**Univerzita Karlova v Praze**

**1. lékařská fakulta**

Autoreferát disertační práce

# Molekulární patologie vybraných porfyrií s kožní manifestací

# Molecular pathology of selected porphyrias with skin manifestation

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## SOUHRN

Porfyrie jsou skupinou heterogenních dědičných metabolických poruch způsobených defekty enzymů biosyntézy hemu, které vedou k nadprodukci prekurzorů porfyrinů hemu v různých tělesných orgánech. Tyto enzymy jsou kódovány specifickými geny a patogenní změny v jejich sekvenci podmiňují konkrétní typ porfyrie. V těchto genech byly zjištěny četné mutace, které vedou k poškození funkce enzymu, a tím k rozvoji klinických projevů porfyrie. Cílem této práce bylo zkoumat na molekulární úrovni gen *UROD* u pacientů s pozdní kožní porfyrií (PCT) a hepatoerytropoetickou protoporfyrií (HEP) a dále gen *FECH* u pacientů s erytropoetickou protoporfyrií (EPP). Identifikovali jsme řadu mutací v genech *UROD* a *FECH* ve třech různých populacích - české, slovenské a egyptské. Popsali jsme nové mutace v genu *UROD* u pacientů egyptského původu s onemocněním HEP a mutace v genu *FECH* u pacientů českého a slovenského původu s onemocněním EPP. Studovali jsme mutovaný enzym UROD na úrovni proteinu a určili, že jeho enzymová aktivita představuje 19 % zdravé kontroly. Stávající studie předkládá poprvé frekvenci sestřihové varianty IVS3-48c genu *FECH* u zdravých kontrol z české populace. Analyzovali jsme 624 alel u nepříbuzných jedinců z běžné české populace a zjistili, že frekvence alely C v české populaci je 5,5 %, podobná jako je v kavkazské západoevropské populaci.



**Klíčová slova:** pozdní kožní porfyrie, Hepatoerythropoietic porfyrie, Erytropoietic protoporfyrie, Uroporfyrinogen dekarboxylázy, ferrochelatázy.



## ABSTRACT

Porphyria is a group of inherited metabolic disorders due to enzymatic defect of the heme biosynthesis resulting in the overproduction of the heme precursors' porphyrins in different body organs. The enzymes of the heme biosynthesis are encoded by corresponding genes in which any defect in any of these genes lead to a specific type of porphyria. Numerous mutations were detected in these genes leading to impairment in the enzyme function and therefore developing of the clinical manifestations of porphyria. The aim of the present work was to investigate the *UROD* gene in patients with porphyria cutanea tarda (PCT) and hepatoerythropoietic protoporphyria (HEP) as well as the *FECH* gene in patients with erythropoietic protoporphyria (EPP) on a molecular level. We identified numerous mutations in the *FECH* and the *UROD* genes in three different populations, Czech, Slovak, and Egyptian. We described the novel mutations in the *UROD* gene in HEP Arabic patients from Egypt as well in the *FECH* gene in patients with EPP of Czech and Slovak origin. We expressed mutatted UROD protein in prokaryotic system and found 19 % of the wild-type enzymatic activity. Moreover, the current study presents for the first time the frequency of the low expression allele IVS3-48c in the *FECH* gene in healthy controls from the Czech population. We analyzed 624 alleles in unrelated individuals from the general Czech population and it was found out to be 5,5 % among the Czech population, similar as in Caucasians from West Europe.


## INTRODUCTION

### Heme and Hemeproteins

Heme, a tetrapyrrole molecule formed of four pyrrole rings linked together by methine bridges with an iron ion in the center of the heterocyclic ring. Hemeprotiens have a wide

range of significant functions inside the human body. They transport divalent gases like oxygen, carbon dioxide and nitric oxide. They carry out the transfere of the electron ion via cytochromes in the mitochondrial respiratory chain. They have an important function in the drug metabolism and the detoxification through the enormous system of Cytochrome P450 (CYPs) (White and Marletta, 1992).

**Heme biosynthesis**

The heme biosynthetic cascade starts in the mitochondrial matrix by the condensation and decarboxylation reactions of the amino acid glycine and the citric acid cycle metabolite succinyl CoA forming the D aminolevulinic acid (δ-ALA). This reaction is catalyzed by the mitochondrial enzyme delta amino levulenic acid synthase (ALAS) with the pyridoxal phosphate as a co-enzyme. Two molecules of (δ-ALA) are transported to the cytoplasm to be condensed by the catalytic activity of the cytosolic enzyme ALA dehydratase (ALAD) to form the first intermediate with a pyrrole ring; porphobilinogen (PBG). Four molecules of PBG are polymerized successively by the elimination of the amino group to form the first tetrapyrrole intermediate Hydroxymethyl bilane (HMB). This deamination reaction is catalyzed by the cytosolic enzyme Hydroxymethyl bilane synthase (HMBS). The uroporphyrinogen III synthase (UROS), convert the linear tetrapyrrole Hydroxymethyl bilane into the cyclic tetrapyrrole uroporphyrinogen III by a hydrolysis reaction. Uroporphyrinogen decarboxylase (UROD), a cytosolic enzyme, that catalyzes the decarboxylation of the four acetate side chains to methyl groups producing coproporphyrinogen III. Two of the four propionyl radicals of the coproporphyrinogen III carry out oxidative decarboxylation in the cytosol by the coproporphyrinogen oxidase (CPOX) converting them to vinyl radicals yielding protoporphyrinogen IX. Then the oxidation of protoporphyrinogen III takes place in the mitochondrial matrix by the enzyme protoporphyrinogen oxidase (PPOX) producing protoporphyrin IX. Finally, the mitochondrial enzyme ferrochelatase (FECH) incorporates ferrous ion into protoporphyrin IX to produce heme (Kappas, et al., 1990).

**Regulation of heme biosynthesis**

The key regulatory enzyme in the heme biosynthetic cascade is the intra-mitochondrial enzyme ALAS which exists in two isoforms (ALAS1) and (ALAS2). ALAS1 is the ubiquitous isoform which is available in all cells and particularly in the liver. ALAS2 is predominantly present in the developing bone marrow erythroids (May, et al., 1995).

**Heme degradation**

Heme degradation takes place in the reticulo-endothelial system (RES) to keep the intracellular homeostasis of heme. Heme catabolism is catalyzed by the endoplasmic enzyme heme oxygenase (HOXG) that converts heme to biliverdin using nicotine amide adenine dinucleotide phosphate hydrogen (NADPH) as a reducing agent. Ferric iron is released to enter the iron pool for reuse. Biliverdin is then reduced by the Biliverdin reductase (BVR) using NADPH as a reducing agent to a less toxic molecule in human called bilirubin. The unconjugated bilirubin (UCB) is then bound to the albumin to be transported in the plasma to the liver. The liver uptakes the UCB and bound it to ligandin in the hepatocyte cytoplasm. UCB is transported to the endoplasmic reticulum (ER) where conjugation with diglucuronides occur by the enzyme uridine diphosphate glycosyltransferase 1A1 to become hydrophilic and easily excreted in the aqueous bile (Shibahara, et al., 2002).

# Porphyria

### Porphyrins and porphyrinogens

These are heterocyclic organic macromolecules formed of four pyrrole rings (A, B, C, D/I-IV) linked together through methine bridges at their alpha carbon atoms in a highly conjugated system.The eight side hydrogen atoms are replaced by special side radicals that determines their physio-chemical properties. Porphyrinogens are biological intermediates and functional precursors for the heme biosynthesis.

### Porphyria

Porphyrias are a group of inherited metabolic disorders due to enzymatic defect of the heme biosynthesis resulting in the overproduction of the heme precursor's porphyrins in different body organs. This leads to both the deposition of the porphyrin precursors in different organs and their excretion in the urine and in the stool (Anderson, et al., 2001).

### Classification of porphyrias

Porphyrias are broadly classified whether according to the main site of the heme precursor's overproduction into hepatic and erythropoietic or classified clinically into neuro-visceral, cutaneous and mixed.

