

# Molecular and Biochemical Studies of Acute Intermittent Porphyria in 196 Patients and Their Families

RAILI KAUPPINEN\* and MIKAEL VON UND ZU FRAUNBERG

**Background:** Acute intermittent porphyria (AIP) is a metabolic disease with clinical manifestations that mimic other abdominal, neurologic, or mental crises. We studied the diagnostic accuracy of current laboratory tests during an acute attack and in remission.

**Methods:** Since 1966, we have studied all known Finnish AIP patients (n = 196) and their families (n = 45) and identified the porphobilinogen deaminase (PBGD) mutation in each family. Diagnoses or exclusions of AIP were based on clinical data (including family history), biochemical tests, and in 239 cases, mutation testing. We retrospectively evaluated the diagnostic accuracy of erythrocyte PBGD activity, urinary excretion of porphobilinogen (PBG) and  $\delta$ -aminolevulinic acid, and urinary and fecal excretion of porphyrins in these patients.

**Results:** Measurement of urinary PBG identified all 35 AIP patients studied during an acute attack. The mean excretion of PBG was 50-fold above the reference interval, although the intraindividual increases were modest (1.6- to 4.0-fold). In the mutation-screened population, urinary PBG analysis identified only 85% of 81 AIP patients studied during remission, but by ROC curve analysis it was nonetheless the best of the biochemical tests. It was increased  $\leq 2$ -fold in 29% of healthy relatives. Erythrocyte PBGD activity was decreased in only 84% of AIP patients, with results within the reference interval mainly in the variant form of AIP; it was decreased in 23% of healthy relatives.

**Conclusions:** Measurement of urinary PBG is the best biochemical test for AIP, although it is unspecific and does not distinguish AIP from other acute porphyrias. Because the acute increase in PBG is often modest, the medical history, signs, and symptoms must be evaluated

carefully during an acute attack. In addition, because biochemical analyses often remain indeterminate in remission, mutation analysis is needed to exclude or confirm the diagnosis of AIP.

© 2002 American Association for Clinical Chemistry

Acute intermittent porphyria (AIP)<sup>1</sup> is a metabolic disease that results from the partial deficiency of porphobilinogen deaminase (PBGD; also known as hydroxymethylbilane synthase; EC 4.3.1.8; MIM No. 176000) (1). PBGD, which is the third enzyme in heme biosynthesis, catalyzes the polymerization of porphobilinogen (PBG), yielding a linear tetrapyrrole, preuroporphyrinogen (hydroxymethylbilane). This is closed by uroporphyrinogen III synthase to form uroporphyrinogen III or, alternatively, nonenzymatically to form uroporphyrinogen I.

The *PBGD* gene (GenBank accession no. M95623; 10 kb) has been assigned to chromosome 11q24, and the coding sequences are spread over 15 exons (2–6). Two tissue-specific isoforms have been characterized; the two transcripts arise from two separate promoters via alternative splicing of exons 1 and 2. The mRNA of the housekeeping (nonerythropoietic) isoform contains exons 1 and 3–15, which code for an enzyme of 361 amino acids ( $M_r \sim 42\,000$ ), whereas the erythroid isoform is encoded by exons 2–15 and lacks the first 17 amino acids of the  $NH_2$  terminus ( $M_r \sim 40\,000$ ).

AIP is inherited as an autosomal dominant trait displaying incomplete penetrance (1). Despite  $\sim 200$  different mutations described in the *PBGD* gene (Fig. 1.), the clinical manifestation is relatively uniform among AIP patients. Clinical symptoms include abdominal pain, vomiting, and constipation accompanied by hypertension and tachycardia, which are signs of sympathetic activity. Peripheral neuropathy is manifested as pain in the ex-

Department of Medicine, Division of Endocrinology, University Hospital of Helsinki, 00029 HUS Helsinki, Finland.

\*Address correspondence to this author at: Department of Medicine, University Central Hospital of Helsinki, Biomedicum-Helsinki, Box 700, 00029 HUS Helsinki, Finland. E-mail: rai.li.kauppinen@hus.fi.

Received January 24, 2002; accepted August 9, 2002.

<sup>1</sup> Nonstandard abbreviations: AIP, acute intermittent porphyria; PBGD, porphobilinogen deaminase; PBG, porphobilinogen; and ALA,  $\delta$ -aminolevulinic acid.

