

CHARLES UNIVERSITY, PRAGUE, 1ST FACULTY OF MEDICINE
LABORATORY FOR STUDIES OF MITOCHONDRIAL DISORDERS
DEPARTMENT OF PEDIATRICS



**STRUCTURAL-FUNCTIONAL CORRELATIONS
OF
HYDROXYMETHYLBILANE SYNTHASE**

PhD thesis

Dana Douděrová, MSc.

Prague 2009

ACKNOWLEDGEMENTS

This study was carried out during the years of 2003-2009 at the Laboratory for Studies of Mitochondrial Disorders, Department of Pediatrics, 1ST Faculty of Medicine, Charles University, Prague, Czech Republic.

I would like to thank to all those who gave me the opportunity, mental support, and assistance to complete this thesis and, particularly, to the following people:

Firstly to my supervisor, Prof. MUDr. Pavel Martásek, DrSc., for all the experience I gained while working in his lab, and for introducing me to the Porphyrin world.

To all my colleagues from Laboratory for Study of Mitochondrial Disorders, especially to Mgr. Danka Zahoráková and Ing. Petra Lelková, for everyday intellectual and mental support and constantly cheering me up.

To all from abroad I worked with, especially to Xiaoye Schneider-Yin and Elisabeth I. Minder from Central Laboratory, Triemli Hospital, Zurich, Switzerland and to Rivka Mamet and Nili Schoenfeld from National Laboratory for the Biochemical Diagnoses of Porphyrins, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel, for excellent and very beneficial cooperation.

To my family for the inspiration and the great exemplar in both professional and personal life.

And most importantly, to my husband for his endless support, patience and tolerance, and always keeping me busy outside of my professional life.

I appreciate the participation of the patients and their relatives in the study. This research was supported by the Ministry of Education, Sport and Youth of the Czech Republic, and The Granting Agency of the Charles University; contract grant number: GA UK 10/2004, GAUK 257540 54007, MSM0021620806, 1M6837805002. I would also like to thank to American Journal Experts (AJE) for proof-reading of this thesis.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	3
TABLE OF CONTENTS	4
LIST OF ABBREVIATIONS	6
INTRODUCTION	7
Background.....	7
Purpose of the study.....	8
Initiation of the study	9
REVIEW OF THE LITERATURE	10
Heme and hemoproteins.....	10
Heme biosynthesis	12
Heme degradation	13
The mechanism of heme biosynthesis control.....	13
Porphyrias.....	17
Acute intermittent porphyria	19
Clinical manifestation	21
Triggering factors of an acute attack.....	22
Pathogenesis of porphyric neuropathy	22
Diagnosis	26
Treatment.....	28
Gene	30
Protein	32
Structure-function correlations	36
LIST OF THE ORIGINAL PUBLICATIONS.....	38
AIMS OF THE STUDY.....	39
RESULTS AND DISCUSSION.....	40
Publication A	40
Publication B	42
Publication C	43
Publication D	45
Publication E.....	47
Publication F.....	49
Publication G	50
Report of a novel mutation identification.....	52

Publication H	52
Publication I.....	53
Publication J	53
CONCLUSIONS	55
Future challenges	56
REFERENCES	57
SUPPLEMENTS: ORIGINAL PUBLICATIONS	65

LIST OF ABBREVIATIONS

AA	Amino acid
AIP	Acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
ALAS1	Hepatic ubiquitous aminolevulinic acid synthase
ALAS2	Erythroid aminolevulinic acid synthase
ATP	Adenosine triphosphate
CAR	Constitutively active receptor
cDNA	Coding deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CEP	Congenital erythropoietic porphyria
CPOX	Coproporphyrinogen oxidase
CYP	Cytochrome P450
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DPM	Dipyrromethane
EEG	Electroencephalography
EPI	European Porphyria Initiative
EPP	Erythropoietic protoporphyria
FECH	Ferrochelatase
GABA	Gamma-aminobutyric acid
gDNA	Genomic deoxyribonucleic acid
GST	Glutathione S-transferase
HCP	Hereditary coproporphyria
HMB	Hydroxymethylbilane
HMBS	Hydroxymethylbilane synthase
HOXG	Heme oxygenase
HRM	High-resolution melting
IRP	Iron-regulatory protein
K _m	Michaelis constant
LS	Lichen sclerosus
mRNA	Messenger ribonucleic acid
NA	Nucleic acid
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
PDB	Protein database
PPOX	Protoporphyrinogen oxidase
PXR	Pregnane xenobiotic receptor
RNA	Ribonucleic acid
SDS	Sodium dodecylsulfate
URO	Uroporphyrin
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen III synthase
V _{max}	Maximal velocity
VP	Variegata Porphyria

INTRODUCTION

Background

The porphyrias are a group of predominantly inherited disorders in heme biosynthesis in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. With the exception of the first enzymatic step, seven deficiencies linked to seven enzymes in the heme biosynthesis pathway have been described. Porphyrias can be divided into hepatic or erythropoietic categories, depending on the anatomical origin, and acute or cutaneous groups, depending on the clinical presentation. The two types of clinical expression can occur separately or together: acute life-threatening neurovisceral attacks, which are associated with the overproduction of aminolevulinic acid (ALA) and porphobilinogen (PBG); and cutaneous symptoms that result from photosensitisation caused by porphyrins.

The acute intermittent porphyria (AIP) is an autosomal dominant disorder, classified as acute hepatic porphyria. It is characterised by a deficiency of hydroxymethylbilane synthase (HMBS), the third enzyme in heme biosynthesis. Where an affected allele is present, the activity of the enzyme is decreased by about 50% in all tissues. The prevalence of symptomatic disease varies from 1 to 10 per 100 000, but due to frequent misdiagnosis and low penetrance the rate may be much higher (Badminton and Elder, 2002; Meyer, et al., 1972; Strand, et al., 1970).

The clinical manifestation of the disease is associated with an acute neurological syndrome characterised mainly by acute attack (Albers and Fink, 2004; Meyer, et al., 1998). Acute attacks are manifested by a wide variety of clinical features including autonomic neuropathy with arrhythmias, central nervous system impairment, peripheral motor neuropathy with muscular weakness and paresthesia or less common sensory symptoms, and the most common clinical presentation, a severe pain caused by neurovisceral crises, usually of abdominal or back origin (Meyer, et al., 1998; Nordmann and Puy, 2002). The porphyric manifestation usually occurs in the third decade in life, though in rare cases it can manifest before puberty or after menopause (Elder, et al., 1997). Clinical expression is, in general, highly variable and ~90% of AIP heterozygotes remain asymptomatic throughout life (Elder, et al., 1997; Petrides, 1998). Symptoms may occur recurrently, lasting from a few days to a few weeks, or occur as a single sporadic incident.

Human HMBS is determined by a single gene located on chromosome 11 (Meisler, et al., 1980). The HMBS gene was characterised in 1986 (Raich, et al., 1986), which enabled further studies at the molecular level. The gene is divided into fifteen exons and spans approximately 10 kb of DNA (Yoo, et al., 1993).

Hydroxymethylbilane synthase (also known as porphobilinogen deaminase or uroporphyrinogen I synthase, EC 4.3.1.8 or EC 2.5.1.61) is a monomeric protein with a dipyrromethane (DPM) cofactor in a single catalytic active site (Louie, et al., 1992). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isoenzymes have been isolated and characterised (Grandchamp, et al., 1987). Relatively high amino-acid sequence conservation has been found, amounting to at least 32% to the proteins from bacteria, fungi, plants and mammals. Crystallographic structures of *E. coli* HMBS and recently of human housekeeping HMBS have been determined (Louie, et al., 1992; Song, et al., 2009).

The diagnosis of AIP is made on the basis of characteristic clinical symptoms, such as elevated levels of urinary porphyrin precursors ALA and PBG and a decreased erythrocytic HMBS activity. Diagnosis has been aided in recent years by the identification of the causal mutations in the HMBS gene. Biochemical analyses remain the preferred choice in diagnostic laboratories because of their simplicity and rapidity, however identification of the causal mutation provides the ultimate proof of AIP occurrence.

Over 300 mutations in the HMBS gene have been identified (<http://www.hgmd.cf.ac.uk>) (Stenson, et al., 2009). Mutations are equally distributed within the HMBS gene and no prevalent site for mutation has been identified.

Purpose of the study

The purpose of this study was first to perform molecular analysis of the AIP patients. Once a mutation is detected in a patient, molecular testing is offered to family members. In each affected family, this becomes an important tool for individualised medicine, allowing for careful drug prescription; in addition, it is very important for the asymptomatic carriers to be warned of precipitating factors, thus avoiding an acute attack.

The proper DNA diagnostics can be achieved by a combination of a robust and effective pre-screening method and a confirmatory DNA sequencing step. We decided to establish a new generation pre-screening method, which will be highly sensitive and relatively time- and

cost-effective. Our method of choice was high-resolution melting (HRM) analysis using the LightScanner instrument.

Another important aspect of this project was to study the molecular heterogeneity of AIP in relation to the HMBS protein. We aimed at characterisation of the impact of the HMBS gene mutation on the structure and function of the enzyme, and demonstration of how this aids the interpretation of clinical, biochemical and genetic data in establishing an AIP diagnosis. To demonstrate this, we used expression and characterisation of mutant HMBS enzymes in the prokaryotic system together with the use of predictive computer-assisted structure-function correlation studies.

Initiation of the study

The idea for this study originated in a laboratory with clinical, diagnostic and scientific experience in most types of porphyria. At the beginning of this study in 2003, only a few AIP-causing mutations had been identified within Czech porphyric families. For the molecular diagnosis, the traditional, but time-consuming method of denaturing gradient gel electrophoresis (DGGE) was performed. Furthermore, very little was known about the exact detrimental effects of specific AIP-causing mutations on the enzyme structure.

The study began in 2003 at the Laboratory for Studies of Mitochondrial Disorders, Department of Pediatrics, 1ST Faculty of Medicine, Charles University, Prague, Czech Republic. In 2007, we initiated collaborations with Porphyria centres abroad, namely Central Laboratory, Triemli Hospital, Zurich, Switzerland and National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel.

REVIEW OF THE LITERATURE

Heme and hemoproteins

Heme serves as a prosthetic group for a wide range of proteins, either covalently or non-covalently bound to the protein itself; the biological functions diverge with heme type. The core structure of a heme molecule is tetrapyrrole, a substance significantly called 'pigment of life' (Leeper, 1989) since it gives the specific colour to green leaves and red blood.

The heme compound contains four pyrrole rings connected in a cyclic fashion. The four pyrrole rings are interconnected through one-carbon (methine or methylene) bridges to form the protoporphyrin. The macrocycle is a highly conjugated system and therefore deeply coloured. Since tetrapyrroles tend to incorporate metals to form complexes, heme consists of iron. The iron in the heme substance is capable of undergoing oxidation and reduction reactions.

Recent protein databank surveys identifies at least several hundred metalloproteins with heme as their subunit, all involved in diverse basic cellular functions. Among the most significant functions, hemoproteins act in: transportation of and sensing of diatomic gases such as oxygen, carbon monoxide and nitric oxide (White and Marletta, 1992); electron transfer through cytochromes in mitochondrial transport chain, performing roles of detoxification and drug metabolism (in the enzyme system of cytochromes P450); and play roles in synthesis of regulatory or signalling molecules such as guanylate cyclase (Stone and Marletta, 1996) and nitric oxide synthase (White and Marletta, 1992). Another important role of hemoproteins is its role in the oxidative stress response where hemoproteins are partially responsible for the destruction of invading bacteria and virus, in the biosynthesis of the thyroid hormones and in the metabolism of tryptophan. Moreover, heme acts as a regulatory molecule by mediating gene expression at the level of transcription (Hach, et al., 1999; Ogawa, et al., 2001; Reddy, et al., 1996; Schmitt, 1999), translation (Chen and London, 1995), protein targeting (Lathrop and Timko, 1993), protein stability (Qi, et al., 1999; Wang, et al., 1999) and cell differentiation (Nakajima, et al., 1999).

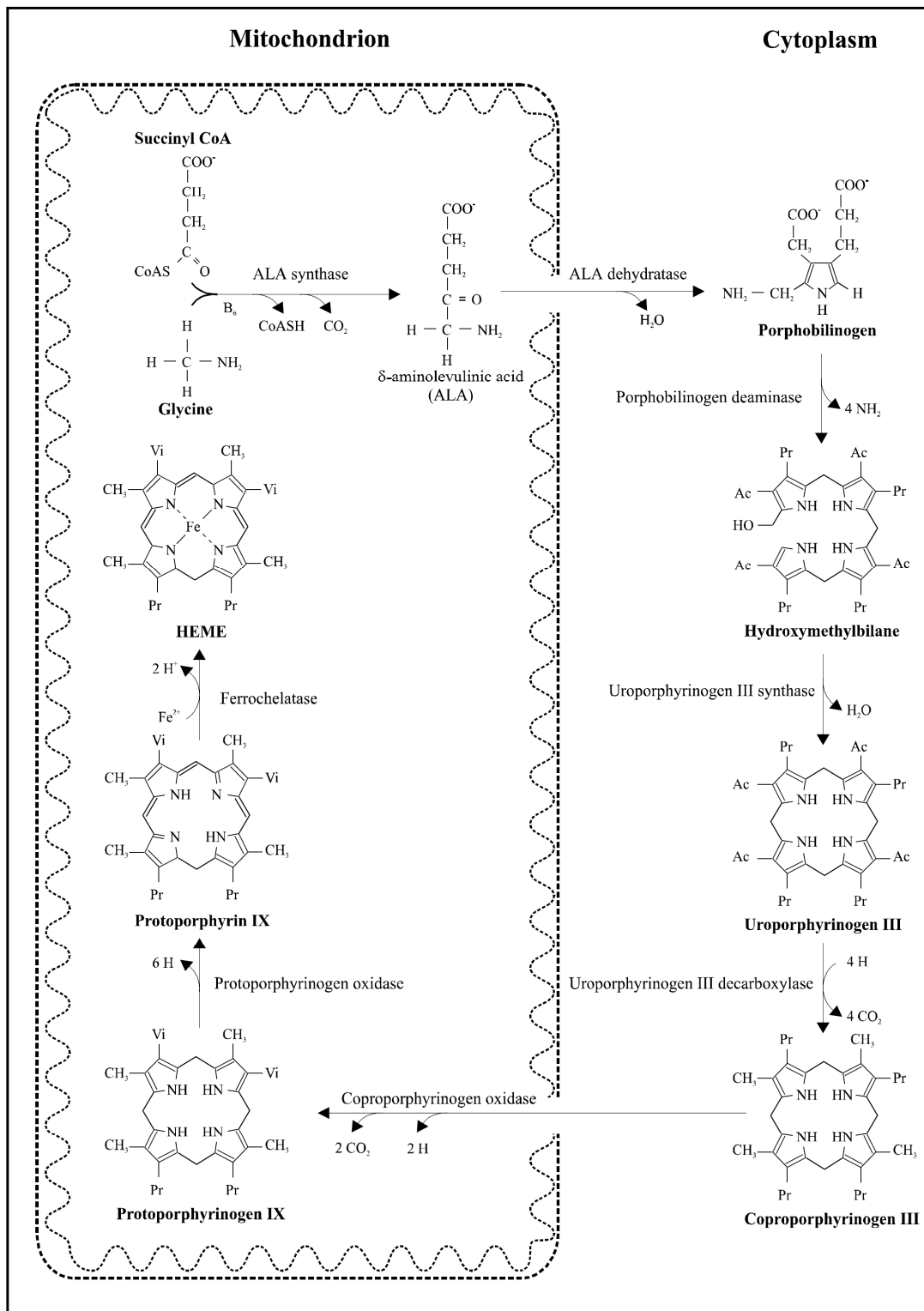


Figure 1 The heme biosynthetic pathway in mammals

Intermediate products and enzymes involved in the synthetic cascade of the heme, which takes place in the mitochondria and cytosol. Ac= $-\text{CH}_2\text{COOH}$, Pr= $-\text{CH}_2\text{CH}_2\text{COOH}$, Vi= $-\text{CH}=\text{CH}_2$ (Mustajoki, 1999)

Heme biosynthesis

Heme biosynthesis is widely distributed process in eukaryotic cells although considerable quantitative variations exist between different cell types. The synthesis takes place in all living cells, but predominantly in the liver and bone marrow. The major fraction is synthesized in erythroid cells, about 85%, where the heme is utilized in hemoglobin formation (Kauppinen, 2005). In the liver, 80% of heme is used for covering great demand for microsomal cytochromes P450.

There are eight enzymatic steps in the heme biosynthesis. The initial enzymatic step take place in the mitochondrion, the following four enzymes operate in the cytosol and the final three steps are directed back into mitochondria, or more precisely, into the inter-membrane space and into the inner membrane. Enzymes in the heme pathway are encoded by nuclear genes, all which have been widely studied and characterized (Anderson, 2001). Initial four enzymes of the biosynthesis contain tissues-specific promoters. Two different transcripts are presented, one being ubiquitous, the other one being erythroid-specific. For the first enzyme, aminolevulinic acid synthase (ALAS), isoenzymes are encoded by two different genes (Bishop, et al., 1990). For aminolevulinic acid dehydratase (ALAD), hydroxymethylbilane synthase (HMBS) and uroporphyrinogen III synthase (UROS), different transcripts are synthesized via tissue-specific promoters and the use of alternative splicing (Anderson, 2001).

The biosynthetic cycle begins with condensation and decarboxylation of two amino acids, glycine and succinyl-CoA, coming from the citric acid cycle (Krebs cycle), to form D-aminolevulinic acid (5-ALA or δ -ALA). This reaction is the rate-limiting step of the overall synthesis as the enzyme responsible for this reaction, ALAS, is highly sensitive to intracellular iron and heme levels and, therefore, strictly regulated by its concentration. The following reaction, catalyzed by ALAD, combines two molecules of ALA to give porphobilinogen (PBG), the first pathway intermediate which contains a pyrrole ring. In the next step, initiated by elimination of the amino group, the porphyrin ring is formed by condensation of four molecules of PBG, in the reaction running under control of HMBS. The reaction proceeds through deamination into hydroxymethylbilane (HMB). HMB can be non-enzymatically converted into uroporphyrinogen I and, in the second step, to coproporphyrinogen I. Enzymatically, UROS hydrolyses the linear tetrapyrrole HMB creating the macrocyclic asymmetric tetrapyrrole uroporphyrinogen III. Decarboxylation of

all four acetyl side chains of uroporphyrinogen III, converting them to methyl groups, is catalyzed by uroporphyrinogen decarboxylase (UROD), forming coproporphyrinogen III as the next porphyrin intermediate. Following the oxidative decarboxylation of propionyl side chains, converting them to vinyl groups, it is catalyzed by coproporphyrinogen oxidase (CPOX), and forms protoporphyrinogen IX. Final enzyme of this porphyrin conversion, protoporphyrinogen oxidase (PPOX), adds more double bonds in oxidative reaction, yielding protoporphyrin IX. In the last biosynthetic step, Fe^{2+} is inserted into protoporphyrin IX via ferrochelatase (FECH) to generate heme (**Figure 1**).