Congenital Erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP) are examples of the erythropoietic type of Porphyria where the porphyrin precursors accumulate mainly in the bone marrow. The remaining sub-types of porphyria are considered as examples of hepatic porphyria as the most clinically affected organ is the liver due to the accumulation of the porphyrin precursors in the hepatocytes. Moreover, the hepatic porphyrias are further classified to acute and chronic according to the severity of the clinical picture. Acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), variegate porphyria (VP) and Plumboporphyria are usually presented as acute hepatic porphyria. Porphyria cutanea tarda (PCT) and hepatoerythropoietic porphyria (HEP) are presented as chronic hepatic porphyria. (Schmid, et al., 1954). Plumboporphyria and AIP manifest mainly with neuropsychiatric symptoms. CEP, fPCT and HEP manifest mainly by dermatological symptoms. HCP and VP manifest by both neuropsychiatric and dermatological signs. (Elder, et al., 1990).

The enzymes of the heme biosynthestic pathway are encoded by specific corresponding genes (Anderson, et al., 2001). Numerous mutations were detected in these genes that can lead to the impairment of the protein function resulting in the above mentioned clinical subtypes of porphyria. All subtypes of porphyria are inherited as autosomal dominant except the plumboporphyria, CEP and HEP which are inherited as an autosomal recessive. Porphyrias with autosomal dominant inheritance receive one copy of the diseased allele from one parent that can lead to diminishing of the enzyme activity while the normal allele is just adequate to maintain the vital cell metabolism (Gouya et al., 2004).

# Uroporphyrinogen decarboxylase (UROD) defect disorders

Uroporphyrinogen decarboxylase (UROD) (UROD; E.C.4.1.1.37) is a cytosolic enzyme that catalyzes the decarboxylation of the acetate radicals of the uroporphyrinogen III to produce coproporphyrinogen III (Elder, 1998). The decrease of the UROD enzyme activity results in three metabolic disorders: sporadic porphyria cutanea tarda (s-PCT), familial porphyria cutanea tarda (f-PCT), and hepatoerythropoietic porphyria (HEP) (de Verneuil, et al., 1984; Kappas, et al., 1995). Sporadic porphyria cutanea tarda (s-PCT) is the most common clinically existing form worldwide. It results from the slight decrease in the UROD activity and it is only limited to the hepatic UROD. In s-PCT, there are no mutations associated in the *UROD* gene while there are predisposing factors that lead to the symptoms

to develop such as smoking, alcohol, certain drugs and viral infections. In familial porphyria cutanea tarda (f-PCT), an autosomal dominant trait, the UROD enzyme activity decrease to half of that of normal in all tissues. The decrease in the enzyme activity is due to heterozygous mutation in the *UROD* gene (de Verneuil, et al., 1978; Elder, et al., 1980). In hepatoerythropoietic protoporphyria (HEP), an autosomal recessive trait, the UROD enzyme activity decrease between 5 % to 30 % of that of normal in all tissues due to homozygous mutation or compound heterozygous mutation in the *UROD* gene (de Verneuil, et al., 1984; Sassa, 2000).

**Genetics**

The human *UROD* gene is assigned to the short arm p34 on chromosome 1 spans over 3kb (Dubart, et al., 1986). The gene is formed of a single promoter, 10 exons, and a polyadenylation signal with a canonical AATAAA element (Morán-Jiménez, et al., 1996). The *UROD* gene codes for a primary polypeptide precursor formed of 367 amino acyl residues. The human UROD enzyme is a homodimer with a molecular weight of around 40.8 kDa (Phillips, et al., 1997).

**Protein**

The human UROD enzyme is a homodimer with the two subunits arranged head to head with their active sites situated in the interface between the two dimers.

**Pathophysiology**

The defect in the UROD catalytic activity results in the accumulation of the porphyrins especially the uroporphyrins that will be excreated in urine (a biochemical diagnostic tool in the disease). The accumulated porphyrin precursors deposite in the subcutaneous tissue in these patients with f-PCT and HEP causing photogenic dermatitis (Lim, 1989).

**Clinical picture**

PCT is the most common subtype of porphyria worldwide with onset in the middle age while HEP is the rarest form of porphyria and with early onset during childhood. HEP has more severe symptoms and signs than that of PCT as well as bad prognosis. Moreover, HEP does not require a trigger factor to manifest (Camagna, et al., 1998). Patients with f-PCT and HEP suffer mainly from skin photosensitivity on exposure to the sun light. The photosensitive reactions appear as redness, erosions that are susceptible to infections, crustings, milia, swellings and blisters with clear fluid and inflammation that usually heal slowly by scarring. These manifestations are often observed on the face, back of the hands, forearms and on the lower limbs. Hyperpigmentation and hypertrichosis on the cheeks are also sometimes visible especially in women (Brazzelli, et al., 1999). Patients with fPCT and HEP are often presented by red colour of the urine (Horner, et al., 2013). In late cases especially in patients with HEP, liver manifestations occur due to accumulation of toxic porphyrin metabolites in the hepatocytes. This leads to liver cell inflammation and liver cirrhosis (Smith, 1986).

**Diagnosis**

Uropoprhyrines and hepatocarboxylate porphyrins are markedly elevated in the urine samples of the patients with fPCT during acute attacks and in patients with HEP while PBG remain usually within the normal level (De Matteis, 1998). Plasma porphyrins and isocoproporphyrins in feaces could be also elevated (Anderson, et al., 2001). The plasma samples of the patients with PCT show a charactarestic fluorometry emission between 618-

622 nm (ussually 619 nm) which is highly suggestive of the disease (de Salamanca, et al., 1993).

**Treatment**

Predisposing factors such as alcohol intake or drugs that precipitates acute attacks should be discontinued as well as avoiding exposure to direct sun light. The database for the porphyrinogenic drugs are listed on the following web page (http://www.drugs-porphyria.org/). PCT varies from any other form of porphyria in that it could be easily controlled whether through depletion of iron stores by phlebotomy or using low dose chloroquine that form complexes with uroporphyrinogen and facilitates its release from the liver (Nordmann, et al., 2002).

## Ferrochelatase (FECH) defect disorder

Erythropoietic protoporphyria (EPP; OMIM 177000) is an inherited metabolic disorder caused by the deficient activity of the final enzyme of the heme biosynthesis ferrochelatase (FECH; EC4.99.1.1) (Bloomer, 1982).

**Genetics**

The *FECH* gene was primarily sequenced in 1990 (Nakahashi, et al., 1990) and a year later was mapped to the chromosome number 18 (18q22.31) (Whitecombe, et al., 1991). It comprises 11 exons, spans over 45kb of genomic DNA and has an open reading frame of 1269 bp. The gene encodes a primary polypeptide precursor of 423 amino acyl residues (GenBank D00726) which later undergoes proteolysis to the mature protein that consists of 369 amino acids (Nakahashi, et al., 1990; Taketani, et.al., 1992). It was described that EPP has an autosomal recessive mode of inheritance (Balwani, et al., 2012) and recently, it was found out in many patients that it has an autosomal dominant inheritance with incomplete penetrance conjoint with a wild type low expression allele (Anstey and Hift, 2007). It has been firstly reported in France that the EPP clinical expression requires two molecular defects; mutation in the *FECH* gene with the coinheritance trans to a hypomorphic FECH*IVS3-48c Allele that affects a splice site. The inheritance of the intronic low expression allele leads to an invisible acceptor splice site (63 bases upstream of the usual splicing site) transcripting abberantly spliced mRNA that carries out rapid and easy degradation resulting in the decrease of the enzyme activity and EPP expression (Gouya, et al., 2002). In 2007, another compound hetero-zygosity for mutations in the promoter (-251G) and in intron 1 (IVS1-23T) of the *FECH* gene were suggested to decrease the allele expression (Di Pierro, et al., 2007).

**Protein**

FECH (protoheme ferro-lyase, E.C. 4.99.1.1) is a homodimeric enzyme (86 kDa); consists of two identical subunits, each subunit contains a four-stranded parallel $\beta$ -sheets surrounded by $\alpha$ –helices. The mature enzyme contains an iron sulphar [2Fe-2S] cluster per subunit (Chia-Kuei, et al., 2001).

**Pathophysiology**

For the EPP to manifest, the FECH activity should decrease to 10 % - 35 % of the normal level (Kong, et al., 2008). The deficiency in the enzyme activity leads to protoporphyrin accumulation in tissues especially in the bone marrow reticulocytes, erythrocytes and in the liver. The porphyrins are excited when exposed to sun light and cause tissue and endothelial damage (Schneider-Yin, et al., 2000).