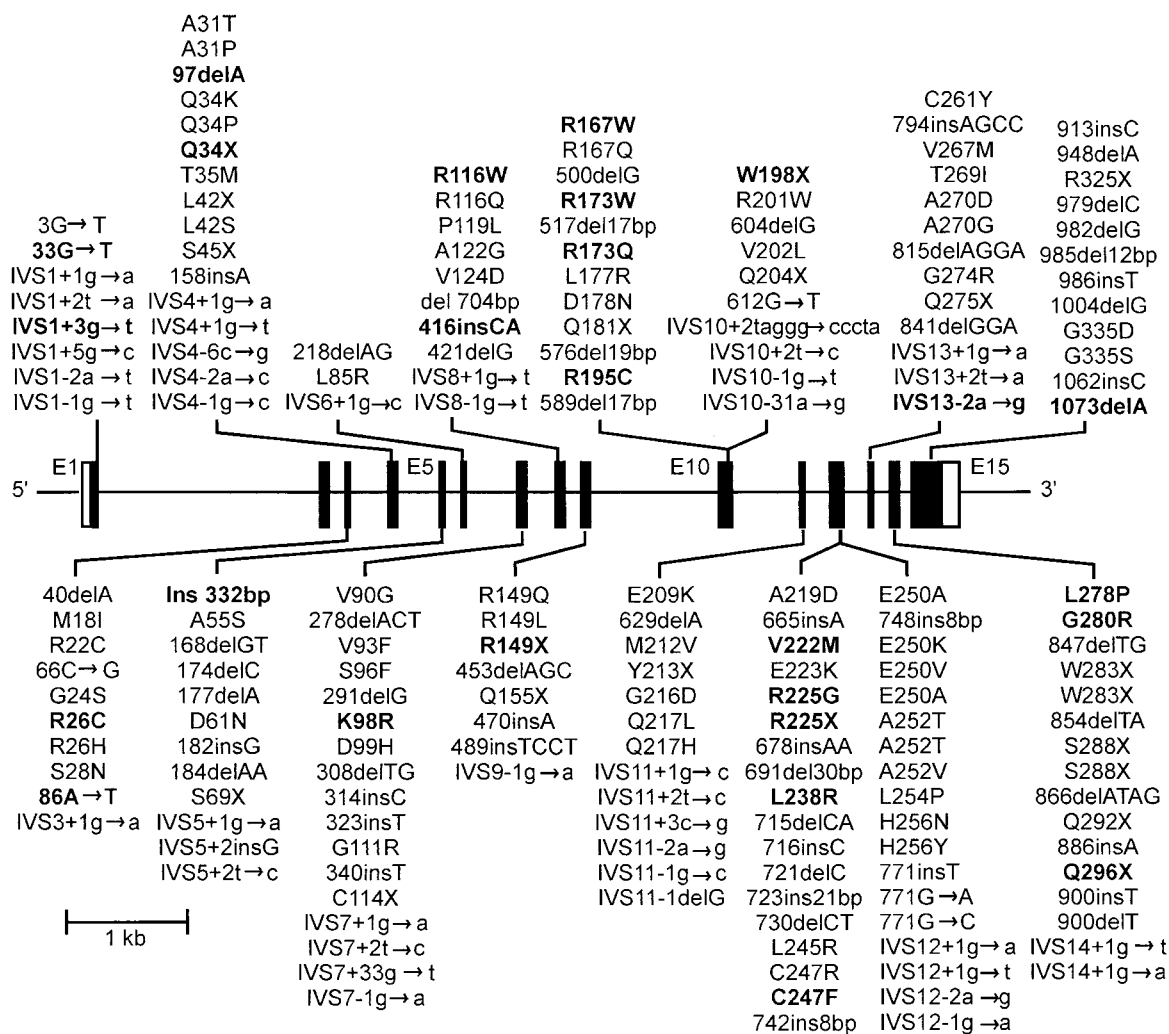


Fig. 1. Exon/intron organization of the human *PBGD* gene and reported mutations responsible for AIP.

Exons are indicated by boxes: closed and open boxes represent protein-coding and untranslated regions, respectively. Finnish mutations are shown in bold.

tremities, and it may progress to a severe motor neuropathy. Cranial nerve palsies, epileptic seizures, respiratory insufficiency, abnormal sphincter function, hallucinations, and mental changes may be present. An acute attack precipitated by AIP is indistinguishable from attacks produced by variegate porphyria or hepatic coproporphyrin. Acute attacks are precipitated by various exogenous factors, such as drugs, alcohol, infections, and fasting, or endogenous changes in the sex-hormone balance (7). A few homozygous patients with more severe and chronic neurologic dysfunction have been reported (8, 9).

PBGD activity is usually decreased to 50% of normal in patients' erythrocytes (10). In the variant form of AIP, patients have a mutation in the region of the first exon or intron; thus, measurement of PBGD activity in erythrocytes is uninformative (11). In addition, AIP patients manifest overproduction and increased excretion of porphyrins and their precursors: proto- and coproporphyrins

in feces; uro- and coproporphyrins in the urine and plasma (12).

In this study we used DNA technology in the diagnosis of AIP and compared the results with the data from biochemical analyses and clinical findings to evaluate the diagnostic accuracy of current laboratory tests during an acute attack and in remission.

## Materials and Methods

### PATIENTS

Since 1966, we have conducted a systematic follow-up of all Finnish patients known to have AIP (13). After the diagnosis of AIP in an index case, family members were enrolled in the study during the years 1966–2001. The patients represent 45 Finnish AIP families, and the ancestry for the majority of them could be traced back to at least the 19th century by checking church registers (13). All individuals analyzed were members of the families in which AIP was confirmed by mutation analysis.

**Table 1. Results of mutation and biochemical analysis of Finnish AIP patients in remission and their healthy relatives.**

Mutation	Urine <sup>a</sup>																		Feces <sup>a</sup>					
	Erythrocyte PBGD (>50 pmol · h <sup>-1</sup> · mg <sup>-1</sup> )			PGB (<9 μmol/L)			ALA (<34 μmol/L)			Uroporphyrin (<36 nmol)			Coproporphyrin (<230 nmol/day)			Coproporphyrin (<100 nmol/g)			Protoporphyrin (<130 nmol/g)					
	n <sup>b</sup>	↓ <sup>c</sup>	N	n	↑	N	n	↑	N	n	↑	N	n	↑	N	n	↑	N	n	↑	N			
Positive	85	71	14	81	69	12	78	48	30	67	48	19	70	47	23	55	2	53	55	11	44			
Negative	43	10	33	41	12	29	41	4	37	30	1	29	31	4	27	26	0	26	25	1	24			
Total	128			122			119			97			101			81			80					

<sup>a</sup> Values in parentheses are the reference intervals.

<sup>b</sup> n, number of patients studied.

<sup>c</sup> ↓, decreased; ↑, increased; N, within the reference interval.

Of the 489 family members, AIP was diagnosed in 196 patients (84 males, 112 females; age range, 15–78 years; 75 symptomatic and 121 symptom free) and excluded in 293 healthy relatives (146 males, 147 females; age range, 15–61 years). We examined 239 individuals with mutation analysis and 381 individuals with biochemical testing, and 131 individuals were tested by both methods.

The diagnosis of AIP was based on mutation analysis in 120 individuals. In 66 cases (35 symptomatic and 31 symptom free), the diagnosis was based on low erythrocyte PBGD activity (<55 pmol uroporphyrin · h<sup>-1</sup> · mg<sup>-1</sup>) with increased (>9 μmol/L; n = 54), normal (n = 2), or undetermined (n = 10) urinary PBG excretion. In seven cases, the diagnosis was based on increased urinary PBG excretion (>47 μmol/L) with characteristic clinical symptoms (n = 5) or without symptoms (n = 2). In three cases (one symptomatic, two symptom free), the diagnosis was based on pedigree analysis. The exclusion of AIP was based on mutation analysis in 119 cases. In 139 symptom-free individuals, the erythrocyte PBGD activity was within the reference interval and the urinary PBG excretion was 0–22 μmol/L (n = 124) or undetermined (n = 15). In 35 cases, the exclusion was based on absence of clinical symptoms and normal excretion of porphyrins and their precursors. Erythrocyte PBGD activity could not be used in 16 of the individuals studied because they had the variant form of AIP. Exclusion of these family members did not affect the mean results of urinary and fecal excretions of porphyrins and their precursors in healthy relatives.

The biochemical data included data collected retrospectively and prospectively since 1966. Informed consent was obtained for all DNA testing, and the study protocol was approved by the Ethical Committee of the Department of Medicine, University Central Hospital of Helsinki.

The participants were classified into four groups based on a comparison of urinary and fecal excretion of porphyrins and their precursors with clinical manifestations: (a) AIP patients in an acute attack; (b) symptomatic AIP patients in remission; (c) symptom-free AIP patients; and (d) healthy relatives. Information about acute attacks was obtained from hospital records for all patients who had

had acute attacks requiring hospitalization since 1929. The criteria for an acute attack were the acute nature of symptoms, urinary excretion of PBG that was at least five times above the upper limit of the reference interval, and severe abdominal or other pain associated with one or more additional typical porphyric symptoms (7, 11).

#### BIOCHEMICAL METHODS

PBGD activity was determined from red cells separated from venous blood by centrifugation (1000g for 10 min) with heparin added as an anticoagulant (13, 14). Red cells were washed twice with 9 g/L NaCl solution and hemolyzed by freezing and thawing three times. The red cell solution was diluted with 50 mmol/L Tris-HCl, pH 8.0 (1:20 by volume). A total of 500 μL of the red cell dilution and 1 mL of 10 mmol/L PBG in H<sub>2</sub>O were mixed before incubation for 60 min at 37 °C. The reaction was stopped with 1.5 mL of 2 mol/L perchloric acid. Coproporphyrin I formation was measured in the supernatant by spectrofluorometry (Hitachi F-4010) at an emission wavelength of 598 nm. The amount of uroporphyrin I (pmol · mg protein<sup>-1</sup> · h<sup>-1</sup>) formed was calculated as follows: 0.75 (conversion factor from copro- to uroporphyrin) × 1.527 (pmol/ng) × 3 (sample volume in mL) × the amount of coproporphyrin I measured (μg/L):protein (mg). The amount of protein was measured with the Bio-Rad DC Protein Assay. The reference interval was determined and monitored regularly using series of erythrocytes from 20 healthy volunteers. Two samples of erythrocytes having PBGD activities of ~70 and 40 pmol · h<sup>-1</sup> · mg protein<sup>-1</sup> were used as a quality control to monitor the precision of PBGD measurements.

