# *Molecular pathology of porphyrias*

**MUDr. Eva Flachsová**

**1st Faculty of Medicine**
**Charles University**
**Prague, Czech Republic**

**PhD thesis**
Biochemistry and Pathobiochemistry

Prague 2007

This PhD thesis was elaborated at the Laboratory for Studies of Mitochondrial Disorders, Department of Pediatrics, 1st Faculty of Medicine in Prague, Czech Republic. The dissertation is based on the results of four scientific papers published in the prestigious journals, one manuscript accepted for publication, and two manuscripts prior to submission for publication.

I would like to express my greatest gratitude to all of you who, have been involved in this work in one way or another. It has been my pleasure working together with you, with joyful moments in the lab, as well as outside. Special thanks are due to:

Prof. Pavel Martásek, M.D., DrSc, my supervisor for directing me into the field of porphyrinology and for his scientific and material support throughout my PhD studies;

Prof. Jiří Zeman, M.D., DrSc, for numerous inspiring talks and ideas;

My friends and colleagues from the Laboratory for Studies of Mitochondrial Disorders for their help during my experimental work and for making a pleasant and friendly ambiance;

Colleagues from The Centre Français des Porphyries in Colombes for their help during my fellowship in their laboratory;

And most of all, my husband Pavel, my son Jáchym, my parents and my entire family, who provided me with moral support and lots of love.

**This thesis is based on the following articles, reffered to in the text by their capital letter as indicated here:**

A) Lamoril J, Andant C, Gouya L, **Malonova E,** Grandchamp B, Martasek P, Deybac JC, Puy H. **Hemochromatosis (HFE) and transferrin receptor-1 (TFRC1) genes in sporadic porphyria cutanea tarda (sPCT)**. Cell Mol Biol (Noisy-le-grand). 2002 Feb;48(1):33-41.

B) Schmitt C, Gouya L, **Malonova E,** Lamoril J, Camadro JM, Flamme M, Rose C, Lyoumi S, Da Silva V, Boileau C, Grandchamp B, Beaumont C, Deybach JC, Puy H. **Mutations in human CPO gene predict clinical expression of either hepatic hereditary coproporphyria or erythropoietic harderoporphyria**. Hum Mol Genet. 2005 Oct 15;14(20):3089-98. Epub 2005 Sep 13.

C) Lee DS, **Flachsova E**, Bodnarova M, Demeler B, Martasek P, Raman CS. **Structural basis of hereditary coproporphyria**. Proc Natl Acad Sci U S A. 2005 Oct 4;102(40):14232-7. Epub 2005 Sep 21.

D) Ulbrichova D, **Flachsova E**, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. **De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study**. Physiol Res. 2007;55 Suppl 2:S145-54.

E) **Flachsova E**, Verma IC, Ulbrichova D, Saxena R, Zeman M, Saudek V, Raman CS, Martasek P: **New mutation within porphobilinogen deaminase gene leading to the truncated protein as a cause of acute intermittent porphyria in extended Indian family** (Folia Biologica, accepted for publication).

F) **Flachsová E**., Keslová P., Sedláček P., Plavka R., Zeman J., Starý J., Martásek P. **Treatment of congenital erythropoietic porphyria (Morbus Günther) by cord blood stem cell transplantation, long term follow-up with 7-11% chimerism and prenatal diagnosis in a sibling** (prior to submission).

G) **Flachsova E,** Robreau A-M, Da Silva V, Deybach J-Ch, Martasek P., Puy H **Search for molecular determinants responsible for different phenotypic expression of porphyria variegata** (manuscript in preparation).

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | δ-aminolevulinic acid synthase deficiency |
| AIP | acute intermittent porphyria |
| ALA | δ-aminolevulinic acid |
| ALAD | δ-aminolevulinic acid dehydratase |
| ALAS | δ-aminolevulinic acid synthase |
| ALAS1 | δ-aminolevulinic acid synthase - housekeeping isoenzyme |
| ALAS2 | δ-aminolevulinic acid synthase erythroid isoenzyme |
| ATP | adenosin triphosphate |
| BMT | bone marrow transplantation |
| CBST | cord blood stem cells transplantation |
| CEP | congenital erythropoietic porphyria |
| COPRO | coproporphyrin |
| COPROgen | coproporphyrinogen |
| CPO | coproporphyrinogen oxidase |
| DGGE | denaturing gradient gel electrophoresis |
| EPP | erythropoietic protoporphyria |
| FAD | flavin adenine dinucleotide |
| FECH | ferrochelatase |
| fPCT | familiar porphyria cutanea tarda |
| GABA | gamma-aminobutyric acid |
| GST | glutathione S-transferase |
| HC | hereditary coproporphyria |
| HCA | hydrophobic cluster analysis |
| hemF | oxygen dependent bacterial coproporphyrinogen oxidase |
| hemG | oxygen independent bacterial protoporphyrinogen oxidase |
| hemN | oxygen independent bacterial coproporphyrinogen oxidase |
| hemY | oxygen dependent bacterial protoporphyrinogen oxidase |
| HEP | hepatoerythropoietic porphyria |
| HFE | hemochromatosis |
| HMB | hydroxymethylbilane |
| HT | hereditary tyrosinemia |
| $NAD(P)^+$ | nicotinamide adenine dinucleotide (phosphate) |
| PBG | porphobilinogen |
| PBGD | porphobilinogen deaminase |
| PCT | porphyria cutanea tarda |
| pGEX vectors | glutathione S-transferase gene fusion system |
| PPOX | protoporphyrinogen oxidase |
| PROTO | protoporphyrin |
| PROTOgen | protoporphyrinogen |
| PV | porphyria variegata |
| SDS PAGE | SDS polyacrylamid gel electrophoresis |
| SNP | single nucleotide polymorphism |
| sPCT | sporadic porphyria cutanea tarda |
| TFRC1 | transferrin receptor-1 gene |
| URO | uroporphyrin |
| UROD | uroporphyrinogen decarboxylase |
| UROgen | uroporphyrinogen |
| UROS | uroporphyrinogen III synthase |

# CONTENTS

# 1. INTRODUCTION

Porphyrias are mostly inherited metabolic disorders caused by decreased activities of the enzymes in the heme biosynthetic pathway [1] The term porphyria describes the purple-red crystalline porphyrins named from the Greek *"πορφυροσ"* purphuros (purple). Porphyrins are essential components of a wide range of chemical reactions that are required for life. These macrocyclic tetrapyrroles act as cofactors for multiple enzymes that perform a variety of processes within the cell, such as methionine synthesis (vitamin B12), oxygen transport (heme) and photosynthesis (chlorophyll).

Heme is a prosthetic group of a number of cellular hemoproteins which have various biological functions. Among them are hemoglobin, myoglobin, drug metabolizing cytochrome P450, cytochromes involved in oxidative phosphorylation (cytochrome c, cytochrome c oxidase, cytochrome reductase), catalases, peroxidases, nitric oxide synthase and guanyl cyclases. As heme biosynthesis is one of the essential pathways of life, it occurs in all metabolically active cells.

Heme is synthesized by an orchestrated cascade of eight enzymes [2]. The biosynthetic pathway starts in mitochondria, and after passing through four cytoplasmic stages, re-enters the mitochondria for the final steps of heme formation.

With the exception of the first enzyme of heme biosynthesis, deficiency of each one can be assigned to a certain type of porphyria. Each type of porphyria is characterized by typical spectra of accumulated and excreted porphyrins and their precursors, namely δ-aminolevulinic acid and porphobilinogen Most of them can be classified as inborn errors of metabolism, while only a minority of them are acquired. The inheritance is either autosomal dominant or autosomal recessive. Knowledge of the structure of genes of heme biosynthesis will allow molecular diagnosis of porphyrias.

## 1.1. PORPHYRINS AND PORPHYRINOGENS

The structural basis of porphyrins is the tetrapyrrole ring (Figure 1). The four single pyrrole rings (A, B, C, D/I-IV) are joined together via methine bridges to form a highly conjugated structure: the porphyrin. Its eight hydrogen side atoms (1-8) are substituted with characteristic side chains.

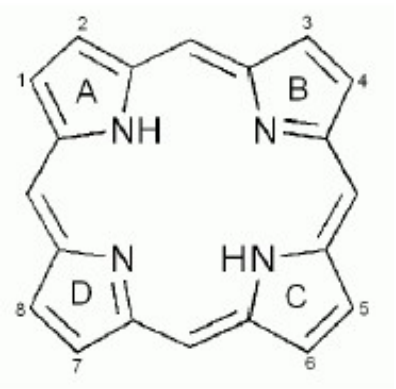


Figure 1: The chemical structure of porphyrins

The biological intermediates of heme synthesis are hexahydroporphyrins, porphyrinogens, with the four methine bridge carbon atoms and the two pyrrolenine nitrogen atoms hydrogenated. They are highly unstable molecules that, when exposed to air, rapidly oxidize to porphyrins. Porphyrinogens are functional precursors of heme, and oxidation to the corresponding porphyrins removes them from the pathway. Protoporphyrin IX (PROTO) is the only oxidized porphyrin that serves as a substrate for heme-pathway enzymes. Porphyrinogens are colorless and do not fluoresce, but highly conjugated porphyrins are colored molecules with unique photochemical features.

The type of the side chain of porphyrinogens determines physico-chemical properties of subsequently oxidized porphyrins. The first of the porphyrinogens synthesized by the heme pathway has eight carboxyles groups. The stepwise decarboxylation of the side chains from eight to two occurs along the pathway. Thus, porphyrins with higher numbers of carboxyl groups are hydrophilic, a quality that facilitates their excretion in urine; while porphyrins with fewer carboxyl

moieties as side chains have lipophilic properties and are excreted by the hepatobiliary route.

An important characteristic of porphyrins is metal-binding capability. The most commonly bound metals are iron and magnesium. Heme, the iron-containing complex, is central to most biological oxidation and oxygen transport. Chlorophyl is a magnesium porphyrin compound, which is critical in utilization of solar energy in the biosphere. However, porphyrins are also used in pigmentation such as reddish brown observed in egg shells, and in turacin, the red copper-uroporphyrin III complex that colors feathers of some birds [3]. The Nobel Laureate Hans Fischer described porphyrins as the compounds that “made grass green and blood red”

### 1.1.1. PHOTOCHEMICAL FEATURES OF PORPHYRINS

The porphyrin macrocycle is highly conjugated. The presence of this conjugated double-bond system makes porphyrins resonating compounds, with a typical dark red color (Figure 2) and characteristic absorption bands in the near ultraviolet and visible regions of the electromagnetic spectrum [4].

Porphyrins have absorption spectra consisting of a major band near 400 nm (the Soret band) and multiple smaller bands (depending upon the solvent), between 500 and 630 nm. Porphyrin absorption spectra vary with the solvent in which the porphyrin is dissolved. Solutions of porphyrins in acid have two peaks of absorbance compared to four peaks for porphyrins dissolved in chloroform.

When excited by specific wavelengths within the Soret band, porphyrins emit a characteristic red fluorescence (Figure 2, 3). In acid solution, they show two strong emission bands, one between 580 and 610 nm and the other between 640 and 680 nm. Absorbance and fluorescence of porphyrin solutions are also altered by changes in metal binding and side chains.

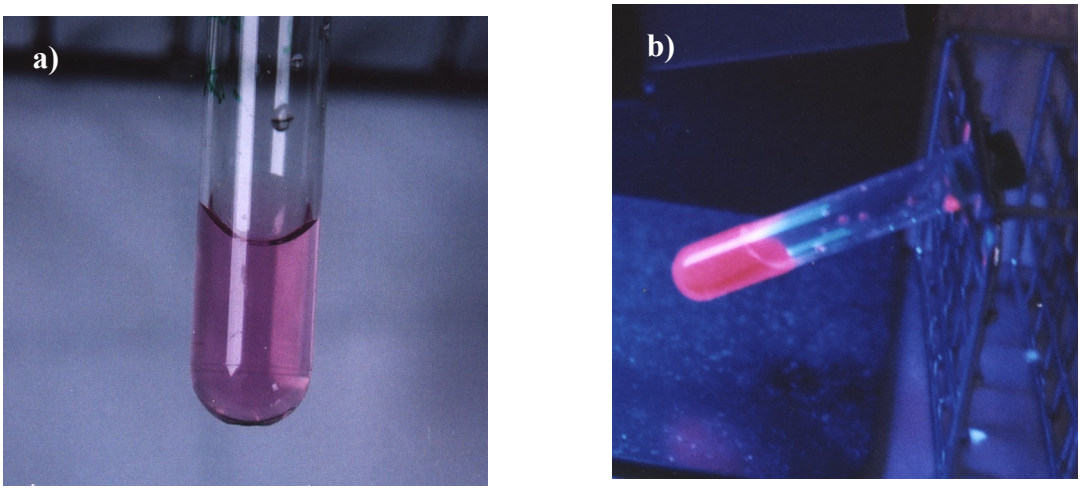


Figure 2: The porphyrins extracted from urine from the patient with congenital erythropoietic porphyria a) in the day light, b) the fluorescence in the “Soret band” light

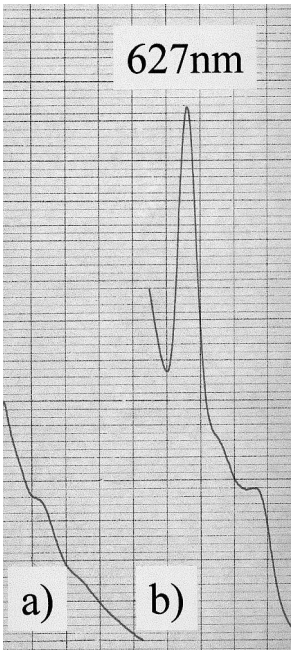


Figure 3: Direct fluorescence of plasma. a) control, b) patient with porphyria variegata who shows typical fluorescence in 627 nm.

## 1.2. HEME BIOSYNTHESIS

Heme synthesis is one of the essentials pathways of life, and occurs in nearly all metabolically active cells. The same pathway is also used by plants, bacteria, and algae to make the other principal pigments of life, chlorophyll and vitamin $B_{12}$. For anaerobic organisms or facultative organisms growing under anaerobic conditions, an oxygen independent enzyme exists that catalyzes the antepenultimate and penultimate step in heme biosynthesis [5].

Heme biosynthesis is most active in erythropoietic tissue, where hemoglobin synthesis takes place, and in the liver where heme forms the basis of various heme- containing enzymes such as cytochromes P450, catalase, tryptophan pyrrolase and others. These two major sites of heme production differ in heme synthesis regulation[2]. In erythropoietic tissue heme biosynthetic enzymes are regulated to permit a high steady-state level of synthesis and regulation is tied to the availability of iron. On the contrary in the liver heme biosynthesis is turned over rapidly, to be able to quickly answer changes in metabolic requirements.

For the first and rate-limiting enzyme of the pathway, two separate genes coding housekeeping and erythroid isoforms exist. The next three steps have dual promoters, allowing both erythroid-specific and non erythroid-specific regulation (Figure 4). The remaining genes have single promoters but exhibit erythroid and non-erythroid differences [6].

Heme is built from the simple raw materials glycine and succinic acid from which δ-aminolevulinic acid (ALA) is formed. In the next steps, six enzymes catalyze formation and tetramerization of porphobilinogen (PBG), and in the final step, ferrous ion is inserted to form heme. The heme biosynthetic pathway consists of a series of eight functionally interlocking enzymes. The first and the three last enzymatic reactions take place in mitochondria, while the remaining four reactions occur in cytosol.