**Clinical picture**

The photosensitivity to sun light usually starts to appear in infancy and in the early childhood between 2 and 5 years (Gross, et al., 2000). The acute attacks are characterized by burning, itching, redness, inflammation and skin friability especially on the sun exposed areas. Subsequently after sun exposure, patients complain of tingling, prickling and burning sensations (Holme, et al., 2006). In late stages, the progressive accumulation of the protoporphyrins in the hepatocytes leads to hepatitis, chronic liver cirrhosis and finally liver failure. The insoluble protoporphyrins precipates in the biliary tract resulting in gall stones and cholestasis (Went, et al., 1984). Progressive polyneuropathy has been reported especially among EPP patients with liver complications due to protoporphyrin accumulation in the peripheral nerve cells (Muley, et al., 1998).

**Diagnosis**

The protoporphyrins in the red blood corpuscles are shown to be highly elevated by the high-performance liquid chromatography and mostly in the free form (not conjugated with Zinc) (Patel, et al., 2000). The detection of the fluorescent red blood corpuscles (RBCs) in an unstained blood smear by the fluorescence microscopy is diagnostic in the EPP patients. Subsequently, the red plasma fluorescence emission is performed and shows a peak at 634 nm with excitation at 405 nm (Whatley, et al., 2008).

**Treatment**

Predisposing factors and direct exposure to sun light should be avoided. Symptomatic treatment such as topical anti-inflammatory drugs could be used to relieve the sunlight induced dermatitis. Oral administration of β-carotene enhance somehow tolerance to sun light in these patients. α-melanocyte-stimulating hormone analog is recently recommended to decrease the photosensitivity reactions that result from the sun exposure (Harms, et al., 2009). Recently, two approaches are recommended for the management of EPP; the first is Cholestyramine (bile sequestrating agent) on daily basis that prevent the re-absorption of the porphyrins from the entero-hepatic circulation by binding to them and facilitating the faecal excretion (Frank, et al., 1995). The second approach is the exchange transfusion or hypertransfusion but it is not suitable for long term management (Dobozy, et al., 1983).

## AIMS OF THE STUDY

Specific aims of the study:

To optimize appropriate analytical methods for detection of the DNA variations in the *FECH* and in the *UROD* genes.

To conduct genetic diagnosis for patients with f-PCT, HEP and EPP from Slavic and Arabic origin.

To perform the expression, the purification and the activity measurement of the human mutant UROD enzyme with introduced pathological mutation of interest in the *E.coli*.

In the case a molecular defect will be found in HEP, to construct graphical representation of UROD structure and prepare an alignment of a segment surrounding the mutated defect in human *UROD* gene with corresponding orthlogous sequences identified in selected metazoa, plants, fungi, bacteria and archea species.

To study extended Czech family with EPP.

To study the frequency of the low expression allele IVS-48c in the *FECH* gene in the healthy controls from the Czech population.

## HYPOTHESES

To test the hypothesis that in biallelic defects UROD needs to keep residual activity to sustain acceptable clinical status.

To test the hypothesis that the severity of metabolic phonotype depends on catalytic activity of UROD.

To test the hypothesis on extended family that clinical manifestation of EPP is always connected with the appearance of the low expression allele and the mutation within *FECH* gene.

To test the hypothesis that low incidence of EPP in the Czech Republic is caused by low frequency of low expression allele IVS3-48C in the Czech population.

## MATERIALS AND METHODS

### DNA Samples

***Patients examined***

Fourteen patients (7 males and 7 females) with porphyria were investigated for the mutations in the *UROD* and the *FECH* genes.

***Healthy controls***

The study enrolled a total of 312 healthy controls from the Czech population. 149 were males and 163 were females. All the samples in the study group were unrelated adults and were obtained through out the longitudinal collection of the control samples of healthy individuals from west Slavic origin in our laboratory.

### Genomic DNA isolation

The genomic DNA was extracted from the peripheral white blood cells by the common salting out procedure. Genomic DNA was isolated from the low volume blood samples using the QIAamp DNA Blood Minikit (QIAGEN, Hilden, Germany) according to manufacturer´s protocol.

### Polymerase chain reaction

Matching pairs of primers were designed using the GeneBank reference sequence in the intronic area encircling the desired exons to avoid missing of the splicing site mutations for both genes *UROD* and *FECH*. The PCR amplification cycles were done on DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories).

### DNA sequencing

The sequence of the purified samples was processed on the automatic sequencer ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster city, CA, USA).

### Site directed mutagenesis

The plasmid Vector for the mutagenesis and the further expression of the wild type of the human UROD was kindly constructed and supplied by prof. de Verneuil Hubert from Biotherapies des Maladies Genetiques et Cancers at the University of Bordeaux (France). Vector pGEX-2T (Amersham Biosciences, Orsay, France) was used to construct the vector pGEX-UD for the expression of the human enzyme UROD. Site-directed mutagenesis was created to produce the required mutation using Quik Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol using the designed mutagenic primers.

**Protein expression**

The human UROD proteins were expressed in the *E. coli* strain codon plus BL21 (Stratagene) and cultured in Terrific Broth medium (TB) containing 100 µM riboflavin and 125 µM ampicillin (Sigma-Aldrich). The dividing cells with the wild type and the mutated constructs were incubated overnight at 37°C in (TB) medium containing 100 µM riboflavin and 10 µg/ml of kanamycin (Sigma-Aldrich). The cells were induced to produce the desired proteins by adding 2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) (Fisher HC, Houston, TX, USA). The bacterial pellets were grown under aerobic conditions for 24 hours at 18°C and subsequently were harvested by centrifugation at 4°C for 10 minutes at 7000 g.

**Protein purification**

Lyzozymes and sonication were used to lyze the bacterial cell harvest. Centrifugation was done at 4°C to obtain the desired soluble protein in the supernatant (Beckman J25, Brea, CA, USA) for one hour at 100 000 x g. Equilibrated Glutathion Sepharose 4B (GE Healthcare) is applied to the supernatant soluble protein after being purified from the cell debris for 1 hour at 4°C. Elution of the desired protein was done to obtain a highly purified fraction of the desired protein. The purity of the generated protein was determined by polyacrylamide gel electrophoresis (SDS-PAGE) and the fractions showing a single band on the gel were extracted and concentrated for enzyme activity measurement (Centriprep YM-30, Millipore Corporation, Billerica, MA, USA).

**Multiplex Ligation-dependent Probe Amplification (MLPA)**

MLPA is a modern, relatively cheap, rapid and reliable assay that we recently introduced in our laboratory to detect the big deletions or duplications in the *FECH* gene that cannot be identified by the usual PCR. This method helped us to amplify many targets by one set pair of primers. We used the SALSA MLPA kit P412 verion A1 (MRC-HOLLAND, Amesterdam, Holland) in which the probes are designed for the hybridization of specific target sequence in the *FECH* gene as well as several other gene sequences. The obtained data were checked by the softwares Peak Scanner v1.0 and Gene Mapper v4.0 (both Applied Biosystems, Foster city, CA, USA).

**Real-time PCR copy-number variation assay**

This technique was used to double check the molecular results of the *FECH* gene that was obtained by MLPA. Therefore, it helped us to verify the obtained data by MLPA. The presence of one copy of the *FECH* gene was confirmed by a real-time PCR copy-number variation assay (Hs00926149_cn; Applied biosystems) according to the manufacturer's instructions. The reference gene used was RNASE P (Device StepOnePlus).

**Restriction Fragment length Polymorphism (RFLP)**

RFLP was used in this study as a quick and reliable method to detect the sequence variants between the different healthy controls in a population. We used it to identify the frequency of the three different variants of the low expression allele IVS-48 T/C, IVS-48 T/T and IVS-48 C/C in the third intron of the *FECH* gene.

## RESULTS

**Molecular analysis of the *UROD* gene in patients with f-PCT**

During our study, we identified the following mutations in the *UROD* gene in Czech patients with fPCT: p.[Gln206*];[=] in exon 6, (c.399-401 delins CCA) (V134Q) in exon 5 and a splice site heterozygous mutation c. 942+1 G>A in intron 9. We consider them to be a disease causing mutations.

**Molecular analysis of the *UROD* gene in patients with extremely rare HEP**

We identified a novel homozygous mutation (c.163T>A) in HEP patients for the first time in the Arabic patients from Egypt as shown in figure 1.