Heme degradation

Heme degradation is of the same importance as the biosynthesis, for being involved in homeostasis of cellular heme pool. The main part of heme catabolism proceeds in two steps, ultimately yielding bilirubin, carbon monoxide, and iron as the ferric ion (Fe^{3+}). In the first step, heme is oxidized to biliverdin by the two isoforms of endoplasmic reticulum enzyme heme oxygenase (HOXG). In the second reaction, biliverdin is converted to bilirubin by biliverdin reductase (BVR). While the substantial reaction is the first step, crucial for the regulation is the fine maintenance of the unsteady balance between induction and repression of HOXG, driven by heme level in case of induction and, by hypoxia, in case of repression. Moreover, HOXG, the rate-limiting step in heme catabolism, appears to play an important role in a number of neurodegenerative disorders, such as Alzheimer disease and aging (Atamna, et al., 2002).

The mechanism of heme biosynthesis control

Due to the special importance of this molecular compound, cellular heme levels are tightly regulated to maintain a balance between heme biosynthesis, demands for heme as a molecular system component, and heme catabolism. Two main tissues are characteristic for its high demand for heme: liver and erythroid bone marrow. Therefore, regulation of heme biosynthesis is split and proceeds in two different tissue-dependent manners (May, et al., 1995).

In liver, the rate-limiting regulatory enzyme of heme synthesis is inducible hepatic ubiquitous ALAS1, which operates in mitochondria initiating the synthesis. The metabolic

activity of this enzyme is determined by the regulatory DNA-binding nuclear proteins affecting ALAS1 gene transcription, and by the post-translational control (Thunell, 2006). Heme, the end-product of this pathway, plays a role as a feedback inhibitor. When the free heme pool decreases under the critical level due to increased utilisation of heme in hemoprotein synthesis, or due to accelerated catabolism through the activation of HOXG, ALAS1 expression is induced (Thunell, 2006). The known feed-back control mechanisms of ALAS1 by heme are as follows: regulation of the translocation of ALAS1 to the mitochondria (Hayashi, et al., 1980), control of the post-translational level of this enzyme by reducing its mRNA-stability (Hamilton, et al., 1991), and repression of the transcription of the housekeeping gene for ALAS1 (Kolluri, et al., 2005). These mechanisms of heme feed-back control are of therapeutic importance; infusion of stabilised exogenous heme, heme arginate or hematin, can abort attacks of porphyria in patients with an inborn error of metabolism in this process, by reducing transcription of ALAS1.

In erythrocytes, the erythroid ALAS2 is expressed only during erythroid differentiation. In the process of erythroid cell differentiation, which is under the general control of a hormone erythropoietin (Spivak, 1986), the highly up-regulated heme synthesis is coordinated with globin synthesis to give rise to hemoglobin. The erythropoietin hormone interacts with specific cell receptors and activates the transcriptional factor GATA-1, a key regulatory element in erythroid differentiation (Orkin, 1992). The GATA-1 factor in turn stimulates transcription of the ALAS2 gene. A further mechanism of ALAS2 translational control is based on the availability of iron, which is mediated by a cell associated transferrin (Hemmaplardh and Morgan, 1974). Translation of the ALAS2 mRNA is prevented by binding of the iron-regulatory protein (IRP) to the iron-responsive element in the 5`prime untranslated region of mRNA. Iron enters the cell in a complex with the transferrin via transferrin receptor pathway. High levels of cellular iron ions converts IRP to its non-RNA binding form and translation of mRNA can then start. The precursor protein of ALAS2 is translocated into mitochondria where it begins heme biosynthesis (Ponka, 1997) (**Figure 2**). In erythroid cells, in contrast to housekeeping ALAS1 protein, ALAS2 is induced by heme (May, et al., 1995).

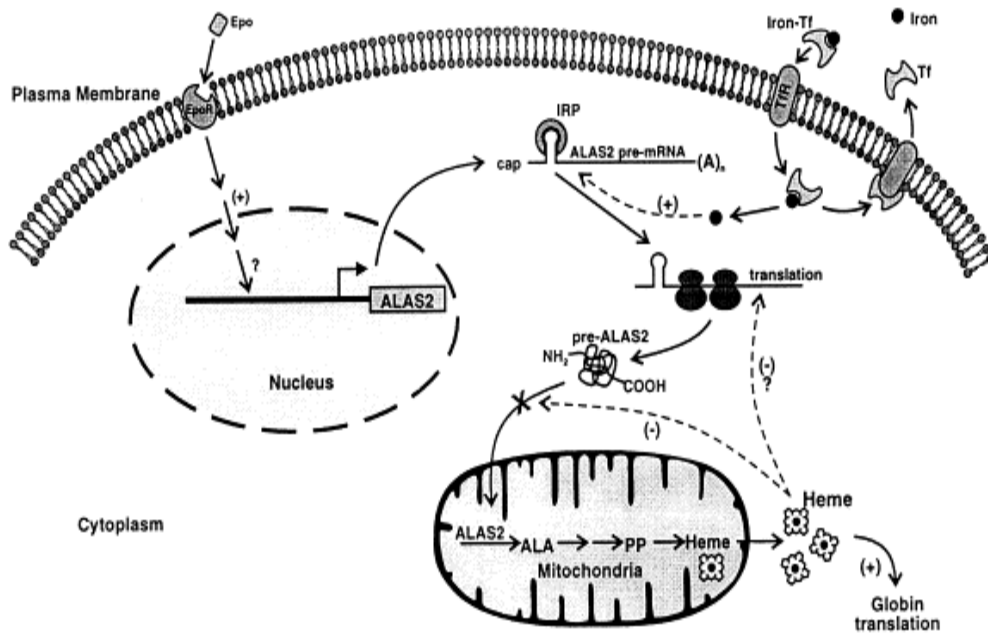


Figure 2 Regulation of the heme biosynthesis in erythropoietic cells

The regulation of ALAS2 expression in erythroid cells is under general control of erythropoietin (Epo). The transcription of the ALAS2 gene is stimulated through the erythropoietin receptor (EpoR). Translation of ALAS2 mRNA is prevented by the binding of the iron regulatory protein (IRP). It is relieved by iron entering as a complex with transferrin (Tf) via the transferrin receptor (TfR). Following translation, the precursor protein (pre-ALAS2) is imported into mitochondria where it becomes functionally active. ALA= aminolevulinic acid, PP= protoporphyrin (Sadlon, et al., 1999)

There has been great improvement in our understanding of the biosynthetic regulation of the heme synthesis pathway, while the connection between some of the endogenous and exogenous stimulatory factors and the mechanism of heme biosynthesis activation has been deciphered. The heme produced by the hepatic ALAS1 is mainly utilised in the synthesis of catalytic proteins. The major fraction of the hepatic hemoprotein pool is a group of cytochromes P450 (CYP). CYPs are responsible for the metabolism of steroid hormones, drugs and other xenobiotics, which are clearly proven accelerating factors (Guengerich, et al., 1995). The identification of the DNA-binding proteins constitutively active receptor (CAR) and pregnane xenobiotic receptor (PXR), which mediate the transcription of CYP genes, explain the concerted transcription of these genes and the ALAS1 gene (Podvinec, et al., 2004). Characterisation of this CAR-PXR transduction mechanism further enhances our understanding of the impact of steroid hormones and/or xenobiotics on the heme biosynthetic pathway. The transcriptional modulation of ALAS1 induction by these and/or other nuclear factors may explain some other biological observations such as the fasting induction of ALAS1, and multiple kinds of stress induction (Handschin, et al., 2005; Thunell, 2006) (Figure 3).

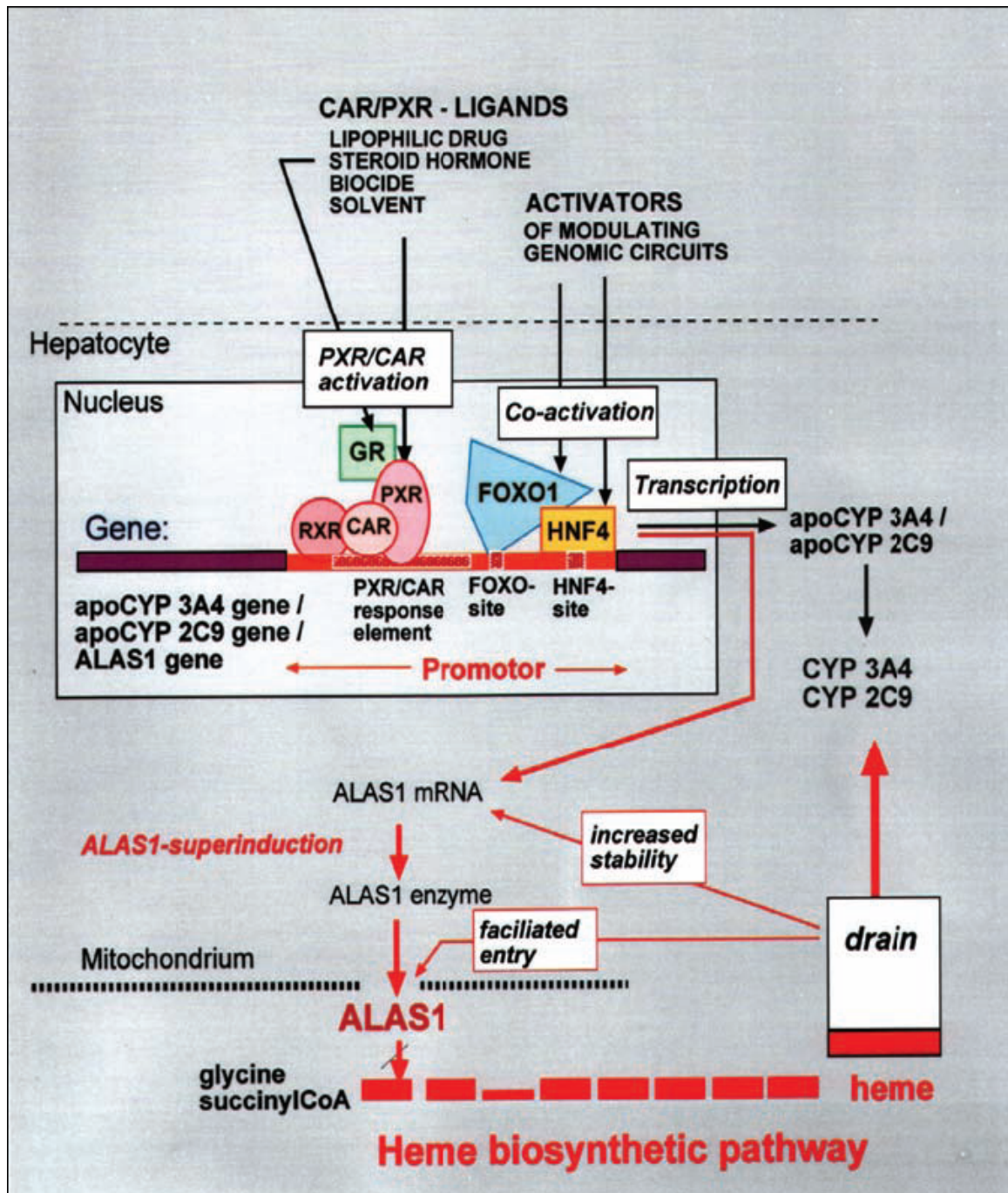


Figure 3 The CAR-PXR transduction mechanism and the superinductive ALAS1 response

Transcriptions of the genes for ALAS1 and apoCYP take place consertedly. Nuclear receptors are activated by several exogenous and endogenous ligands. Co-activators of the nuclear receptors modulate the response of the target genes GR= glucocorticoid receptor, RXR= 9-cis retinoic acid xenobiotic receptor, FOXO= forkhead box class O, HNF= hepatocyte nuclear factor (Thunell, 2006).

Porphyrias

The porphyrias are a group of predominantly inherited disorders of heme biosynthesis in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. With the exception of the first enzymatic step, seven deficiencies linked to seven enzymes of the biosynthetic pathway have been described (Anderson, 2001). Each type of porphyria results from a specific decrease in the activity of a corresponding enzyme. The position of the deficient enzyme in the biosynthetic pathway is crucial for the clinical manifestation with respect to accumulation of specific intermediates (**Table 1**). The genes coding for the enzymes of this pathway have been characterised at the molecular level (Anderson, 2001) and several mutations have been identified. Inheritance of one copy of an affected allele can cause loss of enzymatic activity, but output from the normal allele appears to be sufficient for normal cellular metabolism in individuals (Gouya, et al., 2004). The porphyric manifestation is triggered by several additional factors, and the vulnerability of the genetic background of each affected individual may play important roles in the resulting porphyric phenotype.

Table 1 Excretion pattern of specific porphyrin intermediates in each type of porphyria

Porphyria	Urine	Faeces	Plasma (peak, nm)	Erythrocytes
ALAD deficiency	ALA	-	-	Protoporphyrin (zinc)
Acute intermittent porphyria (AIP)	ALA, PBG	-	615-620	-
Congenital erythropoietic porphyria (CEP)	Uroporphyrin I, Coproporphyrin I	Coproporphyrin I	615-620	Protoporphyrin (zinc and free), Uroporphyrin I
Porphyria cutanea tarda (PCT)	Uroporphyrin, 7-carboxyporphyrin	isocoproporphyrin, 7-carboxy and 5-carboxyporphyrin	615-620	-
Hereditary coproporphyrin (HCP)	ALA, PBG, Coproporphyrin III	Coproporphyrin III	615-620	-
Variegata Porphyria (VP)	ALA, PBG, Coproporphyrin III	Protoporphyrin, Coproporphyrin III	624-627	-
Erythropoietic protoporphyria (EPP)	normal	-	626-634	Protoporphyrin (free)

Most porphyrias are autosomal dominant conditions, only two of them share an autosomal recessive mechanism of inheritance. Low clinical penetrance is an important feature of all of the autosomal dominant porphyrias (Anderson, 2001; Gouya, et al., 2004).

Porphyrias can be divided into hepatic or erythropoietic types depending on the anatomical origin, and further divided into acute or cutaneous types depending on the clinical presentation. Two types of clinical expression can occur separately or in combination: acute life-threatening neurovisceral attacks, which are associated with the overproduction of ALA and PBG; and/or cutaneous symptoms that result from photosensitisation caused by porphyrins (Albers and Fink, 2004; Anderson, 2001; Meyer, et al., 1998).

Porphyrias are panethnic and their prevalence varies from 0.5 to 10 per 100 000 in different population (Anderson, 2001). All types of porphyria are summarised in **Table 2**.

In 2001, in an attempt to improve the accessibility and sharing of information about porphyrias for both the porphyria specialists and patients, the European Porphyria Initiative (EPI) platform was founded by experts from different European countries (Deybach, et al., 2006). The EPI web page (www.porphyrria-europe.com) is constantly updated and important information is made available.

Table 2 Classification of porphyrias

Porphyria	Deficient enzyme	Classification	Inheritance	Clinical	Mutations (HGMD)	Symptoms
ALAD deficiency	ALAD	acute	autosomal recessive	hepatic	12	neurovisceral
Acute intermittent porphyria	HMBS	acute	autosomal dominant	hepatic	309	neurovisceral
Congenital erythropoietic porphyria	UROS	cutaneous	autosomal recessive	erythropoietic	38	photosensitivity
Porphyria cutanea tarda	UROD	cutaneous	sporadic / autosomal dominant	hepatic	103	photosensitivity
Hereditary coproporphyria	CPO	acute + cutaneous	autosomal dominant	hepatic	45	photosensitivity/ neurovisceral
Porphyria variegata	PPOX	acute + cutaneous	autosomal dominant	hepatic	149	photosensitivity/ neurovisceral
Erythropoietic protoporphyria	FECH	cutaneous	autosomal dominant	erythropoietic	120	photosensitivity

Acute intermittent porphyria

With the exception of South Africa and Chile, acute intermittent porphyria (AIP, MIM # 176000) represents the most frequent type of acute porphyria throughout the world (Hift and Meissner, 2005). This predominantly inherited autosomal dominant disorder, classified as acute hepatic porphyria, is characterised by a deficiency of HMBS, the third enzyme in heme biosynthesis. In such cases, the activity of the enzyme is decreased by about 50% in all tissues (Meyer, et al., 1972). A few cases of homozygous and compound heterozygous conditions have been reported, showing a HMBS activity as low as 2% of normal activity. Such cases manifest early in life, often during childhood, and are made obvious by a more severe clinical phenotype (Beukeveld, et al., 1990; Hessels, et al., 2004; Llewellyn, et al., 1992).

Based on biochemical analyses of enzyme activities in erythroid and non-erythroid tissues, AIP is classified as a classic or non-erythroid variant. In the classic variant, both isoforms are deficient, with half-normal activities in all tissues. But in about 5% of examined families, normal enzyme activity in erythrocytes and half-normal activity of the housekeeping enzyme was found, representing non-erythroid variant of AIP (Hessels, et al., 2004; Puy, et al., 1998; Whatley, et al., 2000).

The prevalence of symptomatic disease varies from 1 to 10 per 100 000, but due to frequent misdiagnosis and low penetrance this number may be much higher (Badminton and Elder, 2002; Meyer, et al., 1972; Strand, et al., 1970).

Expression of the disease is highly variable. It is determined in part by environmental, metabolic, and hormonal factors that share the ability to induce hepatic ALAS, and thereby to increase the production of porphyrin precursors, ALA and PBG.

Over 300 mutations in HMBS gene are known (<http://www.hgmd.cf.ac.uk>) (Stenson, et al., 2009). These mutations include one mutation in the promoter region, missense, nonsense, splicing and frame-shift mutations and in-frame deletions and insertions. Mutations are equally distributed along the HMBS gene and no particularly sensitive site for mutations has been identified. Regarding the prevalence of AIP in the Czech Republic, no statistical data exist. To date, 14 different mutations have been identified in the Czech and Slovak population: c.70G>A (p.Gly24Ser) (Puy, et al., 1997; Rosipal, et al., 1997), c.76C>T (p.Arg26Cys) (Kauppinen, et al., 1995), c.77G>A (p.Arg26His) (Llewellyn, et al., 1993), c.87+5G>T (r.spl?) (Luchinina, et al., 2005), c.158_159insA (p.Ile54HisfsX12) (Puy, et al.,

1997; Rosipal, et al., 1997), c.331G>A (p.Gly111Arg) (Gu, et al., 1993), c.518G>A (p.Arg173Gln) (Delfau, et al., 1990), c.610C>A (p.Gln204Lys) (Ulbrichova, et al., 2009), c.675delA (p.Ala226ProfsX28) (Ulbrichova, et al., 2009), c.750A>T (p.Glu250Asp) (Ulbrichova, et al., 2009), c.771+1G>T (r.spl?) (Rosipal, et al., 1997), c.799G>A (p.Val267Met) (Puy, et al., 1997; Rosipal, et al., 1997), c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10) (Douderova and Martasek, 2009) and c.965_966insA (p.Asn322LysfsX36) (Ulbrichova, et al., 2006) (**Table 3, Figure 4**). Some of them are described in this thesis.

