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Molekulární patologie vybraných porfyrií s kožní manifestací

Molecular pathology of selected porphyrias with skin manifestation

PhD thesis

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In Science, self-satisfaction is death; personal self-satisfaction is the death of the scientist. Collective self satisfaction is the death of research. It is restlessness, anxiety, dissatisfaction, agony of mind that nourish science.

Jacques Monod

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1. List of abbreviations

δ-ALA	Delta aminolevulinic acid
AIP	Acute intermittent porphyria
ALAD	Aminolevulinic acid dehydratase
ALAS	Delta amino levulenic acid synthase
BbvI	Bacillus brevis
Bp	Base pair
BVR	Biliverdin reductase
c.	coding
CEP	Congenital erythropoietic porphyria
CNV	Copy number variation
CPOX	Coproporphyrinogen oxidase
CT	Computed tomography
CYPs	Cytochrome P450
del	deletion
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EPP	Erythropoietic protoporphyria
ER	Endoplasmic reticulum
FECH	Ferrochelatase
gDNA	Genomic deoxyribo nucleic Acid
Hb	Hemoglobin
HCP	Hereditary coproporphyria
HEP	Hepatoerythropoietic porphyria
HIV	Human immuno deficiency virus
HMB	Hydroxymethyl bilane
HMBS	Hydroxymethyl bilane synthase
HOXG	Heme oxygenase
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
IRP	Iron regulatory proteins
IVS	Intervening sequence
Kb	Kilobit
kDa	Kilodalton

MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acids
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
OCPs	Oral contraceptive pills
PAGE	Polyacrylamide gel electrophoresis
PBG	Porphobilinogen
PCT	Porphyria cutanea tarda
PGC-1α	Peroxisome proliferator-activated gamma co-activator 1 α
PP	Protoporphyrin
PPOX	Protoporphyrinogen oxidase
Primer F	Forward primer (Sense)
Primer R	Reverse primer (Anti-sense)
RBCs	Red blood corpuscles
RES	Reticulo-endothelial system
RFLP	Restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphism
Taq	Thermus aquaticus
TB	Terrific broth
UCB	Unconjugated bilirubin
UGT1A1	Uridine diphosphate glycosyltransferase
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen III synthase
VP	Variegate porphyria

2. SUMMARY

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 different patients of Czech, Slovak a Egyptian origin. We described novel mutations in the *UROD* gene in HEP patients from Egypt as well as in the *FECH* gene in patients with EPP from Czech and Slovak origins. We studied the enzyme activity of the mutated UROD protein in comparison with the wild type protein. 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. We performed the molecular analysis of the *UROD* and *FECH* genes in patients with PCT, HEP and EPP. In addition, we defined for the first time the frequency of the low expression allele IVS3-48c in the Czech control population.

Keywords: Porphyria cutanea tarda, Hepatoerythropoietic porphyria, Eryhtropoietic protoporphyria, Uroporphyrinogen decarboxylase, Ferrochelatase.

3. 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 typ porfyrie. V těchto genech byly zjištěny četné mutace, které vedou k poškozením 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 nemocných s erytropoetickou protoporfyrií (EPP). Identifikovali jsme řadu mutací v genech *UROD* a *FECH* v 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 nemocných českého a slovenského původu s onemocněním EPP. Studovali jsme mutovaný enzym UROD na úrovni proteinu a jeho enzymovou aktivitu ve srovnání se zdravou kontrolou. 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 bylo zjištěno, že v této populaci byla frekvence alel 5,5 %. Provedli jsme molekulární analýzu genů *UROD* a *FECH* u nemocných s onemocněním PCT, HEP a EPP. Poprvé byla definována frekvence sestřihové varianty IVS3-48c v české kontrolní populaci.

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

4. INTRODUCTION

4.1. 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. The prosthetic ion serves many functions by binding to proteins whether covalently or non-covalently, plays a role in the electron transfer chain and delivering divalent oxygen to the cells (Leeper, 1989).
The organic molecule is mostly found in three different forms Heme a, b, and c. Heme a and c are commonly known to be in the structure of the mitochondrial cytochrome c oxidase (Tsukihara, et al., 1995) and cytochrome c reductase or co-enzyme Q of the mitochondrial respiratory chain (Xia, et al., 1997) respectively. Heme b is the predominant sub-type in human which form the diatomic gas carriers hemoglobin (Hb) (Park, 2006) and myoglobin (Evans and Brayer, 1988).
Hemeproteins 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). They play a role in the synthesis of the intracellular second messengers like nitric oxide synthase (White and Marletta, 1992) and guanylate cyclase (Stone and Marletta, 1996). They also have an anti-oxidant effect protecting the cells from the oxidative damage by the superoxides as Catalase and peroxidase (Munro, et al., 2009). All these varieties of proteins contain heme as their prosthetic group.

4.2. Heme biosynthesis

Heme is synthesized in all biologically active living cells as it is a necessary pathway for life but mainly it takes place in the erythropoietic organs such as the bone marrow developing erthroid cells and the hepatocytes. 85 % of the heme produced in the bone marrow is utilized in the synthesis of hemoglobin (Kauppinen, 2005) while 80 % of the heme produced by the liver is used mainly in the synthesis of the microsomal CYPs and cytochromes of the electron transport chain.
Eight enzymes are involved in the heme biosynthetic cascade. The pathway takes place in the mitochondria and in the cytosol. The first enzyme is a mitochondrial enzyme and is considered to be the rate limiting in the heme biosynthetic pathway. The next enzymes are

cytosolic while the last three enzymes operate in the inter-membranous space and the inner membrane of the mitochondria (Anderson, et al., 2001).

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. D Amino levulenic acid synthase is considered to be the rate limiting enzyme in the heme biosynthetic pathway because of its high sensitivity to the intracellular concentration levels of iron and heme. Two molecules of (δ-ALA) are transported to the cytoplasm through still unidentified channels to be condensed by the catalytic activity of the cytosolic enzyme ALA dehydratase (ALAD) which requires zinc cations 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), a cytosolic enzyme convert the linear tetrapyrrole Hydroxymethyl bilane into the cyclic tetrapyrrole uroporphyrinogen III by a hydrolysis reaction. Uroporphyrinogen decarboxylase (UROD) another cytoslic enzyme 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). The steps of the heme biosynthetic pathway are simplified in Figure 1.

Glycine + Succinyl CoA **Mitochondrion**

↓ *ALA synthase*

Delta aminolevulonic acid (ALA)……

↓ *ALA dehydratase*

Porphobilinogen (PBG)

↓ *PBG deaminase*

Hydroxymethylbilane **Cytosol**

↓ *Uroporphyrinogen III co-synthase*

Uroporphyrinogen III

↓ *Uroporphyrinogen decarboxylase*

Coproporphyrinogen III……..……..