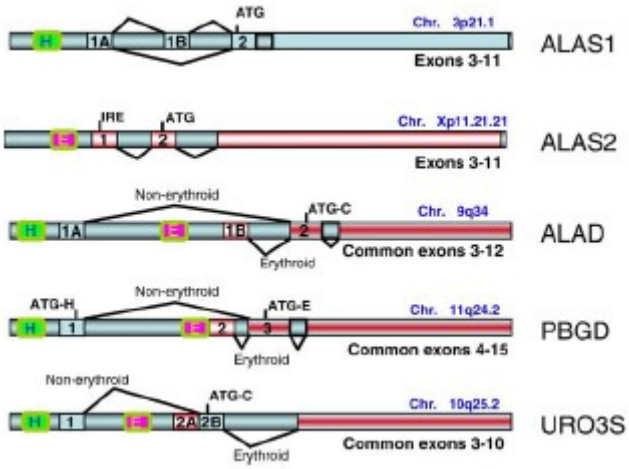


Figure 4: Tissue-specific expression of the "early" genes in the heme biosynthetic pathway. H=Housekeeping promoter. E=Erythroid promoter. Chr.=chromosome location. ATG=translational start site. ATG-C=Common transcriptional start site. ATG-H=Housekeeping transcriptional start site. ATG-E=Erythroid transcriptional start site. The single genes for ALAD, PBGD and URO3S utilize tissue-specific promoters and alternative splicing to generate either identical proteins (ALAD, URO3S) or tissue-specific proteins (PBGD). [2]

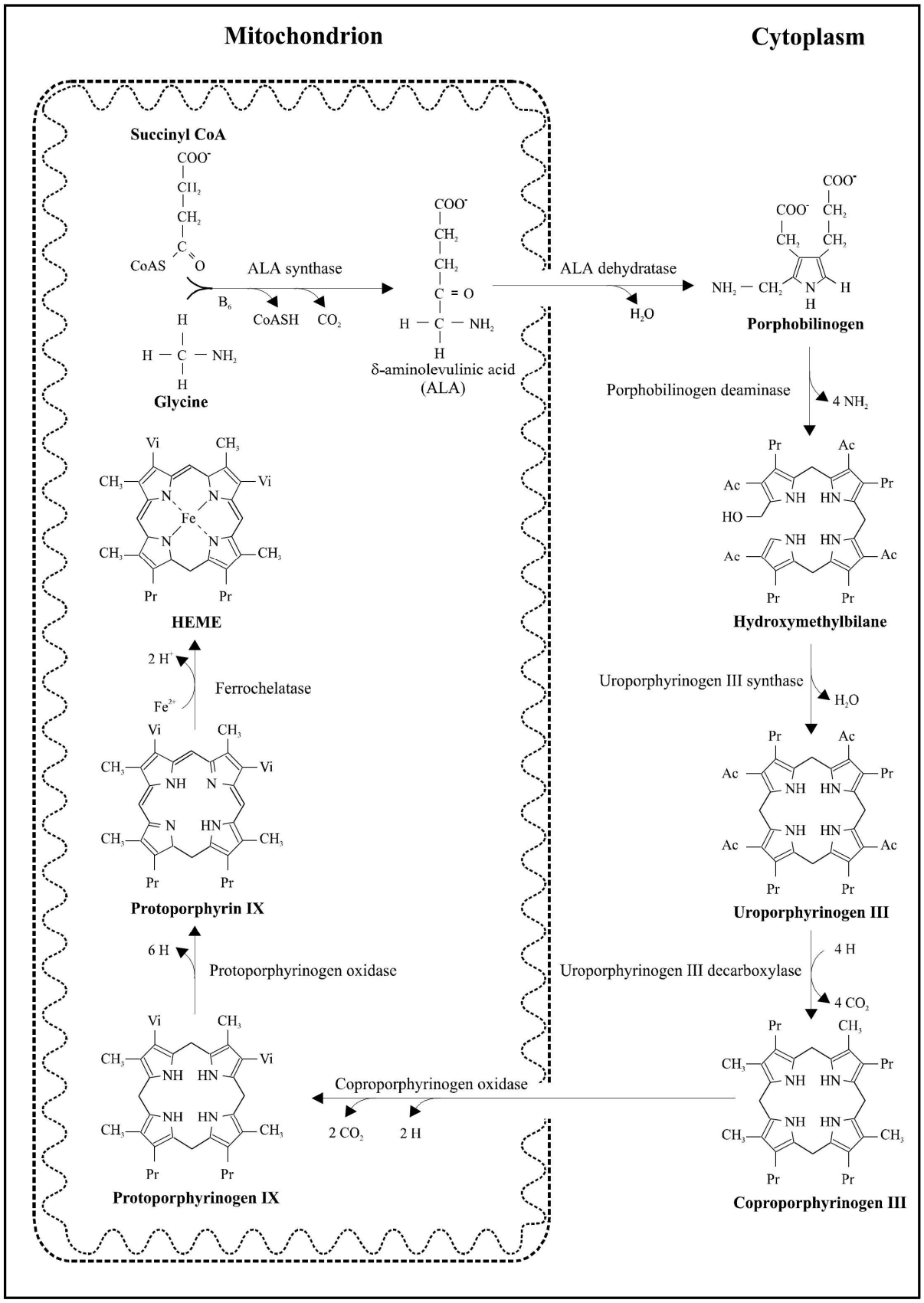


Figure 5: Enzymes and intermediate products in the heme biosynthetic pathway. Ac= - $CH_2COOH$, Pr= -$CH_2CH_2COOH$, Vi= -$CH{=}CH_2$ [7]

## 1.2.1. ENZYMES OF HEME BIOSYNTHESIS

In order to be succinct, only a brief list of each enzyme of heme biosynthesis is described in this chapter (Figure 5). The detailed information about protein structures are provided within the descriptions of appropriate porphyrias, except for δ-aminolevulinic acid synthase, which is summarized in this chapter.

1. In the mitochondrial matrix, **δ-aminolevulinic acid synthase** (EC 2.3.1.37, ALAS) catalyzes the first step of heme synthesis, a condensation reaction between glycine and succinyl coenzyme A resulting in ALA. It is the rate limiting enzyme of whole biosynthetic pathway [2,6]. It exists in two tissue specific isozymes encoded by separate genes, one of which (ALAS1) is expressed ubiquitously, whereas the expression of the other (ALAS2) is specific to erythroid cells. ALAS1 acts like a housekeeping protein in all cells that need heme as a prosthetic group while ALAS2 is synthesized exclusively to cover the needs for hemoglobin biosynthesis. The gene encoding ALAS1 lies on chromosome 3 and ALAS2 is encoded on the chromosome X [8,9]. Both isoforms require pyridoxal 5-phosphate as a cofactor and both are homodimers [10].

In the liver ALAS1 is under negative feedback control by heme [11]. This mechanism is applied in the treatment of acute porphyric attack by administration of exogenous heme arginate. Contrary to the necessity for heme detoxication, certain drugs actually stimulate the activity of ALAS and thus can provoke an acute porphyric attack. The acquisition of Fe from ferritin is suggested to be the rate limiting condition in erythroid cells [11].

ALAS is the only enzyme in heme biosynthesis not associated with a specific porphyria. Mutations in ALAS2 cause X-linked sideroblastic anemia, characterized by a microcytic hypochromic anemia with ringed sideroblasts, hyperferremia and potential death from hemochromatosis.

2. Two molecules of ALA are condensed to form PBG, a monopyrrole, by the cytosolic enzyme **δ-aminolevulinic acid dehydratase** (EC 4.2.1.24; ALAD; porphobilinogen synthase). The mechanism by which ALA exits mitochondria and enters cytosol is unknown.

3. **Porphobilinogen deaminase** (EC 4.3.1.8, PBGD, hydroxymethylbilane synthase, uroporphyrinogen I synthase) catalyzes the head to tail oligomerization of four PBG molecules to form hydroxymetylbilane (HMB). PBGD first binds HMB and then deaminates and polymerizes two additional molecules of PBG to form hexapyrrole. The final step is cleavage of the distal tetrapyrrole and the release of HMB. The proximal dipyrrole remains covalently bound to the enzyme and is not turned over [12].

The secondary control site of heme synthesis lies at the level of PBGD. PBGD has low endogenous activity and is even more inhibited by protoporphyrinogen (PROTOgen) and coproporhyrinogen (COPROgen) [13].

4. Cyclization of HMB is catalyzed by **uroporphyrinogen III synthase** (EC 4.2.1.75, UROS). The goal of this enzymatic process is to convert a regularly substituted linear tetramer into an asymmetrically substituted cyclic tetramer uroporphyrinogen (UROgen III). This is achieved by intramolecular rearrangement of the terminal ring in HMB (Figure 6).

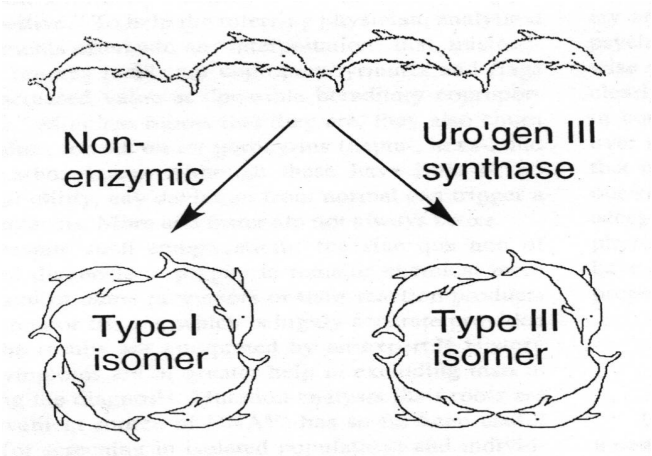


Figure 6: The scheme of synthesis of uroporphyrinogen III [14]

5. Conversion of UROgen III to COPROgen III by **uroporphyrinogen decarboxylase** (EC 4.1.1.37; UROD) involves removal of all four acetate carboxyl groups. This single enzyme can catalyse four successive decarboxylation reactions producing 7-, 6-, 5-, and 4- carboxylated

porphyrinogens. Isomer I or isomer III of UROgen may serve as a substrate but only COPROgen III is a substrate for the next enzyme in the pathway, coproporphyrinogen oxidase. The removal occurs in an orderly manner with the carboxylgroups being removed clockwise starting at ring D

6. **Coproporphyrinogen oxidase** (EC 1.3.3.3, CPO) catalyzes the oxidative decarboxylation of COPROgen III to PROTOgen IX. The enzymatic conversion consists of oxidative decarboxylation of the propionate groups of pyrrole rings A and B to yield vinyl groups. The reaction proceeds via tripropionate monovinyl porphyrinogen – harderoporphyrinogen (2-vinyl, 4 – propionate porphyrinogen) in a stepwise fashion. The decarboxylation of the propionate side chain in the 2-position proceeds first and at a faster rate compared with the subsequent decarboxylation at the 4-position. Under normal conditions, free harderoporphyrinogen is not released from the enzyme.

For anaerobic organisms, an oxygen independent enzyme exists that decarboxylates COPROgen to PROTOgen [5]. There is no sequence similarity between the two types of CPO enzymes. The oxygen-independent CPO (Hem N) is found in a variety of anaerobic bacteria. This enzyme product of the hem N gene has been reported to require $Mg^{2+}$, ATP, $NAD(P)^{+}$ and methionine for its activity. In facultative organisms there exist genes for both oxygen-dependent (Hem F) and independent forms of CPO.

7. **Protoporphyrinogen oxidase** (EC 1.3.3.4, PPOX) catalyses the conversion of PROTOgen to PROTO via six electron oxidation. Three molecules of dioxygen are consumed and three molecules of $H_2O_2$ are produced. Here, the synthesis switches from colorless, flexible, nonplanar, and unconjugated intermediates to a colored, conjugated, and relatively rigid planar structure.

As with CPO, two forms of PPOX exist: one oxygen dependent and one oxygen independent. In bacteria, they are named hem Y for the oxygen dependent form and hem G for the oxygen independent one [5]. Only a single form of PPOX is found within a cell in the facultative organisms, namely hemG, the oxygen independent form. It appears that hem G functions by interacting with a cell's respiratory chain to eliminate the reduction equivalents acquired by PROTOgen oxidation.

8. **Ferrochelatase** (EC 4.99.1.1, FECH) catalyses the final step in heme biosynthesis, the insertion of ferrous iron into PROTO to form protoheme IX (heme). Transition metals other than iron can serve as metal substrates and zinc protoheme (zinc protoporphyrin) is formed when the iron supply is deficient. For the porphyrin substrate, a variety of IX isomers with substituents at the 2,4 position on ring A and B that are uncharged and the size of hydroxyethyl group or smaller can be utilized. On the contrary the position of the propionate groups on the C and D ring is critical for proper orientation within the active site for enzyme catalysis.

## 1.3. PORPHYRIAS

Porphyrias are heterogenous disorders caused by inherited or acquired deficiency of the enzymes in the heme biosynthetic cascade. With the exception of ALAS the deficiency of each of the enzyme of heme synthesis can be assigned to a certain type of porphyria[15]. Each one is characterized by a typical spectrum of accumulated and excreted porphyrins and porphyrin precursors, PBG and ALA. Characteristic spectrophotometric and fluorometric properties of porphyrins allow the diagnosis of porphyrias by their analyses in urine, plasma and feces. Genes encoding all 8 enzymes of the heme biosynthetic pathway were cloned and the molecular background of all inherited porphyrias was established. There is a profound genetic heterogeneity at the molecular level in porphyrias. Most of them are inherited in autosomal dominant fashion with low penetrance. The knowledge of molecular bases of the disease permits more accurate diagnoses.

### 1.3.1. CLASSIFICATION

Porphyrias can be classified and subdivided in several ways, and a variety of names have been used to describe each particular disorder (Table 1).

Table 1: The nomenclature of porphyrias

| Disease / abbreviation | MIM | Synonyms |
|---|---|---|
| Delta-aminolevulinate dehydratase deficienty - ADP | #125270 | Porphobilinogen synthase deficiency |
| | | Doss porphyria |
| Acute intermittent porphyria AIP | #176000 | Porphyria, Swedish type |
| | | Porphobilinogen deaminase deficiency |
| | | Hydroxymethylbilane synthase deficiency |
| Congenital erythropoietic porphyria CEP | #263700 | Gunther disease |
| | | Uroporphyrinogen III synthase deficiency |
| Porphyria cutanea tarda PCT | #176100 | Uroporphyrinogen decarboxylase deficiency |
| | | Hepatoerythropoietic porphyria HEP |
| Hereditary coproporphyria HC | #121300 | Coproporphyrinogen oxidase deficiency |
| | | Coproporphyria |
| Porphyria variegata PV | #176200 | Variegate porphyria |
| | | Porphyria, South African type |
| | | Protoporphyrinogen oxidase deficiency |
| Erythropoietic protoporphyria EPP | #177000 | Erythrohepatic protoporphyria |
| | | Protoporphyria |
| | | Ferrochelatase deficiency |
| | | Heme synthetase deficiency |

Based on the site of major production of porphyrins and their precursors they can be classified as HEPATIC (PCT, AIP, HC, VP, ADP) or ERYTHROPOIETIC (EPP, CEP). For abbreviations see table 1. Another practical classification is due to pathogenesis of the disease as either ACUTE (AIP, HC, PV, ADP) or

CHRONIC (PCT, EPP, CEP) based on the ability of certain drugs to precipitate so-called acute porphyric attack (Table 2, 3). Chronic porphyrias are porphyrin accumulation diseases where enzymatic defect lead to massive accumulation of the preceding porphyrins in various tissues. Conversely, acute hepatic porphyrias are considered to be molecular regulatory diseases of the heme biosynthetic pathway characterized in general by induction of ALAS in the liver by various stimuli such as drugs, sex hormones, fasting, alcohol etc [16].

It is extremely rare to observe a patient with mutation(s) in more than one enzyme, resulting in two types of porphyria in one patient, such as Chester porphyria (AIP and VP) and dual porphyria (VP and PCT). The rare cases of homozygous forms of primarily autosomally dominantly inherited porphyrias with severe clinical manifestations were also described [17].

Table 2: Classification of porphyrias

| **Type** | | **Disease** | **Symptoms** |
|---|---|---|---|
| Hepatic porphyrias | Acute | ADP | neurologic |
| | | AIP | neurologic |
| | | HC | neurologic + cutaneous |
| | | PV | neurologic + cutaneous |
| | Chronic | PCT | cutaneous |
| Erythropoietic | | CEP | cutaneous + hematologic |
| | | EPP | cutaneous |

Table 3: Molecular basis of porphyrias

| **Disease** | **Gene** | **Inheritance** | **Chromosomal location** | **Gene size (kb)** | **Number of exones** |
|---|---|---|---|---|---|
| ADP | ALAD | AR | 9q34 | 13 | 13 |
| AIP | PBGD | AD | 11q24.1-2 | 10 | 15 |
| CEP | UROS | AR | 10q25.2-26.3 | 34 | 10 |
| PCT/HEP | UROD | AD/AR | 1p34 | 3 | 10 |
| HC | CPO | AD | 3q12 | 14 | 7 |
| PV | PPOX | AD | 1q21-23 | 5 | 13 |
| EPP | FECH | AD | 18q21.3 | 45 | 11 |

## 1.3.2. ACUTE HEPATIC PORPHYRIAS

Acute hepatic porphyrias are believed to be molecular regulatory diseases in contrast to nonacute porphyrias. Porphyrins do not accumulate in acute porphyrias whereas in chronic porphyrias they do. The disease process depends on heme pathway dysregulation, namely by derepression of hepatic ALAS following heme depletion [16].

Induction factors cause decrease of regulatory liver heme which is followed by maximized PBG synthesis to provide sufficient hepatic heme biosynthesis. An induction of ALAS causes overproduction of ALA and PBG, but their metabolic conversion into HMB is limited due to decreased activity of PBGD either by genetic defect in AIP or secondarily by high levels of PROTOgen and COPROgen in PV and HC [13]. This limiting function of hepatic PBGD explains increased levels of porphyrin precursors in all three types of acute hepatic porphyrias.

The precipitating factors can be of either endogenous or exogenous origin. They are numerous examples, such as certain drugs and chemicals, especially alcohol having the potential to induce cytochrome P450, endocrine factors including cyclical premenstrual exacerbation, low caloric intake and stress resulting from different origins as emotion surgery illness or social problems [3].

### 1.3.2.1. CLINICAL MANIFESTATION OF ACUTE HEPATIC PORPHYRIAS

Typically, porphyrias do not manifest before puberty. The penetrance of the acute hepatic porphyrias is low as 10-20 % and the affected are mostly women. The clinical features may be divided into two main categories: acute porphyric attack and skin disease.