Table 3 Mutations found in Slavic population

Mutation NA	Exon	Mutation AA	References
c.70G>A	3	p.Gly24Ser	(Puy, et al., 1997; Rosipal, et al., 1997)
c.76C>T	3	p.Arg26Cys	(Kauppinen, et al., 1995)
c.77G>A	3	p.Arg26His	(Llewellyn, et al., 1993)
c.87+5G>T	IVS3	(r.spl?)	(Luchinina, et al., 2005)
c.158_159insA	4	p.Ile54HisfsX12	(Puy, et al., 1997; Rosipal, et al., 1997)
c.331G>A	7	p.Gly111Arg	(Gu, et al., 1993)
c.518G>A	10	p.Arg173Gln	(Delfau, et al., 1990)
c.610C>A	10	p.Gln204Lys	(Ulbrichova, et al., 2009)
c.675delA	12	p.Ala226ProfsX28	(Ulbrichova, et al., 2009)
c.750A>T	12	p.Glu250Asp	(Ulbrichova, et al., 2009)
c.771+1G>T	12	(r.spl?)	(Rosipal, et al., 1997)
c.799G>A	13	p.Val267Met	(Puy, et al., 1997; Rosipal, et al., 1997)
c.899_900delinsTGCCTGCATCTG	14	p.His300LeuFsX10	(Douderova and Martasek, 2009)
c.965_966insA	15	p.Asn322LysfsX36	(Ulbrichova, et al., 2006)



Fig 4 Schematic representation of the HMBS gene with mutations found in Slavic population

Localization of mutations in exons and intron, marked by red circle, clearly demonstrates wide heterogeneity of AIP and the fact that no DNA variation hot spot site for mutations within HMBS gene in Slavic population was found as mutations are evenly dispersed throughout the gene. ATG= translational start site, AATAAA= polyadenylation signal.

Clinical manifestation

The manifestation of the disease is associated with an acute neurological syndrome characterised mainly by an acute attack (Albers and Fink, 2004; Meyer, et al., 1998). The porphyric manifestation usually occurs in the third life decade, though there is the rare occurrence before puberty or after menopause (Elder, et al., 1997). Clinical expression, in general, is highly variable and ~90% of AIP heterozygotes remain asymptomatic through life (Elder, et al., 1997; Petrides, 1998). Individual gene carriers differ from each other in both biochemical and clinical manners. Symptoms may be recurrent and last from a few days to a few weeks, or they may occur as a single sporadic incident in a lifetime.

Acute attacks are manifested by a wide variety of clinical features including: autonomic neuropathy with arrhythmias, central nervous system impairment, peripheral motor neuropathy with muscular weakness and paresthesia or less common sensory symptoms, and most commonly a severe pain caused by neurovisceral crises, usually of abdominal or back origin (Meyer, et al., 1998; Nordmann and Puy, 2002). This general symptom is often associated with nausea, vomiting and constipation. Complications usually include convulsion, hypertension, seizure, tachycardia and hyponatraemia. Hypomagnesaemia can also occur representing another electrolyte abnormality accompanying this disorder (Liamis, et al., 2007). Among psychiatric disturbances, symptoms widely range from minor behavioural changes such as irritability, restlessness, insomnia, anxiety or depression to more severe agitation, hysteria, psychosis with hallucinosis or even delirium (Crimlisk, 1997; Millward, et al., 2001; Regan, et al., 1999). An unmistakable symptom of an acute attack, which can often lead to the correct diagnosis, is the occurrence of red or dark-coloured urine resulting from high concentrations of porphyrins and porphyrin precursors.

Acute attacks can be potentially life-threatening, especially when respiratory paralysis occurs (Goldberg, 1959). In patients with severe AIP, hepatocellular cancer may be a potential and serious complication (Kauppinen and Mustajoki, 1988). The chronic complications include the development of renal disease which eventually leads to kidney failure (Andersson, et al., 2000).

Triggering factors of an acute attack

Clinical presentation appears to require additional factors that affect the heme pathway by increasing demand for heme, by causing an additional decrease in enzyme activity, or by combination of both these factors. This is made possible by the mechanism probably common to all triggering factors, namely the ability of multiple agents to activate the nuclear receptors or their co-activators, responsible for initiating transcription of the ALAS1 gene (Thunell, 2006). These factors include genetic and environmental conditions. The environmental factors can be divided into two main groups: exogenous and endogenous agents. Of the exogenous factors, porphyrinogenic drug are of great importance, since AIP was determined to be pharmacogenetic (Moore and Hift, 1997; Tschudy, et al., 1975). Many common medications which have an effect on either induction of hepatic cytochrome P450, or destructive effect on heme or on inhibition of heme synthesis can provoke an acute attack (McColl and Moore, 1981). Lists of safe and unsafe drugs and recommendations in managing the use of hormonal contraception (Andersson, et al., 2003) and local anaesthetics (Jensen, et al., 1995) are available on the website of a platform European Porphyrria Initiatives (www.porphyrria-europe.com) (Deybach, et al., 2006). Other exogenous agents include tobacco, cannabis, alcohol, organic solvents, terpenes, biocides, infection, major surgery, restricted carbohydrate and calorie intake and various kinds of stress, possibly including oxidative stress (Albers and Fink, 2004; Anderson, et al., 2005; Bonkovsky and Barnard, 2000; Bonkovsky, et al., 1992; Hift and Meissner, 2005; Kauppinen and Mustajoki, 1992; Moore and Hift, 1997; Thunell, et al., 1992). Of the endogenous factors, the use of steroid hormones and the fluctuations in female sex hormones are particularly important, and together explain the higher frequency of disease manifestation in woman (Andersson, et al., 2003). Although it appears that an individual's genetic background can influence susceptibility to acute attacks, the genes involved have not yet been identified.

Pathogenesis of porphyric neuropathy

The symptomatology of porphyric neuropathy consistently includes histological changes in peripheral and autonomic nerves. These changes involve demyelisation, axonal disruption, axonal vacuolisation and degeneration, central chromatolysis, muscle denervation, decreased

motor nerve conduction velocities, reddish fluorescence in white matter and EEG abnormalities (Albers and Fink, 2004; Lin, et al., 2008; Pischik and Kauppinen, 2009).

Despite our advanced understanding of the molecular and biochemical basis of AIP, the mechanism of the development of transient neurovisceral symptoms of AIP remains unclear. Many theories concerning the pathophysiology have been proposed (Meyer, et al., 1998). At present, we believe that more than one mechanism can give rise to neurovisceral symptoms.

One of the main hypotheses suggests the potential depletion of heme that develops in liver and possibly in neuronal tissue during an acute attack. This may impair critical cell processes dependent on hemoprotein formation (Bonkovsky, 1993; Watson, 1975). It has been revealed that certain drugs have the ability to increase demand in hepatic heme by inducing the biosynthesis of cytochrome P450 enzymes that utilise heme as a prosthetic group, in some cases by as much as 40-50-fold in the liver (Albers and Fink, 2004). Another supporting theory is that some of the cerebral manifestations of the disease are due to decreased functioning of signalling molecules such as nitric oxide (NO) produced by nitric oxide synthase or cGMP due to lack of guanylate cyclase, which are another hemoproteins (Kupferschmidt, et al., 1995). The decreased production of NO may explain some of the cerebral manifestations such as vasospasm (Kupferschmidt, et al., 1995; Sze, 1996). Other neurological effects are suggested to be caused by the energy production level in the brain, since heme is an essential component of the mitochondrial electron transport chain and critical to aerobic metabolism and adenosine triphosphate (ATP) production (Yeung Laiwah, et al., 1987). Interestingly, there is growing evidence of an additional globin protein containing heme called neuroglobin. Together with hemoglobin and myoglobin, known for transport of oxygen in blood and muscle respectively, this new protein has a high affinity for oxygen. It is possible that impairment of heme metabolism may lead to an aberrant neuroglobin pathway (Burmester, et al., 2000).

Other findings suggest that some of the neurological symptoms may be secondary effects of heme deficiency. In the case of decreased function of the hepatic tryptophan pyrrolase, a heme-dependent enzyme which controls tryptophan degradation, tryptophan levels in blood increase, and tryptophan is then quickly converted into serotonin and may cause an unbalance of neuro-transmissional processes in the brain (Litman and Correia, 1985). In agreement with this idea, some tryptophane metabolite excretion in the urine of patients with a porphyria attack has been detected, disappearing after administration of heme-arginate (Puy, et al., 1993). This hypothesis on heme depletion in the system is supported by the fact that simple

compensation of the absent heme in the form of heme-arginate is sufficient to control the condition of an acute attack (Kordac and Martasek, 1986; Mustajoki, et al., 1986).

Nevertheless, the leading hypothesis postulates that the symptoms of acute hepatic porphyria are caused by the neurotoxicity of high levels of accumulated ALA and PBG precursors, primarily in neuronal tissues. Under special circumstances and in combination with the deficient HMBS enzyme, heme biosynthesis accelerates through the induction of ALAS1 transcription. Under these conditions, the aberrant enzyme may become rate-limiting. Furthermore, due to negative feedback control, a reduced heme pool may cause additional induction of ALAS1. This may result in the pathological accumulation of metabolites at the impaired biosynthetic step (**Figure 5**).

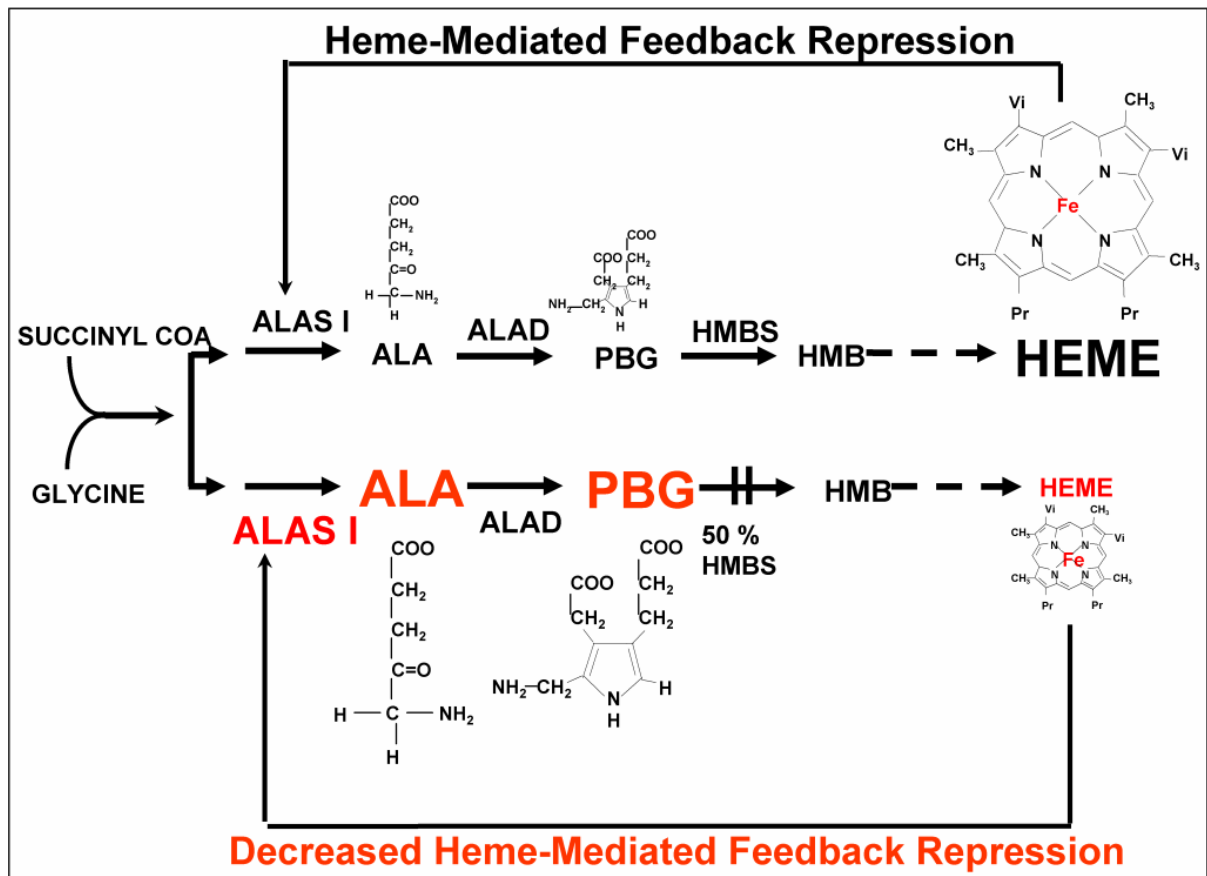


Figure 5 The mechanism of an acute attack

In the presence of HMBS deficiency, availability of the heme from the regulatory heme pool in hepatocytes decreases. It leads to the acceleration of the synthesis due to the heme-mediated feedback repression and, therefore, to the porphyrin precursor overload; Pr= $-\text{CH}_2\text{CH}_2\text{COOH}$, Vi= $-\text{CH}=\text{CH}_2$ (scheme according to (Anderson, 2001)).

It has been well documented that the acute attacks of AIP are accompanied by an increase in porphyrin precursors, ALA and PBG, which accumulate in different tissues such as liver, kidney and cerebrospinal fluid (Aarsand, et al., 2006; Anderson, 2001; Kauppinen, et al., 1995; Kauppinen and von und zu Fraunberg, 2002; Miyagi, et al., 1971; Solis, et al., 2004; Sweeney, et al., 1970; Thunell, 2000; Thunell, et al., 2000). The neurotoxicity of ALA may have a structural basis. One theory suggests that due to similarities in structure, ALA competes with the inhibitory neurotransmitter gamma-amino-butiric-acid (GABA) and excitatory glutamic acid for receptor binding in synaptic plasma membranes (Albers and Fink, 2004; Brennan and Cantrill, 1979; Meyer, et al., 1998; Yeung Laiwah, et al., 1987) (**Figure 6**). This could explain some of dysfunctions in the central nervous system. ALA, undergoing metal-catalysed oxidation, can also mediate lipid peroxidation and induce iron release from ferritin in the liver (Oteiza, et al., 1995). This and other similar principles may be responsible for oxidative damage (Hermes-Lima, et al., 1992). In addition, autoxidation of ALA generates reactive oxygen species (oxygen radicals) that produce oxidative damage on the membranes of the CNS (Batlle, 1993). In the peripheral nervous system it has been suggested that the neuropathic effects of ALA are attributable to its pro-oxidant properties which damage myelinating Schwann cells (Felitsyn, et al., 2008). This hypothesis on neurotoxicity is supported by the fact that other disorders, such as ALAD deficiency, tyrosinaemia type I and lead poisoning all resemble the neurological symptoms of acute porphyria, and share the symptom of increased levels of ALA. On the other hand, this underestimates the possible toxicity of PBG since no increase in PBG levels was observed in such cases (Anderson, 2001). There have been several studies involving the administration of ALA to humans (Dowdle, et al., 1968; Mustajoki, et al., 1992) and to rodents (Edwards, et al., 1984), which failed to reveal any effects linked to these symptoms. These findings are further supported by the observation that many patients excrete constantly excessive amounts of PBG and ALA and still remain asymptomatic (Harper P, 2003). However, direct administration of ALA to cultured cells of various origins and species have demonstrated neurotoxic effects (Meyer, et al., 1998). Moreover, from the observation of a special case of a homozygous AIP patient, the results of full-scale neurological and neuroradiological findings suggest that the neurological manifestations result from porphyrin precursor toxicity (Solis, et al., 2004).

A mouse model of AIP, in which a partial deficiency of HMBS was created, was produced by homologous gene targeting. This animal exhibits some of the neurologic symptoms, such as impaired motor function, ataxia, and increased levels of ALA in plasma

and brain, as well as decreased heme saturation of liver tryptophan pyrrolase. The study of this mouse model supports the hypothesis that nerve damage results from heme depletion as opposed to the neurotoxic effect of porphyrin precursors (Johansson, et al., 2003; Lindberg, et al., 1999; Lindberg, et al., 1996). Clearly, many details of pathogenesis of porphyric neuropathy still remain unresolved and await further explanation.

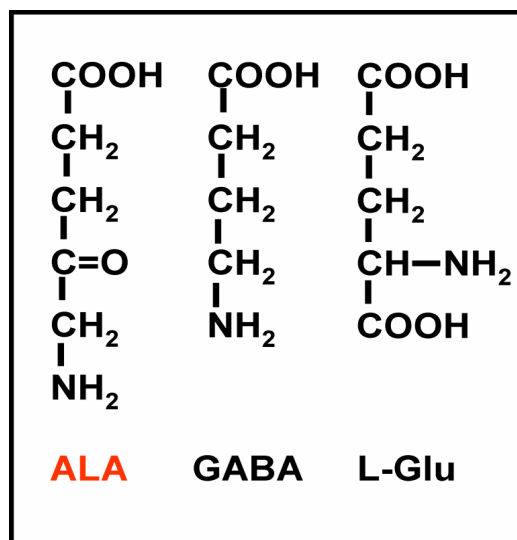


Figure 6 Structural similarity between aminolevulinic acid (ALA) and gamma-aminobutyric acid (GABA) and glutamic acid (Glu)

Diagnosis

Since acute porphyric attack can be potentially life-threatening, prompt and proper diagnosis of AIP heterozygotes is crucial to prevent attacks in both symptomatic and asymptomatic carriers. However, the precise diagnosis of AIP can be precluded by several eventualities. At an early stage, consideration of porphyria as a potential diagnosis is crucial, since life-threatening aspects of acute attacks arise mainly from misdiagnosis followed by inadequate treatment or late diagnosis. Diagnosis is not always transparent, as patients suffering from one of the acute porphyries often develop nonspecific clinical features mimicking other diseases which are more common. Such cases with similar symptoms include appendicitis, pancreatitis, gallstones, encephalitis, and Guillain-Barre syndrome above all (Crimlisk, 1997; Poblete Gutierrez, et al., 2001). Moreover, correct diagnosis can be complicated by the low penetrance of the disease, since only approximately 10% of those who inherit AIP will develop symptoms (Elder, et al., 1997; Petrides, 1998).