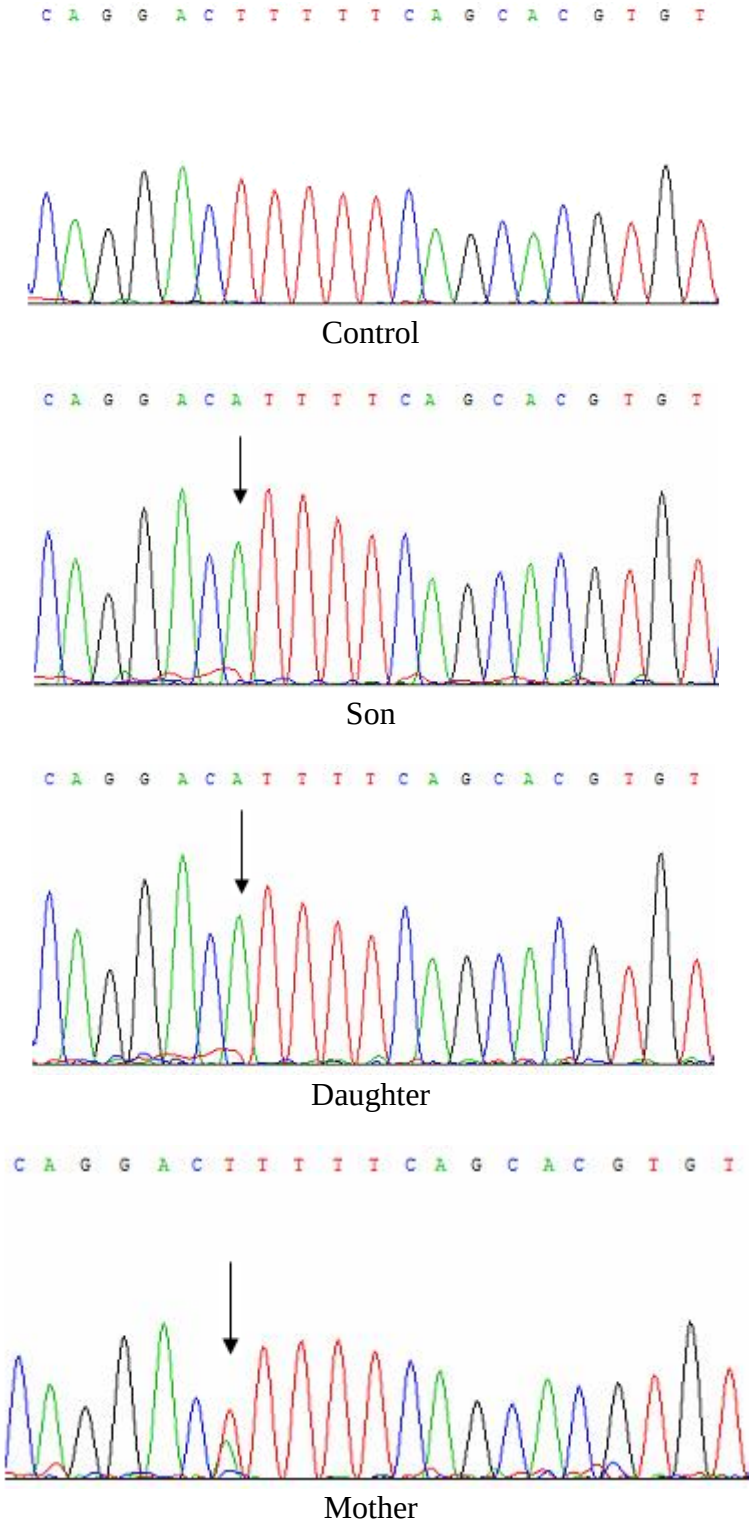



**Figure 1**. Sequencing profiles for the mutation of the *UROD* gene in the different family members. *TTT* indicates the normal allele (phenylalanine) present in the control sample. *ATT*, the mutant allele (Isoleucine) is present at the homozygous state in the probands and heterozygous state (*WTT*) in the mother; W means A and T when both *ATT* and *TTT* alleles are present.

The mutation resulted in the substitution of phenylalanine to isoleucine at the codon 55 (F55I). We expressed mutated UROD protein in prokaryotic vector and the specific activity of the protein was determined and found that F55I mutant UROD aquires 19 % of the activity of the wild-type as shown in table 1. These measurements were done in collaboration with the laboratory of prof. H. de Verneiul in Bordeaux, France.

**Table 1.** Expression of the normal and mutated GST-UROD fusion proteins**.** Data are given as picomoles of coproporphyrinogen I formed per hour and per milligram of protein

| Plasmid | Specific activity [#] | | No of assays | Residual activity (% vs WT) |
|---|---|---|---|---|
| | Mean (SD) | Range | | |
| pGEX-UROD | 3,1± 0,9 | 2,6 - 3,2 | 3 | 100 |
| pGEX-UROD-F55I | 0,6± 0,1 | 0,5 - 0,7 | 3 | 19 |

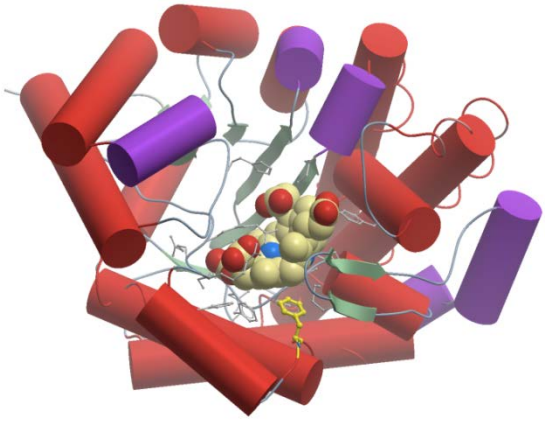


**Figure 2.** Graphical representation of UROD structure (PDB 1r3y, only one monomeric unit is displayed) with coproporphyrinogen product in space-filling rendering. Sidechains participating in the direct binding of the product are displayed in ball and stick. Phe 55 (mutated in the probands to Ile) is highlighted in bold and yellow.

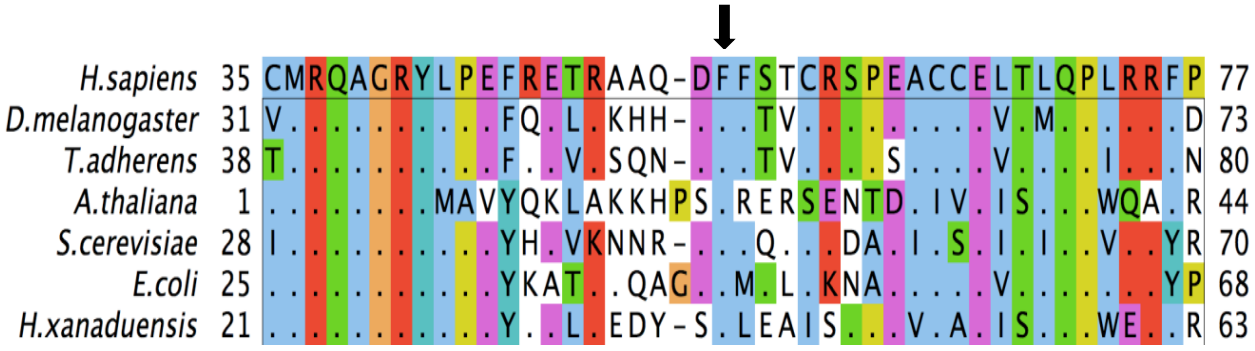



**Figure 3**. Alignment of a segment surrounding F55 (arrow) in human UROD with corresponding orthlobous sequences identified in selected metazoa, plants, fungi, bacteria and archea. Species and UniProt sequence identifiers from top to bottom: *Homo sapiens*, P06132; *Drosophila melanogaster*, Q9V595; ***Trichoplax adhaerens,*** B3S2H7: ***Arabidopsis thaliana*** Q93ZB6; ***Saccharomyces cerevisiae***, P32347; ***Escherichia coli,*** P29680; *Halopiger xanaduensis*, F8DD35. Only conserved amino acids are shown and their functionality is colored according to ClustalW scheme (www.jalview.com). F55 has been found conserved in all sequences inspected (over 1100).

**Molecular analysis of *FECH* in patients with EPP**

In this study, we identified several mutations in the *FECH* gene in three Czech patients and one Slovak patient with EPP. We identified a novel nonsense mutation p.[Ser264*];[=] in exon 7 that resulted in a truncated protein in a Slovak patient. We identified deletion in exon 1 in a Czech patient. A novel mutation p.[Trp28*];[=] in exon 2 of the *FECH* gene was found in two members of an extended Czech family with EPP that is located in an area of mitochondrial targeting sequence spanning amino acid residues 1-62 that is removed during proteolytic cleavage.