Excretion of urinary porphyrin precursors was measured by the δ-aminolevulinic acid (ALA)/PBG Column test (Bio-Rad), which is based on ion-exchange chromatography with measurements made by spectrophotometry. For the measurement of PBG and porphyrins, 50 mg of sodium carbonate was added to 10 mL of urine. For ALA measurements, 0.1 mL of 998 g/L acetic acid was added to 10 mL of urine (pH 5–7). The samples were preserved frozen for a maximum of 7 days before the measurement.

Excretion of urinary and fecal porphyrins was determined by HPLC (15,16). A Varian Vista 5500 liquid chromatograph with a Shimadzu RF 530 fluorescence spectromonitor detector (Shimadzu Corporation) was used and set at an excitation wavelength of 403 nm and emission wavelength of 626 nm. Samples were loaded with an AASP Vac-Elut sample preparation system with C<sub>18</sub>-bonded AASP cassettes (Varian) and run through a 3.9 × 300-mm C<sub>18</sub>  $\mu$ Bondapak column with 10- $\mu$ m particle size (Waters Corporation).

#### SAMPLE PREPARATION

We mixed 100  $\mu$ L of a urine sample and 0.9 mL of 1 mol/L ammonium acetate buffer (pH 5.16) before HPLC analysis. A 10  $\mu$ mol/L porphyrin standard mixture (Chromatographic Marker Porphyrin Acids; Porphyrin Products) was diluted in methanol-H<sub>2</sub>O-acetic acid (6:4:1 by volume; 0.556 pmol/ $\mu$ L for each porphyrin). Coproporphyrin III standard (Porphyrin Products) was diluted with methanol-H<sub>2</sub>O-acetic acid (6:4:1 by volume), and the final concentration was measured spectrophotometrically for each series separately. We mixed 50  $\mu$ L of each standard dilution (1:1 by volume) with 0.9 mL of 1 mol/L ammonium acetate buffer (pH 5.16) before HPLC analysis. The amount of urinary porphyrins (nmol) was calculated: 10 × standard (pmol/100  $\mu$ L) × sample (mm):standard (mm).

We vortex-mixed 100 mg of wet feces with 2 mL of 100 g/L trichloroacetic acid-dimethyl sulfoxide (1:1 by volume) for 3 min and separated the porphyrins by centrifugation (3000g for 10 min). We then mixed 50  $\mu$ L of the supernatant with 0.95 mL of 1 mol/L ammonium acetate acid (pH 5.16) before HPLC analysis. We diluted a 10  $\mu$ mol/L porphyrin standard mixture (Chromatographic Marker Porphyrin Acids; Porphyrin Products) and 2 mmol/L protoporphyrin IX and coproporphyrin III standards (Porphyrin Products) with 50 g/L bovine serum albumin in phosphate-buffered saline (1:3000) and then mixed 50  $\mu$ L of this diluted standard mixture with 0.95 mL of 100 g/L trichloroacetic acid-dimethyl sulfoxide. After centrifugation, we mixed 50  $\mu$ L of the supernatant with 0.95 mL of 1 mol/L ammonium acetate acid before HPLC analysis, as performed for the samples. The amount of porphyrins (nmol/g dry weight) was calculated: 10 × standard (pmol/100  $\mu$ L) × [sample (mm):standard (mm)]:dry weight of feces (mg).

Lyphechek Quantitative Urine Control (Bio-Rad) was used as an assay quality control to monitor the precision of urinary measurements. For fecal measurements, two whole-blood samples having protoporphyrin concentrations of ~700 and 400 nmol/L of red cells were used as a control. The reference values for all laboratory measurements were adapted from the Mayo Medical Laboratories Test Catalog and Interpretive Handbook for 1988 (<http://www.mayo.edu/mmml/testcat.html>) and are shown in Table 1. The biochemical tests and mutation analyses were

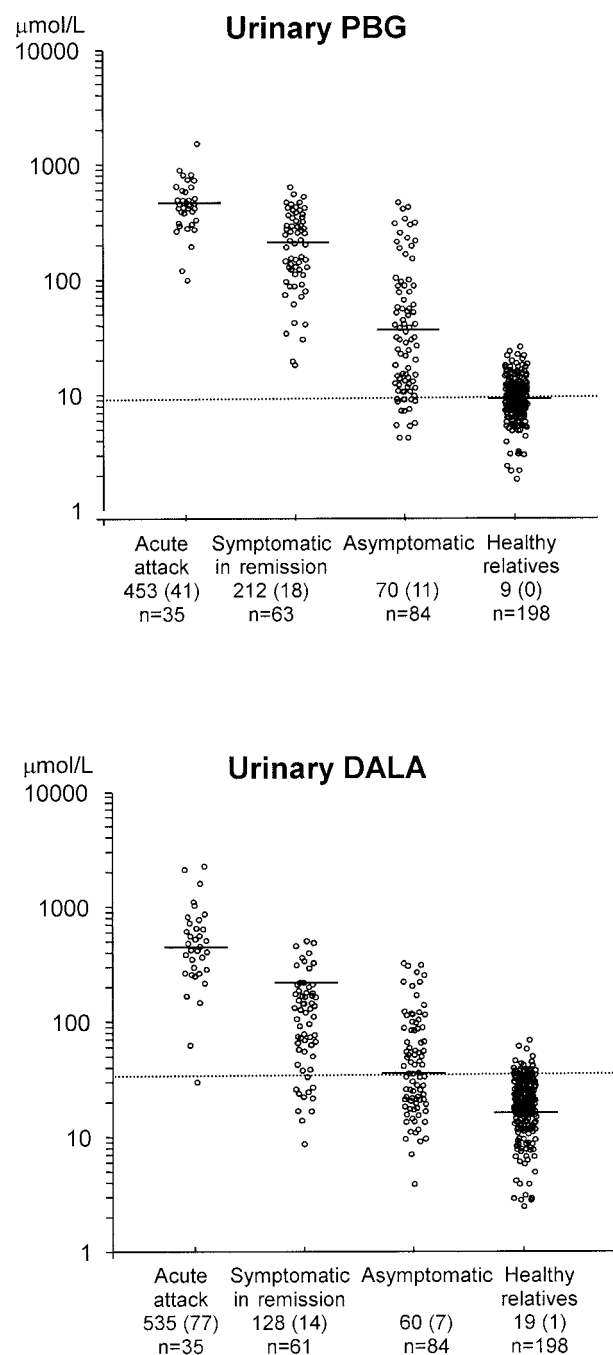


Fig. 2. Comparison of urinary PBG and ALA excretion in symptomatic AIP patients during an acute attack and remission, in symptom-free patients, and in their healthy relatives.