Traditionally, the diagnosis of porphyria is made on the basis of clinical symptoms, characteristic biochemical findings and enzyme assays. Once acute porphyria is suspected based on clinical symptoms, specific types should be distinguished, as different types of porphyria often reveal overlapping clinical and/or biochemical findings. This is especially true in case of AIP, HCP and ALAD deficiency (Kauppinen, 2005). Since accumulated heme precursors differ in precursor type and concentration level according to type of porphyria, the measurement of urinary, fecal, and plasma porphyrins, enzymatic activity in erythrocytes and the analysis of the excretion of and the fluorescence pattern of the plasma porphyrins should be performed (Bonkovsky and Barnard, 1998; Deacon and Elder, 2001; Sassa, 2006).

Of the biochemical findings, the marked increase in urinary PBG accompanied by an increase in ALA levels and some other porphyrins are common features for AIP, HCP, and VP. These precursors, essential for the diagnosis, are highly elevated during an acute attack and high excretion of these metabolites is often detected in the asymptomatic phase. During acute attacks, the first measurement of PBG is generally increased from 25 to 100 times the upper limit of the normal control (Anderson, et al., 2005). In AIP patients, the urinary porphyrins, uro- and coproporphyrin, are usually moderately increased, predominantly uroporphyrin I and III. In the plasma and fecal porphyrin levels, the excretion is usually normal or slightly increased only. In clinically asymptomatic carriers, only about one-third present a slight increase in excretion of PBG (two to five times the normal level), but others can show normal excretion of this precursor (Nordmann, et al., 1999). This means that asymptomatic AIP carriers are rarely detected through the measurement of urinary and fecal porphyrin precursors. The porphyrin measurement results often display a high variability primarily due to significant overlap between measurements made in patients, in clinically asymptomatic carriers, and in normal control individuals. The results of the porphyrin analysis, therefore, are not always conclusive (Bonaiti-Pellie, et al., 1984; Grandchamp, et al., 1996; McColl, et al., 1982; Pierach, et al., 1987). Nowadays, the biochemical detection of porphyrin precursors have been implemented as the first step in analysis of clinical syndromes and of the severity of the disease, e.g. in detecting acute or latent phase (Sassa, 2006).

Measurement of erythrocyte HMBS activity is another component of laboratory diagnostic tools. In most of the AIP patients, HMBS activity is approximately half of the normal activity (Meyer, et al., 1972). Some complications in the measurement of HMBS activity arise from the fact that a measurement of normal erythrocyte activity does not completely exclude AIP, since this enzymatic activity is normal in the case of the non-

erythroid variant of AIP (Gross, et al., 1996; Mustajoki, 1981). Another complication is that the range for values measured is wide and overlaps (Erlandsen, et al., 2000; Gross, et al., 1997). Moreover, the enzymatic activity in erythrocytes is highly age-dependent (Anderson, et al., 1977), and, in some cases, during an acute attack this activity can be normal (Kostrzewska and Gregor, 1986). Therefore, measurement of erythrocyte HMBS activity is still used as a diagnostic tool, but has a lower diagnostic value in screening for AIP in patients with acute symptoms (Anderson, 2001).

Due to inaccuracies in biochemical diagnostic tools, in recent years, several DNA-based screening molecular techniques have been included in the clinical diagnostic process as the final step to confirm the gene carrier status (Frank and Christiano, 1998; Grandchamp, et al., 1996; Kauppinen, 2004; Sassa, 2006). The search for the disease-causing mutation in each affected family is an important tool for individualised medicine, allowing for careful drug prescription and acute attack prevention. The molecular techniques are not only important in diagnostics, but also increase the deep insight into genes and their function in the field of science.

Treatment

An acute porphyric attack requires immediate intervention because it is known to be a life-threatening event and may result in serious neurological damage. Due to modern therapeutic options, the mortality rate nowadays is low.

In management of the porphyric attack, which usually requires hospitalisation, treatment is focused on a specific cure of symptoms and complications. This involves disease-specific therapy, and identifying and removing precipitating factors of an acute attack.

Of the specific therapies, the administration of intravenous heme followed by large amounts of carbohydrates is considered the most powerful treatment (Anderson, et al., 2005; Anderson, 2001; Bonkovsky and Barnard, 2000; Handschin, et al., 2005; Li, 2005; Stein and Tschudy, 1970; Watson, et al., 1978). This restores the regulatory heme pool and therefore suppresses hepatic ALAS1 induction and reduces the overproduction of ALA and PBG precursors. In an event of an acute attack, the heme therapy should be initiated as soon as possible (Mustajoki and Nordmann, 1993).

There are two types of heme derivatives available for use to suppress severe acute attacks - hematicin (Panhematin®, Abbott Laboratories) and heme-arginate (Normosang, Orphan

Europe). Since the early 1970's acute attacks have been treated with hematin (heme hydroxide) preparations (Dhar, et al., 1975). This lyophilised substance is preferentially used by the American population. But, due to its low stability and the evidence of some side effects such as transient anticoagulant effects and thrombophlebitis, a new compound - heme-arginate - with improved qualities, was introduced in Finland and is currently widely used across Europe and South Africa (Bonkovsky, 1993; Goetsch and Bissell, 1986; Mustajoki, et al., 1986; Tenhunen and Mustajoki, 1998; Tenhunen, et al., 1987). Heme-arginate, which is composed of human hemin and L-arginate as an additive, was created to improve both stability and solubility. In addition, no significant changes in coagulation, fibrinolysis and thrombophlebitis or other side effects were observed (Badminton and Elder, 2002; Mustajoki and Nordmann, 1993; Tenhunen and Mustajoki, 1998). When administered, it is used as a short time infusion (15-20 min) in a dosage of 3 mg per kg of bodyweight a day over a period of four days (Mustajoki and Nordmann, 1993). It is not recommended for use of longer than seven days, since long-term prophylactic use of heme-arginate for those having multiple recurrent attacks is being discussed (Tenhunen and Mustajoki, 1998).

However, one disadvantage of the repeated treatment of heme derivatives is the synchronous induction of a crucial enzyme in the heme degradation pathway, heme oxygenase. This may reduce the efficacy of the treatment; therefore some trials of inhibitors of heme oxygenase have been performed. Preliminary observations are promising, but there is still great concern about the drugs' side effects (Elder and Hift, 2001).

In parallel with specific heme therapy, the monitoring of neurologic complications, respiratory function and electrolyte imbalance, identification of and removal of precipitating factors, as well as symptomatic treatment should not be underestimated. Symptomatic treatment usually involves the use of drugs such as narcotic analgesics for abdominal and extremity pain, and phenothiazine for nausea, vomiting and acute psychiatric conditions. Drugs should be selected with great respect to the porphyrinogenicity of such substances. Approximately 1000 therapeutic drugs categorised with regard to porphyrinogenicity are presented on the internet (<http://www.drugs-porphyrin.org>) (Thunell, et al., 2007).

For some patients with severe recurrent attacks and no means of accessible treatment, liver transplantation may be an option (Seth, et al., 2007; Soonawalla, et al., 2004).

Of similar importance as the accurate treatment of acute attacks is the general knowledge to prevent acute attacks and genetic counselling within the families. The detailed procedure of treatment and diagnosis of porphyria has been summarised in several reviews (Anderson, et al., 2005; Kauppinen, 2005).

Gene

Human HMBS is determined by a single gene located on chromosome 11 (Meisler, et al., 1980), assigned to the locus to the long arm in the segment 11q24.1-q24.2 (Namba, et al., 1991). HMBS gene is divided into 15 exons ranging from 39 to 438 bp and 14 introns ranging from 87 to 2913 bp in length and, spans approximately 10 kb of DNA (Chretien, et al., 1988; Yoo, et al., 1993). All exon/intron boundaries conformed to the GT/AG rule (Breathnach and Chambon, 1981) are consist with the 5' and 3' consensus sequence for splice junctions of transcribed genes (Yoo, et al., 1993). Six Alu repetitive elements were identified in or adjacent to the HMBS gene, which makes this 10-kb gene relatively Alu-rich, as these repetitive elements occur on the average every 4kb across the genome (Hwu, et al., 1986; Yoo, et al., 1993).

The HMBS gene was the first gene ever described to be a single gene having dual purpose of being encoded by mRNAs transcribed from two promoters, a housekeeping and an erythroid-specific (Grandchamp, et al., 1987; Chretien, et al., 1988). The housekeeping promoter is in the 5' flanking region and its transcript is encoded by exons 1 and 3 through 15. The erythroid-specific promoter is located 3 kb downstream from the housekeeping promoter in the first intron and its transcript is encoded by exons 2 trough 15 (Grandchamp, et al., 1987; Gubin and Miller, 2001; Chen, et al., 1994) (**Figure 7**).

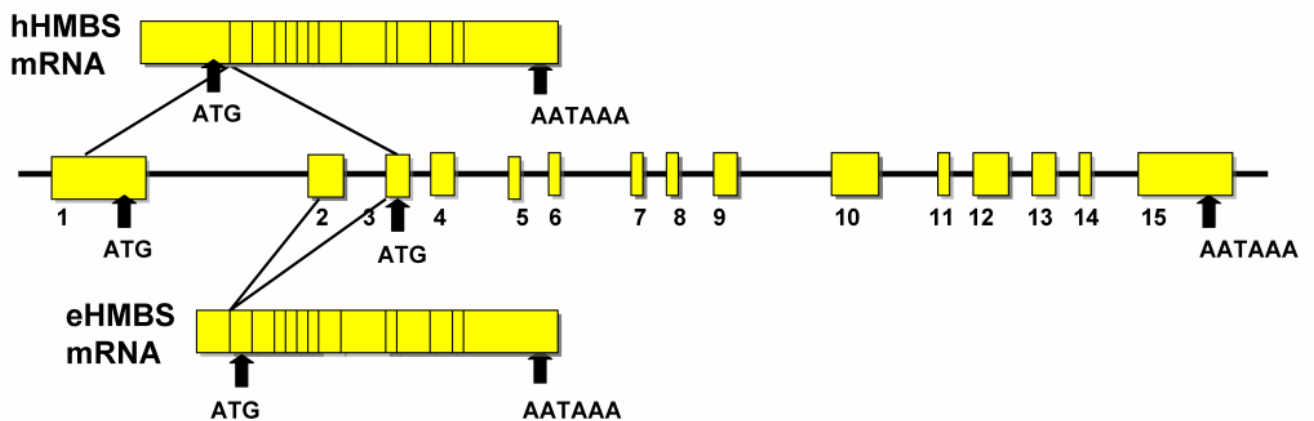


Figure 7 Genomic organization of the HMBS gene and alternative splicing of the transcripts

Tissue-specific expression of the HMBS gene is under control of two separate promoters: housekeeping promoter (hHMBS) and erythroid-specific promoter (eHMBS). The housekeeping transcript is encoded by exons 1 and 3 through 15. The erythroid-specific transcript is encoded by exons 2 trough 15. ATG= translational start site, AATAAA= polyadenylation signal.

The housekeeping promoter has certain features characteristic of housekeeping promoters, while comparison of the human beta-globin genes with the erythroid-specific promoter of HMBS gene suggests a modular organization, which might be common to a set of erythroid-specific promoters (Chretien, et al., 1988; Mignotte, et al., 1989a; Raich, et al., 1989). Several putative transcriptional factor binding sites for both promoter regions have been suggested based on sequence similarities (Yoo, et al., 1993). The sequence of housekeeping promoter region of human HMBS gene is GC rich and displays neither the classical TATA box, nor the CAAT box (Benoist, et al., 1980). Computer-assisted analysis for housekeeping gene region revealed several putative binding sites for a variety of transcriptional factors including AP1, AP4, thyroid responsive element, core enhancer element, SP1 element binding sites and CAC box (Yoo, et al., 1993). All the housekeeping genes bearing this modular organization (no TATA and CAAT, but cluster of SP1 binding sites) are either regulated during the cell cycle (Farnham and Schimke, 1986) or subjected to the feedback regulation by a product of the biosynthetic pathway in which they are involved (Luskey, et al., 1983).

The structural organization of the erythroid promoter of HMBS gene is very similar to that of beta-globin gene promoters, containing several separate sequence elements necessary for efficient transcription. There are at least four known motives common for beta-globin promoter region and erythroid promoter region of HMBS gene being very similar, i.e. ATAAAA box, CAAT box, CAAC box and CAC box (Chretien, et al., 1988). Furthermore, erythroid-specific trans-acting factors, two NF-E1 binding sites and one NF-E2 binding site, were identified (Frampton, et al., 1990; Chretien, et al., 1988; Mignotte, et al., 1989a; Mignotte, et al., 1989b). Nuclear factor NF-E1, which is now termed as GATA-1, is erythroid-specific trans-acting factor that recognizes sequence in erythroid promoter (Mignotte, et al., 1989a; Mignotte, et al., 1989b). A sequence with matching to the consensus CAC box (CACCC motif) was found close to the GATA-1 binding site in erythroid-specific promoter region of HMBS. It was suggested that CAC-GATA-1 association is probably involved in erythroid-specific initiation, since no consensus TATA box was found (Frampton, et al., 1990; Mignotte, et al., 1989a). Another trans-acting factor NF-E2, erythroid-specific enhancer binding protein, was suggested to cooperate together with GATA-1 in correct regulation of this promoter in erythroid cells (Mignotte, et al., 1989a; Mignotte, et al., 1989b). Computer-assisted analysis showed other putative regulatory elements like NF-1, AP1, AP4 and topoisomerase binding site (Yoo, et al., 1993). The expression of beta-globin and HMBS mRNA are among the earliest events characterizing

erythroid cell differentiation and, their structural similarities indicate that both genes can be activated in a coordinate manner during this process (Fibach, et al., 1979; Mignotte, et al., 1989a).

Moreover, during the studies of erythroid cell gene expression patterns, another distinct erythroid-specific HMBS mRNA was found and the existence of this transcript was confirmed in primary tissue (Gubin and Miller, 2001). This alternative erythroid-specific transcript is encoded by exons 2 through 15. This finding suggests that the erythroid-specific mRNAs contain an alternate 5' region and that the erythroid-specific cell development is accomplished at the genomic level via multiple mechanisms, including shared patterns of gene organization, transactivation, and RNA maturation (Gubin and Miller, 2001). The existence of this second erythroid HMBS isoform has not been reported elsewhere after originally published, despite extensive analyses of this gene over years. The linkage between the system and function of this third transcript of HMBS gene should be further investigated.

Protein

Hydroxymethylbilane synthase (also known as porphobilinogen deaminase or uroporphyrinogen I synthase, EC 4.3.1.8 or EC 2.5.1.61), is the third enzyme of the heme biosynthetic pathway. This monomeric protein with a single catalytic active site (Louie, et al., 1992) is organised into three domains approximately equal in size (Louie, et al., 1996). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isoenzymes have been isolated and characterised (Grandchamp, et al., 1987; Raich, et al., 1986). The housekeeping isoform of the protein consist of 361 amino acids, with an additional 17 amino acid residues at the N-terminus compared to the erythroid variant of 344 amino acids (Grandchamp, et al., 1987; Raich, et al., 1986). Both isoforms catalyse the same reaction. HMBS from several different species has been studied and its enzymatic kinetic properties have been identified. For HMBS isolated from human erythrocytes, optimal pH at 8.2 and K_m 6 μ M was determined (Anderson, 1980; Jordan and Shemin, 1973; Miyagi, et al., 1979).

In the HMBS reaction, the enzyme cooperates together with uroporphyrinogen III cosynthase to form uroporphyrinogen III from PBG (**Figure 8**). At the same time, the enzyme catalyses the formation of its dipyrromethane (DPM) prosthetic group. In the first catalytic cycle, the apo-protein catalyses step-wise head-to-tail polymerisation of six units of PBG into an enzyme-bound straight-chain haexapyrrole, before a hydrolytical cleavage event

releases the tetrapyrrole intermediate hydroxymethylbilane (Battersby, et al., 1979). The intermediate is rapidly converted into uroporphyrinogen III, closing the tetrapyrrole ring. A DPM cofactor, which is not subjected to catalytic turnover, remains covalently bound to the enzyme to form the active holo-enzyme (Jordan, et al., 1988; Shoolingin-Jordan, 1995). The apo-enzyme of HMBS is unstable and heat-labile while the holo-enzyme is much more stable and resistant to heat (Awan, et al., 1997).

The catalytic reaction proceeds through four covalent enzyme-intermediate complexes, ES, ES₂, ES₃ and ES₄, which are sequentially generated during the course of the tetrapolymerisation. These are stable to various degrees and are isolable (Lander, et al., 1991; Warren and Jordan, 1988). There is evidence that the relative movement of the domains may be important during the catalytic cycle to accommodate the intermediates (Lander, et al., 1991; Warren and Jordan, 1988). Only the monopyrrolic porphobilinogen, and not di- or tripyrroles, can be efficiently used as a substrate (Louie, et al., 1996).

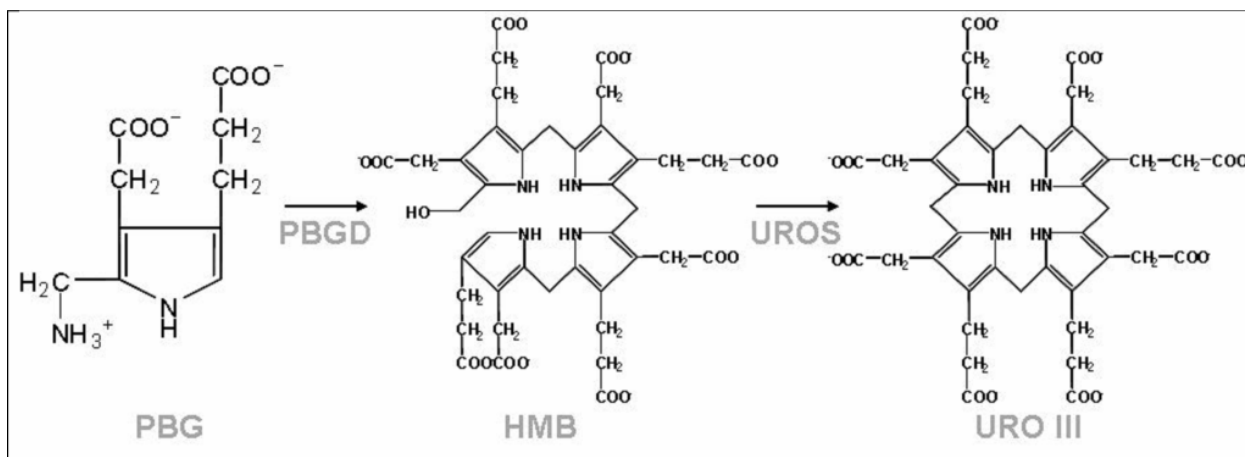


Figure 8 Enzymatic reaction of the hydroxymethylbilane synthase

In rapid conversion of porphobilinogen (PBG) into the uroporphyrinogen III (URO III), two enzymes cooperate. The first enzyme, hydroxymethylbilane synthase (HMBS), catalyzes the condensation of four molecules of PBG to form a linear unstable tetrapyrrole, hydroxymethylbilane (HMB). HMB is then enzymatically converted by uroporphyrinogen III synthase (UROS) to a cyclic tetrapyrrole, uroporphyrinogen III.