**Molecular results**

Molecular genetic analyses of the ferrochelatase gene revealed a novel heterozygous G84A transition in exon 2 as shown in figure 4. The point mutation leads from a tryptophan to a stop codon substitution (p.W28*). The amino acid tryptophan at position 28 is located in area of mitochondrial targeting sequence spanning amino acid residues 1-62 that is removed during proteolytic processing. Mutation analyses were carried out on eight members of proband´s family. We found the same mutation in both probands (the mother & the son). Two other family members also showed the G84A mutation but are meanwhile clinically silent as shown in the pedigree in figure 5. The mother and the son with EPP are both heterozygous for intronic single nucleotide polymorphisms (SNP) IVS3-48C/T which is required for the expression of the EPP and IVS1-23 T/C with a presumed role in EPP pathogenesis (Gouya et al., 1999). Given the available data, it seems that the *CAT* haplotype (IVS1-23 T/C; G84A; IVS3-48C/T) combined with *TGC* haplotype precipitated in EPP manifestation in the two probands, whereas the combination of *TGC* or *CAT* with the wild-type *CGT* did not lead to any clinical consequences.

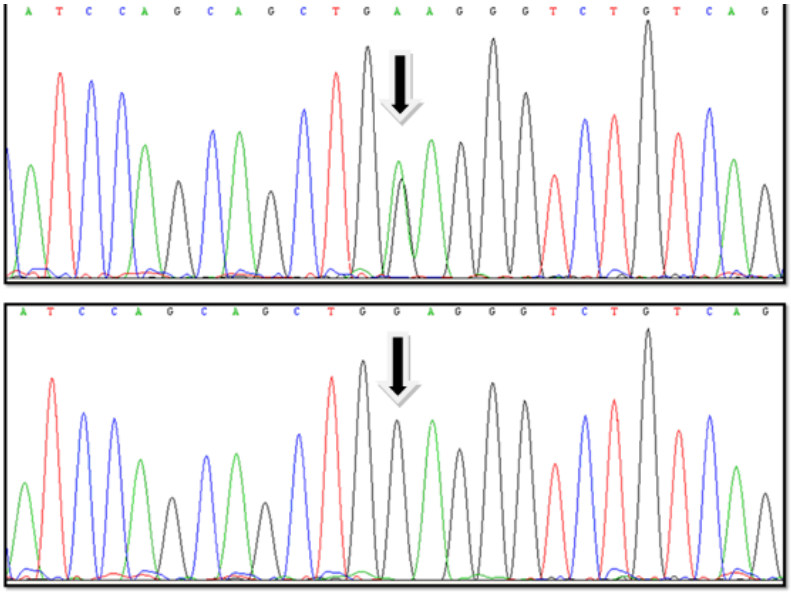


**Figure 4**. G84A mutation in exon 2 in the *FECH* gene with comparison to a control sequence below.

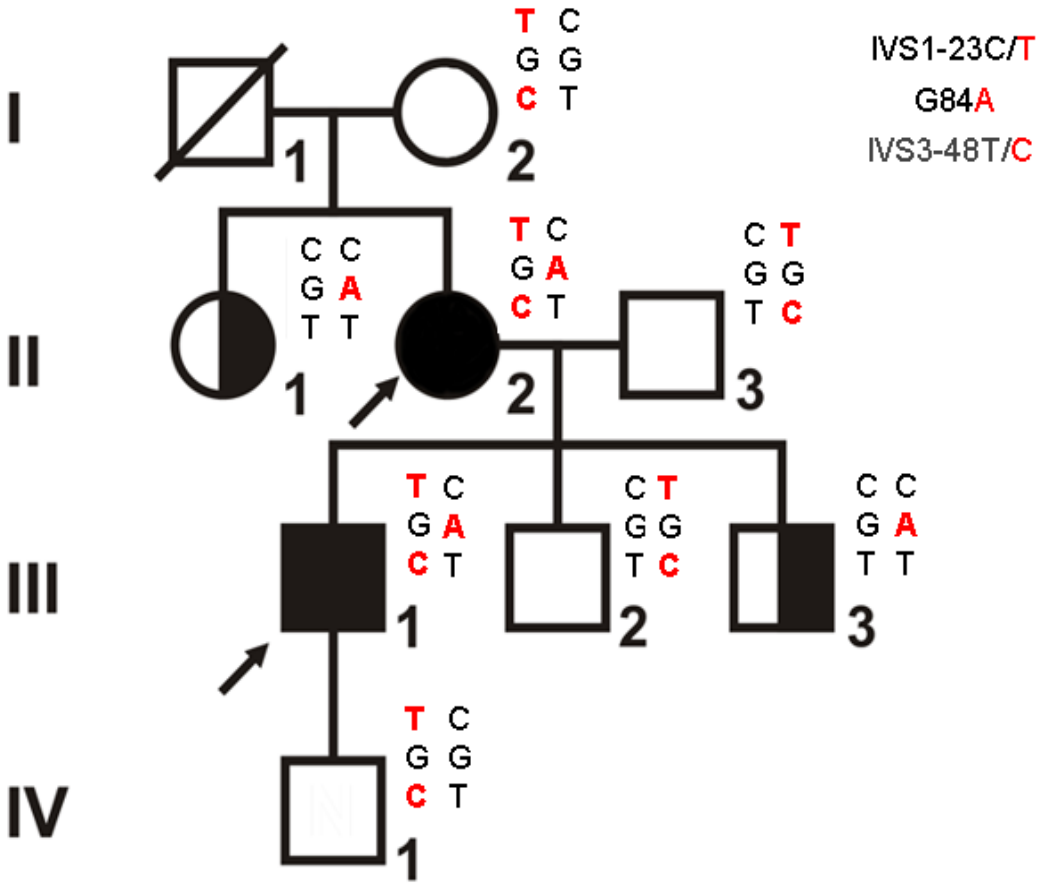
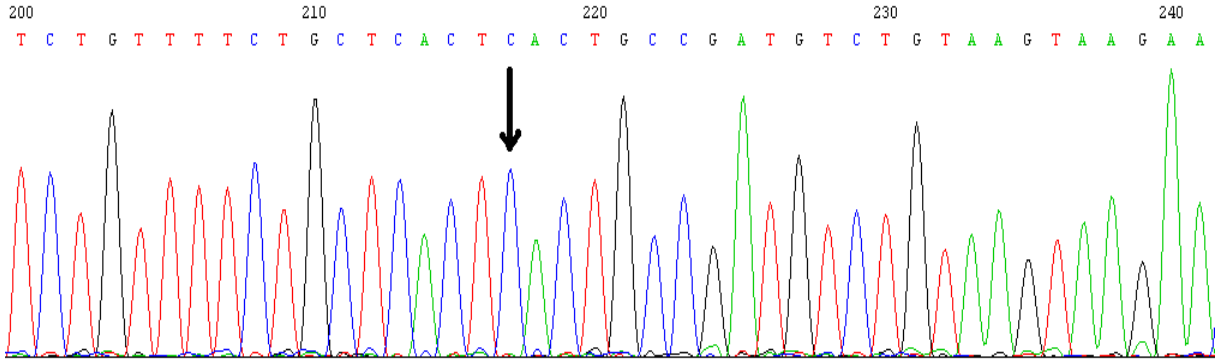



**Figure 5.** Genealogical tree of the EPP family with p.W28* mutation. A pedigree of the proband´s family. ○ - female, □ - male, full filled - clinically manifest porphyria, half-filled - clinically silent. Polymorphisms are shown as haplotypes (left and right columns) with the mutant alleles in red, from top to bottom: IVS1-23C/T, mutation within *FECH* exon 2 G84A m; and the low expression allele polymorphism IVS3-48C/T.

### Description of a new mutation within *FECH* gene as a cause of EPP

A female Slovak patient was clinically diagnosed as porphyria and the blood sample was sent to our laboratory for molecular analysis. Analysis of the whole *FECH* gene was performed and revealed a novel heterozygous nonsense mutation C→A transition at position 264 in exon 7 of the *FECH* gene leading to TAA instead of TCA ( UAA is a stop codon). The point mutation leads from Serine to a stop codon substitution (S264X) that results in a truncated protein as shown in figure 6. Unfortunately, no relatives of the proband were availbale for the molecular study of the *FECH* gene.The low expression allele IVS3-48 T>C was found to be heterozygous. A missense mutation S264L, affecting the same codon has been previously reported, in an Italian patient (Aurizi, et al., 2007).