↓ *Coporpophyrnogen oxidase*

Protoporphyrinogen IX

↓ *Protopoprhyrinogen oxidase* **Mitochondrion**

Protoporphyrin IX

↓ *Ferrochelatase*

Heme

Figure 1. Heme biosynthetic cascade in Homo sapiens.

4.3. Regulation of heme biosynthesis

The intra-cellular levels of heme are finely regulated to keep a balance between the heme production, cellular needs and its breakdown. As mentioned before, 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. The heme biosynthesis is controlled in the liver and in the bone marrow in two different fashions. This is because of the variation in regulation between the two isoforms (May, et al., 1995).

In non-erythrocytes, particularly in hepatocytes, the catalytic activity of ALAS1 is controlled by the regulatory DNA-binding nuclear proteins which affects the transcription of *ALAS1* gene (Thunell, 2006). Heme, as an end product of the biosynthetic cascade, causes negative feedback inhibition to ALAS1 enzyme and down regulates the transcription of the *ALAS1* gene. On the other aspect, decrease of the intra-cellular levels of heme whether because of increase utilization or increased catabolism results in the expression of *ALAS1* gene (Thunell, 2006). The heme molecule in human post-transcriptionally regulates the stability of the major form of the ALAS1 mRNA (missing exon 1B) by stimulating its breakdown while the minor form (retain exon 1B) oppose the heme mediated breakdown (Roberts, et al., 2001) (Roberts, et al., 2005). On the post translational level, heme attach to a putative heme binding site at the N-terminal mitochondrial targeting sequence of the pre ALAS1 preventing its transport to mitochondria where the premature protein is cleaved to the mature form (Munakata, et al., 2004) (Dailey, et al.,2005).

Based on the previous information comes the importance of the therapeutic administration of heme analogues to decrease the severity of the acute attacks in patients with porphyria. Heme analogues like heme arginate or hematin are given intra-venous during the acute attack to abort it. Nevertheless, Drugs that induce Cytochromes P450 should be contraindicated in patients with porphyria because they will increase the rate of heme biosynthesis to supply heme for the hemeproteins cytochrome P450 (Podvinec, et al., 2004).

Hexoses such as glucose decrease the expression at the transcriptional level of the *ALAS1* gene operating via the peroxisome proliferator-activated co-activator 1 α (PGC-1α), a co-activator of nuclear receptors and transcription factors (Doss, et al., 1981) (Handschin, et al., 2005). Hypoglycemia during fasting up-regulates the expression form PGC-1α and therefore increases the expression of *ALAS1* gene leading to induction of heme production (Scassa, et

al., 2001). This interpretes why fasting predisposes to acute attacks in patients with porphyria. Moreover it explains the importance of the intra-venous glucose infusion to reduce the severity of the acute attacks in these patients (Anderson, et al., 2005; Phillips, et al., 2005).
In the erythroid bone marrow developing cells, ALAS2 in contrast to ALAS1 enzyme is induced by heme and only during erythroid differentiation. (May, et al., 1995). In developing erythroid cells, heme binds with globin to produce hemoglobin under the influence of the erythropoietin hormone (Spivak, 1986). Binding of the erythropoietin hormone to its specific surface receptors in the erythroid cells stimulates the transcriptional factor GATA-binding factor 1 which induces the transcription of the *ALAS2* gene (Orkin, 1992).
Morever, the availability of the intra-cellular iron transferrin complexes regulates the *ALAS2* gene translation (Hemmaplardh and Morgan, 1974). The iron ions in the erythroids cells attach to the iron regulatory proteins (IRP) when their intra-cellular level increases and therefore the IRP detach from the iron responsive element in the 5'prime untranslated region of the mRNA of *ALAS2* fascilitating its translation (Ponka, 1997).

4.4. Heme degradation

Heme degradation takes place in the reticulo-endothelial system (RES); the liver and the spleen to keep the intracellular homeostasis of heme. The first step in 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 and carbon monoxide, a potent vasodilator, is generated.The previous reaction is the rate limiting step in heme degradation. 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), a hydrophobic molecule is then bound to the albumin to be transported in the plasma to the liver. By an unknown mechanism, the liver uptakes the UCB and bounds 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 (UGT1A1; EC2.4.1.17) to become hydrophilic and easily excreted in the aqueous bile (Shibahara, et al., 2002).

4.5. Porphyrines and porphyrinogens

These are heterocyclic organic macromolecules formed of four pyrrole rings (A, B, C, D/I-IV) linked together through methine bridges (=CH−) at their alpha carbon atoms in a highly conjugated system as shown in figure 2.The eight side hydrogen atoms are replaced by special side radicals that determine their physio-chemical properties.

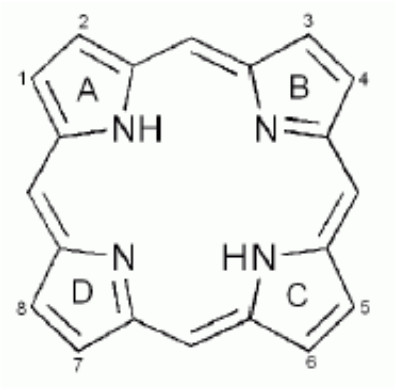


Figure 2. The chemical structure of porphyrines (adapted from Flachsova E, Thesis, 2007).

Porphyrinogens are biological intermediates and functional precursors for the heme biosynthesis with hydrogenated the four methine bridge carbon and the two pyrrolenine nitrogen atoms. These macro-molecules are unstable that quickly carry out oxidation to their corresponding porphyrin when exposed to air. Porphyrinogens are charachterized by being colourless and with no fluorescence emission while porphyrines are coloured compounds with characteristic photochemical properties. The chemical structure of the side chain in the different porphyrinogens designates both the physical and the chemical features of the subsequently oxidized porphyrins. During the heme biosynthetic pathway, there is a process of stepwise decarboxylation of the side radicals from eight to two. Therefore, porphyrines with more carboxylic moieties are highly solouble in water and thus easily excreated in the urine through the renal tubules, on the contrary porphyrines with less number of carboxylic groups are less hydrophilic and therefore they are excreated in the bile through the bilary tract.

Nevertheless, porphyrines are characterized by their capability to bind ion metals that play an important role in their function. The commonly bound metals to porphyrines are magnesium and iron. The chlorophyll is the porphyrin that contains magnesium which is necessary for its biological function to utilize the sun light energy. Hemoglobines and myoglobines, the heme

containing proteins, use the iron to perform their biological function. Hemoglobin uses iron for gas transport in the blood and myoglobin uses the iron atom for oxygen storage in the muscle.