The values for individual persons were calculated as the means of one to eight single measurements in adulthood. Dashed lines denote the upper limits of the reference intervals (9 and 34  $\mu$ mol/L for PBG and ALA, respectively). Means (SE) are indicated. DALA,  $\delta$ -ALA.

performed independently by different laboratory technicians without knowledge of participants' clinical status.

Plasma samples were diluted with phosphate-buffered saline (1:4 by volume) before analysis by spectrofluoro-

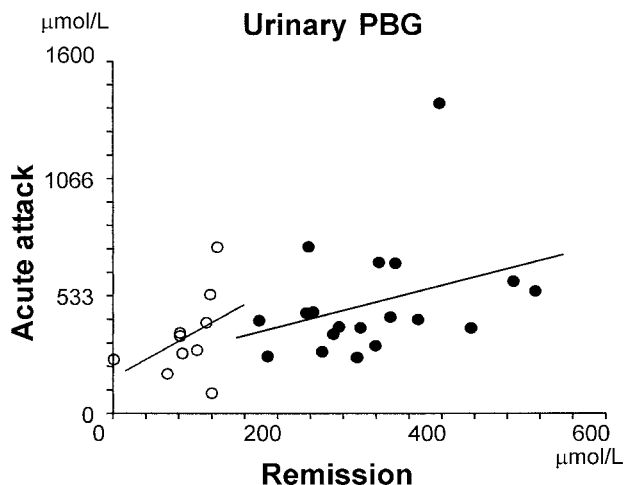


Fig. 3. Correlation between urinary PBG measured from AIP patients in remission and during an acute attack.

○, patients with low excretion (<150  $\mu\text{mol/L}$ ) in remission; ●, patients with high excretion (>150  $\mu\text{mol/L}$ ) in remission.

photometry (17). In AIP, the fluorescence emission maximum is at 619 nm.

#### DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Leukocyte DNA was extracted from the venous blood samples with EDTA as an anticoagulant using the QIAamp Blood Kit (Qiagen). The PCR mixture contained 100 ng of DNA, 0.2 mM deoxynucleoside triphosphates, 20 pmol of primers, and 1 U of DNA polymerase (DynaZyme II; Finnzymes) in 50  $\mu\text{L}$  of the enzyme buffer (18). Primer sequences have been published previously (19). The temperature profile for the PCR reactions was 2 min at 94  $^{\circ}\text{C}$  for the first denaturing step, followed by 30 s at 94  $^{\circ}\text{C}$ , 30 s at 56–60  $^{\circ}\text{C}$ , and 30 s at 72  $^{\circ}\text{C}$  for 30 cycles. The PCR products were purified by use of the QIAquick PCR Purification Kit (Qiagen). The samples were directly sequenced using dideoxynucleotide chain termination method (20) with the Amplicycle Sequencing Kit (Perkin-Elmer) and including 5  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]dATP (Amersham Pharmacia Biotech) in each sample. After electrophoresis, gels were dried (30 min at 80  $^{\circ}\text{C}$ ) and autographed with Kodak Biomax MR film (Eastman Kodak). The analyses were repeated at least twice for each sample studied in the presence of negative and positive controls.

#### RESTRICTION DIGESTION

We incubated 10  $\mu\text{L}$  of PCR product and 20 U of restriction enzyme in 30  $\mu\text{L}$  of the enzyme buffer for 1 h at the temperature specific for the enzyme (New England Biolabs). The resulting fragments were run in an ethidium bromide-stained 1–4% agarose gel (Amersham Pharmacia) and visualized with ultraviolet light. The cleavage was repeated at least twice with different samples from each patient in the presence of positive and negative controls in each series analyzed.

#### STATISTICAL ANALYSES

The Mann–Whitney test was used to assess the differences in urinary excretion of porphyrins and their precursors. ROC analyses were used to assess the validity of different laboratory tests in the diagnosis of AIP. Statistical calculations were performed using SPSS, Ver. 10.04, and NCSS 2000.

#### Results

##### MUTATION TESTING

Shown in Fig. 1 are 196 mutations identified in the *PBGD* gene, including 26 mutations identified among Finnish AIP patients [bold; Human Gene Mutation Database (<http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html>)]. These mutations cover 87% of the 45 AIP families in the Finnish population of 5 million. Of the 239 individuals tested, 120 were mutation-positive, and in 119 cases, AIP could be excluded. The probability is small that a healthy relative would have a disease-causing mutation in the *PBGD* gene that is different from that in the proband; we thus considered that mutation analysis excluded the diagnosis of AIP in symptom-free relatives when a mutation was known in the family. Of the 131 individuals (Table 1) who underwent mutation analysis and biochemical analyses, the diagnosis or exclusion of AIP was based solely on mutation testing in 3 cases (2%).

Mutation testing had some problems. In four cases, the proband was the only patient in the family and was deceased or could not be traced back. In two additional cases, the clinical manifestations and biochemical patterns best suited AIP, but long PCR fragments and sequencing of all exons, exon-intron boundaries, and 5'- and 3'-untranslated regions did not reveal a genetic defect. In one of the latter two cases, erythrocyte *PBGD* activity was decreased. In the other case, six of the eight enzymes in the heme biosynthetic pathway were within the reference intervals. Her uroporphyrinogen III synthase activity was not measured, and the coproporphyrinogen oxidase activity was increased threefold during a psychotic period, suggesting secondary porphyrinuria. No other family members were affected. Although mutations could not be identified in these patients, AIP could not be excluded because of acute attacks and suitable biochemical abnormalities.

##### BIOCHEMICAL METHODS

The first PBG analysis on each patient detected 84% of 147 AIP patients screened in remission. When the analyses were repeated one to eight times during follow-up in 73 of the patients, the overall sensitivity was increased only to 88%. In the patients with increased PBG excretion ( $n = 129$ ; 62 symptomatic by medical history, 67 symptom free), variation among individuals was wide (9.8–590  $\mu\text{mol/L}$ ). The mean (SD) excretion for all patients was 145 (138)  $\mu\text{mol/L}$ , which was 10-fold above the upper limit of the reference interval. Excretion of PBG was also increased in 40% of 198 healthy relatives screened [ $n = 79$ ;