**Control**




**Mutation:**

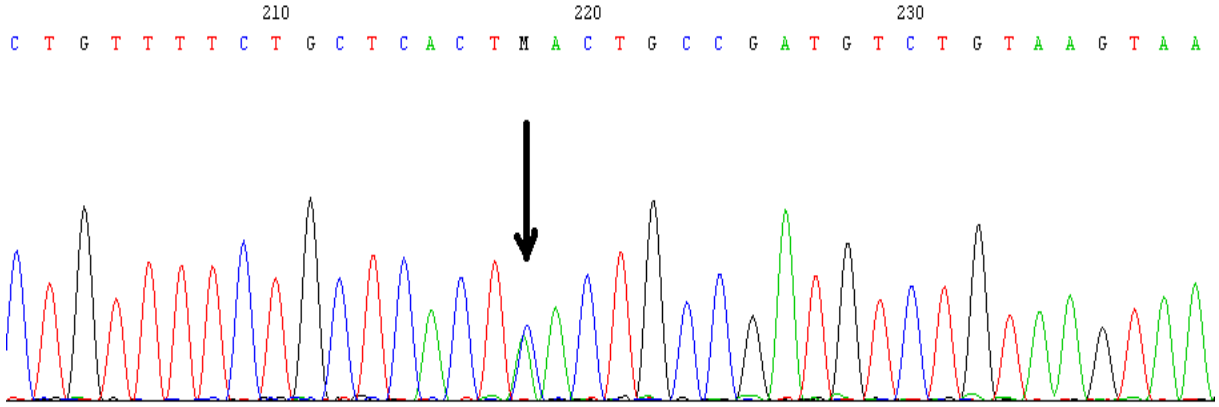



**Figure 6.** S264X mutation in exon 7 of the *FECH* gene with comparison to a control sequence above.

**Screening the frequency of the low expression allele IVS-48C in the *FECH* gene in the Czech population**

There is no knowledge about the frequency of low expression allele in Slavic population. We, therefore, screened the frequency of the splice site modulator IVS3-48c in 624 alleles of 312 controls, 149 controls were males and 163 females. The Czech healthy control identified 277 homozygotes IVS3-48t/t and 35 heterozygotes IVS3-48c/t. Therefore, the frequency of C allele was estimated as 5,5 % in the Czech population, 5 % among males and 6 % among females as shown in table 2.

**Table 2.** Describes the number of controls, the percentages of the different genetic variation of the low expression allele IVS3-48c in males, females and the total Czech controls.

| | Total male control screened (n=149) | Total female control screened (n=163) | Total Czech control screened (n=312) |
|---|---|---|---|
| Genotype | | | |
| IVS-48T/C | 15 subjects (5 % individuals have C allele) | 20 subjects (6 % of screened individuals have C allele) | 35 subjects (5,5 % of all screened subjects have C allele) |
| IVS-48T/T | 134 subjescts | 143 subjects | 277 subjects |
| IVS-48C/C | 0 | 0 | 0 |

# DISCUSSION

## Study of molecular pathology of new case of rare HEP in Arabic family from Egypt

We described a new missense mutation of the *UROD* gene at the homoallelic state in young patients in an Egyptian family, characteristic of the HEP. The observed mutation in the probands was present at the homozygous state and their mother showed to be a heterozygous carrier. There was no possibility to obtain the blood from the father, but he should be bearing the same mutation as the mother did. This is more evident because father and mother of the affected siblings were cousins.

The mutation in the probands leads to the substitution of phenylalanine to isoleucine at position 55 of UROD. According to the crystal structure (Phillips et al., 2003), this residue participates in the loop between Helix 2 and 3 and interacts tightly with the substrate in the active site (Figure 2). Moreover, F55 is conserved in both eukaryota and prokaryota (Figure 3). This offers a possibility to study this new mutation in UROD active site with a specific human phenotype and enzyme activity using a prokaryotic expression system. The F55I mutant protein expressed in bacteria exhibits 19 % of the wild-type protein activity (Table 1). We did not have possibility to investigate biological samples of the affected individuals to measure directly UROD enzyme activity. However, the relatively moderate skin problems of the children correlate well with the high residual activity of the UROD.

The mutation identification helps us to better understand the disease and helps in counselling the affected families. We report the first cases of HEP in the Egyptian population based on molecular diagnosis of the *UROD* gene. Because of the very low number of the HEP patients worldwide, the identification of a novel *UROD* mutation and its characterization broaden our current knowledge on the molecular heterogeneity of the HEP worldwide.

In the mutation described in this paper, an aromatic residue is replaced by a bulky aliphatic one. The stacking interaction of three aromatic residues (Figure 2) forming the bottom of the substrate-binding site is thus disrupted and the substrate binding becomes probably much looser. The absolute conservation of the mutated residue indicates that the optimal arrangement in the vicinity of the substrate has been reached early in the evolution and no further diversion in this region was possible.

To date, there are 109 known mutations in the *UROD* gene in humans. Their detailed molecular analysis is likely to contribute to discoveries of new biological roles and functions of this evolutionarily conserved enzyme.

## EPP in extended Czech family

To date, more than 130 mutations in *FECH* gene have been reported worldwide. We identified a novel missense mutation in the *FECH* gene in four members in a Czech family, a transition of G84A in exon 2 leading from a tryptophan to a stop codon substitution causing premature ending of translation. For clinical manifestation of EPP, a synergy of a private mutation within *FECH* gene and the presence of low expression IVS3-48C allele in trans is needed in majority of cases (Gouya et al.,1999; Richard et al., 2008, Tahara et al., 2010) Indeed, the two patients with manifest EPP inherited, apart from the G84A variant, also the hypomorphic allele IVS-48C (and the IVS1-23T intronic variant) which is necessary for the EPP phenotype (Tahara et al., 2010). As both intronic variants are apparently inherited in a single haplotype block (Figure 2), it is impossible to assess the distinct impact of IVS1-23T in combination with G84A mutation.

**Screening the frequency of the low expression allele IVS-48C in the *FECH* gene in the Czech control cohort**

So far, just few patients with EPP were diagnosed in the Czech Republic despite good availability of a laboratory diagnosis of this disorder. According to our knowledge, it was only 5 families in the last three decades, in three patients molecular defect was shown (including one described in this thesis, Martasek P., personal observation).

We, therefore, performed a screening for the frequency of low expression allele in control Czech (west Slavic) Caucasian population. Such a study was not performed yet in any Slavic population. Previously, it has been reported that the frequency of the IVS3-48C allele in the Japanese population was 45 % (Nakano et al., 2006), in Chinese (Han) 41 % (Kong et al., 2008), in southeast Asian 31 %, in white French 6.4 % (Gouya et al., 2006), in British 13 % (Berroeta et al., 2007), in Ashkenazi Jews 8 % (Schneider-Yin et al., 2008), in Swiss population 7 % (Schneider-Yin et al., 2009), in Spanish 5 % (Herrero et al., 2007), in north African 2.7 %, in black west African populations <1 % (Gouya et al., 2006) and in Italians 1% (Aurizi et al., 2007).

Our results from the first Slavic Caucasian screening of 624 alleles in Czech population thus indicate the overall IVS3-48C allele frequency of 5.5 %, comparable to the above mentioned reports from other West Caucasian populations. While the frequency of IVS3-48C allele is most likely not the reason for the low incidence of EPP in the Czech Republic, it remains to be determined whether distinct protective variant or complex rearrangements of *FECH* or other genes involved in EPP pathogenesis underlie this phenomenon.

# CONCLUSION

Genotyping both *FECH* and *UROD* genes which are responsible for EPP, f-PCT and HEP, respectively, the heterogeneity of the *FECH* and *UROD* genes were shown in patient of Slavic and Arabic origin.

We described two novel mutations in the *FECH* gene and one novel mutation in the *UROD* gene.

We report the first cases of HEP in the Egyptian population based on molecular diagnosis of the *UROD* gene. Because of the very low number of the HEP patients worldwide, the identification of a novel *UROD* mutation and its characterization broaden our current knowledge on the molecular heterogeneity of the HEP worldwide.

We confirmed the importance of the inheritance of the low expression allele IVS3-48C for the EPP manifestation in the Czech population. The importance of the low expression allele IVS3-48C for the clinical manifestations was confirmed by analysis of the extended Czech EPP family as well in one single case.

Our results from the first Slavic Caucasian screening of 624 alleles in Czech control population thus indicate the overall IVS3-48C allele frequency of 5.5 %, comparable to the reports from other West Caucasian populations.