The presence of the double bonds in the highly conjugated porphyrins is responsible for their charactarestic dark reddish colour as well as the typical absorption bands in the ultraviolet light (Zaider, E at al., 1998). Porphyrines are characterized by absorption spectra of a major band around 400 nm (the soret band) and numerous smaller bands (according to the type of solvent) between 500 and 630 nm. Porphyrin absorption spectra vary from two peaks of absorbance when dissolved in an acid to four peaks when dissolved in chloroform.

4.6. Porphyria

The term porphyria is an ancient Greek name *"πορφυροσ"* purphuros (purple) which describes the charactarestic reddish-purple colour of the tetrameric porphyrins. 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. Being rare diseases, they are quite often mis-diagnosed or wrongly diagnosed by the health care professionals. Seven sub-types of human porphyria are identified according to the enzyme defect in the heme biosynthesis cascade. Each subtype is characterized by the specific decrease in the activity of one of these enzymes in the heme anabolic pathway as well as by typical spectra of the accumulation and excretion of porphyrin precursor as shown in Table 1 (Anderson, et al., 2001).

Biochemical detection of the spectrophotometric and the fluorometric properties of the different prophyrines in the different body fluids as in plasma, urine and in the stools help in the biochemical diagnosis of porphyria. Moreover, the molecular analysis of the eight coding genes of the eight enzymes in the heme biosynthesis pathway confirm the diagnosis of porphyria and identify which subtype of porphyria it is.

4.7. 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 ussually 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 biosynthetic pathway are encoded by specific corresponding genes as shown in table 1 (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 an 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). The genes coding for the enzymes in the biosynthetic pathway are listed in table 2 with their chromosomal location, gene size and the number of the coding exons for each gene.

4.8. Enzymatic defects in different types of porphyria

Table 1. The different types of porphyria, the defective enzyme, the gene responsible for the disease, the mode of inheritance of each type and the OMIM reference number.

porphyria sub-type	Enzyme defect	Gene name and location	Mode of Inheritence	OMIM
ALAD deficiency porphyria (Plumbopophyria)	δ-aminolevulinate dehydratase (ALAD)	ALA dehydratase, 9q34	Autosomal recessive	#125270
Acute intermittent porphyria	Porphobilinogen deaminase	Porphobilinogen deaminase, 11q24.1-q24.2	Autosomal dominant	#176000
Congenital erythropoietic porphyria	Uroporphyrinogen III synthase (UROS)	Uroporphyrinogen III synthase, 10q25.3-q26.3	Autosomal recessive	#263700
Hepatoerythropoietic porphyria	Uroporphyrinogen decarboxylase (UROD)	Uroporphyrinogen decarboxylase, 1p34	Autosomal recessive	#176100
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase (UROD)	Uroporphyrinogen decarboxylase, 1p34	Autosomal dominant	#176100
Hereditary coproporphyria	Coproporphyrinogen oxidase	Coproporphyrinogen oxidase,3q12	Autosomal dominant	#121300
Variegate porphyria	Protoporphyrinogen oxidase	Protoporphyrinogen oxidase, 1q22-23	Autosomal dominant	#176200
Erythropoietic protoporphyria	Ferrochelatase	Ferrochelatase, 18q21.3	Autosomal dominant	#177000

Table 2. The genes coding for the enzymes in the heme biosynthetic pathway, chromosomal location, gene size and the number of the coding exons.

Gene name	Chromosomal location	Gene size (kb)	Number of coding exons
ALAD	9q34	13	13
PBGD	11q24.1-2	10	15
UROS	10q25.2-26.3	34	10
UROD	1p34	3	10
CPO	3q12	14	7
PPOX	1q21-23	5	13
FECH	18q21.3	45	11

4.9. Identifying susceptible subjects

The biochemical investigations that are done in laboratories for porphyric cases are a bit complicated because plenty of them show mild or sometimes no biochemical abnormalities during the asymptomatic stages. The laboratory biochemical investigations that detect porphyrin levels in plasma, erythrocytes, urine and stool differ in their potency to identify the porphyric patients depending on the clinical status of these patients and whether they are in remission or suffering an acute attack. Therefore, here comes the significant importance of the molecular analysis of the DNA in the potentially porphyric patients. An accurate diagnosis can be done in patients with clinical manifestations and positive family history even if they showed normal biochemical investigations (James and Hift, 2000). It has been stated that prepubertal children with abnormalities in any of the genes coding for heme biosynthesis could appear asymptomatic but still vulnerable to develop an acute attack or symptoms of porphyria at any time if not properly managed or exposed to a predisposing factor (Paslin, 1992).

4.10. Uroporphyrinogen decarboxylase (UROD) 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 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. These predisposing factors could be smoking, alcohol intake, drugs like oral contraceptive pills and viral infections like hepatitis B or C and human immuno deficiency virus (HIV) (Rocchi, et. al., 1986; Fargion, et al., 2003). Recently, it was reported that Tamoxifen; an estrogen receptor antagonist in the breast parenchyma (Cruz, et al., 2010) and olmesartan; Angiotenisn II receptor antagonist trigger manifestations of PCT (Mas-Vidal, et al., 2010). In familial porphyria cutanea tarda (f-PCT), an autosomal dominant trait (Kushner, et al., 1976), The UROD enzyme activity almost decreases 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; Munoz-Santos, et al., 2010). However, not all family members that inherit the *UROD* heterozygous mutation express the manifestations of the disease, so other predisposing factors like alcohol intake and drugs could play a role in the decrease activity of the enzyme (Hindmarch, 1986). In hepatoerythropoietic protoporphyria (HEP), an autosomal recessive trait, the UROD enzyme activity decrease between 5 % to 30 % of that of normal in all tissues. The decrease in the catalytic activity is due to homozygous mutation or compound heterozygous mutation in the UROD gene. It is a rare form of porphyria that usually appears from early childhood (De verneuil, et al., 1984; Smith, 1986, Sassa, 2000). Nevertheless, parents of HEP patients have their enzyme activity usually around 50 % (Koszo, et al., 1990).

4.10.1. 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 (Romana, et al., 1987; 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).

4.10.2. Protein

The human UROD enzyme is a homodimer with a molecular weight of around 40.8 kDa (Phillips, et al., 1997). The two subunits are arranged head to head with their active sites situated in the interface between the two dimers resembling one large active site surrounded by the polypeptide chains of the enzyme protecting it from the solvent as shown in figure 3.

