC gene mutation from her mother, whereas they inherited different alleles from the father (Table 2A and Fig. 3). Thus, it is possible that a different level of expression of the two apparently normal alleles from the father caused the marked differences in phenotype expression of disease between her and her brother. Similar haplotype studies previously reported for

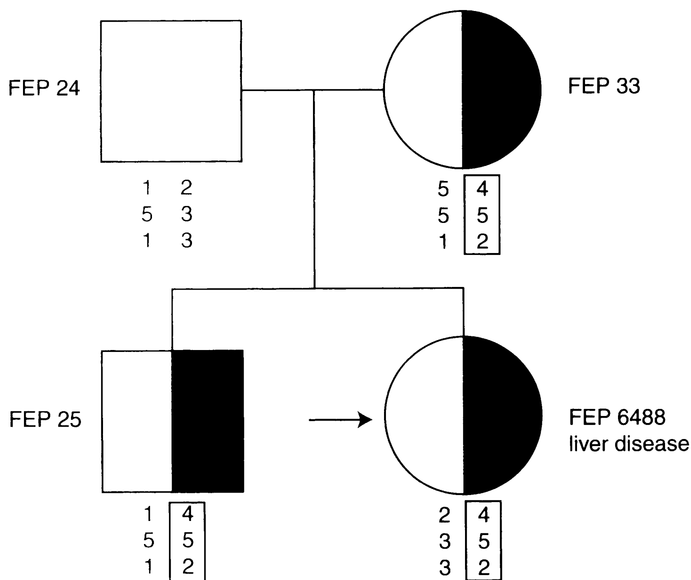


FIG. 3. Haplotype analysis of chromosome 18 in a family in which an 18 year old female developed protoporphyric liver disease and required liver transplantation. The proband is shown by the arrow, and levels of red cell protophyrin (FEP) in mcg/dl are shown for each member of the family. The patient, her asymptomatic brother, and her asymptomatic mother all had the mutation 1135A → T as shown by the black shading. The patient and her brother inherited different haplotypes of chromosome 18 from the father.

families with both symptomatic and asymptomatic members heterozygous for a ferrochelatase gene mutation, but without liver disease, also support this concept (31). In every family, the asymptomatic carriers were shown to have different haplotypes for the nonmutant FC allele compared to symptomatic relatives, while symptomatic siblings always shared the same haplotypes for their nonmutant alleles. Additional haplotypes studies in families with protoporphyric liver disease would be of interest in this regard, since several siblings with liver disease have been reported (22,28,35,36).

The mutations in the patients with advanced protoporphyric liver disease shared the property of causing a major structural alteration in the FC protein (Table 2A). However, the mutations found in 24 patients from 18 families without liver disease also would cause major structural alterations in the FC protein (Table 2B). In 10 families the mutations caused deletion of exons, in 3 families the formation of stop codons, and in 5 families a frame shift. In another 4 families the effect of the mutation on the FC protein was uncertain. One of the families (Family 23) was found to have a missense mutation which caused a substitution of arginine for proline at amino acid position 62. Another patient (Patient 5) had a 463 G $\rightarrow$ C transversion that caused a deletion of exon 4, but also caused a missense mutation to occur at a low level of frequency. The amino acid substitution which resulted (proline for alanine at amino acid position 155) almost completely inactivated the FC protein. Brenner et al previously described another missense mutation in an American patient with EPP, which resulted in the substitution of serine for phenylalanine at amino acid position 417 (14). This point mutation rendered the mutant FC protein inactive.

SUMMARY

In summary, FC gene mutations in patients with protoporphyric liver disease typically cause major structural alterations in the FC protein. However, the gene mutations by themselves do not satisfactorily account for the severe phenotype, as the same mutations are found in asymptomatic family members, and similar mutations are found in patients who do not develop liver disease. Thus there may be unidentified factors in the FC gene locus, or factors outside the locus, which are also important in determining the degree of protoporphyrin accumulation that occurs in an individual patient, hence, the potential for developing significant liver disease. Further studies are needed to clarify this possibility and identify those factors.

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DISCUSSION

MITCH, Atlanta: One question: I was struck by the very high levels of porphyrins that you showed. Then you went on to tell us about the liver problem. I wonder if you

have ever done clearance studies so that you could actually test your hypothesis that the accumulation is due to a defective clearance. The other possibility is reminiscent of the uric acid story and maybe there is an overproduction problem.

BLOOMER: That has been very controversial. Studies were done in the past using radiolabelled compounds and it looked as if the liver contributes, but on a small order of magnitude, maybe 10–20%. In those that develop liver damage, of course, it would be more. I think the most compelling evidence is that after liver transplantation, porphyrin levels remain high in red cells in these patients, not to the same degree you see before transplant, but they still remain high.

STEVENSON, Stanford: As you mentioned to me before, you work on the "upside" of the heme story, and I work on the "downside" of the heme story. Obviously, these people have some heme, otherwise they would have a lot of other problems. The question I have is: are there effects on this condition if heme degradation increases under pathologic circumstances and you put pressure on this defective synthetic pathway?

BLOOMER: David, I was hoping the hematologists in the audience would help me out with this problem. A portion of the patients develop mild anemia, indicating some heme deficiency. In terms of whether a heme deficiency state leads to any of the problems, we have administered heme to these patients intravenously and improved liver function; so I think heme deficiency may be playing a role. Certainly increased heme degredation, which would presumably necessitate more heme biosynthesis, would worsen the situation.

KOHLER, Oregon: Joe, I have one question, a very broad general one. The diagnosis of porphyria seems to be made very frequently, at least in certain parts of the country. I don't understand whether this represents an ambiguity with regard to our diagnostic techniques or not. Can you shed any light on this particular problem?

BLOOMER: Most abnormalities of porphyrin metabolism aren't true porphyrias. It is what is called secondary porphyrinuria. People excrete increased levels of coproporphyrin in urine for a variety of reasons, including hepatobiliary disease, diabetes, and various anemias. If one screens for porphyria in a patient and finds only increased coproporphyrin in the urine, the diagnosis of porphyria thus made may be incorrect. Unfortunately, this was carried further by measuring an enzyme called coproporphyrinogen oxidase, which is a very difficult enzyme to assay, and showing in some of these patients that it was low. This led to a flurry of activity that there are a whole host of conditions, including chemical sensitivity, which are due to porphyria. I don't think that the diagnosis of porphyria was probably established in most of these patients.