In clinical genetics settings, the mutation identification helps us to better understand the disease and helps in treating and counselling the affected families.

# REFRENCES


Anderson KE, Sassa S, Bishop DF, Desnick RJ. 2001. Disorders of heme biosynthesis: X-linked sideroblastic anemia and the porphyrias. *In:*The metabolic and molecular basis of inherited disease. (Scriver, C. R., Beaudet A. L., Sly, W. S., Valle, D. Eds). Ed 8. pp 2961–3062. McGraw-Hill, New York.

Anstey AV and Hift RJ. 2007. Liver disease in erythropoietic protoporphyria: insights and implications for management. Postgrad Med J. 83(986), 739–748.

Aurizi C, Schneider-Yin X, Sorge F, Macri A, Minder EI, Biolcati G. 2007. Heterogeneity of mutations in the ferrochelatase gene in Italian patients with erythropoietic protoporphyria. Mol Genet Metab. 90, 402–407.

Balwani M, Bloomer J, Desnick R. 2012. Erythropoietic Protoporphyria, Autosomal Recessive. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle.

Berroeta, L., Man, I., Goudie, D. R., Whatley, S. D., Elder, G. H., Ibbotson, S. H. 2007. Late presentation of erythropoietic protoporphyria: case report and genetic analysis of family members. Br J Dermatol. 157, 1030–1031.

Brazzelli V, Chiesa MG, Vassallo C, Ardigò M, Borroni G. 1999. Clinical spectrum of porphyria cutanea tarda. Haematologica. 84(3):276-7.

Camagna A, Del Duca P, Petrinelli P, Borelli LG, Ciancio L, Cipollone L, Misasi G, Manfredi MR, Dionisi S, de Martinis C. 1998. Erythrocyte uroporphyrinogen decarboxylase activity: diagnostic value and relationship with clinical features in hereditary porphyria cutanea tarda. Am J Med Sci. 315(1):59-62.

Chia-Kuei Wu, Harry A Dailey1, John P Rose1, Amy Burden, Vera M. Sellers and Bi-Cheng Wang. 2001. The 2.0 Å structure of human ferrochelatase, the terminal enzyme of heme biosynthesis. nature structural biology. volume 8, number 2, 156-160.

De Matteis F. 1998. Porphyria cutanea tarda of the toxic and sporadic varieties. Clin Dermatol. 16(2):265-75.

de Salamanca RE, Sepulveda P, Moran MJ, Santos JL, Fontanellas A, Hernández A. 1993. Clinical utility of fluorometric scanning of plasma porphyrins for the diagnosis and typing of porphyrias. Clin Exp Dermatol.18(2), 128-30.

de Verneuil H, Aitken G, Nordmann Y. 1978. Familial and sporadic porphyria cutanea tarda, two different diseases. Hum Genet. 44, 145-51.

de Verneuil H, Beaumont C, Deybach JC, Nordmann Y, Safar Z, Kastally R. 1984. Enzymatic and immunological studies of uroporphyrinogen decarboxylase in familial porphyria cutanea tarda and hepatoerythropoietic porphyria. Am J Hum Genet. 36,613-622.

Di Pierro E, Brancaleoni V, Moriondo V, Besana V, Cappellini MD. 2007. Co-existence of two functional mutations on the same allele of the human ferrochelatase gene in erythropoietic protoporphyria. Clin Genet. 71(1):84-8.

Dobozy A, Csató M, Siklósi C, Simon N. 1983. Transfusion therapy for erythropoietic protoporphyria. Br J Dermatol. 109, 571–576.

Dubart A, Mattei MG, Raich N, Beaupain D, Romeo PH, Mattei JF, Goossens M. 1986. Assignment of human uroporphyrinogen decarboxylase (URO-D) to the p34 band of chromosome 1, Hum. Genet. 73, 277–279.

Elder GH. 1998. Porphyria cutanea tarda. Semin. Liver Dis. 18, 67–75.

Elder GH, Smith SG, Jane Smyth S. 1990. Laboratory investigation of the Porphyrias. Ann Clinical Biochemistry 27, 395-412.

Frank M, Doss MO. 1995. Liver and liver cirrhosis in protoporphyria: bile acid therapy. Gastroenterology. 33, 399–403.

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), 256-262.

Gouya L, Puy H, Lamoril J, Silva VD, Grandchamp B, Nordmann Y and Deybach JC. 1999. Inheritance in erythropoietic protoporphyria: a common wild-type ferrochelatase allelic variant with low expression accounts for clinical manifestation. Blood. 93, 2105-2110.

Gouya L, Martin-Schmitt C, Robreau AM, Austerlitz F, Da Silva V, Brun P, Simonin S, Lyoumi S, Grandchamp B, Beaumont C, Puy H, Deybach JC. 2006. Contribution of a common single-nucleotide polymorphism to the genetic predisposition for erythropoietic protoporphyria. Am J Hum Genet. 78(1), 2-14.

Gross U, Hoffmann GF, Doss MO. 2000. Erythropoietic and hepatic porphyrias. J. Inherit. Metab Dis. 23, 641-661.

Harms J, Lautenschlager S, Minder CE, Minder EI. 2009. An alpha-melanocyte-stimulating hormone analogue in erythropoietic protoporphyria. N Engl J Med. 360(3), 306–307.

Herrero, C., To-Figueras, J., Badenas, C., Méndez, M., Serrano, P., Enríquez-Salamanca, R., Lecha, M. 2007. Clinical, biochemical, and genetic study of 11 patients with erythropoietic protoporphyria including one with homozygous disease. Arch Dermatol. 143, 1125–1129.

Holme SA, Anstey AV, Finlay AY, Elder GH, Badminton MN. 2006. Erythropoietic protoporphyria in the U.K.: clinical features and effect on quality of life. Br J Dermatol. 155, 574-581.

Horner ME, Alikhan A, Tintle S, Tortorelli S, Davis DM, Hand JL. 2013. Cutaneous porphyrias part I: epidemiology, pathogenesis, presentation, diagnosis, and histopathology. Int J Dermatol. 52(12),1464-1480.

Kappas A, Sassa S, Galbraith RA, Nordmann Y. 1990. The porphyrias. In: Scriver C, Beaudet A, Sly W. Valle D, eds. The metabolic basis of inherited disease. Ann Clin Biochem. 27, 395-412.

Kappas A, Sassa S, Galbraith RA, Nordmann Y. 1995. The porphyrias. In: Scriver CR, Beaudet AL, Sly W, eds. The metabolic basis of inherited disease, 7th ed. McGraw Hill, New York, pp 2103-2159.

Kong XF, Ye J, Gao DY, Gong QM, Zhang DH, Lu ZM, Lu YM, Zhang XX. 2008. Identification of a ferrochelatase mutation in a Chinese family with erythropoietic protoporphyria. J Hepatol. 48, 375-379.

Lamoril J, Boulechfar S, de Verneuil H, Grandchamp B, Nordmann Y, Deybach JC. 1991. Human erythropoietic protoporphyria: two point mutations in the ferrochelatase gene. Biochem Biophys Res Commun. 181(2), 594-599.

Lim HW, 1989.Mechanisms of phototoxicity in porphyria cutanea tarda and erythropoietic protoporphyria. Immunol Ser. 46, 671-685.

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.

Morán-Jiménez MJ, Ged C, Romana M, Enríquez de Salamanca R, Taiëb A, Topi G, D'Alessandro L, de Verneuil H. 1996. Uroporphyrinogen decarboxylase: complete human gene sequence and molecular study of three families with hepatoerythropoietic porphyria. Am. J. Hum. Genet. 58, 712–721.

Muley SA, Midani HA, Rank JM, Carithrs R, Parry GJ. 1998. Neuropathy in erythropoietic protoprophyrias. Neurology. 51, 262-265.

Nakahashi Y, Taketani S, Okuda M, Inoue K, Tokunaga R. 1990. Molecular cloning and sequence analysis of cDNA encoding human ferrochelatase. Biochem Biophys Research Comm. 173, 748-755.

Nakano, H., Nakano, A., Toyomaki, Y., 2006. Novel ferrochelatase mutations in Japanese patients with erythropoietic protoporphyria: high frequency of the splice sitemodulator IVS3–48C polymorphism in the Japanese population. J Invest Dermatol. 126, 2717–2719.

Nordmann Y, Puy H. 2002. Human hereditary hepatic porphyrias. Clin. Chim. Acta. 325, 17-37.