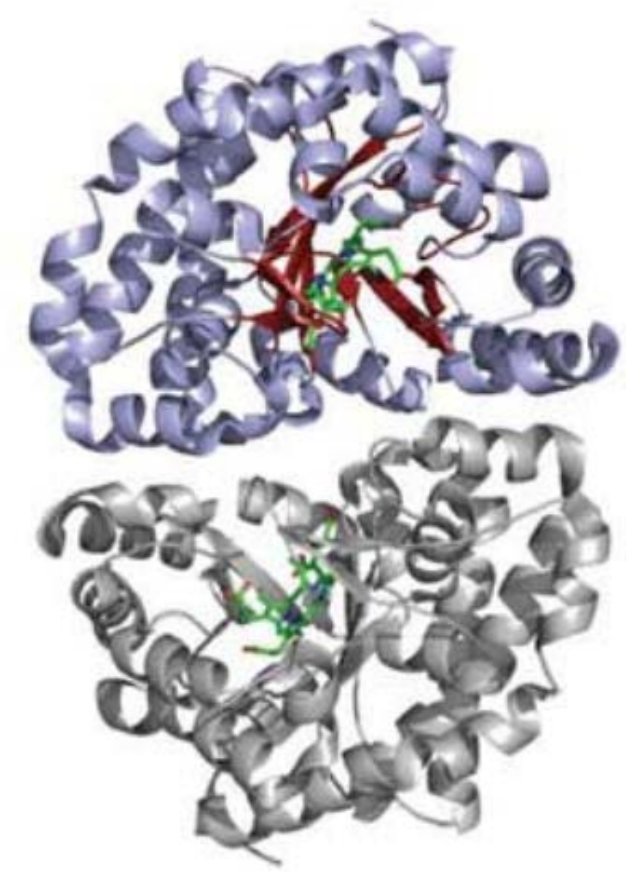


Figure 3. The structure of the UROD enzyme (Layer, et al., 2010).

UROD catalyzes the fifth decaroxylation reaction in the heme biosynthetic cascade. The first report describing the decarboxylation of uroporphyrinogen to coproporphyrinogen by the UROD enzyme was by Mauzerall and Granick in 1958. In 1976, it was suggested that the carboxyl groups are removed from the acetate radicals in a clockwise manner starting from

the pyrrole ring D then sequentially from the rings A, B and C (Jackson, et al., 1976). The UROD has high efficiency in the substrate decarboxylation without neither a co-factor nor a prosthetic group, that's why it was described as a ‘‘benchmark for the catalytic proficiency of enzymes’’ (Lewis, et al., 2008). In 2001, it was proposed according to the crystal structure of the UROD, that the decarboxylation reaction of the acetate radical in the pyrrole ring D takes place in the active site of one monomer then the produced intermediate moves to the active site of the second subunit where the rest of the acetate radicals in the pyrrole rings A, B and C are sequentially decarboxylated (Martins, et al., 2001). Recently, it was shown that this movement of the substrate between the active sites of the two subunits is not necessary (Philips, et al., 2009).

4.10.3. Pathophysiology

The defect in the UROD catalytic activity results in the accumulation of the porphyrins especially the uroporphyrins in different soft tissues. The excess uroporphyrins will be excreated in urine (a biochemical diagnostic tool in the disease) and will be deposited subcutaneous in these patients with f-PCT and HEP as well as in the hepatic parenchyma in late stages. Deposition of the photogenic uroporphyrins under the skin activates the mast cells that will secrete their proteases resulting in the separation between the two layers of the skin (the dermis and the epidermis). This is observed clinically as skin fragility and vesicular formation. Moreover, unkown interaction between the activated mast cells and the fibroblasts leads to cutaneous fibrosis. Also uroporphyrins activate the synthesis of the collagen by the fibroblasts leading to sclerodermoid changes (Lim, 1989).

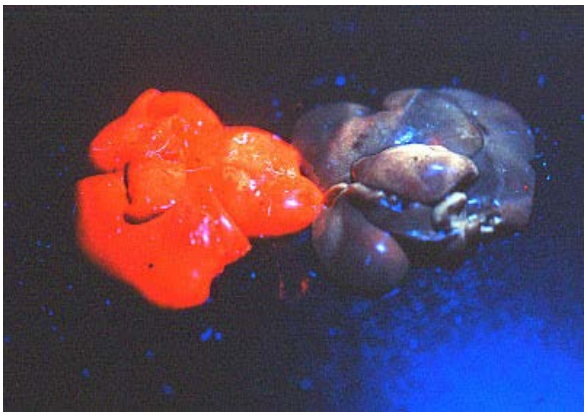


Figure 4. Deposits of uroporphyrin in liver of a rat treated with hexachlorobenzene, mimicking human disese PCT (red fluorescence under UV light) (Schmid, 1960).

4.10.4. Epidemiology

In general, both males and females are equally vulnerable to PCT (Aarsand, et al., 2009) although some studies assume that the disease in the aquired form is predominat in males (Bulat, et al., 2007). PCT is the most common subtype of porphyria with prevalence: 1:25,000 in USA, 1:20,000 in Caucasians, 1:5,000 in the Czech and Slovak populations (Lambrecht, et al., 2007), 1:10,000 in Sweden (Rossmann-Ringdahl, 2005), 1:100,000 in Norway (Aarsand, et al., 2009), 2-5:1000,000 in the United Kingdom (Bleasel, et al., 2000). On the contrary, HEP is a very rare sub-type. fPCT and HEP are presented at any age, most propably with an early onset while sPCT is commonly presented around fourties. The frequency of mutations in 21 independent HEP families are listed in table 3.

Table 3: Frequency of Mutations in 21 Independent HEP Families.

Mutation	Ancestry	n	Allelic status	Allele frequency	References
-M1V	Hungrian	1	Hetero	1/42	Remenyik et al 2008
-F46L	Spanish	2	Homo	4/42	Ged et al 2002
	British				Armstrong et al 2004
-P62L*	Portuguese	1	Homo	2/42	Moran-Jimenez et al 1996
-Q71X	Northern European	1	Hetero	1/42	Phillips et al 2007
-V134Q	British/German	1	Hetero	1/42	Meguro et al 1994
-V166A	Puerto Rican/	1	Hetero	1/42	Cantatore-Francis et al 2010
	Dominican				
-E167Q*	Italian	1	Homo	2/42	Romana et al 1991
-G168R	Northern European	1	Hetero	1/42	Phillips et al 2007
-G170D	African	1	Homo	2/42	To-Figueras et al 2011
-H220P*	British/German	1	Hetero	1/42	Meguro et al 1994
-G281E*	Spanish/Tunisian	9	Homo/Hetero**	17/42	Roberts et al 1995
-R292G*	Dutch	1	Hetero	1/42	de Verneuil et al 1992
-P235S*	Argentina	2	Hetero	2/42	Granata et al 2009
	Hungaria				Remenyik et al 2008
-Y311C*	Italian	1	Homo	2/42	Moran-Jimenez et al 1996
-IVS9$^{-1(G>C)}$ *	Argentina	1	Hetero	1/42	Granata et al 2009
-Del	Dutch	1	Hetero	1/42	de Verneuil et al 1992
-645del1053*	Puerto Rican/	1	Hetero	1/42	Cantatore-Francis et al 2010
ins10	Dominican				