An acute porhyric attack can present with heterogenous manifestations [18] (Table 4). In almost all attacks of acute porphyria, severe abdominal pain without signs of peritonism is seen. Frequently, nausea, vomiting, constipation, and signs of partial ileus are noted. Other symptoms are hypertension, tachycardia, excessive sweating, electrolyte dysregulation (hyponatremia), dysuria and bladder dysfunction, as well as pain in other areas. Peripheral neuropathy is predominantly motor and may be manifest as weakness and paresis particularly in distal muscles. Cranial nerves can be affected as well. The neuropathy may

progress rapidly, resulting in bulbar paralysis, respiratory failure and death. Sensory changes with parestesia, dysestesia and loss of sensation may accompany the motor neuropathy. Psychiatric symptoms include anxiety, insomnia, confusion, agitation, mania, depression, hallucinations and schizophrenic-like behaviour. This wide range of often nonspecific clinical signs mimics other diseases and may be easily misdiagnosed. If an acute porphyria is overlooked and not appropriately diagnosed, consequences for the patient can be fatal. In these cases, the symptoms may be treated with analgesics, which prolong or even exacerbate the attack. Even worse, the acute abdominal pain may be confused with acute abdomen resulting with surgical intervention requiring general anesthesia, further graduating the attack [15].

Table 4: Clinical features in the acute hepatic porphyrias [18]

| **Symptom** | **Percentage** |
|---|---|
| Abdominal pain | 95 |
| Vomiting | 90 |
| Constipation | 85 |
| Asthenia | 89 |
| Myalgia | 72 |
| Tachycardia | 70 |
| Hypertension | 36 |
| Mental symptoms | 25 |
| Convulsions | 12 |
| Paresis | 10 |
| Paralysis | 2 |
| Transitory blindness | 1 |

Skin disease can be present in PV and HC but not in AIP (Figure 7). It manifests as classic photodermatosis with affected sun – exposed areas, particularly the dorsa of the hands and forearms, face, neck and dorsa of the feet [19]. The dominant symptoms are skin fragility, erosions, milia, blisters, and abnormal pigmentation leading into thickening, grooving, hirsutes and premature

aging. In light microscopic accumulated porphyrins in the upper layers of the dermis can be found.

Acute hepatic porphyrias are risk factors for hepatocellular carcinoma [20]. Hepatic porphyrias should be examined in patients with hepatocellular cancer without obvious etiology, and a periodic screening for hepatocellular carcinoma should be evaluated in these patients.

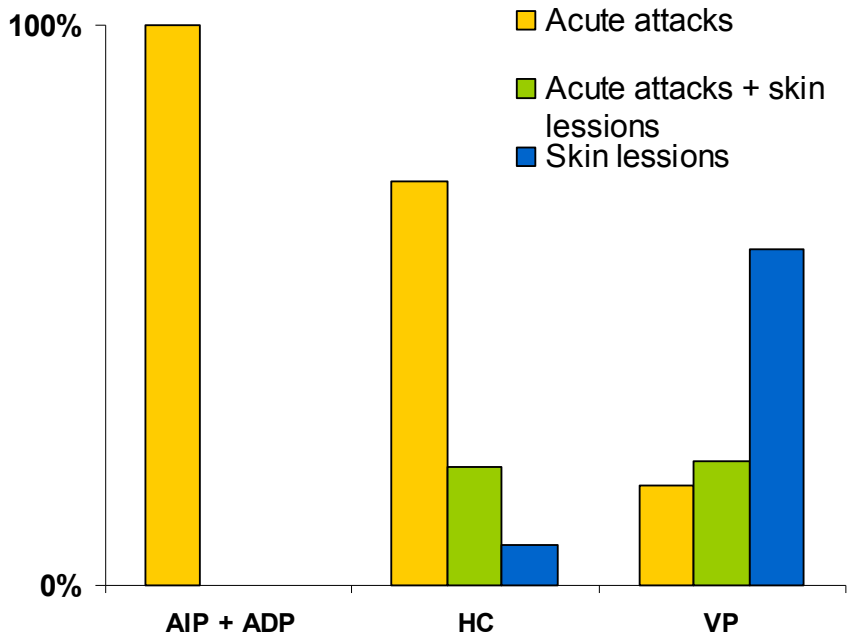


Figure 7: Frequency of symptoms in acute hepatic porphyrias [21]

## 1.3.2.2. PATHOGENESIS OF ACUTE PORPHYRIC ATTACK

The clinical symptoms of an acute attack have been ascribed as dysfunction of the central, peripheral and autonomic nervous system. The pathogenetic mechanism, by which altered heme synthesis results in nervous system dysfunction, remains poorly understood.

Numerous hypotheses have been proposed [22] (Table 5). The present evidence suggests that multiple mechanisms interact in causing the varied symptoms, including ALA interaction with gamma-aminobutyric acid (GABA)

receptors, altered hemoprotein functions, especially tryptophan metabolism, and possibly heme depletion in nerve cells.

ALA is an ω- amino acid with a 5 carbon chain structure similar to the inhibitory neurotransmiter GABA and the excitatory L-glutamic acid. In vitro ALA can act as a partial GABA agonist [23]. In rats the effect of ALA on melatonin production in the pineal gland was demonstrated [24]. The ability of ALA to generate oxygen free radicals in the presence of heavy metal is suspected to make transient mitochondrial damage which can cause some of the symptoms [25].

The second major hypothesis is that deficiency of heme could lead to decreased levels of hemoproteins resulting in direct or indirect effect in the nervous system. Among them, hemoproteins, tryptophan dioxygenase and hepatic cytochromes P450 are altered in acute porhyrias. As a consequence of decreased activity of tryptophan dioxygenae, the levels of tryptophan are increased and result in increased levels of serotonin [26]. Many of the clinical features of the acute hepatic porphyria resemble the effects of incresed serotonergic activity. Other hemoproteins suspected for response to neurologic symptoms are cytochromes P450 which may affect the metabolism of neuroactive compounds, hemoproteins involved in mitochondrial electron transport leading in deficiency in energy production or nitric oxide synthase with consecutive malfunction in production of nitric oxide [22].

Table 5 Hypotheses of the pathogenesis of nervous system dysfunction in the hepatic porphyrias, adapted from [22]

| | |
|---|---|
| 1. | ALA, PBG, or porphyrins overproduced and accumulating in liver or nervous system are neurotoxic |
| 2. | Relative heme deficiency in the liver and/or nervous system leads to decreased hemeprotein function in neural tissues |
| 3. | Abnormal products derived from ALA or PBG are neurotoxic (e.g. free radicals, hydroxyhemopyrroline 2-one, porphobilin) |
| 4. | Depletion of essential substrates or cofactors resulting from the disturbance of heme synthesis cause the symptoms (e.g. depletion of pyridoxal phosphate, zinc or glycin) |

### 1.3.2.3. DIAGNOSIS OF PORPHYRIC SYNDROMES

For the diagnosis and differential diagnosis of acute porphyria, investigation of excretion of porphyrin and porphyrin precursors ALA and PBG are employed using specimens of urine, feces and plasma (Table 6). Acute porphyria attacks are characterized by excessive urinary excretion of ALA and PBG. The specimen may also contain the type I isomeric form of uroporphyrin (URO) and coproporphyrin (COPRO) as a result of spontaneous condensation of PBG by a nonenzymatic reaction favored by light. High levels of precursors are an important diagnostic tool as urinary porphyrin can be increased in several other conditions such as hepatobiliary disease, alcohol abuse and infection, and therefore lacks the specifity. An increase in the intracellular concentration of the substrate of each defective enzyme gives rise to a characteristic pattern of tissue accumulation and excretion of heme precursors. These patterns are useful to differentiate the types of acute porphyria [27,28].

Enzymatic assays and DNA-based testing are accessible and play an important role in definitive accurate diagnosis as well as in diagnosis of asymptomatic carriers.

Table 6: Biochemical features in acute hepatic porphyrias, 1 – acute attack, 2 - remission [21]

| | | Urine | | | Feces | | | Plasma |
|---|---|---|---|---|---|---|---|---|
| | | precursors | URO | COPRO | URO | COPRO | PROTO | fluorescence |
| AIP | 1 | +++ | ++ | ++ | ++ | + | - | 619 nm |
| | 2 | ± | - | + | - | - | - | |
| HC | 1 | +++ | ++ | +++ | ++ | ++++ | + | 619 nm |
| | 2 | ± | - | + | - | +++ | - | |
| PV | 1 | +++ | ++ | +++ | + | ++ | +++ | 627 nm |
| | 2 | ± | - | + | - | + | ++ | |

### 1.3.2.4. THE THERAPY OF PORPHYRIC SYNDROMES

The therapy of an acute attack is identical for all acute hepatic porphyrias [15,16,18] (Table 7). First of all, precipitating factors including underlying infection, hypocaloric diet and particularly drugs should be removed. Complete lists of potentially safe and unsafe drugs are available on the internet e.g. http://www.porphyria-europe.com/. Specific therapy of an attack includes administration of high- dose of carbohydrates and heme arginate. The "glucose effect" frequently leads to reduction of excretion of urinary porphyrin precursors by a mechanism which is not certain. An adequate supplement 300-400 g of glucose/day should be administered usually by slow intravenous infusion with careful management of intravenous fluid to minimize the danger to precipitate hyponatremia. The treatment of a porphyric attack has been greatly improved by the induction of hematin which down-regulates the heme synthesis. Currently, heme stabilization by L-arginine is used [29,30]. Heme arginate (Normosang®, Orphan Europe) is given intravenously in doses 3 mg/kg body weight/24 hours, usually over 4 days (Table 8).

Women with cyclical perimenstrual acute attacks have benefited from administration of low dose contraceptive pills or agonists of luteinizing hormone-releasing hormone [31,32]. In the symptomatic therapy, opiates, beta-blockers, diazepam and phenothiazines e.g. chlorpromazine take place.

Table 7: Therapy of acute clinical porphyria crisis [16]

| |
|---|
| 1. Prerequisite |
| Confirmation of diagnosis by excess urinary ALA an PBG |
| Elimination of all precipitationg factors |
| 2. Regulatory treatment |
| High-dose glucose (400g per day p.o. or i.v.) |
| Hem arginate (3 mg/kg body weight per day i.v. for 4 days) |
| 3. Symptomatic treatment |
| Pain: opiates |
| Hypertension, tachycardia: beta-blockers (e.g. propranolol) |
| Nausea, vomiting: chlorpromazine or other phenothiazines |
| Psychosis: chlorpromazine or other phenothiazines |
| Seizures: diazepam, check for hyponatremia |
| Hyponatremia: saline infusions, fluid restriction if signs of inappropriate secretion of antidiuretic hormone |
| Motor neuropathy: physiotherapy |

Table 8: Albumin protocol for *Normosang*® administration

| |
|---|
| 10 ml *Normosang*® diluted in 100ml of albumin 4% |
| Slow 30 minute perfusion |
| Flush through 4 x 10ml physiological saline using a syringe |
| Use remaining solution in normal perfusion (85ml) |

### 1.3.2.5 ACUTE INTERMITTENT PORPHYRIA

Acute intermittent porphyria (AIP) is the most frequent acute hepatic porphyria. It results from the half- normal activity of **porphobilinogen deaminase** (PBGD), the third enzyme along the heme synthetic pathway. PBGD catalyzes the head-to-tail condensation of four molecules of PBG to form the linear tetrapyrrole,

HMB. PBGD gene is localized to the chromosomal region 11q24.1-q24.2 [33]. It spans about 10 kb and contains 15 exons and two distinct promoters [34,35]. The PBGD gene encodes two isoenzymes: erythroid specific and ubiquitous [36,37]. Both are encoded by one gene by different promoters that generate 2 different transcripts by alternative splicing of exon 1 and 2. The isoforms differ only at their NH2 end where ubiquitous isoform extends for an additional 17 amino acid residues making a polypeptide of 361 amino acids. Eleven of these additional amino acids are encoded by exon 1 which lies 3kb upstream from the erythroid-specific promoter and six are encoded by a short section of exon 3 that immediately precedes the methion codon that initiates translation of the erythroid isoform and encodes the 18th residue of the ubiquitous isoform.

The *E.coli* enzyme has been crystallized and its three dimensional structure has been determined by X-ray diffraction [38]. The structure of human PBGD was predicted from the *E.coli* model. The enzyme chain is folded into three domains of approximately equal size [39]. The dipyrromethane cofactor is covalently linked to domain 3 but it is bound by extensive salt-bridges and hydrogen-bonds within the cleft between domains 1 and 2 [40]. The active site is located between domain 1 and 2. Secondary structure of domains 1 and 2 is modified doubly wound parallel beta sheet which has a similar overall topology. Domain 3 is an open-faced three-stranded antiparallel beta-sheet, with one face covered by three alpha-helices [39]. To date around 300 mutations in PBGD have been identified among AIP patients from various countries. Most of human mutations have been described on exons 10 and 12 (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php). In addition, a total of 16 intragenic single nucleotide polymorphisms (SNP) have been identified [41].

The prevalence of AIP is 1-10/100 000 with exception of northern parts of Sweden with prevalence 100:100 000 [42]. It is transmitted as an autosomal dominant disorder with low penetrance. Only 10-20% of affected individuals develop clinical symptoms.

The disease is manifested by acute porphyric attack with abdominal pain and neurological dysfunction, indistinguishable from that of other hepatic porphyrias. In contrast to PV and HC, skin photosensitivity is never present [20].

Laboratory findings include large amounts of porphyrin precursors during acute attacks and moderately increased URO and COPRO. Asymptomatic carriers may have increased excretion of porphyrins and porphyrin precursors in urine but negative tests do not exclude the asymptomatic individuals. Enzyme activity measurements in erythrocytes can be used to confirm the diagnosis as well as for detection of clinically latent individuals who often do not show evidence of heme precursor overproduction. However, there is an overlap of the two groups that makes interpretation difficult at the lower end of the reference range [43]. To further complicate the situation, a group of patients with AIP have normal PBGD activity in erythrocytes, although the activity of the hepatic enzyme is decreased due to an isolated defect of the ubiquitous isoform [44,45] The molecular analysis of the causal mutation is unambiguous and should be undertaken within the family. Several methods were optimized for screening of mutations in the PBDG gene including denaturing gradient gel electrophoresis (DGGE), heteroduplex analyses and single-strand conformation polymorphisms analyses [46-48]. The therapy for all types of acute hepatic porphyrias is described above.

### 1.3.2.6. PORPHYRIA VARIEGATA

Porphyria variegata (PV) is the second most frequent acute hepatic porphyria. It is caused by a deficient activity of **protoporphyrinogen oxidase** (PPOX), the penultimate enzyme in heme biosynthesis. PPOX catalyzes the six electron oxidation of PROTOgen to the planar fully conjugated macrocycle PROTO. The gene coding PPOX has been mapped to chromosome 1q22-23, contains 13 exons and spans about 5,5 kb [49-51]. The cDNA encodes a protein of 477 amino acids [52]. PPOX is active as a dimer with a subunit molecular mass of approx. 50 kDA. It contains FAD which is associated in part with highly conserved N-terminal dinucleotide motif [53,54]. PPOX belongs to a superfamily of FAD-containing oxidases that also include monoamine oxidases and others. In eukaryotes, the enzyme is anchored to the outer surface of the inner mitochondrial membrane [55] with its active side facing the cytosolic side of the membrane. In plants the enzyme is also found in chloroplasts. Plant's PPOX are strongly inhibited by diphenyl ether herbicides [56]. Currently, almost 150 different mutations in the PPOX gene have been described (Human Gene Mutation Database,

http://www.hgmd.cf.ac.uk/ac/index.php). Furthermore 15 SNPs in the PPOX gene are known, most of them are localized in introns or in the untranslated first exon. In addition, two amino acid substitutions were identified in the general population without clinical correlate: in exon seven P256R [57] and in exon nine R304H [58]. The frequency of named allelic variants, established in French Caucasian population, was 0,95:0,05 for 256 P/R and 0,61:0,39 for 304R/H [57].

The world prevalence of PV is much lower than of AIP, with the exception of in South Africa, where calculations based on population growth theories predict up to 20 000 carries of the gene. Genealogic studies suggest that PV families in South Africa were descended from a Dutch couple who married at Cape of Good Hope in 1688 [59]. Unlike AIP, the disease is characterized by acute neurovisceral crises usually associated with the presence of a vesiculoerosive photodermatitis. Skin photosensitivity may be the only manifestation of the disease. The term porphyria variegata was coined to reflect the varied presentation of these conditions [19]. Laboratory finding in acute attack includes increased porphyrin precursors in urine, and porphyrins in urine and in stool – predominantly the protoporphyrin. A plasma fluorometric emission at 626 nm (excitation wavelength is set at 405 nm) is typical for PV [60]. However asymptomatic carriers and patients during remission do not necessarily test positive. The activity measurement is provided by a fluorometric assay in lymphocytes since PPOX is not present in erythrocytes. The low level of PPOX activity in lymphocytes coupled with the difficulties of the assay usually requires specilized laboratories [61]. The molecular confirmation of the diagnosis follows.