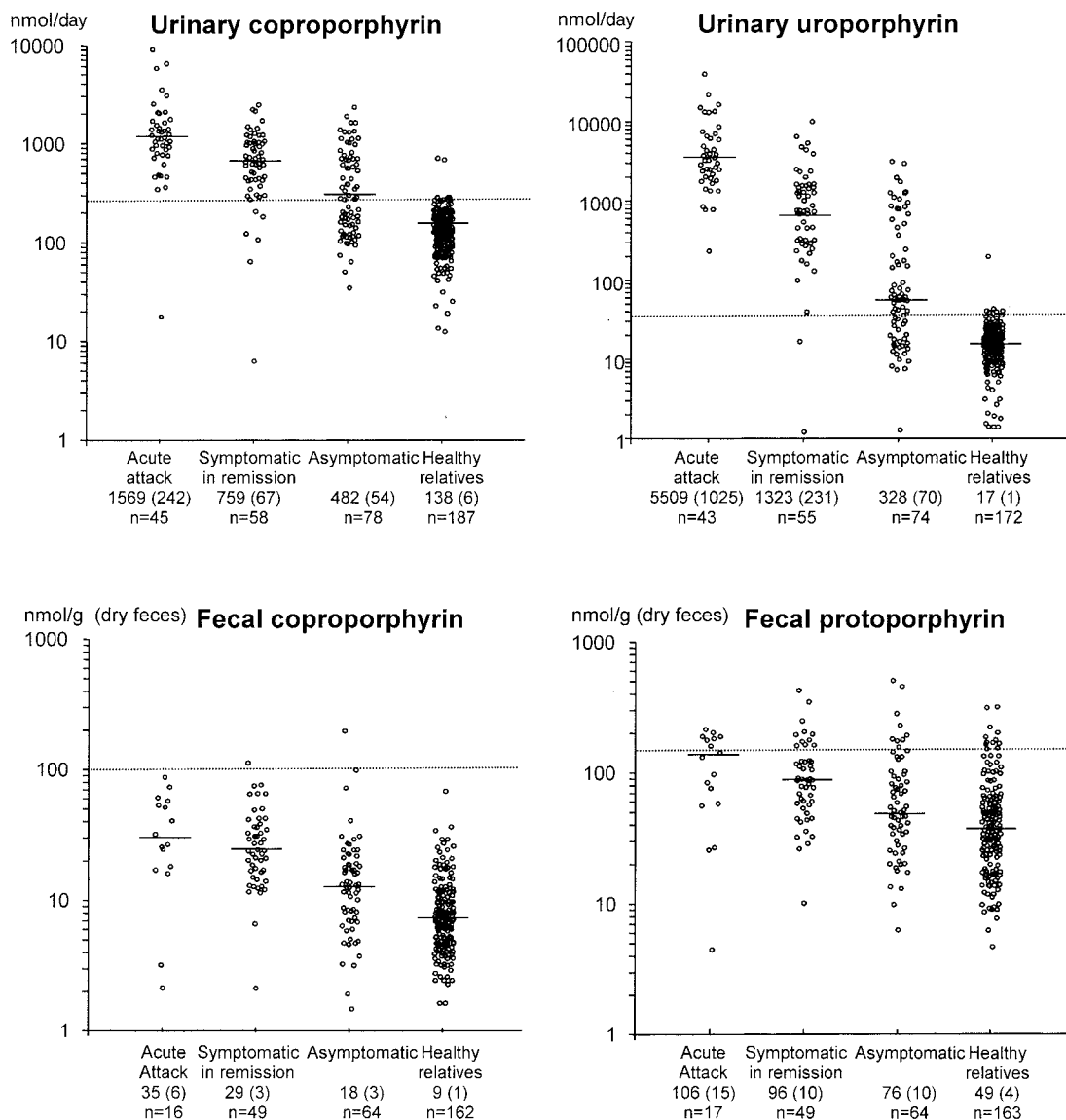


Fig. 4. Comparison of urinary porphyrin excretion in symptomatic AIP patients during an acute attack and remission, in symptom-free patients, and in their healthy relatives.

The values for individual persons were calculated as the means of one to eight single measurements in adulthood. Dashed lines denote the upper limits of the reference intervals (36 nmol/day, 230 nmol/day, 100 nmol/g, and 130 nmol/g for urinary uro- and coproporphyrin and fecal copro- and protoporphyrin, respectively). Means (SE) are indicated.

range, 9.1–24  $\mu\text{mol/L}$ ; mean (SD), 13 (3.2)  $\mu\text{mol/L}$ ], but the mean increase was markedly less (twofold). Urinary ALA was increased in 61% of 145 AIP patients screened in remission [ $n = 89$ ; 50 symptomatic by medical history, 39 symptom free; range, 34–453  $\mu\text{mol/L}$ ; mean (SD), 130 (96)  $\mu\text{mol/L}$ ], which is a 3.8-fold increase above the upper limit of the reference interval, and in 6.6% of 198 healthy relatives screened [ $n = 13$ ; range, 34–62  $\mu\text{mol/L}$ ; mean (SD), 42 (8.8)  $\mu\text{mol/L}$ ], which is a 1.2-fold increase.

Shown in Fig. 2 are the mean PBG and ALA excretions for symptomatic AIP patients during an acute attack and in remission compared with symptom-free patients and their healthy relatives. During an acute attack, excretion

of PBG was increased approximately twofold ( $n = 29$ ; mean values, 237–449  $\mu\text{mol/L}$ ) compared with the values in remission, but there was a wide interindividual variation in excretion (range, 92–1411  $\mu\text{mol/L}$ ). The patients with urinary PBG <150  $\mu\text{mol/L}$  in remission ( $n = 10$ ) had a relatively greater increase in PBG excretion during an acute attack (3.9-fold) than those who excreted high amounts of PBG (>150  $\mu\text{mol/L}$ ;  $n = 19$ ) even in remission (1.6-fold; Fig. 3).

Never-symptomatic patients ( $n = 80$ ) had significantly lower PBG excretion than symptomatic patients ( $n = 63$ ) in remission [mean (SE) 72 (11)  $\mu\text{mol/L}$  vs 212 (18)  $\mu\text{mol/L}$ ;  $P < 0.000001$ ]. Among healthy relatives, urinary

excretion of PBG was significantly higher than in the general population (Fig. 2). This difference was also confirmed among mutation-screened healthy relatives ( $n = 43$ ). This may reflect the influence of common modifying polymorphisms in other metabolic genes in these families.

Among 129 AIP patients screened in remission, 75% ( $n = 97$ ; 53 symptomatic and 44 symptom free) excreted high amounts of uroporphyrins [range, 38–9440 nmol/day; mean (SD), 975 (1409) nmol/day], of which ~50% were I and III isomers. Among 172 healthy relatives, 4% [ $n = 7$ ; range, 36–189 nmol/day; mean (SD), 60 (57) nmol/day] excreted abnormally high amounts of uroporphyrins. Fig. 4 shows the urinary excretion of uro- and coproporphyrins in sometimes-symptomatic and always-symptom-free patients and their healthy relatives. In symptomatic patients ( $n = 32$ ), uroporphyrin excretion increased 4.2-fold [mean (SD), 1395 (1432) nmol/day vs 5921 (7513) nmol/day] during an acute attack, which was similar to values found in porphyria cutanea tarda patients. Moreover, symptomatic patients ( $n = 58$ ) excreted significantly higher amounts of uroporphyrin, even in remission, compared with symptom-free patients [ $n = 74$ ; mean (SE), 759 (67) nmol/day vs 460 (50) nmol/day;  $P = 0.0005$ ].

Urinary coproporphyrins were increased in 69% of 136 AIP patients in remission [ $n = 94$ ; 52 symptomatic, 40 symptom free; range, 263–2392 nmol/day; mean (SD), 810 (476) nmol/day; 70–90% III isoform] and in 8.6% of 187 healthy relatives [ $n = 16$ ; range, 235–689 nmol/day, mean, 311 (144) nmol/day], respectively. In AIP patients, excretion of urinary uroporphyrin exceeded that of coproporphyrins and 7-, 6-, and 5C-porphyrins.