* Mutations were identified in both HEP and f-PCT

** 8 patients (7 Spanish and 1 Tunisian) were homoallelic and one Spanish heteroallelic

4.10.5. 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. Rarely, few patients complain that their hair colour is getting dark and of scarring alopecia (Brazzelli, et al., 1999; Shaffrali, et al., 2002). In 18 % of the PCT patients, facial sclerodermoid changes develop due to the chronic irritation of the skin of the face by the sun exposure producing a clinically mask-face appearance (Fritsch, et al., 1998, Giunta, et al., 2009). Some patients were reported with ocular manifestations because of the porphyrins deposition on the eye lids and the lacrimal glands. These manifestations could be seen as periorbital dermatitis, scarring of the eye lid and/or the lacrimal gland (Zaborowski, et al., 2004). Ophthalmic complications as scleral necrosis were also described but rarely reported (Altiparmak, et al., 2008). Patients with fPCT and HEP are sometimes presented by reddish discolouration 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). It was found out that 30 % to 40 % of the patients with PCT suffer from hepatic cirrhosis (Lambrecht, et al., 2007). Neurological manifestations are usually not present in these patients.

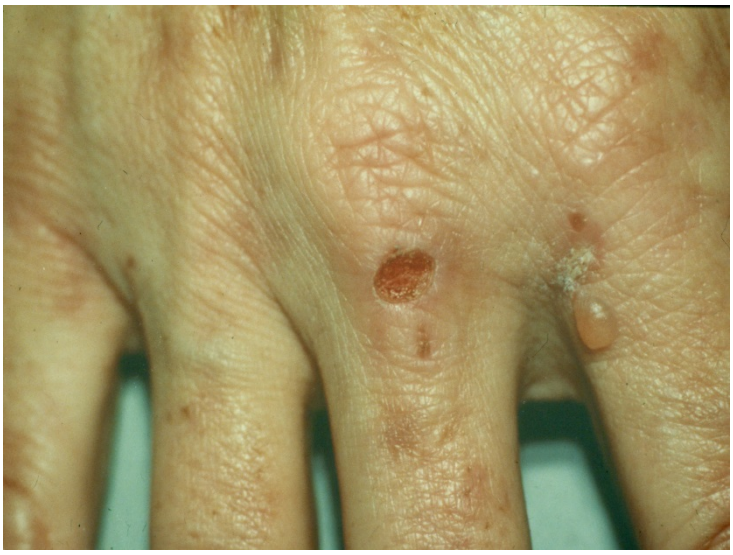


Figure 5. Skin defects in patient with PCT

4.10.6. Diagnosis

The biochemical identification of the different porphyrin precursors is nowadays recommended as the first procedure to diagnose which subtype of porphyria and to analyse the severity of the disease (Sassa, 2006). 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 porphyrines and isocoproporphyrines in feaces could also be elevated (Anderson, et al., 2001). The fluorometric scanning upon excitation at 405 nm of clear non-hemolytic plasma is a useful laboratory technique to diagnose the different subtypes of porphyria. The plasma samples of the patients with PCT show a characteristic fluorometry emission between 618-622 nm (ussually 619 nm) which is highly suggestive of the disease (Enriquez De salamanca, et al., 1993).

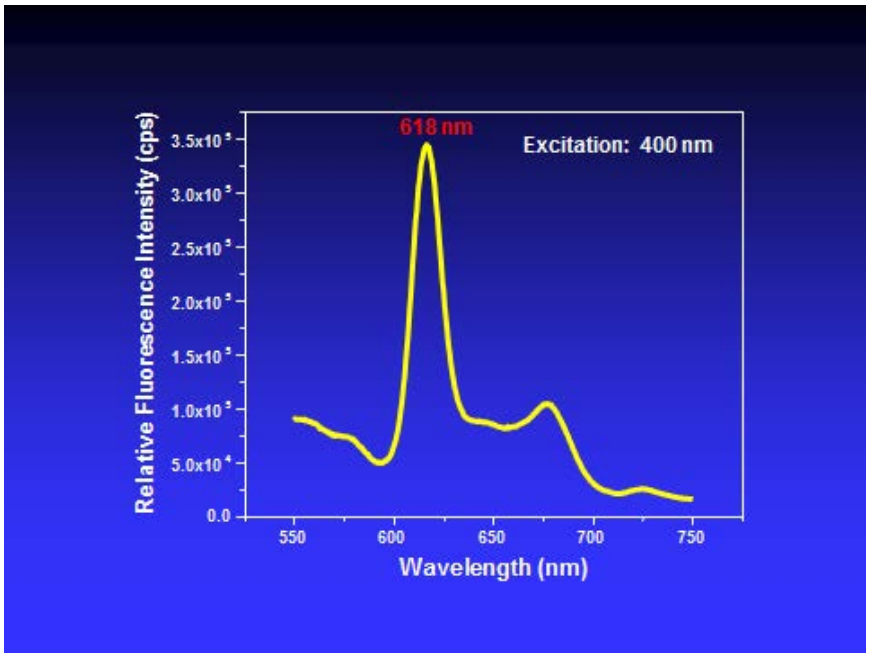


Figure 6. Plasma fluorometry, patient with PCT

4.10.7. Treatment

Predisposing factors such as alcohol intake or drugs like Oral contraceptive pills (OCPs) with high estrogen content should be discontinued as well as avoiding exposure to direct sun light. Drugs that induce porphyria should be contra-indicated in these patients. The database for the porphyrinogenic drugs are listed on the following web page (http://www.drugs-porphyria.org/) (Thunell, et al., 2007). PCT varies from any other forms 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 realease from the liver (Nordmann, et al., 2002). Repeated phlebotomy (withdrawal of 400 – 500 ml of whole blood every 2 weeks for 3 or 4 months) is highly recommended to decrease the serum iron levels to the lower limit of the norm (< 25 ng/mL) which could be achieved after 5 to 6 times. Hemoglobin levels should be always monitored to avoid incidence of anemia in these patients (Thunell, et al., 2000). Chloroquine, the drug of choice in treatment of malaria could be used as well in low doses to avoid hepato-toxicity in treatment of PCT. Twice a week dose of chloroquine could be administrated between the attacks to facilitate porphyrins excretion (Balwani, et al., 2012).

4.11. Ferrochelatase (FECH) defect

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). The mitochondrial enzyme catalyzes the addition of the divalent iron to the protoporphyrin IX to produce heme. Lack of the FECH catalytic activity leads to accumulation of protoporphyrins in tissue leading to EPP (Magnus, et al., 1961).