### 1.3.2.7. HEREDITARY COPROPORPHYRIA

Hereditary coproporphyria (HC) represents another acute hepatic porphyria resulting from diminished activity of **coproporphyrinogene oxidase** (CPO), an antepenultimate step in heme biosynthesis [62]. CPO catalyzes the conversion of two propionate side chains into two vinyl groups; the stepwise oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, via a tricarboxylic intermediate known as “harderoporphyrinogen”. The gene is

localized to chromosome 3q12, spans about 14kb, and consists of seven exons and six introns [63,64]. The cDNA encodes a protein of 354 amino acids [65,66]. The eukaryotic CPO is a nearly globular homodimer with a subunit molecular weight of approx. 39kDA [67]. No cofactor nor metal has been identified in CPO [68]. The enzyme from mammalian cells is localized to the cytoplasmic side of the inner mitochondrial membrane [69] while in *Sacharomyces cerevisiae* its location is cytosolic [70]. It is initially synthesized in the cytosol as a preprotein with an unusually long (110 amino acids) N-terminal targeting sequence required for its import into the mitochondria, which is subsequently proteolytically processed. The human genome contains a single CPO gene with multiple transcriptional initiation sites. It appears that a single promoter may be differentially regulated in erythroid and nonerythroid cells. Over 44 mutations in the CPO gene have been identified (Human Gene Mutation Database). So far, mutations are family specific without any hotspot or phenotype/genotype correlations [71].

Of all the autosomal dominant acute hepatic porphyries, HC is the least common [72]. As in other acute hepatic porphyries, HC patients present with an acute neurovisceral crisis. Skin photosensitivity occurs in a minority of cases. The biochemical picture of acute attacks of HC is dominated by a dramatic increase in fecal coproporphyrin III (10-200 times compared with controls). Typical intensive red fluorescence of feces under UV light is a specific and easily performed diagnostic test for acute attacks of HC [73]. The confirmation of the diagnosis is performed by enzymatic assay in lymphocytes and by molecular investigation of the CPO gene [74,75].

### 1.3.2.8. δ-AMINOLEVULINATE DEHYDRATASE DEFICIENCY PORPHYRIA

δ-Aminolevulinate dehydratase (ALAD) deficiency porphyria is caused by deficiency of **δ-aminolevulinate dehydratase** (ADP), the second enzyme in heme biosynthesis. It catalyzes the formation of PBG, the monopyrrole from two molecules of ALA, which is the obligate precursor for porphyrins, heme and other tetrapyrroles [76]. The gene encoding ALAD is localized at chromosome 9q34 [77]. The gene contains two alternative noncoding exons 1A and 1B and 11 coding exons 2 to 12 [78]. The housekeeping transcript includes exon 1A while the

erythroid specific transcript contains exon 1B. The human ALAD gene contains two promoter regions that generate housekeeping and erythroid-specificity by alternative splicing, analogous to the expression of the human PBGD. Currently, 12 different mutations in the ALAD gene have been described.[79-81]. The human enzyme is a homo-octamer with a subunit size of 36kDa. The enzyme requires an intact sulhydryl group and one zinc atom ($Zn^{2+}$) per subunit. This soluble enzyme is localized in cytosol.

In mammalian cells, this enzyme is present in vast abundance, compared with other enzymes in the heme biosynthetic pathway, especially ALAS, the rate limiting enzyme in heme biosynthesis. Thus a partial deficiency of this enzyme does not usually result in any clinical consequence.

Lead and succinylaceton are the best known ALAD inhibitors. Lead inhibits ALAD activity by displacing $Zn^{2+}$ from the enzyme. Patients with lead poisoning often develop neurological disturbances resembling those of ALAD porphyria [82]. Succinylaceton is a structural analogue of ALA[83]. It is the most potent inhibitor of the enzyme. It is found in large quantities in patients with tyrosinemia, thus approximately 40% children with tyrosinemia exhibit symptoms of ALAD porphyria.

ADP is the only acute hepatic porphyria inherited in autosomal recessive fashion. Homozygous ALAD deficiency results in almost complete lack of ALAD activity. In addition to deficiency of ALAD, it is believed that the overexpression of ALAS due to a loss of heme–mediated repression also plays a role in acute porphyria hepatic syndrome [84]. ADP is the rarest of the porphyrias. The first two cases were reported in 1979 by Doss et al.[85] Only 6 unrelated cases have been reported to date [79,81,85-87]. Interestingly, all described cases were male, unlike other acute hepatic porphyries, where a strong female prevalence is described. Of these patients three were of German origin, one Swedish, one Belgian and one American of Columbian parents. The clinical symptoms of ADP are similar to those in other acute hepatic porphyrias. Four patients suffered from typical acute porphyric attacks with neurovisceral symptomatology with commencement in adolescence. The Belgian patient was an elderly man who developed disease at the age of 63 years together with a myeloproliferative disorder probably polycythemia vera. Since only one mutation was found in this man, the disease

was thought to be due to an expansion of a polycythemic clone which carried the mutant ALAD allele. In the Swedish case, the symptomatology was uniquely present already at birth. At the age of 3, he had recurrent attacks of pain, vomiting, hyponatremia and symptoms of polyneuropathy including respiratory muscles. As attemps to treat the porphyria syndrome of the patients by glucose, hematin or blood were unsuccessful, at the age of 6 he underwent liver transplantation. The postoperative period was complicated by severe paralysis with respiratory failure requiring respiratory support, but his condition gradually improved. Later he needed intermittently assisted ventilation and he died at the age of 9 years due to pneumonia. In addition, an asymptomatic healthy heterozygous Swedish girl was serendipitously found [88].

In all described patients, a larger amount of ALA was excreted in urine, but unlike other acute hepatic porphyries, PBG was either within the normal range or only slightly elevated. Porphyrins in urine and plasma were also elevated, particularly COPRO. Fecal porphyrin excretion was within the normal range. The activity of ALAD in erythrocytes, as well as in lymphocytes, was markedly decreased to less then 2% and about one-half in heterozygous parents and relatives [84].

### 1.3.2.9. HOMOZYGOUS FORMS OF ACUTE HEPATIC PORPHYRIAS

In addition to *"classic"* acute hepatic porphyrias with autosomal dominant inheritance, several homozygous or compound heterozygous cases have been described. They present various clinical features starting in early childhood. It is expected that at least one of mutated allele must retain some residual activity to maintain some heme biosynthesis.

The clinical symptoms are ascribed to porphyrin precursor toxicity rather than heme deficiency [89].

In AIP, five cases, including two siblings, have been described so far [17,90]. The clinical features include mental retardation of different gravity, porencephaly, seizures, cataracta, optic nerve hypoplasia, partial agenesis of cerebellum with subsequent ataxia with intention tremor and dysarthria, hepatosplenomegaly and teeth dyscoloration. No typical acute attack has been described in these children. The children had markedly elevated urinary ALA and PBG and URO. The

molecular analyses showed homozygosity or compound heterozygosity for mutations in exon 6 (L81P) and in exon 10 (R167Q, R167W, R173W)

The first case of a homozygous form of PV was described in a Czech patient by Kordač and colleagues in 1984 [91]. After that, other cases were identified around the world. So far, more than 10 patients with homozygous PV were described and molecularly characterized [92-96]. The homozygous form of the disease is characterized by early childhood onset with photosensitivity, mental retardation, convulsions, nystagmus, growth retardation, abnormality of the hand particulary clinodactyly, brachydactyly and flexion deformity of the fingers. The syndrome is highly variable, not all patients suffer mental retardation, other neurological symptomatology and skeletal abnormalities are present. Except for one case [93], no acute attack was described in a patient with homozygous PV.

A few patients have been reported who are homoallelic for CPO mutations. In such patients the presence of a specific mutation (K404E) on one or both alleles produce neonatal hemolytical anemia that is known as harderoporphyria. Mutations on both alleles elsewhere in the gene give rise to the homozygous variant of HC.

At this time, two cases of homozygous HC have been described. The first case was a girl who at the age of four years presented with growth retardation, hypertrichosis and pigmentation [97]. Later on, she had two acute attacks of porphyria at the age of 10 and 20. This patient was homozygous for the missense mutation R231W in CPO gene resulting in decreased activity and stability of the enzyme [98]. The second recently described case was a 10 year old girl who presented with skin symptoms of easy fragility, scars on hands and feet and local reddening. Another symptom was light brown coloration of teeth with red fluorescence under long-wave ultraviolet light [99]. The enzymatic investigation of CPO activity exhibit the diminution to 2% of normal and molecular analyses determined compound heterozygosity of two point mutations H307R in exon 4 and H327R in exon 5.

Harderoporphyria is a rare erythropoietic variant form of HC. It is characterized by neonatal hemolytic anemia with hyperbilirubinemia sometimes accompanied by skin lesions caused by porphyrin-induced photosensitization [100,101]. After the neonatal period, the course is uneventful with only chronically observed mild residual anemia and mild photosensitivity during childhood and adulthood. The

finding of a massive accumulation of harderoporphyrin in feces is typical for harderoporphyria. All affected individuals described thus far are homozygous or compound heterozygous for the same mutation in the CPO gene (K404E encoded by exon 6) [102,103].

## 1.3.3. CHRONIC HEPATIC PORPHYRIAS

### 1.3.3.1. PORPHYRIA CUTANEA TARDA

Porphyria cutanea tarda (PCT) is a chronic skin disease resulting from decreased activity of UROD[104].

**Uroporphyrinogen decarboxlase**

UROD catalyzes the fifth step in heme biosynthesis, the decarboxylation of the 4 acetate side chain of UROgen to form COPROgen. A single gene encoding UROD is localized on chromosome 1p34 [105,106], spanning 3.5kb with 10 exons [107]. There is no evidence for the existence of tissue specific isoforms. The crystal structure of human UROD has been determined [108]. Human UROD is a homodimer with a monomeric molecular weight about 41kDa [109]. The enzyme does not require cofactors or prosthetic groups, but the mechanism of decarboxylation remains unknown.

**Pathogenesis**

PCT is a heterogenous group of disorders with two distinguishable forms which differ in several ways; the sporadic form (sPCT, type I) accounts for about two thirds of PCT patients, while the familiar form comprises the other third (fPCT, type II) [110]. The main and crucial difference between those two types is the specificity of tissue origin of decreased activity of UROD, which in sPCT is decreased only in the liver, while in fPCT, the enzyme deficiency is ubiquitous. Mutations in the UROD gene cause fPCT with autosomal dominant inheritance with incomplete penetrance. More than 30 mutations in the UROD gene have been reported [111].

The origin of sPCT remains unclear. The liver specific enzyme defect does not appear to be caused by mutation of the UROD gene as the sequence of cDNA for hepatic and extrahepatic UROD and the corresponding promoter region are normal in sPCT.

There are numerous extrinsic factors that precipitate the development of sPCT such as infection, especially hepatitis C, alcoholism, estrogens and drugs [112]. Other discussed factors are the role of iron in disease pathogenesis. The role of hemochromatosis (HFE) in the development of the disease has been studied, revealing mutations in the HFE gene and the transferin receptor gene-1 (TRFC1), which were candidates due to observed abnormal iron metabolism [113,114].

**Clinical manifestation**

PCT is the most common form of porphyria. The prevalence of the disease was estimated to 1 to 5 per 25 000 [112]. Clinical manifestation is identical in both forms and is characterized by skin lesions similar to those seen in VP and HC [21,104]. The most specific features are photosensitivity and skin fragility. On light-exposed areas on the backs of the hands and on the face, a subepidermal blister forms after sun exposure. A minimal trauma is followed by a superficial erosion cover by a crust. The skin lesions heal slowly, leaving scars with hypo and hyperpigmentations. Increased uniform pigmentation of sun-exposed areas is common. Hypertrichosis may develop particularly in the upper part of cheeks and in the ears and arms. Acute photosensitivity is not common. The onset is usually in middle age with the mean between 40-60 years in different countries. The familiar type presents at younger age than the sporadic one but there is a wide overlap between groups. Otherwise, the types of PCT are clinically indistinguishable. The cutaneous symptomatology is clinically and histologically identical to lesions that are seen in PV, HC, porphyria caused by porphyrin-producing hepatic tumors, and in drug induced pseudoporphyria which may be mistaken for PCT. Also in patients receiving chronic dialysis for renal failure, skin changes resembling those in PCT may occur.

The liver in PCT always contains greatly increased concentration of URO and heptacarboxylic porphyrin. Needle biopsy shows red fluorescence in ultraviolet light. In about 15% of patients with PCT, liver cirrhosis develops with greater risk of developing hepatocellular carcinoma than other types of cirrhoses. The risk is

increased by a long symptomatic period (around 10 years) and decreased by effective treatment soon after onset.

Acute porphyric attacks do not occur in PCT. The approach to avoidance of suspected dangerous drugs in acute hepatic differs. Some authors have experiences of precipitating or exacerbating PCT after “porphyrinogenic” [18] drugs while others do not prohibit them [112]. The chloroquine in antimalarial doses has been shown to cause severe hepatotoxic reaction with massive uroporphyrinuria and photosensitivity.

**Diagnosis**

For biochemical diagnosis, it is crucial to know that UROD catalyzes four successive decarboxylation reactions producing 7-, 6-, 5-, and 4- carboxylated porphyrinogens. All of these intermediates have been identified in urine, feces or plasma [115] (Table 9). In urine, patients excrete increased concentrations of URO I and III, heptacarboxylic porphyrin and other acetic-acid substituted porphyrins. Concentration of urinary PBG is always normal. In feces, the main abnormalities are increased concentration of heptacarboxylic porphyrin and isocoproporphyrin. In plasma, porphyrins are increased, with the main component being URO. During clinical remission, porphyrin concentrations in plasma, urine and feces decrease, and eventually normalize. Similar urinary porphyrin profile may sometimes be found in patients with PV, therefore, urine analysis is not sufficient for diagnosis of PCT, so determination of porphyrins in feces or plasma must be performed. To distinguish porphyria variegata and pseudoporphyria the plasma must be analyzed by fluorescence emission [116]. However, patients undergoing long term hemodialysis are better identified by fecal porphyrin analysis since their plasma porphyrin concentration may be increased as well [117].

Enzymatic assay of UROD is performed in erythrocytes and shows about 50% decrease in enzyme activity in fPCT. The molecular diagnostic can be used in fPCT as well.

Table 9: Biochemical features in porphyria cutanea tarda [21]

| | | Urine | | | Feces | | | Plasma |
|---|---|---|---|---|---|---|---|---|
| | | precursors | URO | COPRO | URO | COPRO | PROTO | fluorescence |
| PCT | 1 | - | +++ | + | + | ++ (Isocoproporphyrin) | - | 619 nm |
| | 2 | - | + | - | - | + | - | |

**Therapy**

PCT differs from other porphyrias in the way that an effective treatment is accessible [21]. Either depletion of iron stores or low-dose chloroquine produce prolonged remission in most patients. First of all, avoiding precipitating factors and minimizing the exposure to sunlight until the clinical and biological remission is obtained is advised.

The treatment of choice for PCT is the phlebotomy. Venesections of 300 ml spaced at 10-12 day intervals are continued for 2 months or until the serum iron falls to 60-70% of original value. Clinical and biological remissions are usually obtained within 4-6 months.

When phlebotomy is contraindicated, the low dose chloroquine therapy is the alternative therapy. The principle of the therapy relies on the fact that chloroquine complexes with URO and promote its release from the liver. Oral chloroqiune is taken in a 250 mg weekly dose or 125 mg biweekly dose for adults. Clinical improvement is usually achieved in about 4 months but the therapy should be continued until urinary URO excretion is 100 nmol/l or less. High dose therapy has to be avoided because it causes hepatitis–like syndrome in PCT patients. Chloroquine and venesections may be combined.

## 1.3.3.2. HEPATOERYTHROPOIETIC PORPHYRIA

Hepatoerythropoietic porphyria (HEP) is a rare form of cutaneous porphyria caused by severe deficiency of UROD resulting from its homozygous deficiency[112]. About 30 patients have been reported worldwide [17]. The disease clinically resembles CEP disease with severe photosensitivity starting in early

infancy, usually before six years of age. Onset during adult age was documented only in one case [118]. The porphyrin excretion pattern is similar to those found in PCT patients but PROTO concentration is increased in erythrocytes, mainly as zinc chelate. UROD activity is usually less than 10% of normal, ranging from 3% to 25%, parents of patients with HEP have erythrocyte activities close to 50% [119]. Patients are homozygous or compound heterozygous for mutation of the UROD gene with at least one mutation yielding sufficient residual enzyme activity to maintain heme biosynthesis. The most frequent mutation G281E is associated with early onset of severe skin lesions [120]. The therapy is largely symptomatic.

## 1.3.4. ERYTHROPOIETIC PORPHYRIAS

### 1.3.4.1. ERYTHROPOIETIC PROTOPORPHYRIA

Erythropoietic protoporphyria (EPP) results from partially reduced activity of FECH, the terminal enzyme of the heme biosynthetic pathway.