Fecal coproporphyrins were increased in only 2% of 113 AIP patients screened [ $n = 2$ ; range, 106–185 nmol/g; mean (SD), 145 (56) nmol/g; 30–60% III isoform], and none of 163 healthy relatives had increased fecal excretion of coproporphyrins (Fig. 4). In contrast, fecal protoporphyrins were increased in 19% of 113 AIP patients in remission [ $n = 21$ ; range, 132–454 nmol/g; mean, 212 (95) nmol/g] and in 7% of 163 healthy relatives [ $n = 12$ ; range, 137–286 nmol/g; mean, 180 (52) nmol/g; Fig. 4].

#### SENSITIVITY AND SPECIFICITY

The sensitivity and specificity of the biochemical screening for AIP were studied among 131 family members for whom the results of both the DNA and biochemical analyses were available (Tables 1 and 2). Of the 85 AIP patients, 24 were symptom free and 35 had experienced acute attacks. The measurements performed during acute attacks were excluded. To avoid potential bias from patient selection (e.g., if complex tests were ordered only for the most difficult cases to diagnose), ROC curve analysis was performed on the subset of 75 patients for whom the results of all biochemical tests were available.

Urinary PBG is not a very sensitive or specific screening test for AIP in remission, but according to the ROC curve analysis (Fig. 5), it is as good as any of the other biochemical tests studied. The areas under the curves were 0.94 (95% confidence interval, 0.91–0.97) for urinary PBG, 0.92 (0.88–0.96) for urinary uroporphyrin, 0.92 (0.87–0.96) for erythrocyte PBGD, 0.88 (0.83–0.93) for urinary coproporphyrin, 0.84 (0.78–0.89) for urinary ALA, 0.78 (0.72–0.84) for fecal coproporphyrin, and 0.68 (0.62–0.75) for fecal protoporphyrin.

Fifteen percent of the symptom-free mutation-positive patients had falsely negative PBG at the current cutoff value of 9  $\mu\text{mol/L}$ , and 29% of mutation-negative relatives had falsely positive results. Because the measurement of urinary PBG in remission is unspecific in the diagnosis of AIP, the cutoff value should be higher to exclude false-positive family members. With a cutoff value of 25  $\mu\text{mol/L}$ , 23% of the AIP patients would remain undiagnosed in remission, but no false-positives should be obtained. Patients with low PBG excretion in remission could be screened with mutation analysis for the certain diagnosis of AIP.

Urinary ALA was less sensitive but more specific, but the combination of both urinalyses did not increase overall sensitivity or specificity. Although the sensitivity and specificity of erythrocyte PBGD activity were similar to those of urinary PBG excretion, the combination of these tests did not improve the accuracy of the diagnosis. When the enzyme activity was measured in erythrocytes, 16% of AIP patients were false negative mainly because of the

**Table 2. Diagnosis of AIP.**

	Acute attack	Symptom free	Sensitivity $\pm$ 95% CI, <sup>a</sup> %	Specificity $\pm$ 95% CI, %
Mutation analysis		+ <sup>b</sup>	95	100
Erythrocyte PBGD		$\downarrow$ /N <sup>c</sup>	84 $\pm$ 8	77 $\pm$ 13
Urinary PBG	$\uparrow \uparrow \uparrow / \uparrow \uparrow$	$\uparrow \uparrow / \uparrow / N$	85 $\pm$ 8	71 $\pm$ 14
Urinary ALA	$\uparrow \uparrow \uparrow / \uparrow \uparrow$	$\uparrow / N$	62 $\pm$ 11	90 $\pm$ 9
Urinary uroporphyrin	$\uparrow \uparrow \uparrow / \uparrow \uparrow$	$\uparrow \uparrow / \uparrow / N$	72 $\pm$ 11	97 $\pm$ 6
Urinary coproporphyrin	$\uparrow \uparrow / \uparrow$	$\uparrow \uparrow / \uparrow / N$	67 $\pm$ 11	87 $\pm$ 12
Fecal coproporphyrin	$\uparrow \uparrow / \uparrow$	$\uparrow / N$	4 $\pm$ 5	100
Fecal protoporphyrin	$\uparrow \uparrow / \uparrow$	$\uparrow / N$	20 $\pm$ 11	96 $\pm$ 8
Plasma fluorescence	+/-	-		

<sup>a</sup> CI, confidence interval.

<sup>b</sup> +, mutation positive.

<sup>c</sup>  $\downarrow$ , decreased, N, within the reference interval;  $\uparrow \uparrow \uparrow$ , increased >10-fold;  $\uparrow \uparrow$ , increased 5- to 10-fold;  $\uparrow$ , increased 2- to 5-fold.

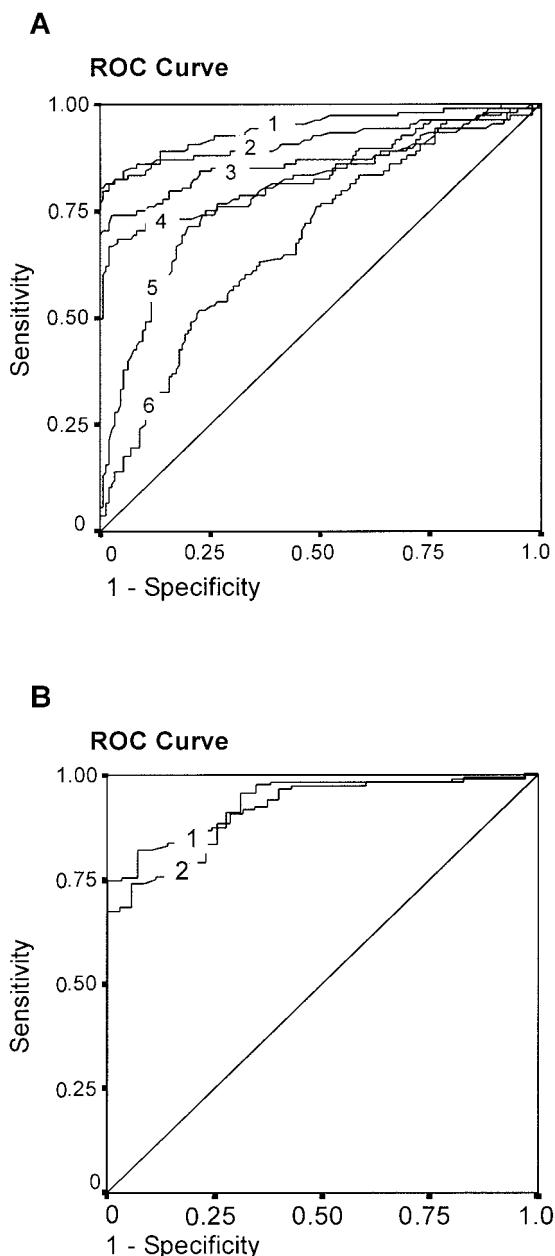


Fig. 5. Comparison of sensitivity and specificity characteristics for different biochemical tests for AIP.