4.11.1. 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; Brenner, et.al. 1992). The amino acyl residues 1–62 in each subunit constitutes the mitochondrial targeting sequence that is proteolyticaly cleaved during the protein processing.

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 (Gouya, et al., 1999). Subsequently, this phenomenon of EPP inheritance was separately confirmed by many studies worldwide like in Japan (Nakano, et al., 2006), North America (Risheg, et al., 2003), Sweden (Wiman, et al., 2003), Israel (Schneider-Yin, et al., 2008), South Africa (Parker, et al., 2008) and in the United Kingdom (Whatley, et al., 2004). 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).

The *FECH* gene mutations found in EPP patients could be classified broadly into three main groups; single nucleotide substitutions: including both the missense and the non-sense mutations, splice site mutations resulting in abnormal mRNA producing shortening of the protein and finally the frameshift mutations creating premature stop codon and rapid degradation of the RNA (Anstey and Hift 2007).

Few number of EPP patients were reported by aquired somatic *FECH* mutations. These patients usually start to manifest for the first time after the age of 40. In these cases, EPP is usually accompanied by the hematological disease myelodysplastic syndrome (Sarkany, et al., 2006) or with myeloproliferative diseases (Goodwin, et al., 2006).

The available knowledge is still not enough to correlate the genotype with the phenotype to the extent that sometimes there is a clear difference in the presentation of EPP even among siblings (Sellers, et al., 1998). An example of the genotype-phenotype correlation, the seasonal palmer keratoderma has been only described in EPP patients that inherits compound heterozygotes or homozygotes *FECH* gene abnormalities (Mendez, et al., 2009). To date EPP as a result of FECH deficiency, has not been reported in the black south African race (Parker, et al., 2008).

4.11.2. Genetic counselling

As previously mentioned, the classical EPP inheritance requires a *FECH* mutation on one allele and a hypomorphic trans allele IVS3-48C. Therefore, the probability in these patients to inherit the disease to their children is less than 2.5 % (Gouya, et al., 2006). Of course, screening of the patient's partner for the low expression allele povides more accurate estimation of this probability.

4.11.3. Epidemiology

EPP is considered the most common sub-type of porphyria in children and has been described in plenty of patients in different populations all over the world. EPP prevalence ranges between: 1:75,000 in Holland (Went, et al., 1984), 1:79,000 in Northern Ireland (Todd, 1994), 1.75:100,000 in Slovenia (Marko, et al., 2007), 1:180,000 in Sweden (Wahlin, et al., 2011) and 1:200,000 in Wales in the UK (Elder, et al., 1990). To date, no prevalence studies in the Czech population have been done. It was assumed by (Holme, et al., 2006) that both males and females are equally vulnerable to the disease while other studies suggest that the disease is slightly predominant in males (Chantorn, et al., 2012; Lecha, 2003).

4.11.4. Protein

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

During catalysis, the FECH enzyme disrupts the planar structure of the substrate protoporphyrin into a saddle conformation to allow the ferrous iron insertion (Medlock, et al., 2007; Karlberg, et al., 2008; Wang, et al., 2009). The enzyme desolves the divalent metal during the chemical reaction and creates a bond between the ferrous iron and the two nitrogen atoms of the pyrrole rings producing ‘‘sitting-atop’’ complex. The remaining two amino groups of the other pyrrole rings are depronated leading to a metalo-porphyrin (Al-Karadaghi, et al., 2006). The subunits of the human FECH enzyme have two identical active site pockets situated on the same surface within the internal mitochondrial membrane. The bottom of the active site pocket contains polar hydrophylic amino acyl residues while the margins of the pocket are lined by hydrophobic residues. The crystal structure of the human enzyme substrate complex showed that the enzyme engulfs the subtrate, so that it is totally surrounded by the active site pocket (Sigfridsson, et al., 2003). Figure 7 shows the crystal structure of the FECH enzyme.

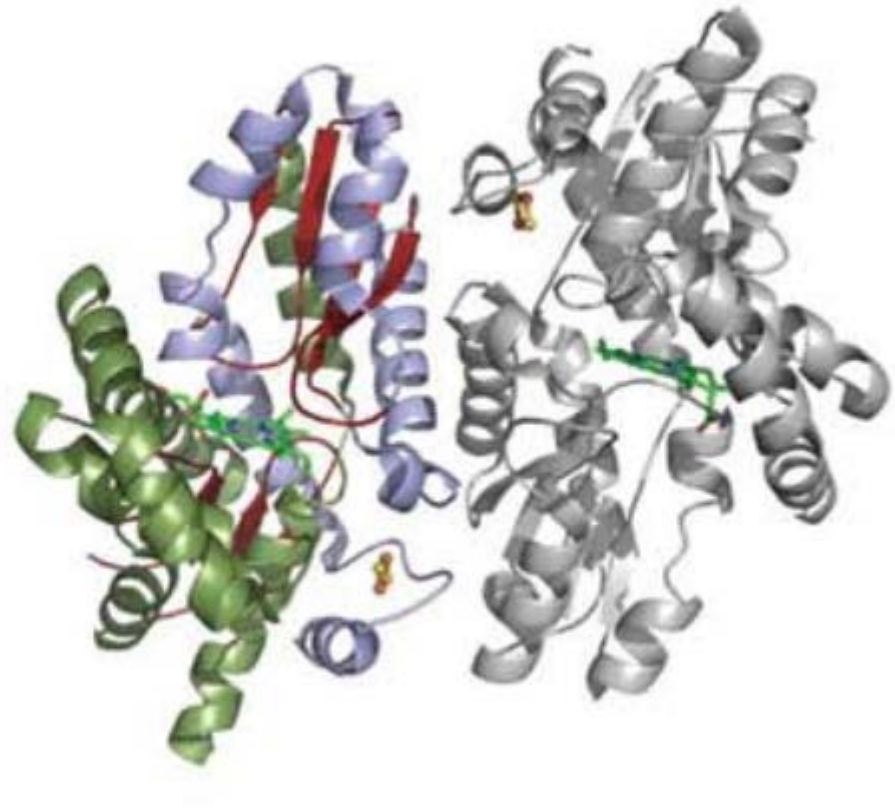


Figure 7. The structure of the FECH enzyme (Layer, et al., 2010).