**Ferrochelatase**

FECH catalyzes chelation of iron into the open ring of the PROTO to form heme [121]. The FECH gene is assigned to chromosome 18q21.3, contains 11 exons and spans about 45kb [122,123]. The same FECH gene encodes two mRNAs, the housekeeping form and the erythroid specific form differing in their 3´end [124]. The mRNAs encode a FECH precursor of 423 amino acids, which is processed into a final protein of 369 residues[125]. More than 85 different mutations have been identified to date, and are spread over the entire gene with no particular mutation “hot spot”. The types of mutations vary, and range from point mutations to complete FECH gene deletions[126,127]. EPP has long been considered as an autosomal dominant disorder with incomplete penetrance. In most patients, a single mutation was identified on one FECH gene allele. However results of enzymatic studies have shown that FECH activity was decreased to 30% of normal in patients, while in symptom free carriers, the FECH activity was decreased to 50% of normal value [128]. Recently, there was identified co-

inheritance of FECH gene mutation with low output of another normal FECH allele [129]. The molecular nature of the low input allele was determined as a result of the presence of an intronic SNP IVS3-48T/C that interferes with the splicing process. The aberrantly spliced mRNA is degraded by a nonsense mediated decay mechanism, producing a decreased steady-state level of mRNA. This SNP is normally presented in 10% of population. Its recognition in affected families has brought a major improvement in genetic counselling since its presence enables prediction of overt disease in individuals carrying a FECH mutation [130].

The eukaryotic FECH enzyme is localized in the inner mitochondrial membrane, with the active site facing the mitochondrial matrix. Human FECH is a homodimeric protein of molecular mass 86 kDa, with each unit containing a nitric oxide sensitive [2Fe-2S] cluster [131,132]. In plants, two isoforms of FECH have been described [133]. One isoform is found in non-photosynthetic tissues and is not light-responsive, while a second isoform is in thylakoid membranes and exhibits light-responsive expression.

**Pathogenesis**

The clinical manifestation is directly related to the excessive production of PROTO as a result of FECH deficiency [134]. The site of PROTO overproduction lies in the erythropoietic tissue. PROTO is mostly accumulated as a free base instead of a zinc-PROTO complex, as is found in lead intoxication or in iron deficienct anemia. Free PROTO diffuses rapidly from erythrocytes into the skin. Protoporphyrin accumulated in cutis becomes phototoxic upon excitation by visible light (Soret wavelength 400-410 nm with subsidiary activation by green light) by generating reactive oxygen species that cause cell damage and lead to cutaneous photosensitivity. Rarely, the disorder is associated with hepatic injury [135]. Because PROTO is a dicarboxylic porphyrin, it is a non water soluble compound which is not filtered by kidneys, but is excreated by the liver into bile and enters enterohepatic circulation. As a result of insufficient elimination of the excessive amount of PROTO from the body, cholestatic liver injury may develop.

**Clinical manifestation**

EPP is the most common erythropoietic porphyria. The prevalence was estimated to range between 1 in 75 000 to 200 000 [134]. The disease is panethnic. Clinical manifestations include skin lesions and liver disease.

The main symptom is painful cutaneous photosensitivity presenting in infancy, usually evident by 2-5 years of age [16,104,134]. Shortly after exposure to visible light, patients complain of a severe burning pain in the skin mostly of the face and hands. The symptoms usually last several days and are more frequent during spring and summer. The pain can be very distressing. On exposed areas, erythema and diffuse edema can occur, but unlike in other photosensitive porphyries, vesicules and blistering are unusual. Pigmentation or skin lichenification can occur especially over the knuckles. Characteristically, there is fine linear scarring around exposed skin, such as fissures around upper lip and cicatrizing in the malar region. There are no changes in teeth or urine. Marked disparity between symptoms and physical signs often leads to delayed diagnosis.

PROTO is a strongly hydrophobic molecule which is cleared from the body exclusively by liver through bile secretion. In a minority of patients, hepatic disease can result from the accumulation of PROTO in the liver [135-137]. Two major complications can develop. Firstly, gallstone formation is fairly common in EPP patients and often occurs at an early age. Secondly, in less than 2% of EPP patients, a massive deposition of protoporphyrin in the liver tissue and bile canaliculi causes cholestatic liver damage that leads to rapidly progressive and life-threatening liver failure.

Mild anemia with hyperchromia and microcytosis and ring sideroblasts may be occasionally seen [138].

**Diagnosis**

Laboratory findings include greatly elevated concentrations of free PROTO in erythrocytes, increased plasma PROTO with appearance of an absorption peak at 620 nm, and usually elevated stool PROTO [115]. Of note, urinary porphyrin excretion is normal unless cholestatic jaundice develops. Enzymatic assay of FECH is performed in peripheral lymphocytes. Typically FECH activity is reduced to 30% of the normal value in EPP patients, and to around 50% in carriers [128].

**Therapy**

Therapeutic measures of photosensitivity focus on minimizing excessive light exposure [134]. As conventional absorbent sunscreens are not effective due to poor protection in longer UVA and visible wavelengths, reflective sunscreens incorporating zinc oxide or titanium dioxide are a mainstay of the therapy. For many years, β-caroten has been prescribed to patients with EPP. It plays a role as a free radical quenching agent and also absorbs the appropriate light, but in a controlled trial, the clinical efficacy was not established. The use of narrow-band UVB phototherapy to induce UV tolerance in patients with EPP was documented[139]. Liver function of all EPP patients should be tested regularly. For patients with terminal liver failure, liver transplantation is the only choice of therapy [140]. In patients with EPP needing surgery (including liver transplantation), the surgeons must be warned about potential hazards of exposure of internal organs to prolonged visible light as severe burns and wound dehiscence may occur [141]. In a few cases, surgery has been complicated by development of a neuropathic syndrome reminiscent of an acute hepatic porphyria. The pathological basis for this syndrome is unclear, although it is hypothesized that it may be provoked by administration of an anaesthetic [142].

## 1.3.4.2. CONGENITAL ERYTHROPOIETIC PORPHYRIA

Congenital erythropoietic porphyria (CEP) is an autosomal recessive disease caused by deficient activity of UROS, the fourth enzyme in the heme biosynthetic pathway [143].

**Uroporphyrinogen synthase**

UROS catalyses the conversion of the linear tetrapyrrole HMB by inversion of the pyrrole ring, followed by its cyclization into UROgen III. In UROS deficiency, the HMB is non-enzymatically conversed to isomer I UROgen, which can be metabolized into COPROgen I. Further metabolism cannot proceed due to the stereospecificity of CPO for isomer III. The defect results in overproduction of type I isomers of porphyrinogens which are oxidized into porphyrins and accumulate in erythrocytes, skin teeth, bones, eyes and other tissues, and are excreted in urine and feces [144].

The gene encoding UROS is localized on chromosome 10q25.3→q26.3 [145]. The cDNA encodes a protein of 265 amino acids [146]. The gene has two alternative promoters that generate two transcripts, housekeeping and erythroid-specific [147]. The crystal structure of recombinant human UROS was determined. The monomeric protein folds into two α/β domains connected by a β-ladder [148]. Location and possible structural ramifications of clinical mutations have been examined. UROS knock-in mice have recently been associated with the CEP phenotype [149,150]. To date, more than 36 mutations have been described, including 4 mutations in the erythroid promoter [151,152]. The majority of mutations were observed in only a few families, but the missense mutation C73R was observed in about one-third of the CEP alleles [153]. This mutation is in homoallelic patients associated with severe phenotype with non-immune hydrops fetalis and/or transfusion dependency from birth. The expression of this mutation shows less than 1% of expressed wild type activity [154].

**Clinical manifestation**

Clinical demonstration of the disease corresponds with accumulation of porphyrins and includes photosensitivity, skin fragility, hemolyses with secondary hypersplenism, red coloured urine, hypertrichosis, alopetia, erythrodontia, eye lesion and others [155]. The clinical severity of CEP is highly variable and ranges from a mild form with only mild cutanous symptoms in adulthood to severe disease. In severe cases, the clinical picture includes non-immune hydrops fatalis, transfusion dependent anemia, severe cutaneous photosensitivity and extreme sensitivity to trauma with consequential mutilations and hirsutism [156,157]. The diagnosis is based on finding of excessive amount of type I porphyrin isomers in urine and plasma, and decreased activity of UROS [158] with subsequent identification of the pathognomonic mutation. Prenatal diagnosis is possible by measurement of UROgen I in amniotic fluid, as well as by direct detection of the gene mutation [159].

**The therapy**

The therapy of CEP includes avoidance of sunlight, high level transfusion, splenectomy, administration of hydroxyurea and oral administration of adsorbents[160]. In 1991, the first case of treatment by bone marrow transplantation

(BMT) was reported. Although the girl died from cytomegalovirus infection, her response to the therapy was significant [161]. Since then, eight other successful cases of correction of CEP by stem cell transplantation have been reported[162-168]. A promising treatment for the future may be gene therapy, which is currently in early stages of experimental testing [169].

## 1.3.5. SECONDARY PORPHYRIAS

### 1.3.5.1. LEAD INTOXICATION

Lead has been a known toxicant for thousands of years, and it remains a persistent environmental health threat [170]. Exposure to lead can result in significant adverse health effects to multiple organ systems. There are many risks of lead poisoning. The most common sources of non professional lead intoxication are old pipes with lead solder, which can contaminate water supplies, and lead-based paint in old houses. Occupational exposure to lead is most often encountered at lead smelters and battery manufacturing facilities. However, poisoning by lead does not have to be clearly evident when lead arises from unexpected sources as Chinese herbal tea and others.

Lead is one of the most famous inducers of chemical porphyria. The clinical picture of patients with lead poising resembles an acute crisis in acute hepatic porphyria, although no cutaneous photosensitivity is present. The typical biochemical signs of lead poisoning are δ-aminolevulinic aciduria, coproporphyrinuria, and accumulation of free and mostly zinc protoporphyrin in erythrocytes [171]. According these findings, the inhibition of three enzymes of heme synthesis was proposed: ALAD, CPO, and FECH.

The mechanism of ALAD inhibition was cleared [82,172]. Lead displaces a zinc ion at the metal binding site, not the active site, producing inhibition through a change in the enzyme's quaternary structure.

Lead also inhibits FECH activity in vitro; however the magnitude of inhibition in vitro is less than in vivo [173]. It has been suggested that the inhibition of FECH by lead is attributable to the decrease in iron-reducing activity. As FECH tends to

form zinc PROTO in a condition of deficient iron supply, the mechanism of accumulation of mostly zinc-PROTO, instead of free PROTO, in lead poisoning needs to be elucidated. Consequently, a zinc-PROTO complex remains bound at heme binding sites in erythrocytes, thus explains the lack of photosensitivity in lead intoxication – in contrast to EPP where PROTO is mostly accumulated as a free base and diffuses rapidly from erythrocytes into the skin.

The third enzyme suspected to be inhibited by lead CPO was not demonstrated in vitro to be inhibited by lead [171].

### 1.3.5.2. HEREDITARY TYROSINEMIA I

Hereditary tyrosinemia type I (HT) is an inborn error of tyrosine metabolism characterized by progressive liver disease and renal tubular defects. HT results from inherited deficiency of fumarylacetoacetate hydrolase (EC 3.7.1.2), the last enzyme in the degradation of tyrosine. As the consequence of enzymatic deficiency, an abnormal metabolite, succinylaceton (4,6-dioxoheptanoic acid) accumulates. Succinylacetone is an extremely potent competitive inhibitor of ALAD in human as well as in animal tissues [83].

In patients with HT, acute neurological symptoms resembling those of AIP have been reported [174]. Patients excrete excessive amounts of urinary ALA and have low ALAD in erythrocytes and in liver.

# 2. SPECIFIC AIMS OF THE WORK

This thesis is focused on the molecular analyses of porphyrias. The following specific aims were addressed:

1. to investigate the associations between sPCT and mutations in the hemochromatosis (HFE) gene and SNPs in the transferrin receptor-1 gene (TRFC1).

2. to characterize a new case of harderoporphyria, to evaluate iron metabolism in harderoporphyric patients and to investigate the molecular bases of the harderoporphyric phenotype.

3. to characterize the crystal structure of CPO and to explain how naturally occuring mutations in CPO gene alter enzyme activity.

4. to identify and characterize the pathognomic mutation within the PBGD gene of a newly diagnosed AIP patient.

5. to perform genetic analysis in the Nepal family suffering symptoms of porphyria, and to characterize the found mutation.

6. to perform the porphyrin analyses and molecular investigations in a first diagnosed Czech patient with congenital erythropoietic porphyria, to monitor the patient after cord blood stem cells transplantation by the decrease in the amount of porphyrins in bodily fluids and to perform the prenatal diagnosis in the next pregnancy.

7. to identify and characterize mutations from Czech and French patients with heterozygous and homozygous forms of porhyra variegata and to analyze the factors which can modulate the clinical manifestation and the phenotype of porphyria variegata.

Each specific aim is related to one of the publications, on which my thesis is based (Publications A-G).

# 3. SUMMARY OF PUBLISHED DATA

## *3.1. Hemochromatosis (HFE) and transferrin receptor-1 (TFRC1) genes in sporadic porphyria cutanea tarda (sPCT) (Publication A)*

The origin of sPCT remains unclear. Among other precipitating factors, iron was implicated in the pathogenesis of sPCT. The association between sPCT and HFE has been suggested, but existing results are ambiguous and other susceptibility genes could be involved. Among them the TFRC1, the receptor used by cells to obtain iron in the form of diferric transferin, could be a candidate gene.

The associations between sPCT and HFE gene mutations in a large cohort of French Caucasian patients was studied and the association between TFRC1 genotypes and sPCT in a case –control study using all known and novel intragenic SNPs was examined.

In the HFE gene, the C282Y, H63D and S65C mutations were studied. The C282Y mutation was present on 12% of chromosomes from patients with sPCT compared with 2% of the controls ($p< 10^4$). No statistically significant difference was found between sPCT patients and controls for the allelic frequencies of H63D and S65C mutations in the HFE gene.

In the TFRC1 gene, three previously reported SNPs were studied: in exon 4, the 519A/G leading to the S142G; in exon 7, the 884C/G; and the X+86G/C in exon 19. In addition, two novel and common SNPs were identified and further analyzed: in intron 4, the IVS4+198G/T and, in exon 19, the X+89A/G. For the IVS4+198G/T SNP, the frequency was 66% of chromosomes from patients with sPCT compared to 46% of chromosomes from controls ($p< 10^3$). No statistical difference was found for the frequencies of the other polymorphisms among sPCT patients and controls.

In summary, our results in French patients underlined the importance of the C282Y mutation as a risk factor in sPCT. We identified a new polymorphism, IVS4+198G/T, in the TFRC1 gene which was found to be associated with sPCT and seems to be a predisposition factor to sPCT as well. Our data suggest that sPCT should be considered as a multifactorial disorder in which several iron metabolism genes could be involved.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. My particular contribution to this publication was to determine the frequency of SNP in the TFRC1 gene using the denaturing gradient gel electrophoresis and restriction enzyme digestion.

## *3.2. Mutations in human CPO gene predict clinical expression of either hepatic hereditary coproporphyria or erythropoietic harderoporphyria (Publication B)*

The proband developed neonatal icterus, which resolved spontaneously, and during adulthood, he suffered from mild anemia and photosensitive cutaneous lesions. Laboratory investigation showed massive accumulation of harderoporphyrin in feces. The residual activity of CPO was 18% of normal and the molecular analysis showed homozygosity for the previously described point mutation K404E in exon 6.

One of the clinical symptoms of harderoporphyria in this newly diagnosed patient, as well as in other patients diagnosed by us, is iron loading anemia. The reason for iron accumulation in these patients has not been elucidated yet. A detailed study of iron metabolism and possible causes of iron overload was performed. No additional disorder was found in these patients. We conclude that the iron overload is secondary to the marked dyserythropoiesis. Two mechanisms could contribute. First, harderoporphyrin accumulation and/heme deficiency in red cell progenitors may cause maturation arrest which is known to stimulate iron absorption. Second, overproduction of porphyrins may account for hemolytic symptoms from photolysis in dermal capillaries.

Prediction of the 2D structure of CPO using hydrophobic cluster analysis (HCA) was performed. The HCA plot of CPO exhibited a region around residue K404 which formed a hinge between two secondary structures. Five amino acids of this region (D400, R401, G402, T403, and K404) and three surrounding amino acids (F395, Y399, F405) were studied. These eight amino acids in exon 6 of CPO gene were individually mutated by site-directed mutagenesis using the pGEX-2T:CPO bacterial expression system. Six amino acids close to the region of interest (Y399-K404) were replaced by amino acids with different polarity or charge. The Y399L

mutant showed only a minor defect without significant harderoporphyrin accumulation. Mutation of the other five amino acids resulted in variable loss of enzymatic activity (1-75%) with major harderoporphyrin accumulation (20-44%). F395 and F405 were replaced by a glycine, which was expected to destabilise the secondary structure. This resulted in loss of enzymatic activity (4 and 1% of residual activity, respectively) without significant accumulation of harderoporhyrin.

To further analyze whether the aberrant harderoporphyrin production is restricted to mutations in the investigated region in exon 6, all point mutations described thus far in CPO patients so far were expressed in E. coli. The residual activities were 1-66%, and no significant accumulation of harderoporhyrin was found, with the exception of mutations R401W and K404E identified in harderoporphyric patients.

Our data strongly support the hypothesis that the region extending from amino acid 400 to 404 is important for the catalyses of oxidative decarboxylation of harderoporphyrinogen. This region may be important in preventing diffusion of harderoporhyrinogen away from the catalytic site during the sequential reaction.