(A), urinary and fecal porphyrins and precursors: curve 1, urinary PBG; curve 2, urinary uroporphyrin; curve 3, urinary coproporphyrin; curve 4, urinary ALA; curve 5, fecal coproporphyrin, and curve 6, fecal protoporphyrin. (B), erythrocyte PBGD activity: curve 1, patients with variant form of AIP excluded; curve 2, all AIP patients.

normal erythrocyte PBGD activity in the variant form of AIP. If those patients ( $n = 8$ ) were excluded, measurement of PBGD activity detected 71 (92%) of the 77 patients with the classic form of AIP. Even more troubling was the result that 23% of healthy relatives were false positive.

### Discussion

Measurement of urinary PBG identified all AIP patients during an acute attack, and the mean excretion of PBG

was markedly increased above the reference interval, although the intraindividual increases were modest, emphasizing the importance of careful evaluation of clinical manifestations. Urinary PBG analysis identified the majority of patients in remission, but ~10% remained undetected. The test is sensitive for acute porphyrias in general and does not distinguish AIP from other acute porphyrias. Although many healthy relatives experienced mild increases in PBG excretion, by ROC curve analysis it was nonetheless the best of the biochemical tests for AIP. Erythrocyte PBGD activity was decreased in the majority of AIP patients, with results within the reference interval mainly in the variant form of AIP, but it was also decreased in one-fourth of healthy relatives. Because biochemical analyses remain often indeterminate, mutation analysis is needed to exclude or confirm the diagnosis of AIP in remission.

AIP is the commonest type of acute porphyria in most countries (1). The prevalence varies from 3:100 000 estimated in Finland and Western Australia (13, 21) and 5–10:100 000 in the US to 1:10 000 in Sweden (22). In northern Sweden, the prevalence exceeds 0.5–2% because of a founder effect in two small municipalities. Low erythrocyte PBGD activity is more common, ~1:500 when estimated among healthy Finnish and French blood donors (23, 24). Penetrance varies between 10% and 50% within the families; approximately one-half of the patients stay symptom free throughout their lives, 30–40% experience mild symptoms, and only 10–20% experience acute attacks (7). Thus, the prevalence may be much higher than previously expected. Expanded mutation screening may provide more accurate prevalence rates.

Our series includes both patients with symptoms and phenotypically normal carriers; it thus provides information about outcomes among patients with AIP in general. The proportion of the patients who had acute attacks (41%) is greater than in some extensive family studies, in which up to 80% of the diagnosed patients have been phenotypically normal carriers (25, 26). The personal contacts with every patient at the time of diagnosis and afterward explain the good collaboration, the high number of included individuals, and the relatively low number of nontraced and excluded individuals, which increased the reliability of these results. Thus, we believe that the results of our series are accurate enough to serve as a basis for conclusions concerning the diagnosis of AIP.

In our series of mutation screenings, many new symptom-free patients were identified and many healthy relatives could be excluded despite slightly increased excretion of porphyrins and porphyrin precursors. Thus, DNA analysis is the only reliable way to screen symptom-free patients to facilitate correct treatment and proper genetic counseling of family members at risk. The certain diagnosis of AIP in the early stage as well as identification of symptom-free individuals is essential for the prevention of acute attacks, which may be potentially fatal (7). The diagnosis should be confirmed in youth, so that patients



can avoid precipitating factors, which may induce acute attacks after puberty. The mutations reported in the *PBGD* gene are usually family specific and include single-base substitutions, splicing defects, insertions, and deletions (Fig. 1) that lead to structural impairment or loss of function of *PBGD*. The identification of a mutation at the DNA level is a convenient and powerful tool providing a certain diagnosis, but the value of DNA analysis is reduced in cases in which a mutation is unknown. Moreover, the molecular heterogeneity limits the potential of DNA diagnostics in AIP.

The use of DNA technology has its own special demands. The PCR method is prone to contamination and requires special laboratory equipment and thorough quality assurance. Mutations can be detected by various DNA methodologies, which can be chosen according to the experience of the laboratory personnel, although sequencing is recommended. In symptom-free individuals, DNA analysis is sufficient for the diagnosis of AIP. Measurement of urinary PBG in adulthood and in remission has been shown to have predictive value (7). Thus, those whose urinary PBG excretion rates are within the reference interval are more likely to remain symptom free throughout their life span. However, a proper clinical investigation, detailed family history, and biochemical measurements are essential before the diagnosis of AIP can be confirmed in symptomatic patients.

In our participants, biochemical urinary and erythrocyte measurements revealed the majority of the AIP patients in remission, and urinary excretion of PBG detected all patients during an acute attack. Thus, measurement of urinary PBG is the method of choice at the symptomatic phase. PBG excretion is usually increased 20- to 50-fold during an acute attack compared with reference values. However, two-thirds of the patients excrete high amounts of PBG even in remission, and the increase may be modest during an attack, making differential diagnosis of abdominal pain more difficult. Measurement of uroporphyrin excretion during an attack provided no additional information in those patients who excreted high amounts of uroporphyrins in remission (data not shown). The plasma porphyrin fluorescence spectrum is usually only transiently characteristic for AIP during an attack. Thus, the importance of the clinical manifestations, including symptoms of porphyria, signs of neuropathy, and a history of precipitating factors, should be emphasized. Correct diagnosis is essential, and differential diagnosis of abdominal pain should be evaluated each time in AIP patients.

Although biochemical measurements of excreted porphyrins and porphyrin precursors reveal acute porphyria in the majority of cases, none of these tests is specific for AIP. Moreover, acute symptoms and signs are indistinguishable from those of other acute porphyrias, although patients with AIP and ALA dehydratase deficiency do not manifest photosensitivity, which is common in patients with variegate porphyria and hereditary coproporphyrin.

Only ~40% of patients with variegate porphyria experience skin symptoms, and these appear independently from acute attacks (27). Measurement of the plasma porphyrin fluorescence spectrum is easy to perform and specific, although not very sensitive for variegate porphyria (28). Excretion of fecal protoporphyrin is increased in 20% of AIP patients even in remission; thus it cannot be used to distinguish variegate porphyria from AIP. Excretion of uroporphyrin is increased in AIP, similar to porphyria cutanea tarda, which is the commonest cutaneous porphyria accompanied by mild liver failure and photosensitivity but no acute attacks. In addition, urinary excretions of coproporphyrins can be increased in many liver diseases and in heavy metal intoxication (12).

Erythrocyte *PBGD* activity is relatively sensitive and specific for AIP, but it may be affected by various conditions, such as anemia (29), liver disease (30), malignancies (31), uremia (32), chronic polyarthritis (30), and hemodialysis, or by any other conditions in which erythropoiesis is either induced or inhibited (12). The decrease in *PBGD* activity may be secondary, as reported in variegate porphyria patients (33). Furthermore, *PBGD* activity can be within the reference interval in AIP patients during an acute attack (34). In the variant form of AIP, the erythrocyte *PBGD* activity is within the reference interval in both the symptomatic and symptom-free phases (11).

This study was supported by grants from the Magnus Ehrnrooth Foundation, the Finnish Cultural Foundation, the Instrumentarium Research Foundation, Jalmari and Rauha Ahokas Foundation, the Research Funds and the Clinical Research Institute of the Helsinki University Central Hospital, the Biomedicum Helsinki Foundation, and the University of Helsinki. The study has been accepted by the Ethical Committee of the University Hospital of Helsinki according to the Helsinki Declaration of 1975 as revised in 1996.