Relatively high amino acid sequence conservation is found in HMBS and its homologs, amounting to at least 32% in the proteins from bacteria, fungi, plants and mammals. It has been reported that the *E. coli* and human HMBS amino acid sequences have 43% identity and more than 60 % similarity (Brownlie, et al., 1994; Jordan and Warren, 1987; Shoolingin-Jordan, et al., 2003). Several crystallographic structures of *E. coli* HMBS have been

determined (PDB 1gtk, 1pda (Helliwell, et al., 2003; Louie, et al., 1996; Louie, et al., 1992)) (Figure 9).

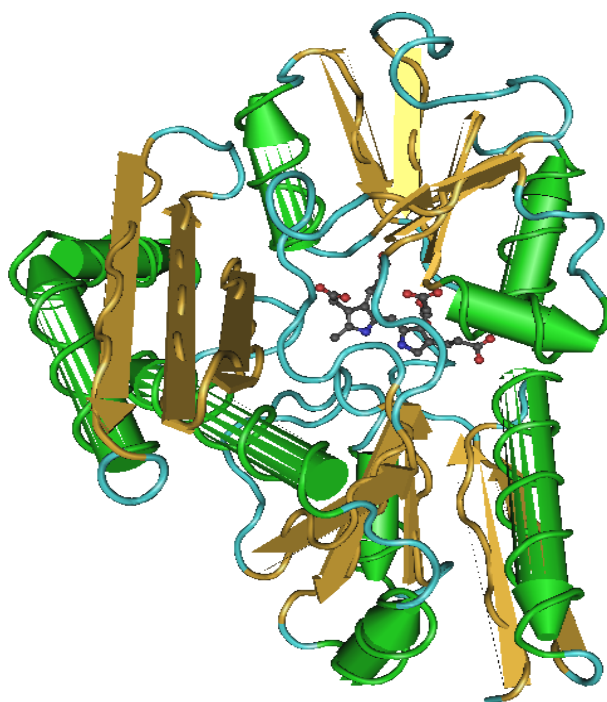


Figure 9 3D structure of the HMBS enzyme (PDB ID 1pda, (Louie, et al., 1992))

The monomeric protein with a single catalytic active site is organized in three domains approximately equal in size. The active site cleft contains the dipyrromethane cofactor.

Recently, the crystallographic structures of human housekeeping HMBS and human mutant protein Arg167Gln HMBS have been determined (PDB 3ecr and 3eq1; (Gill, et al., 2009; Song, et al., 2009)).

The best resolution attained so far for a three-dimensional structure of wild-type HMBS is a resolution of 1.66 Å for *E. coli* HMBS (Helliwell, et al., 2003) and of 2.2 Å for the human enzyme (Song, et al., 2009). Both the *E. coli* and human HMBS molecules have approximate dimensions of 57 x 43 x 32 Å, and have overall similar topology and other features in common (Gill, et al., 2009; Louie, et al., 1992). Approximately 38% of residues have been assigned a helical conformation, 25% an extended conformation, 8% turn, and 29% coil (Louie, et al., 1996). The polypeptide chain is folded into three domains of approximately equal size, each containing beta-strands and alpha-helices in secondary structure as well as a discrete hydrophobic core (Louie, et al., 1996; Louie, et al., 1992). Domain 1 consist of residues 20-115 and 214-238 (residues 3-99 and 200-217 in *E. coli*) and domain 2 consist of

116-213 (residues 105-193 in *E. coli*). These two domains are broadly similar in possessing the same structure of a doubly-wound, mainly parallel beta-sheet of five strands. The alpha-helical segments pack against each face of the beta-sheets. The C-terminal domain 3 comprising residues 239-356 (222-307 in *E. coli*) is an open-faced triple-stranded antiparallel beta-sheet with three alpha-helical segments covering one of the faces (Gill, et al., 2009; Louie, et al., 1992). Domains are connected by polypeptide chain connections. Besides the polypeptide-chain connections, there are only a few direct interactions between domains (Louie, et al., 1996). The active site cleft containing the DPM cofactor occurs at the large positively charged cleft region between domains 1 and 2 (Jordan and Warren, 1987).

Amino acid sequences from a wide range of organisms have been determined, and the 58 invariant residues (18% of the total protein) have been identified (Brownlie, et al., 1994). The majority of the invariant residues are clustered immediately around the active-site cleft. These are involved in the catalysis of the bond-making reactions, and in the formation of direct interactions with the DPM cofactor and the porphobilinogen substrate. Furthermore, the extensive network of hydrogen-bonding in this area may be of importance in stabilising the protein fold (Louie, et al., 1996). Two of these invariant amino acids are at the hot spot; the first is the cysteine Cys261 (Cys242 in *E. coli*) which is responsible for the covalent thioether bond of the DPM cofactor located on the loop of domain 3 and placed at the mouth of a deep cavity formed between the structurally related domains 1 and 2 (Brownlie, et al., 1994; Jordan and Warren, 1987; Shoolingin-Jordan, et al., 2003). The second invariant residue aspartate Asp99 (Asp84 in *E. coli*) creates hydrogen bonds with the cofactor, and was labelled as the key catalytic residue (Louie, et al., 1992). The acetate and propionate side groups of the DPM cofactor forms extensive salt bridge and hydrogen bond interactions with the HMBS protein and with bound water molecules. The basic side chains of Arg149, Arg150, Arg173, Ser96, Ser146, Ser147, Lys98, Asp99, Ala189 and Gly218 (Arg11, Arg131, Arg132, Arg149, Arg155, Lys83, Ser81, Ser129, Thr127 in *E. coli*) residues are, in particular, involved in these interactions.

In the human HMBS enzyme crystal structure, the DPM cofactor was found in the native reduced conformation with a hydrogen from a sulphate ion bonded to Arg26 and Ser28 at the proposed binding site (Gill, et al., 2009; Louie, et al., 1996). Analyses of the potential hinge region, which may assist in the opening and closing of the active site, revealed three amino acids Ser96, His120 and Lys238 as potential hinge residues (Song, et al., 2009).

The substrate is thought to interact with the positively charged side chains of Arg11, Arg149 and Arg155 at the binding site (Lambert, et al., 1994). The overall structure

possesses a large number of other ion pairs that may contribute to the considerable heat stability of the enzyme (Brownlie, et al., 1994; Louie, et al., 1992).

The main difference between the human and *E. coli* enzyme structures is that both the ubiquitous and erythroid human enzymes contain a 29-residue insertion in domain 3 and some other insertions in the loop regions as opposed to the *E. coli* structure (Brownlie, et al., 1994; Gill, et al., 2009). Although the insertion is located at a distance from the active site, it is expected to play a role in modulating enzyme conformational, which is associated with enzymatic action. There are only 148 identical residues between the human and *E. coli* structure, partly located around the active site and partly distributed evenly across the protein structure. Despite the many differences between the two structures at the amino acid level, the similarity in overall structure is achieved by subtle substitutions in the case of all substantial changes in side chains accompanied by compensatory changes and adjustments in adjacent residues (Gill, et al., 2009).

Structure-function correlations

To predict the impact of pathological mutations on the protein structure and function of human HMBS, the homology between human HMBS and the *E. coli* enzyme was used to build a structural model (Brownlie, et al., 1994). Now, the discovery of the human structure further facilitates an understanding of the structural correlations. No prevalent site for HMBS mutations has been determined; mutations are equally dispersed throughout the enzyme structure. Of these mutations, over 120 of them are missense/nonsense single base changes that result in one amino acid substitution or in the formation of a premature stop codon and subsequent protein truncation (<http://www.hgmd.cf.ac.uk>) (Stenson, et al., 2009). These mutations are of special interest, since single amino acid change can provide information on the functional or conformational importance of the wild-type residue.

Pathological mutations can be divided into three broad groups according to their molecular basis. The first group represents a change of amino acid that impacts on protein folding and stability. Such residues are usually located in close proximity to the hydrophobic core and in conformational restricted areas. The second group consists of residue changes with an effect on the binding, reaction and assembly of the DPM cofactor. If the DPM cofactor, important not only for the enzyme function, is absent in the enzyme structure, its stabilising ability is missing and the unstable apo-enzyme is therefore rapidly degraded.

Finally, the last group consists of mutations that affect the catalytic or substrate binding residues and thereby result in inactive proteins (Gill, et al., 2009; Song, et al., 2009).

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by capital letters (A-J).

A. Ulbrichova D, Flachsova E, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. 2006. *De Novo* mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study. *Physiol Res* 55 Suppl 2:S145-54. (IF2006 2.09)

B. Flachsova E, Verma IC, **Ulbrichova D**, Saxena R, Zeman J, Saudek V, Raman CS, Martasek P. 2007. A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family. *Folia Biol (Praha)* 53(6):194-201. (IF2007 0.6)

C. Schneider-Yin X¹, **Ulbrichova D**¹, Mamet R, Martasek P, Marohnic CC, Goren A, Minder EI, Schoenfeld N. 2008. Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria. *Mol Genet Metab* 94(3):343-6. (IF2008 2.63)

¹Both authors contributed equally to this work.

D. Ulbrichova D, Hrdinka M, Saudek V, Martasek P. 2009. Acute intermittent porphyria--impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties. *FEBS J* 276(7):2106-15. (IF2008 3.14)

E. Ulbrichova D, Schneider-Yin X, Mamet R, Saudek V, Martasek P, Minder EI, Schoenfeld N. 2009. Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria. *Blood Cells Mol Dis* 42(2):167-73. (IF2008 2.75)

F. Ulbrichova Douderova D, Martasek P. Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis. *In press in Anal Biochem* 2009 (IF2008 3.09)

G. Ulbrichova Douderova D, Schneider-Yin X, Lautenschlager S, Saudek V, Hofbauer GFL, Dziunycz PJ, Martasek P, Minder EI. **Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation.** *Prior to submission*

Report of the novel mutation identification

H. Ulbrichova D, Kurt I, Zeman J, Martasek P. 2008. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. *Hum Genet* 124(3):315. (IF2008 4.04)

I. Ulbrichova Douderova D, Mamet R, Munter G, Martasek P, Schoenfeld N. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. *Sent to Hum Genet* 2009 (IF2008 4.04)

J. Ulbrichova Douderova D, Zeman J, Martasek P. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. *Sent to Hum Genet* 2009 (IF2008 4.04)

AIMS OF THE STUDY

In general, the aim of this study was to enable the proper molecular diagnosis of AIP patients at the DNA level in order to investigate the structural-functional consequences of mutations at the protein level.

The specific aims were as follows:

- The molecular diagnosis of newly diagnosed AIP patients and the molecular diagnosis of affected families using an established method.
- The optimisation of the next generation diagnostic method, High-Resolution Melting, using the LightScanner instrument for detection of DNA variations in the HMBS gene.
- The expression, purification and biochemical characterisation of human mutant HMBS enzymes with introduced pathological mutations of interest in the prokaryotic (*E. coli*) system.
- The structure-function correlation studies - the assessment of detrimental effects of DNA variations on enzyme function.

RESULTS AND DISCUSSION

Publication A

De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study (Ulbrichova D et al., 2006)

The patient, a 15-year-old boy, was hospitalised while having his first acute attack, characterised by severe abdominal pain, hyponatraemia, cognitive failure, hypertension, tachycardia and subsequent neurological symptomatology, accompanied by the highly elevated porphyrin precursors ALA and PBG in urine, all clinical features typical of AIP.

The prescreening molecular method of denaturing gradient gel electrophoresis (DGGE) revealed a characteristic pattern indicating DNA variation in the HMBS gene. DNA sequencing confirmed the presence of a novel heterozygous mutation, the c.965_966insA. The molecular screening of the HMBS gene in family members was performed, but failed to reveal any DNA variation. This was in agreement with the fact that all of the other five family members from two generations were asymptomatic. Therefore, we suggest that 966insA is a *de novo* mutation since nonpaternity was excluded by DNA microsatellite analysis.

The mutation c.965_966insA is localised in exon 15 of the HMBS gene. At the protein level, it results in a frameshift and production of a STOP codon after expression of 36 completely different amino acids compared to the original sequence (p.Asn322LysfsX36). The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. Usually, the truncation is expected to lead to an unstable and inactive protein.

To investigate the impact of this small insertion mutation on the protein structure and to further study the functional consequences, we decided to express wild-type and mutant protein in the prokaryotic system. For the construct preparation, we used cDNA sequences obtained by reverse transcription of total RNA extracted from peripheral leukocytes. We cloned the cDNA for the HMBS protein into the pGEX-4T-1 expression vector. We used the *E. coli* BL21 (DE3) expression system. We then introduced the insertion mutation into the construct using site-directed mutagenesis. The HMBS proteins were expressed as GST-fusion proteins and purified by affinity chromatography using Glutathione Sepharose 4B columns. We performed the residual activity measurement.

In agreement with the premise that protein truncation would lead to instability, the SDS-PAGE analyses of the mutant protein revealed several bands, in contrast to a single homogenous band in the case of wild-type protein. Wild-type as well as mutant HMBS enzymes were similar in size, measuring approximately Mr 68 kDa with the GST-tag and Mr 42 kDa without the GST-tag. The purified mutant enzyme had a relative activity level of 0.18% of level achieved by wild-type enzyme. This result is in good agreement with the observation that the erythrocytic HMBS activity in the AIP-affected individual is decreased to about 50% of the healthy average, as the patient was heterozygous for the mutation.

We designed the mutant protein structure using the computer-assisted structure prediction program, using the 3D structure of *E. coli* HMBS as a template. The c.965_966insA mutation is localised in the β_3 sheet of domain 3. Due to the incurred frameshift, part of the third enzyme domain has a different formation; two helices α_2 and α_3 of domain 3 are completely missing compared to the wild-type protein. In the wild-type protein, the C-terminal helices protect the beta-strands from being exposed to solvent. This is in agreement with the severely decreased stability of the mutant HMBS.

In summary, the *de novo* mutation c.965_966insA (p.Asn322LysfsX36) was found in a young patient with AIP. Due to a truncated protein sequence with an abnormal C-terminus domain, this small insertion mutation c.965_966insA leads to an almost complete loss of the enzymatic function and decreases the stability of the protein. These results further broaden our understanding of the detrimental effects of mutations in a case of relatively early AIP manifestation. This case is of particular interest as the identification of a *de novo* mutation is a rare event.

In this study, we cooperated with Second Pediatric Department in Košice, Slovak Rep., and Laboratory of Structural Biology in Houston, USA. I contributed to this study by the cooperation on the identification of the proband's mutation in the HMBS gene using DGGE analysis and direct sequencing as well as by the DNA screening of his family members. I performed protein expression, purification, protein analyses and enzyme activity measurements.

Publication B

A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family (*Flachsova E et al., 2007*)

Our laboratory was contacted by a 50-years-old proband from Nepal who suffered from severe abdominal pain accompanied by dark urine. After considering the possibility of having acute intermittent porphyria, he, as a non-health professional, searched the internet for help with treatment and molecular diagnostic confirmation of his clinical status. He arranged to send samples to us- either blood or gDNA - from himself and from 15 members of his family.

Molecular testing revealed a novel heterozygous mutation c.972_973insG in exon 15 of the HMBS gene. Analysis of the protein sequence indicated that following the insertion mutation, four amino acids were different. After this, a premature stop codon occurred, leading to a prematurely truncated protein in which 44 amino acids of the C-terminus of HMBS was missing. Usually, and as observed in our previous study, the truncation was predicted to result in an unstable and inactive protein.

To further investigate the detrimental effects of this small insertion mutation on the protein structure, we decided to express the mutated gene in the prokaryotic system and to perform biochemical testing of the purified enzyme.

In agreement with our previous study results, the SDS-PAGE analyses of the mutant protein displayed again several bands, suggesting high protein instability. The purified mutant enzyme had an activity level 0.5% of the average wild-type level.

Using the computer-assisted structure prediction, the 3D structure of the mutant protein was designed. The c.972_973insG mutation is located in domain 3 in close proximity to the terminal helix, which is much more independent of the rest of the structure and points towards the solvent. The mutations in the penultimate helix or its removal destabilise the whole C-terminal domain; in turn, the N-terminal domain cannot fold in a stable unity and therefore the whole protein is destabilised. Even point mutations in the penultimate helix can lead to AIP, underlining the importance of this helix for enzyme stability.

After DNA screening in the proband's family members, the same mutation was subsequently found in 12 of them. In agreement with the observation that most AIP carriers are without symptoms throughout their life, 7 out of the 12 were asymptomatic.

In summary, a novel mutation c.972_973insG within the HMBS gene was identified in 12 members of an extensive Indian family from Nepal. This mutation results in a truncated and

highly unstable protein with loss of enzymatic function. The uniqueness of this case lies in the fact that the proband diagnosed himself based on information from the internet. This highlights the importance of the accessibility of online information especially in the case of rare diseases. Molecular screening within the proband's family was used for genetic counselling.

In this study, we cooperated with Department of Genetic Medicine, Sir Ganga Ram Hospital, New Delhi, India and Laboratory of Structural Biology in Houston, USA. I contributed to this study by the protein expression, purification, protein analyses and enzyme activity measurements.

Publication C

Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria (Xiaoye Schneider-Yin et al., 2008)

In this study, we report on mutational analysis and *in vitro* characterisation of HMBS variants identified in two individuals who were suspected of having AIP.

The first patient, a 17-year-old Ashkenazi Jewish female, had been experiencing menstruation-related recurrent episodes of severe abdominal pains accompanied by vomiting, tachycardia and hypertension for 3 years. Biochemical analyses failed to show elevations in urinary PBG and ALA levels during acute and latent phases. However, erythrocyte HMBS activity showed an average activity that was 60% of normal. The patient was treated with either glucose or heme-arginate (Normosang, Europe) for acute attacks. While lacking full AIP-confirming biochemical evidence, the diagnosis of acute porphyria was questionable.

The second patient, a 30-year-old Ashkenazi Jewish female, was suffering from recurrent acute attacks of abdominal pain, hyponatraemia and urinary retention for 12 years. Biochemical analyses showed increased urinary ALA and PBG, and reduced erythrocyte HMBS activities about 50% of normal. She was successfully treated with glucose.