In summary, a new case of harderoporphyria was characterized and the previously described mutation K404E was detected. We propose that iron overload in harderoporphyric patients is secondary due to the marked dyserythropoiesis. An accumulation of harderoporhyrin was found when amino acids in the region 400-405 were mutated, while mutations in other parts of CPO gene did not result in harderoporphyrin accumulation.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. My contributions to this publication were to introduce the particular mutations into the pGEX-2T:CPO bacterial expression system using site-directed mutagenesis and to optimize this system.

### *3.3. Structural basis of hereditary coproporphyria (Publication C)*

The crystal structure of human CPO at 1.58-Å resolution was determined. The tertiary topology is characterized by a large seven stranded β-sheet that is flanked on both sides by α- helices. Contrary to other enzymes containing seven stranded β-sheets which are usually curved, the β sheet in CPO is flat probably due to the high abundance of glycine residues.

As was predicted, the homodimer is the biologically relevant form of CPO. The dimensions of the dimer are 80x60x60 Å. The corresponding β strands from the CPO monomers generate key contacts at the dimer interface in an antiparaller fashion. The interface is made up of 64% nonpolar atoms and 36% polar atoms. Ten intersubunits H bonds also contribute to the dimer stability. Taken together, the interacting surface on the CPO monomer is a hydrophobic patch.

CPO structure lacks a transitional metal center. Neither the energy scans nor the native anomalous differences Fourier maps provide evidence for bound transition metal ions in CPO. The invariant His residues are far apart and cannot serve as ligands.

The structure of the active site and catalytic mechanisms were described. The unexpected finding of citrate at the active site allows us to assign Ser-244, His-258, Asn-260, Arg-262, Asp-282, and Arg-332 as a residues mediating substrate recognition and decarboxylation. We favor a mechanism in which oxygen serves as the immediate electron acceptor, and a substrate radical or carbanion with substantial radical character participates in catalysis.

There are >20 naturally occuring HC mutations, and a majority of these mutations lead to substitution of amino acid residues within the structural framework of CPO. The molecular basis for how these alterations diminish enzyme activity is unknown. We have focused on mutations in the CPO gene that are distant from the active site although with little to no activity. The deletion of residues 392-418 encoded by exon six disrupts dimerization. W427R also affect dimerization, H327R and R327C perturb the interaction between helix α7 and the dimerization helix α9, and R331W substitution abolishes the hydrogen bonds between the guanidinium group and the carbonyl of Leu-446 and Arg 447.

The harderoporphyria - causing mutation K404E precludes a type Iβ-turn from retaining the substrate for the second decarboxylation cycle. The R401W mutation is

also defective in the second decarboxylation step. The enzyme most likely prevents the release of the intermediate by an ionic interaction with a propionate group that does not undergo decarboxylation.

In summary, the crystal structure of CPO at 1.58 Å was determined. We conclude that the homodimer is the biologically relevant form of CPO. CPO catalyses an unusual metal- and cofactor-independent oxidative decarboxylation. The structure of the active site and catalytic mechanisms were described. The effects of naturally occuring mutations in the CPO gene were determined. The deletion of residues 392-418 encoded by exon six disrupts dimerization. The primary role for the 401-405 segment is to deter the premature release of intermediate.

This paper was created in cooperation with the Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas, USA. My contributions to this study were to prepare the CPO expression vectors with the desired mutation and to prepare crystallization quality protein.

## *3.4. De novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study (Publication D)*

The proband was a boy having his first attack of AIP at the age of 15 years. The molecular analyses revealed a novel mutation in exon 15, the 966insA. The proband was the only member of the family having this mutation. The mutation was suggested to be a *de novo mutation* as nonpaternity was excluded by DNA microsatellite analyses.

This mutation leads to the formation of a STOP codon after 36 completely different amino acids, compared to the original sequence. The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids.

To investigate the impact of the mutation, we expressed the normal and mutated PBGD in a procaryotic expression system. We inserted the cDNA of PBGD into the pGEX4T-1 expression vector. Using site directed mutagenesis, the mutation was introduced into the construct.

The analysis of the purified mutant protein using the SDS PAGE showed several bands, in contrast to a single homogenous band in the case of normal protein, suggesting low stability of the mutated protein. Both normal and the mutated PBGD enzymes were similar in size, approximately Mr 68kDa with GST-tag and Mr42 kDa without GST-tag.

Specific activities were estimated for the thrombin-digested protein and for the fusion protein as well. The fusion protein exhibited approximately 22% higher activity than the thrombin digested protein. The purified mutant enzyme had extremely low residual activity, 0.18% of normal.

The structure of the mutant protein was predicted using computer-assisted structure prediction. The mutation is located in the $\beta 3_3$ sheet; two helices, $\alpha 2_3$ and $\alpha 3_3$, of domain 3 are completely missing due to the truncation. In the normal protein, the C-terminal helices protect the beta strands from being exposed to solvent. This is in agreement with the severely impaired stability of mutant PBGD, as detected from SDS PAGE results.

In summary, the *de novo* mutation 966insA was found in a Slovak patient with AIP. Due to the truncated protein sequence with an abnormal C-terminus, this small insertion mutation leads to an almost complete loss of the enzymatic function and decreases the stability of the protein structure.

This study was performed in cooperation with Department of Pediatrics in Prague, Second Pediatric Department in Košice and Laboratory of Structural Biology in Houston. My contribution to this study was to identify the mutation in the proband´s PBGD gene using DGGE analysis and direct sequencing. I performed this investigation in all members of the family, and not find the mutation in either one of the parents. This led to conclusion that the mutation found in the young proband is a *de novo* one. Such a situation always complicates diagnostic outcome in a disease normally inherited in an autosomal dominant fashion.

## *3.5. New mutation within porphobilinogen deaminase gene leading to the truncated protein as a cause of acute intermittent porphyria in extended Indian family (Publication E)*

On the basis of the internet search we were contacted by a 50 years old proband from Nepal suffering typical abdominal form of porphyria. He, not being a health professional, diagnosed himself as having an acute porphyric attack, asked for the hemearginate (Normosang, Orphan Europe) treatment with excellent clinical affect, and arranged sending blood or genomic DNA from 15 family members to our laboratory.

Molecular analyses revealed a novel mutation in exon 15, the 973insG. Subsequently, genetic analysis was performed in all accessible members of this extensive family. The mutation leads to inclusion of four different amino acids and, further, the protein is prematurely truncated by the stop codon.

The effect of this mutation was investigated by expression of the wild type and mutated PBGD in a prokaryotic expression system. The analysis of the purified mutant protein using the SDS PAGE showed several bands, in contrast to a single homogenous band in the case of normal protein, suggesting very low stability of the mutated protein. The purified mutant enzyme had very low residual activity, 0.5 % of the average level of protein expressed by the normal allele. The loss of C-terminal helix in mutant protein would be expected to expose the beta-strand core that could make the protein prone to agregation and loss of enzymatic function.

In summary, a novel mutation within the PBGD gene (973insG), which leads to truncated protein, resulting in instability of the protein and loss of enzymatic function was identified and characterized in an extensive Indian family from Nepal. The proband diagnosed himself based on internet information.

This study was performed in cooperation with Department of Genetic Medicine, Sir Ganga Ram Hospital, New Delphi, India and Laboratory of Structural Biology in Houston. My contribution to this study was to identify the proband´s mutation in PBGD gene using DGGE analyses and direct sequencing.

## *3.6. Treatment of congenital erythropoietic porphyria (Morbus Günther) by cord blood stem cell transplantation, long term follow-up with 11% chimerism and prenatal diagnosis in a sibling (Publication F)*

The proband was a boy who developed severe photosensitivity in the neonatal period after phototherapy for an icterus. The porphyrin analysis showed an excessive amount of porphyrins in urine and plasma and mildly increased fecal porphyrins. The identification of the porphyrin isomer in urine showed nonphysiologically increased isomer I, which is typical for Gunther disease. Molecular investigation of the UROS gene revealed homozygosity for "the hotspot " mutation C73R and thus confirmed the diagnosis. Parents of this child were heterozygous for this mutation.

At the age of 8 ½ months, the boy underwent the cord blood stem cells transplantation (CBST). After the transplantation he prospered well. No new marks of photosensitivity appeared, hepatosplenomegaly resolved, and his psychomotor development continues.

Detailed examination of changes in body fluid porphyrin content was performed. We found a fluctuation in the excretion of total porphyrins in the first 5 months after transplantation; after that period, the quantity of excreted porphyrins was low, but did not reach the reference range, remaining about 100-200 times higher. In samples of urine from the 5th and 11th months after CBST, we analyzed isomers of excreted COPRO. The ratio of isomer I and III remained pathological. In plasma, total porphyrins descended continuously but 13 months after CBST, they still exceeded normal value, by approximately 10-fold. Eleven months after CBST, we measured the activity of UROS in erythrocytes. The activity of UROS in erythrocytes of the child was within reference range, while the activity of UROS of his mother was slightly decreased.

Prenatal diagnosis was performed in the next pregnancy. The examination of porphyrins in amniotic fluid, as well as DNA analysis showed that the fetus was a heterozygous carrier of the mutation C73R and thus excluded from the clinical expression of CEP.

In summary, the first patient with CEP was diagnosed in the Czech Republic. Successful treatment by cord blood stem transplantation was documented by porhyrin analyses in long-term follow-up. A prenatal diagnosis was performed in the next pregnancy within the family

This study was performed in cooperation with Departement of Pediatric Haematology and Oncology, Second Faculty of Medicine, Prague. My contribution was to identify the mutation in the UROS gene in proband and his extended family, to analyze porphyrins in body fluids during long-term monitoring after CBST, and to link 7-11% chimerism with the residual metabolic defect.

## *3.7. A search for molecular determinants responsible for different phenotypic expression of porphyria variegata (Publication G)*

DNA from eight patients with “classical” heterozygous PV and from three patients with the homozygous form was analyzed. In all cases, the pathognomic mutation was identified. The list of mutations found is in table 3A in publication G.

To further investigate the functional consequence of these naturally occuring mutations in PPOX, we expressed normal and mutated human PPOX in *Escherichia coli* using the pGEX 2T vector. We analyzed in detail these mutations: a) new missense mutations in patients with the heterozygous form of PV, aa) all missense mutation found in ours patients with homozygous PV aaa) two already described SNP R304H and P256R and these two polymorphism together in one allele.

The activities of the mutated proteins are summarized in table 3B in this publication. Except for the mutation Y422C, all missense mutations found in patients with heterozygous porphyria variegata lack PPOX residual activity. The point mutation Y422C had residual activity 3%. All of the examined mutations found in patients with homozygous PV demonstrated some residual activity (ranging from 0,42%-10,3%). Both analyzed SNPs demonstrate decreased activity in comparison with wt PPOX. The substitution R304H preserved about 85 % of activity and the allelic variation P256R had 65%. The coexistance of the polymorphism R304H together with P256R in one PPOX allele led to further diminution of the activity, to approximately 10%.

The frequencies of the two SNPs, as well as that of the newly identified SNP IV10+9 G→A, were established in the Czech and French populations. The frequency of the P256R variant was 2,3% in Czech and 4.5 % in French population, that of R304H was 4,5% in Czech and 27% in French population, and that of IV10+9 G→A was 0,9% in both analyzed cohorts.

In summary, a search for the molecular determinants responsible for different phenotypic expression of porphyria variegate led to the identification and characterization of two polymorphisms within the PPO gene that result in lowering PPO activity in addition to the original mutation. Once the critical treshhold for decreased activity is reached, phenotypic expression of an acute attack may occur.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. This work was the main subject of my PhD thesis. My contribution was to optimize, prepare and to express individual mutated PPOX proteins in prokaryotic expression systems, to measure the activity of mutated PPOX proteins, and to estimate the frequency of mutations and polymorphisms in the PPOX gene in the Czech population.

# 4. GENERAL DISCUSSION

Porphyria was mentioned for the first time probably as early as 460 BC by Hippocrates [175] but the greatest discoveries date from the end of the 19 th century. As in other metabolic diseases, the research in porphyrias began in clinical description and headed towards the understanding of the basis of this magical disease to facilitate better care of the patients and to comprehend heme synthesis. The porphyria research history can be divided into several periods regarding the dominant methodology (Table 10).

Table 10: Dominant disciplines in porphyria research [72]

| | |
|---|---|
| 1937-1952 | Chemistry |
| 1952-1967 | Biochemistry |
| 1967-1982 | Enzymology |
| 1982-1997 | Molecular genetics |
| 1997- | Functional genomics and proteomics, gene therapy, animal trangenic models, structural aspects of proteins of heme synthesis |

The end of 20th century was the great entrance of molecular biology. During a 15-year period, the genes coding all enzymes of heme biosynthesis were cloned and expressed. Consequently, the methods for identification of mutations and their screening were optimized for each porphyria. The detection of pathognomic mutations greatly improved the diagnostics and ameliorated the therapy.

Nowadays even more profound discoveries have come involving the heme biosynthetic pathway. Successful crystallization of the enzymes of heme biosynthesis allows us to learn about the tertiary and quaternary structures of these enzymes. The study of the consequence of mutations in different regions of the proteins provides better understanding of the function of the enzyme. This is important, not only for the care of patients with porphyrias, but also for understanding the regulation of heme biosynthesis and the importance of heme in a wide variety of essential biological

processes. The introduction of animal models of certain porphyrias helps us better understand the pathogenesis of these mysterious diseases and improves the therapeutic possibilities. Laboratory trials of bone-marrow transplantation (BMT) on murine models were followed by successful BMT in human patients with CEP. Presently, the promising gene therapy is in progress.

**The outcomes from this PhD dissertation deepen our knowledge about porphyrias and the structure and function of heme biosynthetic pathway enzymes**. In publication A, we analyzed the relationship between sPCT and mutations in the HFE gene and SNPs in the TRFC1. In publication B, we studied the molecular bases of harderoporphyria, the consequence of the localization of mutations in the CPO gene on phenotype variant manifestation, either as HC or as harderoporhyria. In publication C, we report the crystal structure of CPO, we describe the catalytic mechanism of the enzyme and we hypothesize how naturally occurring mutations in the CPO gene alter enzyme activity. In publication D and E, we investigated the consequences of the mutation found in patients with AIP on the structure and function of PBGD. In publication F, we present the outcome of the patient with CEP after CBST. Until now, only ten successful transplantations of haematologic stem cell have been reported. In publication G, we investigate the effect of mutations in PPOX gene found in patients with PV on the function of the enzyme and we analyze the modulation of the phenotype of PV by an additional SNP in PPOX gene.

**In conclusion, our work has provided previously uncharacterized insights for understanding porphyrias at the molecular level.**

# 5. CONCLUSION

1. **An association between the HFE C282Y mutation and sPCT was validated and the new polymorphism IVS4+198G/T in TFRC1 gene, which seems to be predisposition factor to sPCT, was identified in a French population.**

2. **A new case of harderoporphyria was characterized. Harderoporphyric patients exhibit iron overload secondary due to dyserythropoiesis. Only a few missense mutations, restricted to five aminoacids in exon 6, may cause accumulation of significant amounts of harderoporphyrin, thus this region is important for catalysis of oxidative decarboxylation of harderoporphyrinogen.**

3. **The crystal structure of CPO at 1.58 Å was determined. The structure of the active site and catalytic mechanisms was proposed. The consequence of naturally occuring mutation in CPO gene was determined.**

4. **One *de novo* mutation in the PBGD gene (966 insA) was found in a Slovak patient with AIP. This mutation leads to an almost complete loss of enzymatic function and a decrease in the stability of the protein structure.**

5. **A novel mutation within the PBGD gene (973insG), which leads to truncated protein, resulting in instability of the protein and loss of enzymatic function was identified and characterized in an extensive Indian family from Nepal. The proband diagnosed himself based on internet information.**

6. **The first patient with CEP was diagnosed in the Czech Republic. Successful cord blood stem transplantation was documented by porhyrin analyses. A successful prenatal diagnosis was performed in the next pregnancy within the family.**

7. **A search for the molecular determinants responsible for different phenotypic expression of porphyria variegate led to the identification and characterization of two polymorphisms within the PPO gene that result in a protein variant lowering PPO activity in addition to the disease-causing mutation. Once the critical treshhold for decreased activty is reached, phenotypic expression of an acute attack may occur.**

# 6. REFERENCES


1. Anderson,K.E., Sassa,S., Bishop,D.F. & Desnick,R.J. The Metabolic and Molecular Basis of Inherited Diseases. Scriver,C.R., Beaudet,A.L., Sly,W.S. & Valle,D. (eds.), pp. 2961-3062 (McGraw-Hill, NY,2001).

2. Ajioka,R.S., Phillips,J.D. & Kushner,J.P. Biosynthesis of heme in mammals. *Biochim. Biophys. Acta* **1763**, 723-736 (2006).

3. Moore. The biochemistry of heme synthesis in porphyria and in the porphyrinurias. *Clin. Dermatol.* **16,** 203-223 (2006).

4. Zaider,E. & Bickers,D.R. Clinical laboratory methods for diagnosis of the porphyrias. *Clin. Dermatol.* **16**, 277-293 (1998).

5. Dailey,H.A. Terminal steps of haem biosynthesis. *Biochem. Soc. Trans.* **30**, 590-595 (2002).

6. Woodard,S.I. & Dailey,H.A. Multiple regulatory steps in erythroid heme biosynthesis. *Arch. Biochem. Biophys.* **384**, 375-378 (2000).