## References

1. Kappas A, Sassa S, Galbraith RA, Nordmann Y. The porphyrias. In: Scriver CR, Beaudet A, Sly WS, Valle D, eds. *The metabolic and molecular bases of inherited diseases*, 7th ed. New York: McGraw-Hill, 1995:2116–27.
2. Raich N, Romeo PH, Dubart A, Beaupain D, Cohen-Solal M, Goossens M. Molecular cloning and complete primary sequence of human erythrocyte PBG deaminase. *Nucleic Acids Res* 1986; 14:5955–68.
3. Grandchamp B, de Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y. Tissue-specific expression of porphobilinogen deaminase: two isoenzymes from a single gene. *Eur J Biochem* 1987;162:105–10.
4. Chretien S, Dubart A, Beaupain D, Raich N, Grandchamp B, Rosa J, et al. Alternative transcription and splicing of the human porphobilinogen deaminase result either in tissue-specific or in house-keeping expression. *Proc Natl Acad Sci U S A* 1988;85: 6–9.
5. Yoo HW, Warner CA, Chen CH, Desnick RJ. Hydroxymethylbilane

- synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. *Genomics* 1993;15:21–9.
6. Namba H, Narahara K, Tsuji K, Yokoyama Y, Seino Y. Assignment of human porphobilinogen deaminase to 11q24.1–q24.2 by in situ hybridization and gene dosage studies. *Cytogenet Cell Genet* 1991;57:105–8.
  7. Kauppinen R, Mustajoki P. Prognosis of acute porphyria: occurrence of acute attacks, precipitating factors, and associated diseases. *Medicine (Baltimore)* 1992;71:1–13.
  8. Llewellyn DH, Smyth SJ, Elder GH, Hutchesson AC, Rattenbury JM, Smith MF. Homozygous acute intermittent porphyria: compound heterozygosity for adjacent base transitions in the same codon of the porphobilinogen deaminase gene. *Hum Genet* 1992;89:97–8.
  9. Beukeveld GJ, Wolthers BG, Nordmann Y, Deybach JC, Grandchamp B, Wadman SK. A retrospective study of a patient with homozygous form of acute intermittent porphyria. *J Inher Metab Dis* 1990;13:673–83.
  10. Meyer UA, Strand LJ, Doss M, Rees AC, Marver HS. Intermittent acute porphyria—demonstration of a genetic defect in porphobilinogen metabolism. *N Engl J Med* 1972;286:1277–82.
  11. Mustajoki P. Normal erythrocyte uroporphyrinogen I synthase in a kindred with acute intermittent porphyria. *Ann Intern Med* 1981;95:162–6.
  12. Moore MR, McColl KEL, Rimington C, Goldberg A. Disorders of porphyrin metabolism. New York: Plenum Publishing Corporation, 1987:46–61, 227–55.
  13. Mustajoki P, Koskelo P. Hereditary hepatic porphyrias in Finland. *Acta Med Scand* 1976;200:171–8.
  14. Strand LJ, Meyer UA, Felsher BF, Redeker AG, Marver HS. Decreased red cell uroporphyrinogen I synthetase activity in intermittent acute porphyria. *J Clin Invest* 1972;51:2530–6.
  15. Li F, Lim CK, Peters TJ. Analysis of urine and faecal porphyrins by HPLC coupled to an advanced automated sample processor. *Biomed Chromatogr* 1986;1:93–4.
  16. Lim CK, Peters TJ. Urine and faecal porphyrin profiles by reversed-phase high-performance liquid chromatography in the porphyrias. *Clin Chim Acta* 1984;139:55–63.
  17. Poh-Fitzpatrick MB. A plasma porphyrin fluorescence marker for variegate porphyria. *Arch Dermatol* 1980;116:543–7.
  18. Mullis KB, Faloona F. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987;155:335–50.
  19. Mustajoki S, Pihlaja H, Ahola H, Petersen NE, Mustajoki P, Kauppinen R. Three splicing defects, an insertion, and two missense mutations responsible for acute intermittent porphyria. *Hum Genet* 1998;102:541–8.
  20. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-termination inhibitors. *Proc Natl Acad Sci U S A* 1977;74:5463–7.
  21. Tschudy DP, Valsamis M, Magnussen CR. Acute intermittent porphyria: clinical and selected research aspects. *Ann Intern Med* 1975;83:851–64.
  22. Andersson C. Acute intermittent porphyria in Northern Sweden. A population-based study [PhD Thesis]. Sweden: University of Umeå, 1997:22–3.
  23. Mustajoki P, Kauppinen R, Lannfelt L, Koistinen J. Frequency of low porphobilinogen deaminase activity in Finland. *J Intern Med* 1992;231:389–95.
  24. Nordmann Y, Puy H, da Silva V, Simonin S, Robreau AM, Bonaiti C, et al. Acute intermittent porphyria: prevalence of mutations in the porphobilinogen deaminase gene in blood donors in France. *J Intern Med* 1997;242:213–7.
  25. Bottomley SS, Bonkowsky HL, Kreimer-Birnbaum M. The diagnosis of acute intermittent porphyria. Usefulness and limitations of the erythrocyte uroporphyrinogen I synthase assay. *Am J Clin Pathol* 1981;76:133–9.
  26. Lamon JM, Frykholm BC, Tschudy DP. Family evaluations in acute intermittent porphyria using red cell uroporphyrinogen I synthetase. *J Med Genet* 1979;16:134–9.
  27. Mustajoki P. Variegate porphyria. Twelve years' experience in Finland. *QJM* 1980;49:191–203.
  28. Fraunberg M, Kauppinen R. The diagnosis of variegate porphyria—hard to get? *Scand J Clin Lab Invest* 2000;60:605–10.
  29. Anderson KE, Sassa S, Peterson CM, Kappas A. Increased erythrocyte uroporphyrinogen-I-synthetase,  $\delta$ -aminolevulinic acid dehydratase and protoporphyrin in hemolytic anemias. *Am J Med* 1977;63:359–64.
  30. Blum M, Koehl C, Abecassis J. Variations in erythrocyte uroporphyrinogen I synthetase activity in non porphyrias. *Clin Chim Acta* 1978;87:119–25.
  31. Epstein O, Lahav M, Schoenfeld N, Nemesh L, Shaklai M, Atsmon A. Erythrocyte uroporphyrinogen synthase activity as a possible diagnostic aid in the diagnosis of lymphoproliferative diseases. *Cancer* 1983;52:828–32.
  32. Andriolo A, Mocelin AJ, Stella SR, Ajzen H, Ramos OL. Determination of erythrocyte uroporphyrinogen I synthetase activity in chronic renal failure. *Clin Chim Acta* 1980;104:241–4.
  33. Weinlich G, Doss MO, Sepp N, Fritsch P. Variegate porphyria with coexistent decrease in porphobilinogen deaminase activity. *Acta Derm Venereol* 2001;81:356–9.
  34. Kostrzewska E, Gregor A. Increased activity of porphobilinogen deaminase in erythrocytes during attacks of acute intermittent porphyria. *Ann Clin Res* 1986;18:195–8.