4.11.5. 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 parenchyma. The protoporphyrin absorbs light and transfers the excess energy to oxygen producing photodynamic reactions that lead to lipid, protein and DNA damage of the cells (Lim, 1989). The protoporphyrin content in the erythrocytes are more than 90 % free and not conjugated with zinc. The ultraviolet light leads to the release of the free protoporphyrin from the erythrocytes without even rapture of the cell membrane. The diffused free protoporphyrins bound to the albumin in the plasma. Subsequently, the bound protoporhyrins are uptaken by the endothelial cells of the blood vessels. The exposure to the ultra-violet light leads to the excitation of these porphyrins which cause endothelial and tissue damage (Schneider-Yin, et al., 2000). The subcutaneous deposition of the protoporphyrins and the irradiation activate the

mast cell to mediate an inflammatory reaction which is reflected clinically as erythema, oedema and urticuria (Lim, 1989). The excessive production and the progressive accumulation of the protoporphyrins are deposited also in the hepatic parenchyma and canaliculi. The insoluble protoporphyrins tend to form crystalline pigments in the hepatocytes causing mitochondrial damage as well as in the bile ducts forming gall stones (Bloomer, 1988). The deposited protoporphyrins in the hepatocytes are observed as birefringent crystals with Maltese cross shape under polarised light while they appear as amorphous deposits in the biliary canaliculi (Thunell, et al., 2000).

4.11.6. 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; Murphy, 1999; Cox, et al., 1998). The acute attacks are characterized by burning, itching, redness and skin inflammation especially on the sun exposed areas that last for six days after sun exposure. Subsequently after sun exposure, patients complain of tingling, prickling and burning sensations. The severity of the symptoms depends on the intensity and the exposure duration to sunlight (Holme, et al., 2006). Nail lesions like photoonycholysis and transversal leuconycholysis were noted in some patients with EPP. Permanent hyperkeratosis and scarring may develop after recurrent episodes on exposure to sun light. Some EPP patients manifest characteristic linear furrows around the lips. Nevertheless, the seasonal keratoderma that is observed on the palms has been reported in some EPP patients which may denote that the type of EPP inheritance is autosomal recessive (Holme, et al., 2009). Moreover, about 30 to 50 % of EPP patients suffer from microcytic hypochromic anemia due to the decrease synthesis of the hemoglobins (Rademakers, et al., 1993; Holme, et al., 2007). In late stages, the progressive accumulation of the protoporphyrins in the hepatocytes leads to hepatitis, chronic liver cirrhosis and finally liver failure. The insoluble protoporpyrines precipates in the biliary tract resulting in gall stones and cholestasis (Went, et al., 1984). Complications of chronic liver failure like portal hypertension, oesophageal varices, splenomegaly and ascites may develop in end stages. Progressive polyneuropathy has been reported especially among EPP patients with liver complications due to protoporphyrin accumulation in the nerve cells (Muley, et al., 1998). Ophthalmic manifestation as optic nerve atrophy due to vascular occlusion in the fundus was reported in a male japanese patient (Tsuboi, et al., 2007).

4.11.7. 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). Protoporphyrin levels are usually found elevated in the stools of the patients while mostly being within the normal levels in the urine. Morever, the enzyme activity is possible to be measured through its zinc chelation properties but it is not commonly performed because of the difficulties in the technical procedures of the test (Cox, 1997). Most EPP patients develop a heptic liver failure as a complication from progressive deposition of porphyrin precursors in the hepatocytes. That's why frequent liver function tests, haemochromatosis tests, ultrasonography, computed tomography scans (CT) and magnetic resonance imaging (MRI) of the liver, gall bladder and the biliary tract should be considered on regular bases. Routine liver biopsy every 5 years is also recommended in EPP patients with a family or past history of liver disease and/or with abnormal liver function tests (Anstey and Hift, 2007).

4.11.8. Treatment

Predisposing factors and direct exposure to sun light should be avoided. Wearing proper clothes covering the body exposed areas to sunlight together with an adequate sunglasses and a hat are practical ways to avoid manifestations resulting from the sun light exposure. Even the application of yellow filters blocking the radiation under 460 nm is necessary during surgeries and in dental caring clinics to avoid the photosensitive reactions induced by the theatre light in these patients (Meerman, et al., 1994; Wahlin, et al., 2007). Topical anti-inflammatory drugs could be used to relieve the sunlight induced dermatitis. Oral administration of β-carotene (90-120 mg/day in children and 180-300 mg/day in adults) enhance somehow tolerance to sun light in these patients. α-melanocyte-stimulating hormone analog is recently recommended to decrease the photosensitivity reactions that results from the sun exposure (Harms, et al., 2009). The previously mentioned medications are generally used for symptomatic treatment but there are two ways to decrease the elevated levels of protoporphyrins. The first approach is the administration of 4-16 g of Cholestyramine on daily basis. The bile sequestrating agent prevent the re-absorption of the porphyrins from the

entero-hepatic circulation by binding to them and facilitating the faecal excretion resulting in some clinical progress (Frank, et al., 1995; Wells, et al., 1980). Although Cholestyramine has been used for a long time and is still used in the disease management but recently in the United Kingdom it has been reported that it is not an effective therapeutic drug in cases of uncomplicated EPP (Tewari, et al., 2012). The second approach is the exchange transfusion or hypertransfusion but it is not suitable for long term management (Dobozy, et al., 1983; Wahlin, et al., 2007). EPP patients develop mild microcytic anemia which should be considered during management. Oral iron was shown to be ineffective, therefore it should be administrated intra-venous. In some cases, the whole blood transfusion is necessary if anemia is severe and accompanied by thrombocytopenia (Thunell, et al., 2000). The major complication of EPP is the progressive hepatic failure, therefore liver transplantation is now indicated. In 1980, the first EPP patient with an end stage hepatic disease was operated for liver transplantation and showed marked improvement (Wells, et al., 1980). Definitely, EPP patients with liver transplant still show high levels of protoporphyrin levels in blood as the transplantation will neither change the *FECH* gene abnormality nor the consequences of the FECH deficiency. The clinical picture improves after the transplantation but still the symptoms exist. Therefore, bone marrow transplantation should be considered as well in these patients. For that reason, recent studies recommend sequential liver and bone marrow transplantation (Metselaar, 2007; Rand et al., 2006).

4.12. Molecular diagnosis of porphyria

Physicians could sometimes misdiagnose porphyria because of being a rare disease and because of the common manifestations that come underneath a wide spectrum for the differential diagnosis of the disease. Moreover, the biochemical investigations are sometimes inaccurate and vague especially if the patients are not in the acute phase. Therefore, in the last two decades the molecular screening techniques based on the DNA analysis have shown a great significance to confirm the clinical and the biochemical diagnosis of porphyria and to discover the asymptomatic carriers. It gives a detailed vision about the current pathology in the patients as well as it helps to identify other family members who could be diseased or carriers. Nowadays, it is highly recommended as the last diagnostic procedure to confirm the gene carrier status (Frank and Christiano, 1998; Sassa, 2006). To date, hundreds of different mutations have been identified in the *UROD* and in the *FECH* genes http://www.hgmd.cf.ac.uk/ac/index.php. The molecular analysis helps in discovering the underlying genetic cause in every individual patient, appropriate drug prescription, proper management and prevention of the acute attacks. The clinical and the biochemical diagnoses of PCT, HEP and EPP are best confirmed by the mutation analyses of the *UROD* and the *FECH* genes respectively.