7. Mustajoki S. Molecular genetics of acute intermittent porphyria in Finland. 1-79. 1999. University of Helsinki. Thesis/Dissertation

8. Cotter,P.D., Willard,H.F., Gorski,J.L. & Bishop,D.F. Assignment of human erythroid delta-aminolevulinate synthase (ALAS2) to a distal subregion of band Xp11.21 by PCR analysis of somatic cell hybrids containing X; autosome translocations. *Genomics* **13**, 211-212 (1992).

9. Bishop,D.F., Henderson,A.S. & Astrin,K.H. Human delta-aminolevulinate synthase: assignment of the housekeeping gene to 3p21 and the erythroid-specific gene to the X chromosome. *Genomics* **7**, 207-214 (1990).

10. Astner,I. *et al.* Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. *EMBO J.* **24**, 3166-3177 (2005).

11. Ponka,P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* **89**, 1-25 (1997).

12. Shoolingin-Jordan,P.M., Al Dbass,A., McNeill,L.A., Sarwar,M. & Butler,D. Human porphobilinogen deaminase mutations in the investigation of the mechanism of dipyrromethane cofactor assembly and tetrapyrrole formation. *Biochem. Soc. Trans.* **31**, 731-735 (2003).

13. Meissner,P., Adams,P. & Kirsch,R. Allosteric inhibition of human lymphoblast and purified porphobilinogen deaminase by protoporphyrinogen and coproporphyrinogen. A possible mechanism for the acute attack of variegate porphyria. *J. Clin. Invest* **91**, 1436-1444 (1993).

14. McDonagh,A.F. & Bissell,D.M. Porphyria and porphyrinology--the past fifteen years. *Semin. Liver Dis.* **18**, 3-15 (1998).

15. Kauppinen,R. Porphyrias. *Lancet* **365**, 241-252 (2005).

16. Gross,U., Hoffmann,G.F. & Doss,M.O. Erythropoietic and hepatic porphyrias. *J. Inherit. Metab Dis.* **23**, 641-661 (2000).

17. Elder,G.H. Hepatic porphyrias in children. *J. Inherit. Metab Dis.* **20**, 237-246 (1997).

18. Nordmann,Y., Puy,H. & Deybach,J.C. The porphyrias. *J. Hepatol.* **30 Suppl 1**, 12-16 (1999).

19. Kirsch,R.E., Meissner,P.N. & Hift,R.J. Variegate porphyria. *Semin. Liver Dis.* **18**, 33-41 (1998).

20. Kauppinen,R. & Mustajoki,P. Prognosis of acute porphyria: occurrence of acute attacks, precipitating factors, and associated diseases. *Medicine (Baltimore)* **71**, 1-13 (1992).

21. Nordmann,Y. & Puy,H. Human hereditary hepatic porphyrias. *Clin. Chim. Acta* **325**, 17-37 (2002).

22. Meyer,U.A., Schuurmans,M.M. & Lindberg,R.L. Acute porphyrias: pathogenesis of neurological manifestations. *Semin. Liver Dis.* **18**, 43-52 (1998).

23. Brennan,M.J. & Cantrill,R.C. Delta-aminolaevulinic acid is a potent agonist for GABA autoreceptors. *Nature* **280**, 514-515 (1979).

24. Puy,H. *et al.* Increased delta aminolevulinic acid and decreased pineal melatonin production. A common event in acute porphyria studies in the rat. *J. Clin. Invest* **97**, 104-110 (1996).

25. Hermes-Lima,M., Castilho,R.F., Valle,V.G., Bechara,E.J. & Vercesi,A.E. Calcium-dependent mitochondrial oxidative damage promoted by 5-aminolevulinic acid. *Biochim. Biophys. Acta* **1180**, 201-206 (1992).

26. PRICE,J.M., BROWN,R.R. & PETERS,H.A. Tryptophan metabolism in porphyria, schizophrenia, and a variety of neurologic and psychiatric diseases. *Neurology* **9**, 456-468 (1959).

27. Bonkovsky,H.L. & Barnard,G.F. Diagnosis of porphyric syndromes: a practical approach in the era of molecular biology. *Semin. Liver Dis.* **18**, 57-65 (1998).

28. Hindmarsh,J.T. The porphyrias, appropriate test selection. *Clin. Chim. Acta* **333**, 203-207 (2003).

29. Mustajoki,P., Tenhunen,R., Tokola,O. & Gothoni,G. Haem arginate in the treatment of acute hepatic porphyrias. *Br. Med J. (Clin. Res. Ed)* **293**, 538-539 (1986).

30. Kordac,V. & Martasek,P. Haem arginate in acute hepatic porphyrias. *Br. Med J. (Clin. Res. Ed)* **293**, 1098 (1986).

31. Anderson,K.E., Spitz,I.M., Bardin,C.W. & Kappas,A. A gonadotropin releasing hormone analogue prevents cyclical attacks of porphyria. *Arch. Intern. Med* **150**, 1469-1474 (1990).

32. Gross,U. *et al.* Hormonal oral contraceptives, urinary porphyrin excretion and porphyrias. *Horm. Metab Res.* **27**, 379-383 (1995).

33. 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* **57**, 105-108 (1991).

34. Raich,N. *et al.* Molecular cloning and complete primary sequence of human erythrocyte porphobilinogen deaminase. *Nucleic Acids Res.* **14**, 5955-5968 (1986).

35. Yoo,H.W., Warner,C.A., Chen,C.H. & Desnick,R.J. Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. *Genomics* **15**, 21-29 (1993).

36. Chretien,S. *et al.* Alternative transcription and splicing of the human porphobilinogen deaminase gene result either in tissue-specific or in housekeeping expression. *Proc. Natl. Acad. Sci. U. S. A* **85**, 6-10 (1988).

37. Grandchamp,B. *et al.* Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur. J. Biochem.* **162**, 105-110 (1987).

38. Louie,G.V. *et al.* Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. *Nature* **359**, 33-39 (1992).

39. Louie,G.V. *et al.* The three-dimensional structure of Escherichia coli porphobilinogen deaminase at 1.76-A resolution. *Proteins* **25**, 48-78 (1996).

40. Jordan,P.M. & Warren,M.J. Evidence for a dipyrromethane cofactor at the catalytic site of E. coli porphobilinogen deaminase. *FEBS Lett.* **225**, 87-92 (1987).

41. Robreau-Fraolini,A.M. *et al.* Porphobilinogen deaminase gene in African and Afro-Caribbean ethnic groups: mutations causing acute intermittent porphyria and specific intragenic polymorphisms. *Hum. Genet* **107**, 150-159 (2000).

42. Grandchamp,B. Acute intermittent porphyria. *Semin. Liver Dis.* **18**, 17-24 (1998).

43. Bottomley,S.S., Bonkowsky,H.L. & Kreimer-Birnbaum,M. The diagnosis of acute intermittent porphyria. Usefulness and limitations of the erythrocyte uroporphyrinogen I synthase assay. *Am. J. Clin. Pathol.* **76**, 133-139 (1981).

44. Mustajoki,P. & Tenhunen,R. Variant of acute intermittent porphyria with normal erythrocyte uroporphyrinogen-I-synthase activity. *Eur. J. Clin. Invest* **15**, 281-284 (1985).

45. Whatley,S.D. *et al.* Non-erythroid form of acute intermittent porphyria caused by promoter and frameshift mutations distant from the coding sequence of exon 1 of the HMBS gene. *Hum. Genet* **107**, 243-248 (2000).

46. Puy,H. *et al.* Acute intermittent porphyria: rapid molecular diagnosis. *Cell Mol. Biol. (Noisy. -le-grand)* **43**, 37-45 (1997).

47. Kauppinen,R., Mustajoki,S., Pihlaja,H., Peltonen,L. & Mustajoki,P. Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. *Hum. Mol. Genet* **4**, 215-222 (1995).

48. Schreiber,W.E., Fong,F., Nassar,B.A. & Jamani,A. Heteroduplex analysis detects frameshift and point mutations in patients with acute intermittent porphyria. *Hum. Genet* **96**, 161-166 (1995).

49. Puy,H., Robreau,A.M., Rosipal,R., Nordmann,Y. & Deybach,J.C. Protoporphyrinogen oxidase: complete genomic sequence and polymorphisms in the human gene. *Biochem. Biophys. Res. Commun.* **226**, 226-230 (1996).

50. Roberts,A.G. *et al.* Partial characterization and assignment of the gene for protoporphyrinogen oxidase and variegate porphyria to human chromosome 1q23. *Hum. Mol. Genet* **4**, 2387-2390 (1995).

51. Taketani,S. *et al.* The human protoporphyrinogen oxidase gene (PPOX): organization and location to chromosome 1. *Genomics* **29**, 698-703 (1995).

52. Nishimura,K., Taketani,S. & Inokuchi,H. Cloning of a human cDNA for protoporphyrinogen oxidase by complementation in vivo of a hemG mutant of Escherichia coli. *J. Biol. Chem.* **270**, 8076-8080 (1995).

53. Dailey,T.A., Meissner,P. & Dailey,H.A. Expression of a cloned protoporphyrinogen oxidase. *J. Biol. Chem.* **269**, 813-815 (1994).

54. Dailey,T.A. & Dailey,H.A. Human protoporphyrinogen oxidase: expression, purification, and characterization of the cloned enzyme. *Protein Sci.* **5**, 98-105 (1996).

55. Deybach,J.C., da,S., V, Grandchamp,B. & Nordmann,Y. The mitochondrial location of protoporphyrinogen oxidase. *Eur. J. Biochem.* **149**, 431-435 (1985).

56. Arnould,S., Takahashi,M. & Camadro,J.M. Acylation stabilizes a protease-resistant conformation of protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. *Proc. Natl. Acad. Sci. U. S. A* **96**, 14825-14830 (1999).

57. Whatley,S.D. *et al.* Variegate porphyria in Western Europe: identification of PPOX gene mutations in 104 families, extent of allelic heterogeneity, and absence of correlation between phenotype and type of mutation. *Am. J. Hum. Genet* **65**, 984-994 (1999).

58. Deybach,J.C. *et al.* Mutations in the protoporphyrinogen oxidase gene in patients with variegate porphyria. *Hum. Mol. Genet* **5**, 407-410 (1996).

59. Jenkins,T. The South African malady. *Nat. Genet* **13**, 7-9 (1996).

60. da,Silva., V, Simonin,S., Deybach,J.C., Puy,H. & Nordmann,Y. Variegate porphyria: diagnostic value of fluorometric scanning of plasma porphyrins. *Clin. Chim. Acta* **238**, 163-168 (1995).

61. Deybach,J.C., de Verneuil,H. & Nordmann,Y. The inherited enzymatic defect in porphyria variegata. *Hum. Genet* **58**, 425-428 (1981).

62. Elder,G.H., Evans,J.O. & Thomas,N. The primary enzyme defect in hereditary coproporphyria. *Lancet* **2**, 1217-1219 (1976).

63. Cacheux,V. *et al.* Localization of the human coproporphyrinogen oxidase gene to chromosome band 3q12. *Hum. Genet* **94**, 557-559 (1994).

64. Delfau-Larue,M.H., Martasek,P. & Grandchamp,B. Coproporphyrinogen oxidase: gene organization and description of a mutation leading to exon 6 skipping. *Hum. Mol. Genet* **3**, 1325-1330 (1994).

65. Martasek,P. *et al.* Molecular cloning, sequencing, and functional expression of a cDNA encoding human coproporphyrinogen oxidase. *Proc. Natl. Acad. Sci. U. S. A* **91**, 3024-3028 (1994).

66. Taketani,S., Kohno,H., Furukawa,T., Yoshinaga,T. & Tokunaga,R. Molecular cloning, sequencing and expression of cDNA encoding human coproporphyrinogen oxidase. *Biochim. Biophys. Acta* **1183**, 547-549 (1994).

67. Martasek,P. *et al.* Human coproporphyrinogen oxidase. Biochemical characterization of recombinant normal and R231W mutated enzymes expressed in E. coli as soluble, catalytically active homodimers. *Cell Mol. Biol. (Noisy. -le-grand)* **43**, 47-58 (1997).

68. Medlock,A.E. & Dailey,H.A. Human coproporphyrinogen oxidase is not a metalloprotein. *J. Biol. Chem.* **271**, 32507-32510 (1996).

69. Grandchamp,B., Phung,N. & Nordmann,Y. The mitochondrial localization of coproporphyrinogen III oxidase. *Biochem. J.* **176**, 97-102 (1978).

70. Camadro,J.M., Chambon,H., Jolles,J. & Labbe,P. Purification and properties of coproporphyrinogen oxidase from the yeast Saccharomyces cerevisiae. *Eur. J. Biochem.* **156**, 579-587 (1986).

71. Lamoril,J. *et al.* Characterization of mutations in the CPO gene in British patients demonstrates absence of genotype-phenotype correlation and identifies relationship between hereditary coproporphyria and harderoporphyria. *Am. J. Hum. Genet* **68**, 1130-1138 (2001).

72. Martasek,P. Hereditary coproporphyria. *Semin. Liver Dis.* **18**, 25-32 (1998).

73. Blake,D., McManus,J., Cronin,V. & Ratnaike,S. Fecal coproporphyrin isomers in hereditary coproporphyria. *Clin. Chem.* **38**, 96-100 (1992).

74. Grandchamp,B. & Nordmann,Y. Decreased lymphocyte coproporphyrinogen III oxidase activity in hereditary coproporphyria. *Biochem. Biophys. Res. Commun.* **74**, 1089-1095 (1977).

75. Rosipal,R. *et al.* Systematic analysis of coproporphyrinogen oxidase gene defects in hereditary coproporphyria and mutation update. *Hum. Mutat.* **13**, 44-53 (1999).

76. Sassa,S. Delta-aminolevulinic acid dehydratase assay. *Enzyme* **28**, 133-145 (1982).

77. Potluri,V.R., Astrin,K.H., Wetmur,J.G., Bishop,D.F. & Desnick,R.J. Human delta-aminolevulinate dehydratase: chromosomal localization to 9q34 by in situ hybridization. *Hum. Genet.* **76**, 236-239 (1987).

78. Kaya,A.H., Plewinska,M., Wong,D.M., Desnick,R.J. & Wetmur,J.G. Human delta-aminolevulinate dehydratase (ALAD) gene: structure and alternative splicing of the erythroid and housekeeping mRNAs. *Genomics* **19**, 242-248 (1994).

79. Akagi,R. *et al.* delta-Aminolevulinate dehydratase (ALAD) porphyria: the first case in North America with two novel ALAD mutations. *Mol. Genet. Metab* **87**, 329-336 (2006).

80. Maruno,M. *et al.* Highly heterogeneous nature of delta-aminolevulinate dehydratase (ALAD) deficiencies in ALAD porphyria. *Blood* **97**, 2972-2978 (2001).

81. Doss,M.O. *et al.* The third case of Doss porphyria (delta-amino-levulinic acid dehydratase deficiency) in Germany. *J. Inherit. Metab Dis.* **27**, 529-536 (2004).

82. Kelada,S.N., Shelton,E., Kaufmann,R.B. & Khoury,M.J. Delta-aminolevulinic acid dehydratase genotype and lead toxicity: a HuGE review. *Am. J. Epidemiol.* **154**, 1-13 (2001).

83. Sassa,S. & Kappas,A. Hereditary tyrosinemia and the heme biosynthetic pathway. Profound inhibition of delta-aminolevulinic acid dehydratase activity by succinylacetone. *J. Clin. Invest* **71**, 625-634 (1983).

84. Sassa,S. ALAD porphyria. *Semin. Liver Dis.* **18**, 95-101 (1998).

85. Doss,M., von Tiepermann,R., Schneider,J. & Schmid,H. New type of hepatic porphyria with porphobilinogen synthase defect and intermittent acute clinical manifestation. *Klin. Wochenschr.* **57**, 1123-1127 (1979).

86. Hassoun,A., Verstraeten,L., Mercelis,R. & Martin,J.J. Biochemical diagnosis of an hereditary aminolaevulinate dehydratase deficiency in a 63-year-old man. *J. Clin. Chem. Clin. Biochem.* **27**, 781-786 (1989).

87. Thunell,S., Holmberg,L. & Lundgren,J. Aminolaevulinate dehydratase porphyria in infancy. A clinical and biochemical study. *J. Clin. Chem. Clin. Biochem.* **25**, 5-14 (1987).

88. Akagi,R., Yasui,Y., Harper,P. & Sassa,S. A novel mutation of delta-aminolaevulinate dehydratase in a healthy child with 12% erythrocyte enzyme activity. *Br. J. Haematol.* **106**, 931-937 (1999).

89. Solis,C. *et al.* Acute intermittent porphyria: studies of the severe homozygous dominant disease provides insights into the neurologic attacks in acute porphyrias. *Arch. Neurol.* **61**, 1764-1770 (2004).

90. Hessels,J. *et al.* Homozygous acute intermittent porphyria in a 7-year-old boy with massive excretions of porphyrins and porphyrin precursors. *J. Inherit. Metab Dis.* **27**, 19-27 (2004).

91. Kordac,V. *et al.* Homozygous variegate porphyria. *Lancet* **1**, 851 (1984).

92. Kauppinen,R. *et al.* Homozygous variegate porphyria: 20 y follow-up and characterization of molecular defect. *J. Invest Dermatol.* **116**, 610-613 (2001).