After molecular screening of the HMBS gene, two novel heterozygous mutations c.176C>T in exon 5 and c.643G>A in exon 11 was identified separately in each subject. At the protein level, the mutation c.176C>T leads to an amino acid substitution p.Thr59Ile and the mutation c.643G>A leads to an amino acid substitution p.Val215Met. Both p.Thr59Ile

and p.Val215Met were absent in the HMBS gene of 50 non-porphyric Ashkenazi Jewish subjects.

We expressed both variant proteins in the prokaryotic system and we performed biochemical testing of the purified mutant enzymes. Recombinant p.Thr59Ile and p.Val215Met mutant enzymes had residual activity of 80.6% and 19.4%, respectively, compared to that of the wild-type enzyme.

While the clinical symptoms, *in vitro* and *in vivo* biochemical analyses, as well as the changes in Km, Vmax and thermostability observed in the mutant protein of the patient who carried p.Val215Met all suggested a causal relationship between p.Val215Met and AIP, the association between the p.Thr59Ile substitution and AIP is less obvious. In view of the results we received, despite the normal urinary excretion of ALA and PBG during acute attacks, the high residual activity (80.6%) of the recombinant enzyme and the similarities between p.Thr59Ile mutant and the wild-type enzyme in Km value and in the thermostability profile, we conclude that p.Thr59Ile might represent a mutation with a weak effect rather than a mere polymorphism. In contrast to the above-mentioned statements, there was some evidence supporting the association between the p.Thr59Ile mutation and the AIP phenotype, such as successful treatment of the clinical symptoms of AIP with glucose and Normosang. Moreover, the patient's erythrocyte HMBS activity was reduced to half-normal value, and considering the age of the patient (that is, under the age of 18), normal values of ALA and PBG may be observed during symptomatic periods. It may very well be that in this compound case, there is more than one causative factor with clinical relevance, which remains undetected.

In summary, two novel mutations c.176C>T (p.Thr59Ile) and c.643G>A (p.Val215Met) within the HMBS gene were identified. Despite not having full AIP-affirming biochemical evidence in one case, both mutations were associated with AIP. The study demonstrates that *in vitro* characterisation of mutations in the HMBS gene can add valuable information to the interpretation of clinical, biochemical and genetic data in establishing a diagnosis of AIP. It also highlights the fact that there are still many causative aspects to be investigated in AIP.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; National Laboratory for the Biochemical Diagnoses of Porphyrrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel; Department of Biochemistry, The University of Texas Health Science Center at San Antonio, USA; Department of Pediatrics, Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel; Department of

Human Molecular Genetics and Biochemistry, The Sackler Faculty of Medicine, Tel Aviv University, Israel. I contributed to this study by the identification of the proband's mutation c.176C>T (p.Thr59Ile) in the HMBS gene using direct sequencing and by expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication D

Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria (*Ulbrichova D et al., 2008*)

In this study, a total of 26 individuals from six unrelated Israeli AIP families of Caucasian origin underwent biochemical and mutational analysis in order to establish an AIP diagnosis. Variability with respect to the ALA/PBG levels and erythrocytic HMBS activity was found among the index patients. Each family carried a unique mutation in the HMBS gene.

Following the molecular screening of the HMBS gene, one novel heterozygous missense mutation c.95G>C (p.Arg32Pro) was shown to exist *de novo* in one family, along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA identified separately in each family.

We expressed p.Arg32Pro and p.Asp178Asn mutant proteins in the prokaryotic system, since the other missense mutations have been expressed and characterised in our previous study. We performed biochemical testing of the purified mutant enzymes. The structure-function consequences of all mutations were studied at the protein level. We analysed the correlation between biochemical findings, the impact of the mutation on the protein function, and the structural predictions of the affected individuals as part of the study.

Of the four missense mutations, p.Arg32Pro and p.Val215Met had not only detrimental effects on the enzyme *in vitro*, with residual activities of 1% and 19% respectively, but these mutations were also associated with high levels of ALA/PBG comparable with that of frameshift mutations c.730_731delCT and c.982_983delCA in patients. In addition, p.Val215Met was shown to be extremely thermo labile. Therefore we labelled them "strong" mutations. In contrast, the *in vitro* effect of both of the "weak" p.Thr59Ile and p.Asp178Asn mutations was much lower, as demonstrated by the relatively high residual activity of 81%. In accordance with this analysis, a common feature shared by these two patients with "weak"

mutations was their normal or borderline levels of ALA/PBG although they presented characteristic clinical symptoms.

All six HMBS mutations were evaluated at the structural level based on the 3D structure of the *E. coli* enzyme. Based on the 3D structure, the two “strong” missense mutations as well as the two frameshift mutations were all predicted to have detrimental effects on the structure and function of the enzyme either due to their location or due to the nature of the substitution. The two “weak” mutations on the other hand were located at less critical positions and therefore exerted limited impact on the structure and function of the enzyme.

In summary, one novel heterozygous mutation c.95G>C (p.Arg32Pro), along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA, were identified separately in each family. We performed an extensive *in vitro* characterisation of the proteins with the introduced mutations, including residual activity analysis and kinetic activity assays. The results of the *in vitro* study broaden our understanding of the impact of individual mutations on enzyme activity and consequently, our understanding of AIP disease.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; National Laboratory for the Biochemical Diagnoses of Porphyrrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel; Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech Republic; Department of Human Molecular Genetics and Biochemistry, The Sackler Faculty of Medicine, Tel Aviv University, Israel. I contributed to this study by the identification of the proband’s mutation c.95G>C (p.Arg32Pro) in the HMBS gene using direct sequencing as well as by the DNA screening of his family members. I performed expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication E

Acute intermittent porphyria - impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties (Ulbrichova D et al., 2009)

In the present study, six patients who were newly diagnosed with AIP were studied. Five of them were women. The diagnosis of AIP was made on the basis of clinical features typical for AIP and the excretion pattern of porphyrin precursors. The most prominent symptom in all patients was severe abdominal pain.

HMBS genes of all probands were screened for DNA variations by denaturing gradient gel electrophoresis (DGGE). Six samples with abnormal patterns suggesting mutations were detected. Direct DNA sequencing revealed seven mutations in these samples. Of the identified mutations, three were novel, c.610C>A (p.Gln204Lys), c.750A>T (p.Glu250Asp) and c.675delA (p.Ala226ProfsX28); and four mutations were previously reported c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln) and c.771+1G>T (r.spl?). One patient had two mutations, c.[518G>A; 610C>A], located in the same allele, which is a rare molecular defect in the HMBS gene. Overall, 33 individuals from proband's families were screened and nine carriers of an affected HMBS gene were identified.

To study the impact of the various mutations on the protein structure and subsequent functional consequences, mutated proteins were expressed in *E. coli* and the enzymatic properties were characterised. All the recombinant expressed and purified proteins displayed homogeneous bands when inspected by SDS-PAGE, except for two mutants, one with the small deletion mutation p.Ala226ProfsX28 as expected, and surprisingly with the mutation p.Glu250Asp. The residual enzymatic activity measurement of the HMBS proteins with mutant alleles revealed that, with the exception of the p.Gln204Lys mutation (which exhibited ~ 46% of wild-type activity), all mutations lead to little, if any, enzymatic activity. The observation of low residual activity for most mutations is consistent with the expected 50% decrease in final HMBS activity, which is observed in individuals with acute intermittent porphyria. These findings further support the AIP-causality of these mutations in the HMBS gene. In the case of the patient with two combined mutations, both located on the same allele, mutation p.Arg173Gln has a severe effect on enzyme function. From the additional testing of the protein properties, we concluded that the p.Gln204Lys mutation has a milder impact on protein function and structure, but can still be associated with AIP.

To further determine the structure–function relationships for these mutations, the 3D structure of the *E. coli* and newly-determined human proteins, as well as the sequence alignment of prokaryotic and eukaryotic HMBS nonredundant sequences was used. From the structure and sequence information, it can be inferred that the patient’s mutations of Arg26 to Cys or His may lead to the loss of interactions with the cofactor, which explains our observation of the near complete loss of enzyme activity. In the case of the Arg173 to Gln mutation, the change results in an apo form of the enzyme that is incapable of catalysis. Most likely, the mutant is unable to interact properly with the cofactor. In the case of the p.Gln204Lys mutant, the mutant enzyme exhibited ~ 46% of wild-type activity. This can be explained by the fact that the Gln204 residue is exposed on the surface of the central domain, remote from the active site of the protein. It is likely that the introduction of the positive charge of the lysine amino group changes the configuration of the two surface loops, which may destabilise the enzyme. The small deletion p.Ala226ProfsX28 causes a truncation which leads to an unstable and inactive protein, which is likely to be rapidly degraded by the proteasome. In mutant p.Glu250Asp, the Glu250 residue is conserved in all sequences with no exception. It creates an interaction that fixes the C-terminal domain to the interdomain hinge whose mobility is important for access of the substrate to the active site. Nevertheless, the effect of the new mutation p.Glu250Asp is unexpected because the change from Glu to Asp results only in a subtle change of the molecule structure: The abolition of the activity demonstrates the importance of exact geometry in the interior of the enzyme. In the case of such mutations as c.771+1G>T, the deletion of the entire exon 12 is expected, and the function of this mutant is expected to be completely abolished.

In summary, we identified four previously reported mutations c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln), c.771+1G>T (r.spl?); and three novel ones c.610C>A (p.Gln204Lys), c.675delA (p.Ala226ProfsX28), c.750A>T (p.Glu250Asp) in Czech AIP patients. Of particular interest, one patient had two mutations (c.518G>A; c.610C>A), both located in the same allele. We performed an extensive *in vitro* characterisation of the mutant proteins by introducing the mutation of interest and performing residual activity measurements, kinetic studies on recombinant enzymes, and structure–function correlations. These findings provided further insights into the causal relationship between HMBS mutations and AIP.

In this study, we cooperated with Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech

Republic. I contributed to this study by the identification of the mutations in the HMBS gene of AIP probands using DGGE analysis and direct DNA sequencing as well as by the DNA screening of all AIP families. I performed expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication F

Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis (*Ulbrichova Douderova D et al., in press in Anal Biochem 2009*)

In this report, we tested the high-resolution melting (HRM) procedure on the LightScanner instrument as a method of screening DNA variations in the polymorphic HMBS gene.

In the selected subjects tested, the diagnosis of porphyria was made based on the clinical features typical for each porphyria type and the related specific porphyrin excretion pattern. The samples were amplified in the presence of a 1x concentration of the saturating DNA dye LC-Green PLUS dye. Straight afterwards, the PCR plate was transferred to the LightScanner in which the HRM analyses were performed. Collected data were analysed with the commercial LightScanner software 1.5 with the Call-IT function. Identified DNA variations were confirmed by independent sequencing of the second PCR product.

To start off, we determined whether the presence of previously detected polymorphisms would adversely interfere with further testing of the gene using this method. In all four cases of polymorphisms consistently detected in our patients, we detected three discrete groups of genotypes (two homozygous and one heterozygous).

The ability of the HRM method to detect DNA variations in the HMBS gene was tested on DNA samples with ten known mutations using a curve shape scan generated with the LightScanner instrument. Each of the ten mutations tested had an altered melting profile compared to the melting profile of the controls. Even if the mutation was localised in the amplicon together with the other known polymorphisms, the mutation was identified correctly.

Finally, we evaluated the HRM method on the group of 97 subjects with suspected acute hepatic porphyria. From the DNA variations identified, three were previously described mutations: c.70G>A (p.Gly24Ser), c.87+5G>T (r.(spl?)), c.[518G>A; 610C>A] (p.[Arg173Gln; Gln204Lys]), one was a novel mutation c.899_900delins TGCCTGCATCTG

(p.His300LeuFsX10), two were previously described polymorphisms g.3119T/G (rs1006195) and g.7998G/A (rs1799997), and three were novel, rare DNA variations g.2922T>G, g.3059G>A and g.7175A>G (found in one subject). The finding of these three novel rare DNA variations requires further investigation, since these variations are localised in the intronic part of the HMBS gene, but were only present in one allele out of 200. We found the small, indel mutation interesting because there are only four such cases (out of 307 mutations in the HMBS gene) registered in the mutation database. At the protein level, the mutation p.His300LeuFsX10 would cause a frame shift leading to a premature stop codon after ten completely different amino acids. A rare, novel DNA variation, g.2922T>G, found in one subject with porphyria variegata suggests the possibility of a rare case of dual porphyria. This finding requires further investigation, since this variation is localised in the regulatory segment of the erythroid promoter of HMBS.

In summary, screening the group of subjects with suspected porphyria revealed nine different DNA variations, four of which were novel. HRM is a fast, cost-effective pre-screening method for detecting DNA variations in the HMBS gene. Moreover, the screening can be extrapolated to an entire family in the event of possible misdiagnosis or rare dual porphyria. We showed that the HRM method can serve as a useful screening tool to identify DNA variations, even in amplicons with other polymorphisms.

I contributed to this study by the introduction of new prescreening method of HRM using LightScanner instrument into the molecular diagnostic of AIP. I identified the mutations in the HMBS gene of AIP subjects using HRM analysis and direct DNA sequencing.

Publication G

Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation (*Ulbrichova Douderova D et al., prior to submission*)

In this case report, we present a latent AIP patient incidentally identified during clarification of a skin disorder that was eventually diagnosed as lichen sclerosus et atrophicus (LS). A cutaneous porphyria disorder was suspected based on skin problems of a 48-year-old female Swiss patient. Biochemical analyses revealed normal fecal porphyrin content, a normal protoporphyrin concentration in erythrocytes and a negative plasma fluorescence

scan, typical features of cutaneous porphyria. However, repeated measurement of erythrocytic HMBS activity revealed a ~ 50% reduction in enzyme activity and slightly increased urinary ALA and PBG, biochemical findings that are compatible with the latent status of AIP. The skin condition in this patient was subsequently diagnosed as LS by biopsy.

After molecular screening of the HMBS gene, the novel heterozygous substitution mutation c.601C>G in exon 10 was identified and the status of latent AIP was confirmed, since the patient had never experienced an acute attack. At the protein level, this missense mutation leads to a change of Arg201 amino acid residue to glycine residue (p.Arg201Gly).

The mutation p.Arg201Gly was subsequently expressed and the mutant protein was characterised *in vitro*. A residual enzymatic activity of 5.9% of wild-type was measured in the mutant enzyme. Moreover, compared to the wild-type enzyme, the mutant was extremely unstable when faced with heat treatment and exhibited a shift in the optimal pH.

In the 3D structure of human HMBS published recently, two lobes of the central domain are joined by ion-pairing between Arg201 and Asp178. Both of these amino acid residues are highly conserved among prokaryotic as well as eukaryotic HMBS enzyme sequences. Such high degree of conservation suggests that the ion pair plays an important role in the enzyme structure.

In summary, the novel mutation c.601C>G (p.Arg201Gly) was found in a Swiss patient with AIP. The result of the *in vitro* characterisation of the mutant suggested that the p.Arg201Gly mutation has a deleterious effect on the HMBS protein. This result is in accordance with the *in vivo* measurement of decreased erythrocytic HMBS activity. Thus, a latent status, so typical in this disorder, but hardly identifiable without family history, was granted in this patient, suffering simultaneously with lichen sclerosus et atrophicus, taking into account that she has so far not shown any symptoms of AIP.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech Republic; Outpatient Clinic of Dermatology, Triemli Hospital, Zurich, Switzerland; and Department of Dermatology, University Hospital, Zürich, Switzerland. I contributed to this study by protein expression, purification, protein analyses and enzyme activity measurements.

Report of a novel mutation identification

Publication H

Gene symbol: HMBS. Disease: Porphyria, acute intermittent (Ulbrichova D et al., 2008)

From our cooperative laboratory we received whole blood of a Turkish AIP patient. His diagnosis was made based on clinical findings typical for AIP and almost half-normal (57.8%) erythrocytic HMBS activity. After gDNA isolation, we performed molecular screening in the HMBS gene by direct DNA sequencing. The DNA screening revealed a novel heterozygous mutation in exon 4, c.89T>G. At the protein level, this mutation results in the amino acid substitution of leucine to arginine, p.Leu30Arg.

We expressed the mutated gene in the prokaryotic system and performed biochemical testing of the purified enzyme. The purified mutant enzyme had a relative activity 0.03% of the average wild-type level (*unpublished data*). This is in good agreement with the results measurement of half-normal erythrocytic HMBS activity as the patient was heterozygous for the mutation. The detrimental effect of this amino acid substitution on the protein structure is further emphasised by the fact that the Leu30 residue is highly conserved in both the prokaryotic and eukaryotic HMBS sequences and therefore likely of special importance to the enzyme structure.

In summary, the novel mutation c.89T>G (p.Leu30Arg) was found in a Turkish patient with AIP. This finding further confirmed the diagnosis at the molecular level.

In this report, we cooperated with Department of Clinical Biochemistry in Gulhane School of Medicine, Ankara, Turkey. I contributed to this report by the identification of the proband's mutation in the HMBS gene using direct sequencing. I performed protein expression, purification, protein analyses and enzyme activity measurements (*unpublished data*).

Publication I

Gene symbol: HMBS. Disease: Porphyria, acute intermittent (Ulbrichova Douderova D et al., sent to Hum Genet 2009)

The patient, female, was hospitalised while having her first acute attack characterised by severe abdominal pain, hyponatraemia and behavioural disturbances. From the biochemical measurements, the highly elevated level of porphyrin precursors was detected, a distinct peak in the fluorimetric plasma scan at 404/622 nm was identified and her erythrocytic HMBS activity was 71% of the normal value.

DNA sequence analyses revealed a small heterozygous insertion c.184_185insT within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Lys62IlefsX3 causes a frameshift and creates a stop codon after three completely different amino acids, resulting in a truncated protein of 64 amino acids. The effect of such a truncation is expected to be detrimental.

In summary, the novel mutation c.184_185insT (p.Lys62IlefsX3) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

In this report, we cooperated with National Laboratory for the Biochemical Diagnoses of Porphyrrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel and Department of Internal Medicine A, Shaare Zedek Medical Center, Jerusalem, Israel. I contributed to this report by the identification of the proband's mutation in the HMBS gene using direct DNA sequencing.

Publication J

Gene symbol: HMBS. Disease: Porphyria, acute intermittent (Ulbrichova Douderova D et al., sent to Hum Genet 2009)

The patient, a Ukrainian female, was diagnosed with AIP by a clinician while having clinical manifestations typical for acute hepatic porphyria- abdominal pain, dark urine and muscle weakness. During an acute attack, the level of porphyrin precursors PBG and ALA were elevated (16.6 mg/100ml and 18.2 mg/100ml, respectively).