Patel GK, Weston J, Derrick EK, Hawk JL. 2000. An unusual case of purpuric erythropoietic protoporphyria. Clin Exp Dermatol. 25(5), 406-408.

Phillips JD, Whitby FG, Kushner JP and Hill CP. 1997. Characterization and crystallization of human uroporphyrinogen decarboxylase. Protein Sci. 6, 1343-1346.

Philips, J.D., Whitby, F.G., Kushner, J.P., Hill, C.P. 2003. Structural basis for tetrapyrrole coordination by uroporphyrinogen decarboxylase. EMBO J.; 22, 6225-33.

Richard E, Robert-Richard E, Ged C, Moreau-Gaudry F, De Verneuil H. 2008. Erythropoietic porphyrias: animal models and update in gene-based therapies. Curr Gene Ther. 8(3), 176-186.

Sassa S. 2000. Hematologic aspects of the porphyrias. Int J Hematol. 71, 1-17.

Schmid R, Schwartz S, Watson CJ. 1954. Porphyrin content of bone marrow and liver in the various forms of porphyria. AMA Arch. Internal Medicine. 93, 167-190.

Schneider-Yin X, Gouya L, Meier-Weinand A, Deybach JC, Minder EI. 2000. New insights into the pathogenesis of erythropoietic protoporphyria and their impact on patient care. Eur J Pediatr. 159, 719–725.

Schneider-Yin X, Harms J, Minder EI. 2009. Porphyria in Switzerland, 15 years experience, Swiss Med Wkly. 139, 13-14.

Schneider-Yin X, Mamet R, Minder EI, Schoenfeld N. 2008. Biochemical and molecular diagnosis of erythropoietic protoporphyria in an Ashkenazi Jewish family. J Inherit Metab Dis 31 (Suppl 2), S363-S367.

Shibahara S, Kitamuro T, Takahashi K. 2002. Heme degradation and human disease: diversity is the soul of life. Antioxid. Redox Signal. 4, 593–602.

Smith SG. 1986. Hepatoerythropoietic porphyria. Semin Dermatol. 5(2), 125-137.

Tahara, T., Yamamoto, M., Akagi, R., Harigae, H., Taketani, S. 2010. The low expression allele (IVS3-48C) of the ferrochelatase gene leads to low enzyme activity associated with erythropoietic protoporphyria. Int J Hematol. 92(5):769-71.

Taketani S, Inazawa J, Nakahashi Y, Abe T, Tokunaga R. 1992. Structure of the human ferrochelatase gene: exon/intron gene organisation and location of the gene to chromosome 18. Eur J Biochem. 205, 217-222.

Went L, Klasen EC. 1984. Genetic aspects of erythropoietic protoporphyria. Ann Hum Genet. 48, 105–117.

Whatley SD, Gouya L, Grandchamp B, Beaumont C, Badminton MN, Elder GH, Holme SA, Anstey AV, Parker M, Corrigall AV, Meissner PN, Hift RJ, Marsden JT, Ma Y, Mieli-Vergani G, Deybach JC, Puy H. 2008. C-terminal deletions in the ALAS2 gene lead to gain of function and cause x-linked dominant protoporphyria without anemia or iron overload. Am J Hum Genet. 83, 408–414.

White KA, Marletta MA. 1992. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. Biochemistry. 31(29), 6627-6631.

Whitcombe DM, Carter NP, Alvertson DG, Smith SJ, Rhodes DA, Cox TM. 1991. Assignment of the human ferrochelatase gene (FECH) and a locus for protoporphyria to chromosome 18q22. Genomics. 11, 1152–1154.

## LIST OF ORIGINAL PUBLICATIONS

***Publications in extenso related to the thesis:***

**Farrag S.M**, Kučerová J. , Šlachtová L., Šeda O., Šperl J., Martásek P.
A novel mutation in the FECH gene in a Czech family with erythropoetic protoporphyria and a population study of IVS-48C variant contributing to the disease
Folia Biologica, 2015; *in press*.
IF = 1,000

**M.S.Farrag**, I.Mikula, V. Saudek, E. Richard, H. de Verneuil, P. Martasek.
Hepatoerythropoietic porphyria caused by novel homoallelic mutation in uroporphyrinogen decarboxylase gene in Egyptian patients. Folia Biologica, 2015; *in press*
IF = 1,000

**Publications not related to the thesis**:

Stránecký V, Hoischen A, Hartmannová H, Zaki MS, Chaudhary A, Zudaire E, Nosková L, Barešová V, Přistoupilová A, Hodaňová K, Sovová J, Hůlková H, Piherová L, Hehir-Kwa JY, de Silva D, Senanayake MP, **Farrag MS**, Zeman J, Martásek P, Baxová A, Afifi HH, St Croix B, Brunner HG, Temtamy S, Kmoch S. Mutations in ANTXR1 cause GAPO syndrome. American Journal of Human Genetics, 2013 May 2; 92(5):792-9.
IF = 10.931 (cited 14x)

## CONFERENCE ABSTRACTS

***2014***

- **Farrag S.M.**, I.Mikula, E. Richard, H. de Verneuil, Martásek P. *Novel mutation in Uroporphytinogen decarboxylase gene in Egyptian patients with Hepatoreythropoietic protoporphyria.* Gordon research conference, Chemistry and Biology of tetrapyrroles. Newport, Rhode Island, USA.

***2013***

- **Farrag S.M.**, Martásek P. *Novel ferrochelatase gene mutation in a Slovak patient with erythropoietic protoporphyria and the prevelance of common single-nucleotide polymorphism that contribute to the genetic predisposition in Slavic and Jewish populations.* In 12th International congress of Inborn errors of metabolism, 3-6 September 2013 Barcelona, Spain. Journal of inherited metabolic disorders, 2013 ,Volume 36 (Suppl 2): S91-S342, Meeting abstract P-868.

***2012***

- **Farrag S.M.** , Kučerová J., Šlachtová L., Puchmajerová A., Šperl J ,

Martásek P. Erythropoietic protoporphyria: *Novel mutation in the ferrochelatase gene in a Czech family*. In 16 conference DNA diagnosis ,Czech Republic . 28-30 November 2012. P.58.

***2011***

- **Farrag S. M**, Sperl J, Kucerova J, Spucal J. Martasek P: *Molecular patology of erythropoietic protoporphyria in a Czech family*. Annual symposium, Society of the study of inborn errors of metabolism, Geneva, Switzerland, August 30 – september 2, 2011. Journal of inherited metabolic disorders, Volume 34 (Suppl 3): S264-S264, Meeting abstrakt P-542.

***2010***

- **Farrag S. M**, Douderova D, Weshahy H, Martasek P: *Novel mutation in uroporphyrinogen decarboxylase in Egyptian patients with porphyria cutanea tarda*. Annual symposium, Society of the study of inborn errors of metabolism, Istanbul, Turkey, August 31 – september 3, 2010. Journal of inherited metabolic disorders, 33 (Suppl 1): S180-S180, 201.

***2009***

- **Farrag S.M.**, Dana Douderova, P.Martasek: *Novel mutation in gene for uroporphyrinogen decarboxylase in Egyptian patients with porphyria cutanea tarda.* in XIII conference DNA diagnosis ,Czech Republic . 26-27 November 2009.

***2008***

- Prochazkova J., Sperl J., **Farrag S.M**., Barnincova L., Spicak J., Martasek P.: *Erythropoetic protoporphyria in a Czech family caused by a new 84G>A (W28X) mutation in the ferrochelatase gene.* European human genetics conference 2008, Spain, Barcelona, May 31 - June 3, 2008.
- Sperl, J., Petrasek, J., Frankova, S., **Farrag, S. M**., Subhanova, I., Vitek, L., Jirsa, M., Spicak, J., Martasek, P: *Improvement of liver dysfunction after treatment with N-acetyl cysteine in patient with erythropoietic protoporhyria.* 43rd Annual Meeting of the European Association for the Study of the Liver, Milan, Italy, April 23-27, 2008. European Journal of Human Genetics, Vol 16, Suppl. 2, pp. 246- 247, 2008. Journal of Hematology, Volume: 48 Pages: S333-S333 Supplement: Suppl. 2 , Meeting Abstract: 888 , Published: 2008.
- Prochazkova J., Sperl J., **Farrag S.M**., Spicak J., Martasek P. *Novel mutation in the ferrochelatase gene in Czech family with erythropoetic protoporphyria.*Book of abstracts, 61. Inherited metabolic disorders - 23. Metabolické dny. Senec - Slovakia,14 -16. 5.2008