93. Corrigall,A.V. *et al.* Homozygous variegate porphyria in South Africa: genotypic analysis in two cases. *Mol. Genet Metab* **69**, 323-330 (2000).

94. Roberts,A.G. *et al.* Molecular characterization of homozygous variegate porphyria. *Hum. Mol. Genet* **7**, 1921-1925 (1998).

95. Palmer,R.A., Elder,G.H., Barrett,D.F. & Keohane,S.G. Homozygous variegate porphyria: a compound heterozygote with novel mutations in the protoporphyrinogen oxidase gene. *Br. J. Dermatol.* **144**, 866-869 (2001).

96. Meissner,P.N. *et al.* A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. *Nat. Genet* **13**, 95-97 (1996).

97. Grandchamp,B., Phung,N. & Nordmann,Y. Homozygous case of hereditary coproporphyria. *Lancet* **2**, 1348-1349 (1977).

98. Martasek,P., Nordmann,Y. & Grandchamp,B. Homozygous hereditary coproporphyria caused by an arginine to tryptophane substitution in coproporphyrinogen oxidase and common intragenic polymorphisms. *Hum. Mol. Genet* **3**, 477-480 (1994).

99. Doss,M.O. *et al.* Compound heterozygous hereditary coproporphyria with fluorescing teeth. *Ann. Clin. Biochem.* **36 ( Pt 5)**, 680-682 (1999).

100. Nordmann,Y. *et al.* Harderoporphyria: a variant hereditary coproporphyria. *J. Clin. Invest* **72**, 1139-1149 (1983).

101. Doss,M., von Tiepermann,R. & Kopp,W. Harderoporphyrin coproporphyria. *Lancet* **1**, 292 (1984).

102. Lamoril,J. *et al.* A molecular defect in coproporphyrinogen oxidase gene causing harderoporphyria, a variant form of hereditary coproporphyria. *Hum. Mol. Genet* **4**, 275-278 (1995).

103. Lamoril,J. *et al.* Neonatal hemolytic anemia due to inherited harderoporphyria: clinical characteristics and molecular basis. *Blood* **91**, 1453-1457 (1998).

104. Murphy,G.M. The cutaneous porphyrias: a review. The British Photodermatology Group. *Br. J. Dermatol.* **140**, 573-581 (1999).

105. McLellan,T., Pryor,M.A., Kushner,J.P., Eddy,R.L. & Shows,T.B. Assignment of uroporphyrinogen decarboxylase (UROD) to the pter----p21 region of human chromosome 1. *Cytogenet. Cell Genet* **39**, 224-227 (1985).

106. Dubart,A. *et al.* Assignment of human uroporphyrinogen decarboxylase (URO-D) to the p34 band of chromosome 1. *Hum. Genet* **73**, 277-279 (1986).

107. Romana,M. *et al.* Structure of the gene for human uroporphyrinogen decarboxylase. *Nucleic Acids Res.* **15**, 7343-7356 (1987).

108. Phillips,J.D., Whitby,F.G., Kushner,J.P. & Hill,C.P. Characterization and crystallization of human uroporphyrinogen decarboxylase. *Protein Sci.* **6**, 1343-1346 (1997).

109. Whitby,F.G., Phillips,J.D., Kushner,J.P. & Hill,C.P. Crystal structure of human uroporphyrinogen decarboxylase. *EMBO J.* **17**, 2463-2471 (1998).

110. de Verneuil,H., Aitken,G. & Nordmann,Y. Familial and sporadic porphyria cutanea: two different diseases. *Hum. Genet* **44**, 145-151 (1978).

111. Christiansen,L. *et al.* Screening for mutations in the uroporphyrinogen decarboxylase gene using denaturing gradient gel electrophoresis. Identification and characterization of six novel mutations associated with familial PCT. *Hum. Mutat.* **14**, 222-232 (1999).

112. Elder,G.H. Porphyria cutanea tarda. *Semin. Liver Dis.* **18**, 67-75 (1998).

113. Feder,J.N. *et al.* The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc. Natl. Acad. Sci. U. S. A* **95**, 1472-1477 (1998).

114. Elder,G.H. & Worwood,M. Mutations in the hemochromatosis gene, porphyria cutanea tarda, and iron overload. *Hepatology* **27**, 289-291 (1998).

115. Elder,G.H., Smith,S.G. & Smyth,S.J. Laboratory investigation of the porphyrias. *Ann. Clin. Biochem.* **27 ( Pt 5)**, 395-412 (1990).

116. da,S., V, Simonin,S., Deybach,J.C., Puy,H. & Nordmann,Y. Variegate porphyria: diagnostic value of fluorometric scanning of plasma porphyrins. *Clin. Chim. Acta* **238**, 163-168 (1995).

117. Seubert,S., Seubert,A., Rumpf,K.W. & Kiffe,H. A porphyria cutanea tarda-like distribution pattern of porphyrins in plasma, hemodialysate, hemofiltrate, and urine of patients on chronic hemodialysis. *J. Invest Dermatol.* **85**, 107-109 (1985).

118. Moran-Jimenez,M.J. *et al.* Uroporphyrinogen decarboxylase: complete human gene sequence and molecular study of three families with hepatoerythropoietic porphyria. *Am. J. Hum. Genet* **58**, 712-721 (1996).

119. Koszo,F., Elder,G.H., Roberts,A. & Simon,N. Uroporphyrinogen decarboxylase deficiency in hepatoerythropoietic porphyria: further evidence for genetic heterogeneity. *Br. J. Dermatol.* **122**, 365-370 (1990).

120. de Verneuil,H. *et al.* Prevalence of the 281 (Gly----Glu) mutation in hepatoerythropoietic porphyria and porphyria cutanea tarda. *Hum. Genet* **78**, 101-102 (1988).

121. Ferreira,G.C. *et al.* Structure and function of ferrochelatase. *J. Bioenerg. Biomembr.* **27**, 221-229 (1995).

122. Whitcombe,D.M. *et al.* Assignment of the human ferrochelatase gene (FECH) and a locus for protoporphyria to chromosome 18q22. *Genomics* **11**, 1152-1154 (1991).

123. Taketani,S., Inazawa,J., Nakahashi,Y., Abe,T. & Tokunaga,R. Structure of the human ferrochelatase gene. Exon/intron gene organization and location of the gene to chromosome 18. *Eur. J. Biochem.* **205**, 217-222 (1992).

124. Tugores,A., Magness,S.T. & Brenner,D.A. A single promoter directs both housekeeping and erythroid preferential expression of the human ferrochelatase gene. *J. Biol. Chem.* **269**, 30789-30797 (1994).

125. Nakahashi,Y., Taketani,S., Okuda,M., Inoue,K. & Tokunaga,R. Molecular cloning and sequence analysis of cDNA encoding human ferrochelatase. *Biochem. Biophys. Res. Commun.* **173**, 748-755 (1990).

126. Wang,X., Poh-Fitzpatrick,M., Taketani,S., Chen,T. & Piomelli,S. Screening for ferrochelatase mutations: molecular heterogeneity of erythropoietic protoporphyria. *Biochim. Biophys. Acta* **1225**, 187-190 (1994).

127. Rufenacht,U.B. *et al.* Systematic analysis of molecular defects in the ferrochelatase gene from patients with erythropoietic protoporphyria. *Am. J. Hum. Genet* **62**, 1341-1352 (1998).

128. Gouya,L. *et al.* Inheritance in erythropoietic protoporphyria: a common wild-type ferrochelatase allelic variant with low expression accounts for clinical manifestation. *Blood* **93**, 2105-2110 (1999).

129. Gouya,L. *et al.* The penetrance of dominant erythropoietic protoporphyria is modulated by expression of wildtype FECH. *Nat. Genet* **30**, 27-28 (2002).

130. Deybach,J.C. Porphyria. Painful photosensitivity. *Lancet* **358 Suppl**, S49 (2001).

131. Burden,A.E. *et al.* Human ferrochelatase: crystallization, characterization of the [2Fe-2S] cluster and determination that the enzyme is a homodimer. *Biochim. Biophys. Acta* **1435**, 191-197 (1999).

132. Wu,C.K. *et al.* The 2.0 A structure of human ferrochelatase, the terminal enzyme of heme biosynthesis. *Nat. Struct. Biol.* **8**, 156-160 (2001).

133. Suzuki,T. *et al.* Two types of ferrochelatase in photosynthetic and nonphotosynthetic tissues of cucumber: their difference in phylogeny, gene expression, and localization. *J. Biol. Chem.* **277**, 4731-4737 (2002).

134. Cox,T.M., Alexander,G.J. & Sarkany,R.P. Protoporphyria. *Semin. Liver Dis.* **18**, 85-93 (1998).

135. Doss,M.O. & Frank,M. Hepatobiliary implications and complications in protoporphyria, a 20-year study. *Clin. Biochem.* **22**, 223-229 (1989).

136. Bloomer,J.R. The liver in protoporphyria. *Hepatology* **8**, 402-407 (1988).

137. Meerman,L. Erythropoietic protoporphyria. An overview with emphasis on the liver. *Scand. J. Gastroenterol. Suppl* 79-85 (2000).

138. Rademakers,L.H. *et al.* Accumulation of iron in erythroblasts of patients with erythropoietic protoporphyria. *Eur. J. Clin. Invest* **23**, 130-138 (1993).

139. Warren,L.J. & George,S. Erythropoietic protoporphyria treated with narrow-band (TL-01) UVB phototherapy. *Australas. J. Dermatol.* **39**, 179-182 (1998).

140. Bloomer,J.R. *et al.* Liver transplantation in a patient with protoporphyria. *Gastroenterology* **97**, 188-194 (1989).

141. Meerman,L. *et al.* Perioperative measures during liver transplantation for erythropoietic protoporphyria. *Transplantation* **57**, 155-158 (1994).

142. Herbert,A. *et al.* Erythropoietic protoporphyria: unusual skin and neurological problems after liver transplantation. *Gastroenterology* **100**, 1753-1757 (1991).

143. Romeo,G. & Levin,E.Y. Uroporphyrinogen 3 cosynthetase in human congenital erythropoietic porphyria. *Proc. Natl. Acad. Sci. U. S. A* **63**, 856-863 (1969).

144. Desnick,R.J., Glass,I.A., Xu,W., Solis,C. & Astrin,K.H. Molecular genetics of congenital erythropoietic porphyria. *Semin. Liver Dis.* **18**, 77-84 (1998).

145. Astrin,K.H. *et al.* Regional assignment of the human uroporphyrinogen III synthase (UROS) gene to chromosome 10q25.2----q26.3. *Hum. Genet* **87**, 18-22 (1991).

146. Tsai,S.F., Bishop,D.F. & Desnick,R.J. Human uroporphyrinogen III synthase: molecular cloning, nucleotide sequence, and expression of a full-length cDNA. *Proc. Natl. Acad. Sci. U. S. A* **85**, 7049-7053 (1988).

147. Aizencang,G., Solis,C., Bishop,D.F., Warner,C. & Desnick,R.J. Human uroporphyrinogen-III synthase: genomic organization, alternative promoters, and erythroid-specific expression. *Genomics* **70**, 223-231 (2000).

148. Mathews,M.A. *et al.* Crystal structure of human uroporphyrinogen III synthase. *EMBO J.* **20**, 5832-5839 (2001).

149. Ged,C. *et al.* A knock-in mouse model of congenital erythropoietic porphyria. *Genomics* **87**, 84-92 (2006).

150. Bishop,D.F. *et al.* Uroporphyrinogen III synthase knock-in mice have the human congenital erythropoietic porphyria phenotype, including the characteristic light-induced cutaneous lesions. *Am. J. Hum. Genet* **78**, 645-658 (2006).

151. Solis,C., Aizencang,G.I., Astrin,K.H., Bishop,D.F. & Desnick,R.J. Uroporphyrinogen III synthase erythroid promoter mutations in adjacent GATA1 and CP2 elements cause congenital erythropoietic porphyria. *J. Clin. Invest* **107**, 753-762 (2001).

152. Shady,A.A. *et al.* Congenital erythropoietic porphyria: identification and expression of eight novel mutations in the uroporphyrinogen III synthase gene. *Br. J. Haematol.* **117**, 980-987 (2002).

153. Xu,W., Warner,C.A. & Desnick,R.J. Congenital erythropoietic porphyria: identification and expression of 10 mutations in the uroporphyrinogen III synthase gene. *J. Clin. Invest* **95**, 905-912 (1995).

154. Warner,C.A., Yoo,H.W., Roberts,A.G. & Desnick,R.J. Congenital erythropoietic porphyria: identification and expression of exonic mutations in the uroporphyrinogen III synthase gene. *J. Clin. Invest* **89**, 693-700 (1992).

155. Freesemann,A.G., Bhutani,L.K., Jacob,K. & Doss,M.O. Interdependence between degree of porphyrin excess and disease severity in congenital erythropoietic porphyria (Gunther's disease). *Arch. Dermatol. Res.* **289**, 272-276 (1997).

156. Deybach,J.C. *et al.* Congenital erythropoietic porphyria (Gunther's disease): enzymatic studies on two cases of late onset. *J. Lab Clin. Med* **97**, 551-558 (1981).

157. Moruno,T.A. *et al.* Congenital erythropoietic porphyria, description of a new mutation in two brothers. *Eur. J. Pediatr.* **156**, 817-818 (1997).

158. Freesemann,A.G., Gross,U., Bensidhoum,M., de Verneuil,H. & Doss,M.O. Immunological, enzymatic and biochemical studies of uroporphyrinogen III-synthase deficiency in 20 patients with congenital erythropoietic porphyria. *Eur. J. Biochem.* **257**, 149-153 (1998).

159. Ged,C. *et al.* Prenatal diagnosis in congenital erythropoietic porphyria by metabolic measurement and DNA mutation analysis. *Prenat. Diagn.* **16**, 83-86 (1996).

160. Murphy,G.M. Diagnosis and management of the erythropoietic porphyrias. *Dermatol. Ther.* **16**, 57-64 (2003).

161. Kauffman,L., Evans,D.I., Stevens,R.F. & Weinkove,C. Bone-marrow transplantation for congenital erythropoietic porphyria. *Lancet* **337**, 1510-1511 (1991).

162. Zix-Kieffer,I. *et al.* Successful cord blood stem cell transplantation for congenital erythropoietic porphyria (Gunther's disease). *Bone Marrow Transplant.* **18**, 217-220 (1996).

163. Thomas,C. *et al.* Correction of congenital erythropoietic porphyria by bone marrow transplantation. *J. Pediatr.* **129**, 453-456 (1996).

164. Lagarde,C. *et al.* [Allogeneic bone marrow transplantation in congenital erythropoietic porphyria. Gunther's disease]. *Ann. Dermatol. Venereol.* **125**, 114-117 (1998).

165. Tezcan,I. *et al.* Congenital erythropoietic porphyria successfully treated by allogeneic bone marrow transplantation. *Blood* **92**, 4053-4058 (1998).

166. Shaw,P.H., Mancini,A.J., McConnell,J.P., Brown,D. & Kletzel,M. Treatment of congenital erythropoietic porphyria in children by allogeneic stem cell transplantation: a case report and review of the literature. *Bone Marrow Transplant.* **27**, 101-105 (2001).

167. Harada,F.A., Shwayder,T.A., Desnick,R.J. & Lim,H.W. Treatment of severe congenital erythropoietic porphyria by bone marrow transplantation. *J. Am. Acad. Dermatol.* **45**, 279-282 (2001).

168. Dupuis-Girod,S. *et al.* Successful match-unrelated donor bone marrow transplantation for congenital erythropoietic porphyria (Gunther disease). *Eur. J. Pediatr.* **164**, 104-107 (2005).

169. Geronimi,F. *et al.* Lentivirus-mediated gene transfer of uroporphyrinogen III synthase fully corrects the porphyric phenotype in human cells. *J. Mol. Med* **81**, 310-320 (2003).

170. Mamet,R. *et al.* Lead poisoning: a new biochemical perspective on the differentiation between acquired and hereditary neuroporphyria. *Clin. Chem.* **47**, 1710-1713 (2001).

171. Fujita. Lead, chemical porphyria, and heme as a biological mediator *Tohoku J.Exp.Med* , **196**, 53-64 (2002).

172. Warren,M.J., Cooper,J.B., Wood,S.P. & Shoolingin-Jordan,P.M. Lead poisoning, haem synthesis and 5-aminolaevulinic acid dehydratase. *Trends Biochem. Sci.* **23**, 217-221 (1998).

173. Taketani,S., Tanaka,A. & Tokunaga,R. Reconstitution of heme-synthesizing activity from ferric ion and porphyrins, and the effect of lead on the activity. *Arch. Biochem. Biophys.* **242**, 291-296 (1985).

174. Strife,C.F. *et al.* Tyrosinemia with acute intermittent porphyria: aminolevulinic acid dehydratase deficiency related to elevated urinary aminolevulinic acid levels. *J. Pediatr.* **90**, 400-404 (1977).

175. RIMINGTON,C. Was Hippocrates the first to describe a case of acute porphyria? *Int. J. Biochem.* **25**, 1351-1352 (1993).