DNA sequence analysis revealed a novel heterozygous small insertion c.384_385insT in exon 8 within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Val130CysfsX80 causes a frameshift and creates a stop codon after eighty completely different amino acids resulting in a truncated protein. The effect of such truncation is expected to be detrimental.

In summary, the novel heterozygous mutation c.384_385insT (p.Val130CysfsX80) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

I contributed to this report by the identification of the proband's mutation in the HMBS gene using direct DNA sequencing.

CONCLUSIONS

In the present study, the patients carrying mutation in the HMBS gene were characterised at the molecular level. The major achievements of the work in this thesis are the following:

➤ Twenty-eight DNA variations were identified in patients with AIP. Out of them, thirteen were novel mutations, ten were previously reported mutations, two were previously reported polymorphisms, and three were novel rare DNA variations, which require further investigation. Moreover, out of the novel mutations identified, two were *de novo* mutations, which are rare events in this disorder. To the six mutations known to exist in the Slavic population to date, another nine mutations were identified, broadening the molecular heterogeneity of the HMBS gene in our population.

➤ The comparison of the clinical manifestation of AIP patients disclosed the evidence of the variability with respect to the ALA/PBG levels and erythrocytic HMBS activity among the index patients. This clearly demonstrates that although biochemical measurements should be included as a first diagnostic step, the detection of the causal mutation in the HMBS gene is the ultimate diagnostic criteria for AIP.

➤ In order to improve molecular testing of the HMBS gene, a fast, cost-effective pre-screening method of high-resolution melting using the LightScanner instrument was established.

➤ Fourteen different proteins with introduced mutations were expressed in the prokaryotic system. These mutants were characterised at the biochemical level. Even though most of them exhibited a residual activity close to zero, some of them exhibited as high a residual activity as 81% that of wild-type. Only further characterisation allowed the association of these mutations with AIP. This demonstrates that *in vitro* expression of HMBS mutant genes and characterisation of their structure-function consequences can improve the interpretation of clinical, biochemical and genetic data and the diagnosis of AIP. Moreover, the results suggest

that there is more than one causative factor with clinical relevance, which remains undiscovered.

➤ Based on the identification of the causal mutation in the HMBS gene of the index patient, appropriate genetic counselling based on the DNA diagnostics was applied within the AIP affected families.

Future challenges

One of the unknown aspects in the pathogenesis of AIP is the mechanism of incomplete penetrance and high variability of the clinical manifestation among the porphyria patients. According to the literature, only 10-20% of the mutation carriers will ever develop clinical symptoms. This suggests that the genetic background of individuals may explain differences in susceptibility to an acute attack, though the genes involved have not yet been identified. Future studies may be focused on the identification of such modifier genes which influence the susceptibility of patients with acute hepatic porphyrias to acute attack.

REFERENCES

- Aarsand AK, Petersen PH, Sandberg S. 2006. Estimation and application of biological variation of urinary delta-aminolevulinic acid and porphobilinogen in healthy individuals and in patients with acute intermittent porphyria. *Clin Chem* 52(4):650-6.
- Albers JW, Fink JK. 2004. Porphyric neuropathy. *Muscle Nerve* 30(4):410-22.
- Anderson KE, Bloomer JR, Bonkovsky HL, Kushner JP, Pierach CA, Pimstone NR, Desnick RJ. 2005. Recommendations for the diagnosis and treatment of the acute porphyrias. *Ann Intern Med* 142(6):439-50.
- Anderson KE, Sassa S, Peterson CM, Kappas A. 1977. Increased erythrocyte uroporphyrinogen-I-synthetase, delta-aminolevulinic acid dehydratase and protoporphyrin in hemolytic anemias. *Am J Med* 63(3):359-64.
- Anderson KE, Sassa, S., Bishop, D., Desnick, R.J. 2001. Disorders of heme biosynthesis: X-linked sideroblastic anemia and the porphyrias. In: Scriver CR, Beaudet, A.L., Sly, W.S., Valle, D., editor. *The metabolic and molecular basis of inherited disease*. New York: McGraw-Hill. p 2991-3062.
- Anderson PM. 1980. Purification and properties of the inducible enzyme cyanase. *Biochemistry* 19(13):2882-8.
- Andersson C, Innala E, Backstrom T. 2003. Acute intermittent porphyria in women: clinical expression, use and experience of exogenous sex hormones. A population-based study in northern Sweden. *J Intern Med* 254(2):176-83.
- Andersson C, Wikberg A, Stegmayr B, Lithner F. 2000. Renal symptomatology in patients with acute intermittent porphyria. A population-based study. *J Intern Med* 248(4):319-25.
- Atamna H, Killilea DW, Killilea AN, Ames BN. 2002. Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. *Proc Natl Acad Sci U S A* 99(23):14807-12.
- Awan SJ, Siligardi G, Shoolingin-Jordan PM, Warren MJ. 1997. Reconstitution of the holoenzyme form of *Escherichia coli* porphobilinogen deaminase from apoenzyme with porphobilinogen and preuroporphyrinogen: a study using circular dichroism spectroscopy. *Biochemistry* 36(30):9273-82.
- Badminton MN, Elder GH. 2002. Management of acute and cutaneous porphyrias. *Int J Clin Pract* 56(4):272-8.
- Battle AM. 1993. Porphyrins, porphyrias, cancer and photodynamic therapy--a model for carcinogenesis. *J Photochem Photobiol B* 20(1):5-22.
- Battersby AR, Buckley DG, Staunton J. 1979. Studies of enzyme-mediated reactions. Part II. Experiments on the dismutation of aldehyde to alcohol and carboxylic acid by the complex of liver alcohol dehydrogenase and NAD. *J Chem Soc Perkin 1* 10:2559-62.
- Benoist C, O'Hare K, Breathnach R, Chambon P. 1980. The ovalbumin gene-sequence of putative control regions. *Nucleic Acids Res* 8(1):127-42.
- Beukeveld GJ, Wolthers BG, Nordmann Y, Deybach JC, Grandchamp B, Wadman SK. 1990. A retrospective study of a patient with homozygous form of acute intermittent porphyria. *J Inherit Metab Dis* 13(5):673-83.
- Bishop DF, Henderson AS, Astrin KH. 1990. Human delta-aminolevulinic acid synthase: assignment of the housekeeping gene to 3p21 and the erythroid-specific gene to the X chromosome. *Genomics* 7(2):207-14.
- Bonaiti-Pellie C, Phung L, Nordmann Y. 1984. Recurrence risk estimation of acute intermittent porphyria based on analysis of porphobilinogen deaminase activity: a Bayesian approach. *Am J Med Genet* 19(4):755-62.
- Bonkovsky HL. 1993. Advances in understanding and treating 'the little imitator,' acute porphyria. *Gastroenterology* 105(2):590-4.
- Bonkovsky HL, Barnard GF. 1998. Diagnosis of porphyric syndromes: a practical approach in the era of molecular biology. *Semin Liver Dis* 18(1):57-65.
- Bonkovsky HL, Barnard GF. 2000. The Porphyrias. *Curr Treat Options Gastroenterol* 3(6):487-500.

- Bonkovsky HL, Cable EE, Cable JW, Donohue SE, White EC, Greene YJ, Lambrecht RW, Srivastava KK, Arnold WN. 1992. Porphyrigenic properties of the terpenes camphor, pinene, and thujone (with a note on historic implications for absinthe and the illness of Vincent van Gogh). *Biochem Pharmacol* 43(11):2359-68.
- Breathnach R, Chambon P. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50:349-83.
- Brennan MJ, Cantrill RC. 1979. Delta-aminolaevulinic acid is a potent agonist for GABA autoreceptors. *Nature* 280(5722):514-5.
- Brownlie PD, Lambert R, Louie GV, Jordan PM, Blundell TL, Warren MJ, Cooper JB, Wood SP. 1994. The three-dimensional structures of mutants of porphobilinogen deaminase: toward an understanding of the structural basis of acute intermittent porphyria. *Protein Sci* 3(10):1644-50.
- Burmester T, Weich B, Reinhardt S, Hankeln T. 2000. A vertebrate globin expressed in the brain. *Nature* 407(6803):520-3.
- Crimlisk HL. 1997. The little imitator--porphyria: a neuropsychiatric disorder. *J Neurol Neurosurg Psychiatry* 62(4):319-28.
- Deacon AC, Elder GH. 2001. ACP Best Practice No 165: front line tests for the investigation of suspected porphyria. *J Clin Pathol* 54(7):500-7.
- Delfau MH, Picat C, de Rooij FW, Hamer K, Bogard M, Wilson JH, Deybach JC, Nordmann Y, Grandchamp B. 1990. Two different point G to A mutations in exon 10 of the porphobilinogen deaminase gene are responsible for acute intermittent porphyria. *J Clin Invest* 86(5):1511-6.
- Deybach JC, Badminton M, Puy H, Sandberg S, Frank J, Harper P, Martasek P, Minder E, Parker S, Thunell S and others. 2006. European porphyria initiative (EPI): a platform to develop a common approach to the management of porphyrias and to promote research in the field. *Physiol Res* 55 Suppl 2:S67-73.
- Dhar GJ, Bossenmaier I, Petryka ZJ, Cardinal R, Watson CJ. 1975. Effects of hematin in hepatic porphyria. Further studies. *Ann Intern Med* 83(1):20-30.
- Douderova DU, Martasek P. 2009. Detection of DNA variations in the polymorphic hydroxymethylbilane synthase gene by high-resolution melting analysis. *Anal Biochem*.
- Dowdle E, Mustard P, Spong N, Eales L. 1968. The metabolism of (5-14C)delta-aminolaevulinic acid in normal and porphyric human subjects. *Clin Sci* 34(2):233-51.
- Edwards SR, Shanley BC, Reynoldson JA. 1984. Neuropharmacology of delta-aminolaevulinic acid--I. Effect of acute administration in rodents. *Neuropharmacology* 23(4):477-81.
- Elder GH, Hift RJ. 2001. Treatment of acute porphyria. *Hosp Med* 62(7):422-5.
- Elder GH, Hift RJ, Meissner PN. 1997. The acute porphyrias. *Lancet* 349(9065):1613-7.
- Erlandsen EJ, Jorgensen PE, Markussen S, Brock A. 2000. Determination of porphobilinogen deaminase activity in human erythrocytes: pertinent factors in obtaining optimal conditions for measurements. *Scand J Clin Lab Invest* 60(7):627-34.
- Farnham PJ, Schimke RT. 1986. In vitro transcription and delimitation of promoter elements of the murine dihydrofolate reductase gene. *Mol Cell Biol* 6(7):2392-401.
- Felitsyn N, McLeod C, Shroads AL, Stacpoole PW, Notterpek L. 2008. The heme precursor delta-aminolevulinic acid blocks peripheral myelin formation. *J Neurochem* 106(5):2068-79.
- Fibach E, Gambari R, Shaw PA, Maniatis G, Reuben RC, Sassa S, Rifkind RA, Marks PA. 1979. Tumor promoter-mediated inhibition of cell differentiation: suppression of the expression of erythroid functions in murine erythroleukemia cells. *Proc Natl Acad Sci U S A* 76(4):1906-10.
- Frampton J, Walker M, Plumb M, Harrison PR. 1990. Synergy between the NF-E1 erythroid-specific transcription factor and the CACCC factor in the erythroid-specific promoter of the human porphobilinogen deaminase gene. *Mol Cell Biol* 10(7):3838-42.
- Frank J, Christiano AM. 1998. The genetic bases of the porphyrias. *Skin Pharmacol Appl Skin Physiol* 11(6):297-309.
- Gill R, Kolstoe SE, Mohammed F, Al DBA, Mosely JE, Sarwar M, Cooper JB, Wood SP, Shoolingin-Jordan PM. 2009. Structure of human porphobilinogen deaminase at 2.8 Å: the molecular basis of acute intermittent porphyria. *Biochem J* 420(1):17-25.

- Goetsch CA, Bissell DM. 1986. Instability of hematin used in the treatment of acute hepatic porphyria. *N Engl J Med* 315(4):235-8.
- Goldberg A. 1959. Acute intermittent porphyria: a study of 50 cases. *Q J Med* 28(110):183-209.
- Gouya L, Puy H, Robreau AM, Lyoumi S, Lamoril J, Da Silva V, Grandchamp B, Deybach JC. 2004. Modulation of penetrance by the wild-type allele in dominantly inherited erythropoietic protoporphyria and acute hepatic porphyrias. *Hum Genet* 114(3):256-62.
- Grandchamp B, De Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y. 1987. Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur J Biochem* 162(1):105-10.
- Grandchamp B, Puy H, Lamoril J, Deybach JC, Nordmann Y. 1996. Review: molecular pathogenesis of hepatic acute porphyrias. *J Gastroenterol Hepatol* 11(11):1046-52.
- Gross U, Honcamp M, Doss MO. 1996. Heterogeneity of acute intermittent porphyria: a subtype with normal erythrocyte porphobilinogen deaminase activity in Germany. *Eur J Clin Chem Clin Biochem* 34(8):613-8.
- Gross U, Jacob K, Frank M, Doss MO. 1997. Haem precursors and porphobilinogen deaminase in erythrocytes and lymphocytes of patients with acute intermittent porphyria. *Cell Mol Biol (Noisy-le-grand)* 43(1):29-35.
- Gu XF, de Rooij F, de Baar E, Bruyland M, Lissens W, Nordmann Y, Grandchamp B. 1993. Two novel mutations of the porphobilinogen deaminase gene in acute intermittent porphyria. *Hum Mol Genet* 2(10):1735-6.
- Gubin AN, Miller JL. 2001. Human erythroid porphobilinogen deaminase exists in 2 splice variants. *Blood* 97(3):815-7.
- Guengerich FP, Bell LC, Okazaki O. 1995. Interpretations of cytochrome P450 mechanisms from kinetic studies. *Biochimie* 77(7-8):573-80.
- Hach A, Hon T, Zhang L. 1999. A new class of repression modules is critical for heme regulation of the yeast transcriptional activator Hap1. *Mol Cell Biol* 19(6):4324-33.
- Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE. 1991. Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. *Arch Biochem Biophys* 289(2):387-92.
- Handschin C, Lin J, Rhee J, Peyer AK, Chin S, Wu PH, Meyer UA, Spiegelman BM. 2005. Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1alpha. *Cell* 122(4):505-15.
- Harper P FY, Andersson C, Möller C, Rejkjaer L, Sardh E, Andersson D.E.H. 2003. Correlation between plasma and urinary levels of porphobilinogen (PBG) and 5-aminolevulinic acid (ALA) in ten asymptomatic gene carriers of acute intermittent (AIP) with increased porphyrin precursor excretion. *Physiological Research* 52(1S-3S):10S.
- Hayashi N, Terasawa M, Yamauchi K, Kikuchi G. 1980. Effects of hemin on the synthesis and intracellular translocation of delta-aminolevulinate synthase in the liver of rats treated with 3,5-dicarbethoxy-1,4-dihydrocollidine. *J Biochem* 88(5):1537-43.
- Helliwell JR, Nieh YP, Habash J, Faulder PF, Raftery J, Cianci M, Wulff M, Hadener A. 2003. Time-resolved and static-ensemble structural chemistry of hydroxymethylbilane synthase. *Faraday Discuss* 122:131-44; discussion 171-90.
- Hemmaplardh D, Morgan EH. 1974. The mechanism of iron exchange between synthetic iron chelators and rabbit reticulocytes. *Biochim Biophys Acta* 373(1):84-99.
- Hermes-Lima M, Castilho RF, Valle VG, Bechara EJ, Vercesi AE. 1992. Calcium-dependent mitochondrial oxidative damage promoted by 5-aminolevulinic acid. *Biochim Biophys Acta* 1180(2):201-6.
- Hessels J, Voortman G, van der Wagen A, van der Elzen C, Scheffer H, Zuijderhoudt FM. 2004. Homozygous acute intermittent porphyria in a 7-year-old boy with massive excretions of porphyrins and porphyrin precursors. *J Inherit Metab Dis* 27(1):19-27.
- Hift RJ, Meissner PN. 2005. An analysis of 112 acute porphyric attacks in Cape Town, South Africa: Evidence that acute intermittent porphyria and variegate porphyria differ in susceptibility and severity. *Medicine (Baltimore)* 84(1):48-60.

- Hwu HR, Roberts JW, Davidson EH, Britten RJ. 1986. Insertion and/or deletion of many repeated DNA sequences in human and higher ape evolution. *Proc Natl Acad Sci U S A* 83(11):3875-9.
- Chen CH, Astrin KH, Lee G, Anderson KE, Desnick RJ. 1994. Acute intermittent porphyria: identification and expression of exonic mutations in the hydroxymethylbilane synthase gene. An initiation codon missense mutation in the housekeeping transcript causes "variant acute intermittent porphyria" with normal expression of the erythroid-specific enzyme. *J Clin Invest* 94(5):1927-37.
- Chen JJ, London IM. 1995. Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. *Trends Biochem Sci* 20(3):105-8.
- Chretien S, Dubart A, Beaupain D, Raich N, Grandchamp B, Rosa J, Goossens M, Romeo PH. 1988. 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(1):6-10.
- Jensen NF, Fiddler DS, Striepe V. 1995. Anesthetic considerations in porphyrias. *Anesth Analg* 80(3):591-9.
- Johansson A, Moller C, Fogh J, Harper P. 2003. Biochemical characterization of porphobilinogen deaminase-deficient mice during phenobarbital induction of heme synthesis and the effect of enzyme replacement. *Mol Med* 9(9-12):193-9.
- Jordan PM, Shemin D. 1973. Purification and properties of uroporphyrinogen I synthetase from *Rhodospseudomonas spheroides*. *J Biol Chem* 248(3):1019-24.
- Jordan PM, Thomas SD, Warren MJ. 1988. Purification, crystallization and properties of porphobilinogen deaminase from a recombinant strain of *Escherichia coli* K12. *Biochem J* 254(2):427-35.
- Jordan PM, Warren MJ. 1987. Evidence for a dipyrromethane cofactor at the catalytic site of *E. coli* porphobilinogen deaminase. *FEBS Lett* 225(1-2):87-92.
- Kauppinen R. 2004. Molecular diagnostics of acute intermittent porphyria. *Expert Rev Mol Diagn* 4(2):243-9.
- Kauppinen R. 2005. Porphyrias. *Lancet* 365(9455):241-52.
- Kauppinen R, Mustajoki P. 1988. Acute hepatic porphyria and hepatocellular carcinoma. *Br J Cancer* 57(1):117-20.
- Kauppinen R, Mustajoki P. 1992. Prognosis of acute porphyria: occurrence of acute attacks, precipitating factors, and associated diseases. *Medicine (Baltimore)* 71(1):1-13.
- Kauppinen R, Mustajoki S, Pihlaja H, Peltonen L, Mustajoki P. 1995. Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. *Hum Mol Genet* 4(2):215-22.
- Kauppinen R, von und zu Fraunberg M. 2002. Molecular and biochemical studies of acute intermittent porphyria in 196 patients and their families. *Clin Chem* 48(11):1891-900.
- Kolluri S, Sadlon TJ, May BK, Bonkovsky HL. 2005. Haem repression of the housekeeping 5-aminolaevulinic acid synthase gene in the hepatoma cell line LMH. *Biochem J* 392(Pt 1):173-80.
- Kordac V, Martasek P. 1986. Haem arginate in acute hepatic porphyrias. *Br Med J (Clin Res Ed)* 293(6554):1098.
- Kostrzewska E, Gregor A. 1986. Increased activity of porphobilinogen deaminase in erythrocytes during attacks of acute intermittent porphyria. *Ann Clin Res* 18(4):195-8.
- Kupferschmidt H, Bont A, Schnorf H, Landis T, Walter E, Peter J, Krahenbuhl S, Meier PJ. 1995. Transient cortical blindness and bioccipital brain lesions in two patients with acute intermittent porphyria. *Ann Intern Med* 123(8):598-600.
- Lambert R, Brownlie PD, Woodcock SC, Louie GV, Cooper JC, Warren MJ, Jordan PM, Blundell TL, Wood SP. 1994. Structural studies on porphobilinogen deaminase. *Ciba Found Symp* 180:97-104; discussion 105-10.
- Lander M, Pitt AR, Alefounder PR, Bardy D, Abell C, Battersby AR. 1991. Studies on the mechanism of hydroxymethylbilane synthase concerning the role of arginine residues in substrate binding. *Biochem J* 275 (Pt 2):447-52.
- Lathrop JT, Timko MP. 1993. Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science* 259(5094):522-5.

- Leeper FJ. 1989. The biosynthesis of porphyrins, chlorophylls, and vitamin B12. *Nat Prod Rep* 6(2):171-203.
- Li D. 2005. PGC-1 α : looking behind the sweet treat for porphyria. *Cell* 122(4):487-9.
- Liamis G, Mitrogianni Z, Liberopoulos EN, Tsimihodimos V, Elisaf M. 2007. Electrolyte disturbances in patients with hyponatremia. *Intern Med* 46(11):685-90.
- Lin CS, Krishnan AV, Lee MJ, Zagami AS, You HL, Yang CC, Bostock H, Kiernan MC. 2008. Nerve function and dysfunction in acute intermittent porphyria. *Brain* 131(Pt 9):2510-9.
- Lindberg RL, Martini R, Baumgartner M, Erne B, Borg J, Zielasek J, Ricker K, Steck A, Toyka KV, Meyer UA. 1999. Motor neuropathy in porphobilinogen deaminase-deficient mice imitates the peripheral neuropathy of human acute porphyria. *J Clin Invest* 103(8):1127-34.
- Lindberg RL, Porcher C, Grandchamp B, Ledermann B, Burki K, Brandner S, Aguzzi A, Meyer UA. 1996. Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. *Nat Genet* 12(2):195-9.
- Litman DA, Correia MA. 1985. Elevated brain tryptophan and enhanced 5-hydroxytryptamine turnover in acute hepatic heme deficiency: clinical implications. *J Pharmacol Exp Ther* 232(2):337-45.
- Llewellyn DH, Smyth SJ, Elder GH, Hutchesson AC, Rattenbury JM, Smith MF. 1992. Homozygous acute intermittent porphyria: compound heterozygosity for adjacent base transitions in the same codon of the porphobilinogen deaminase gene. *Hum Genet* 89(1):97-8.
- Llewellyn DH, Whatley S, Elder GH. 1993. Acute intermittent porphyria caused by an arginine to histidine substitution (R26H) in the cofactor-binding cleft of porphobilinogen deaminase. *Hum Mol Genet* 2(8):1315-6.
- Louie GV, Brownlie PD, Lambert R, Cooper JB, Blundell TL, Wood SP, Malashkevich VN, Hadener A, Warren MJ, Shoolingin-Jordan PM. 1996. The three-dimensional structure of *Escherichia coli* porphobilinogen deaminase at 1.76-Å resolution. *Proteins* 25(1):48-78.
- Louie GV, Brownlie PD, Lambert R, Cooper JB, Blundell TL, Wood SP, Warren MJ, Woodcock SC, Jordan PM. 1992. Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. *Nature* 359(6390):33-9.
- Luskey KL, Faust JR, Chin DJ, Brown MS, Goldstein JL. 1983. Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53-kDa protein, in UT-1 cells. *J Biol Chem* 258(13):8462-9.
- May BK, Dogra SC, Sadlon TJ, Bhasker CR, Cox TC, Bottomley SS. 1995. Molecular regulation of heme biosynthesis in higher vertebrates. *Prog Nucleic Acid Res Mol Biol* 51:1-51.
- McCull KE, Moore MR, Thompson GG, Goldberg A. 1982. Screening for latent acute intermittent porphyria: the value of measuring both leucocyte delta-aminolaevulinic acid synthase and erythrocyte uroporphyrinogen-1-synthase activities. *J Med Genet* 19(4):271-6.
- McCull KE, Moore R. 1981. The porphyrias: an example of pharmacogenetic disease. *Scott Med J* 26(1):32-40.
- Meisler M, Wanner L, Eddy RE, Shows TB. 1980. The UPS locus encoding uroporphyrinogen I synthase is located on human chromosome 11. *Biochem Biophys Res Commun* 95(1):170-6.
- Meyer UA, Schuurmans MM, Lindberg RL. 1998. Acute porphyrias: pathogenesis of neurological manifestations. *Semin Liver Dis* 18(1):43-52.
- Meyer UA, Strand LJ, Doss M, Rees AC, Marver HS. 1972. Intermittent acute porphyria--demonstration of a genetic defect in porphobilinogen metabolism. *N Engl J Med* 286(24):1277-82.
- Mignotte V, Eleouet JF, Raich N, Romeo PH. 1989a. Cis- and trans-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene. *Proc Natl Acad Sci U S A* 86(17):6548-52.
- Mignotte V, Wall L, deBoer E, Grosveld F, Romeo PH. 1989b. Two tissue-specific factors bind the erythroid promoter of the human porphobilinogen deaminase gene. *Nucleic Acids Res* 17(1):37-54.
- Millward LM, Kelly P, Deacon A, Senior V, Peters TJ. 2001. Self-rated psychosocial consequences and quality of life in the acute porphyrias. *J Inher Metab Dis* 24(7):733-47.

- Miyagi K, Cardinal R, Bossenmaier I, Watson CJ. 1971. The serum porphobilinogen and hepatic porphobilinogen deaminase in normal and porphyric individuals. *J Lab Clin Med* 78(5):683-95.
- Miyagi K, Kaneshima M, Kawakami J, Nakada F, Petryka ZJ, Watson CJ. 1979. Uroporphyrinogen I synthase from human erythrocytes: separation, purification, and properties of isoenzymes. *Proc Natl Acad Sci U S A* 76(12):6172-6.
- Moore MR, Hift RJ. 1997. Drugs in the acute porphyrias--toxicogenetic diseases. *Cell Mol Biol (Noisy-le-grand)* 43(1):89-94.
- Mustajoki P. 1981. Normal erythrocyte uroporphyrinogen I synthase in a kindred with acute intermittent porphyria. *Ann Intern Med* 95(2):162-6.
- Mustajoki P, Nordmann Y. 1993. Early administration of heme arginate for acute porphyric attacks. *Arch Intern Med* 153(17):2004-8.
- Mustajoki P, Tenhunen R, Tokola O, Gothoni G. 1986. Haem arginate in the treatment of acute hepatic porphyrias. *Br Med J (Clin Res Ed)* 293(6546):538-9.
- Mustajoki P, Timonen K, Gorchein A, Seppalainen AM, Matikainen E, Tenhunen R. 1992. Sustained high plasma 5-aminolaevulinic acid concentration in a volunteer: no porphyric symptoms. *Eur J Clin Invest* 22(6):407-11.
- Mustajoki S. 1999. Molecular genetics of acute intermittent porphyria in Finland. Thesis/Dissertation(University of Helsinki):1-79.
- Nakajima O, Takahashi S, Harigae H, Furuyama K, Hayashi N, Sassa S, Yamamoto M. 1999. Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload. *EMBO J* 18(22):6282-9.
- Namba H, Narahara K, Tsuji K, Yokoyama Y, Seino Y. 1991. Assignment of human porphobilinogen deaminase to 11q24.1---q24.2 by in situ hybridization and gene dosage studies. *Cytogenet Cell Genet* 57(2-3):105-8.
- Nordmann Y, Puy H. 2002. Human hereditary hepatic porphyrias. *Clin Chim Acta* 325(1-2):17-37.
- Nordmann Y, Puy H, Deybach JC. 1999. The porphyrias. *J Hepatol* 30 Suppl 1:12-6.
- Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H and others. 2001. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. *EMBO J* 20(11):2835-43.
- Orkin SH. 1992. GATA-binding transcription factors in hematopoietic cells. *Blood* 80(3):575-81.
- Oteiza PI, Kleinman CG, Demasi M, Bechara EJ. 1995. 5-Aminolevulinic acid induces iron release from ferritin. *Arch Biochem Biophys* 316(1):607-11.
- Petrides PE. 1998. Acute intermittent porphyria: mutation analysis and identification of gene carriers in a German kindred by PCR-DGGE analysis. *Skin Pharmacol Appl Skin Physiol* 11(6):374-80.
- Pierach CA, Weimer MK, Cardinal RA, Bossenmaier IC, Bloomer JR. 1987. Red blood cell porphobilinogen deaminase in the evaluation of acute intermittent porphyria. *JAMA* 257(1):60-1.
- Pischik E, Kauppinen R. 2009. Neurological manifestations of acute intermittent porphyria. *Cell Mol Biol (Noisy-le-grand)* 55(1):72-83.
- Poblete Gutierrez P, Kunitz O, Wolff C, Frank J. 2001. Diagnosis and treatment of the acute porphyrias: an interdisciplinary challenge. *Skin Pharmacol Appl Skin Physiol* 14(6):393-400.
- Podvinec M, Handschin C, Looser R, Meyer UA. 2004. Identification of the xenosensors regulating human 5-aminolevulinic acid synthase. *Proc Natl Acad Sci U S A* 101(24):9127-32.
- Ponka P. 1997. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* 89(1):1-25.
- Puy H, Deybach JC, Baudry P, Callebert J, Touitou Y, Nordmann Y. 1993. Decreased nocturnal plasma melatonin levels in patients with recurrent acute intermittent porphyria attacks. *Life Sci* 53(8):621-7.
- Puy H, Deybach JC, Lamoril J, Robreau AM, Da Silva V, Gouya L, Grandchamp B, Nordmann Y. 1997. Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. *Am J Hum Genet* 60(6):1373-83.

- Puy H, Gross U, Deybach JC, Robreau AM, Frank M, Nordmann Y, Doss M. 1998. Exon 1 donor splice site mutations in the porphobilinogen deaminase gene in the non-erythroid variant form of acute intermittent porphyria. *Hum Genet* 103(5):570-5.
- Qi Z, Hamza I, O'Brian MR. 1999. Heme is an effector molecule for iron-dependent degradation of the bacterial iron response regulator (Irr) protein. *Proc Natl Acad Sci U S A* 96(23):13056-61.
- Raich N, Mignotte V, Dubart A, Beaupain D, Leboulch P, Romana M, Chabret C, Charnay P, Papayannopoulou T, Goossens M and others. 1989. Regulated expression of the overlapping ubiquitous and erythroid transcription units of the human porphobilinogen deaminase (PBG-D) gene introduced into non-erythroid and erythroid cells. *J Biol Chem* 264(17):10186-92.
- Raich N, Romeo PH, Dubart A, Beaupain D, Cohen-Solal M, Goossens M. 1986. Molecular cloning and complete primary sequence of human erythrocyte porphobilinogen deaminase. *Nucleic Acids Res* 14(15):5955-68.
- Reddy SV, Alcantara O, Roodman GD, Boldt DH. 1996. Inhibition of tartrate-resistant acid phosphatase gene expression by heme and protoporphyrin IX. Identification of a heme-responsive inhibitor of transcription. *Blood* 88(6):2288-97.
- Regan L, Gonsalves L, Tesar G. 1999. Acute intermittent porphyria. *Psychosomatics* 40(6):521-3.
- Rosipal R, Puy H, Lamoril J, Martasek P, Nordmann Y, Deybach JC. 1997. Molecular analysis of porphobilinogen (PBG) deaminase gene mutations in acute intermittent porphyria: first study in patients of Slavic origin. *Scand J Clin Lab Invest* 57(3):217-24.
- Sadlon TJ, Dell'Oso T, Surinya KH, May BK. 1999. Regulation of erythroid 5-aminolevulinate synthase expression during erythropoiesis. *Int J Biochem Cell Biol* 31(10):1153-67.
- Sassa S. 2006. Modern diagnosis and management of the porphyrias. *Br J Haematol* 135(3):281-92.
- Seth AK, Badminton MN, Mirza D, Russell S, Elias E. 2007. Liver transplantation for porphyria: who, when, and how? *Liver Transpl* 13(9):1219-27.
- Shoolingin-Jordan PM. 1995. Porphobilinogen deaminase and uroporphyrinogen III synthase: structure, molecular biology, and mechanism. *J Bioenerg Biomembr* 27(2):181-95.
- Shoolingin-Jordan PM, Al-Dbass A, McNeill LA, Sarwar M, Butler D. 2003. Human porphobilinogen deaminase mutations in the investigation of the mechanism of dipyrromethane cofactor assembly and tetrapyrrole formation. *Biochem Soc Trans* 31(Pt 3):731-5.
- Schmitt MP. 1999. Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of heme and hemoglobin. *J Bacteriol* 181(17):5330-40.
- Solis C, Martinez-Bermejo A, Naidich TP, Kaufmann WE, Astrin KH, Bishop DF, Desnick RJ. 2004. Acute intermittent porphyria: studies of the severe homozygous dominant disease provides insights into the neurologic attacks in acute porphyrias. *Arch Neurol* 61(11):1764-70.
- Song G, Li Y, Cheng C, Zhao Y, Gao A, Zhang R, Joachimiak A, Shaw N, Liu ZJ. 2009. Structural insight into acute intermittent porphyria. *FASEB J* 23(2):396-404.
- Soonawalla ZF, Orug T, Badminton MN, Elder GH, Rhodes JM, Bramhall SR, Elias E. 2004. Liver transplantation as a cure for acute intermittent porphyria. *Lancet* 363(9410):705-6.
- Spivak JL. 1986. The mechanism of action of erythropoietin. *Int J Cell Cloning* 4(3):139-66.
- Stein JA, Tschudy DP. 1970. Acute intermittent porphyria. A clinical and biochemical study of 46 patients. *Medicine (Baltimore)* 49(1):1-16.
- Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS, Cooper DN. 2009. The Human Gene Mutation Database: 2008 update. *Genome Med* 1(1):13.
- Stone JR, Marletta MA. 1996. Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* 35(4):1093-9.
- Strand LJ, Felsher BF, Redeker AG, Marver HS. 1970. Heme biosynthesis in intermittent acute porphyria: decreased hepatic conversion of porphobilinogen to porphyrins and increased delta aminolevulinic acid synthetase activity. *Proc Natl Acad Sci U S A* 67(3):1315-20.
- Sweeney VP, Pathak MA, Asbury AK. 1970. Acute intermittent porphyria. Increased ALA-synthetase activity during an acute attack. *Brain* 93(2):369-80.
- Sze G. 1996. Cortical brain lesions in acute intermittent porphyria. *Ann Intern Med* 125(5):422-3.
- Tenhunen R, Mustajoki P. 1998. Acute porphyria: treatment with heme. *Semin Liver Dis* 18(1):53-5.
- Tenhunen R, Tokola O, Linden IB. 1987. Haem arginate: a new stable haem compound. *J Pharm Pharmacol* 39(10):780-6.

- Thunell S. 2000. Porphyrins, porphyrin metabolism and porphyrias. I. Update. *Scand J Clin Lab Invest* 60(7):509-40.
- Thunell S. 2006. (Far) Outside the box: genomic approach to acute porphyria. *Physiol Res* 55 Suppl 2:S43-66.
- Thunell S, Floderus Y, Henrichson A, Moore MR, Meissner P, Sinclair J. 1992. Alcoholic beverages in acute porphyria. *J Stud Alcohol* 53(3):272-6.
- Thunell S, Harper P, Brock A, Petersen NE. 2000. Porphyrins, porphyrin metabolism and porphyrias. II. Diagnosis and monitoring in the acute porphyrias. *Scand J Clin Lab Invest* 60(7):541-59.
- Thunell S, Pomp E, Brun A. 2007. Guide to drug porphyrogenicity prediction and drug prescription in the acute porphyrias. *Br J Clin Pharmacol* 64(5):668-79.
- Tschudy DP, Valsamis M, Magnussen CR. 1975. Acute intermittent porphyria: clinical and selected research aspects. *Ann Intern Med* 83(6):851-64.
- Ulbrichova D, Flachsova E, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. 2006. De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study. *Physiol Res* 55 Suppl 2:S145-54.
- Ulbrichova D, Hrdinka M, Saudek V, Martasek P. 2009. Acute intermittent porphyria--impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties. *FEBS J* 276(7):2106-15.
- Wang L, Elliott M, Elliott T. 1999. Conditional stability of the HemaA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*. *J Bacteriol* 181(4):1211-9.
- Warren MJ, Jordan PM. 1988. Investigation into the nature of substrate binding to the dipyrrromethane cofactor of *Escherichia coli* porphobilinogen deaminase. *Biochemistry* 27(25):9020-30.
- Watson CJ. 1975. Editorial: Hematin and porphyria. *N Engl J Med* 293(12):605-7.
- Watson CJ, Pierach CA, Bossenmaier I, Cardinal R. 1978. Use of hematin in the acute attack of the "inducible" hepatic porphyrias. *Adv Intern Med* 23:265-86.
- Whatley SD, Roberts AG, Llewellyn DH, Bennett CP, Garrett C, Elder GH. 2000. 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(3):243-8.
- White KA, Marletta MA. 1992. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31(29):6627-31.
- Yeung Laiwah AC, Moore MR, Goldberg A. 1987. Pathogenesis of acute porphyria. *Q J Med* 63(241):377-92.
- Yoo HW, Warner CA, Chen CH, Desnick RJ. 1993. Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. *Genomics* 15(1):21-9.

SUPPLEMENTS: ORIGINAL PUBLICATIONS