5.

AIMS OF THE STUDY

Specific aims of the study were as follows:

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

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

5.3 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*.

5.4 In the case a molecular defect will be found in HEP, to construct graphical representation of UROD structure and prepare

5.5 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.

5.6 To study extended Czech family with EPP

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

6.

HYPOTHESES

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

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

6.3 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.

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

7.

MATERIALS AND METHODS

7.1. Ethics

The current research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the Committee of Medical Ethics at the First Faculty of Medicine, Charles University in Prague.

Informed consent whether from the participated adults or from the sponsored parents of the underaged participants was obtained.

7.2.1. Patients and healthy controls examined

More than 50 cases from different populations were examined for the *UROD* and the *FECH* gene disorders on the molecular level in our laboratory during this work. Fourteen patients (7 males and 7 females) with porphyria were investigated for the mutations in the *UROD* and the *FECH* genes. Relatives of the proband were also genetically examined.

7.2.2. 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.

7.3. 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.

7.4. Polymerase Chain reaction

7.4.1. *UROD gene*

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. The sequence of the 8 set pair of the used primers and the the PCR product size are listed in table 4. The reaction was prepared using a commercially available kit PPP master mix (2x concentrated PPP Master Mix contains 150 mMTris-HCl, pH 8.8 (25^{o}C), 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/ml Taq DNA polymerase, dye, stabilizers and additive). Top-Bio s.r.o.; Product No.: P134.

Table 4. List of the primers and the corresponding length of the PCR products of the *UROD* gene.

Fragment	Primer F (5'→3')	Primer R (5'→3')	Length of the PCR product
1	gacgctcttggttccctaca	attaaggccctgggatgaac	360
2	ctgaatcggccttatgaacc	ttgctaggtggcagactgaa	720
3 and 4	ggtaagagtcagggtctggaaa	ccaggaggaaggaaaaggag	600
5	ttctccttttccttcctcct	gagccaccaccctcaacc	540
6	actctggaaggtctggggtag	gtgtcaggatgggcttgg	540
7	agtgctgggatctgaggaaa	ggccttgctacaaccactaatc	540
8 and 9	actggagggcagcagaag	cacaaatgaacaacagcaacaa	780
10	cttcatgcctgggtccata	gtcctggaaacgatcaatca	419

The amplified PCR products were optimized and carried out in a total volume of 25 µl according to the protocols shown in tables 5 and 6.

Table 5. PCR reaction blend (all fragments)

Chemicals	*Stock concentration*	*Final concentration in 1 reaction*
PP mix	2x	1x
*** DMSO**	100%	*4% or 8%
Forward primer	3.2 pmol/µl	0.4 mM
Reverse primer	3.2 pmol/µl	0.4 mM
gDNA	50-100 ng/µl	50-100 ng

* Sigma-Aldrich, St. Louis, MO, USA,*4% used only for the PCR of fragment number 5.

Table 6. PCR conditions

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´30´´
2.	denaturation	94°C	30´´
3.	annealing	*	40´´
4.	extension	72°C	50´´
5.	30 times repeation cycle 2 to 4		
6.	final extension	72°C	10´

*65°C for all the fragments except for the fragment number 5, the annealing temperature was 62°C. The PCR products were amplified using DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Waltham, MA, USA).

7.4.2. *FECH* gene

The promoter and the 11 coding exons of the *FECH* gene with their flanking intronic regions were amplified using the matching primers as shown in table 7.

Table 7. List of the primers and the corresponding length of the PCR products of the *FECH* gene.

Fragment	***Primer F (5'→3')***	***Primer R (5'→3')***	***Length of the PCR product***
1	TAGGAGTCCAGCAGGTTTTG	GTGACAATAACCAAGGCTCT	669 bp
2	GTCAGGAATTATGCTCTGAGG	AGCTATTGAAAGGAAGCCAAG	348 bp
3	AGATTAGAGTTTGCTGGCTG	ACCATTACCAGATACGCATT	320 bp
4	TCTCTGCATGGGTGTTGTGT	AAGGCTAAAGGTCAAGGGATAA	605 bp
5	GTCAGTGCCATAGGAAATTACA	GACTGACCTGAACTCTCGTGT	406 bp
6	CACTAGAACTGACATCAATAATC	AGTAAGGCTCAGAAGGACA	444 bp
7	CAATGCTGAGAGGCTGGACTGT	CTTGCACTGGGCTTAGGACATA	374 bp
8 + 9	TCATCATTGGTGCAGGAGAC	TGAGGACACCGTACATGCAA	947 bp
10	GCGAACAGTTGAAGTCAGAC	CAGACATAGTTATAGGTGGGT	402 bp
11	CCAAGCCAGAGCGCTGACCT	CTCTCCGTACCCTTTCGGGAGG	586 bp

The PCR amplification cycles were done on DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories) in a total volume of 25 µl for the fragments number 3, 5, 6, 7 and 10 while the rest of the fragments was prepared in a total volume of 40 µl according to the protocols shown in tables 8 and 9.

Table 8. PCR reaction blend (all fragments)

Chemicals	Stock concentration	Final concentration in 1 reaction
[a] **PP mix**	2x	1x
[b] **DMSO**	100%	4%
Forward primer	3.2 pmol/µl	0.4 mM
Reverse primer	3.2 pmol/µl	0.4 mM
gDNA	50-100 ng/µl	50-100 ng

[a]Top Bio; [b] Sigma-Aldrich

DMSO is used only in the reactions for the fragments number 1, 7 and 11.

Table 9. PCR conditions of the different fragments.

9.A. PCR conditions for fragments 1, 8+9 and 11.

	Step	***Temperature***	***Time***
1.	initial denaturation	94°C	2´30´´
2.	denaturation	94°C	30´´
3.	annealing	58°C	30´´
4.	extension	72°C	50´´
5.	32 repetition cycle 2 to 4		
6.	final extension	72°C	10´

9.B. PCR conditions for fragment 2.

	Step	***Temperature***	***Time***
1.	initial denaturation	95°C	2´30´´
2.	denaturation	94°C	30´´
3.	annealing	60°C	40´´
4.	extension	72°C	40´´
5.	35 repetition cycle 2 to 4		
6.	final extension	72°C	10´

9.C. PCR conditions for fragment 3.

	Step	***Temperature***	***Time***
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	30´´
3.	annealing	60°C	30´´
4.	extension	72°C	40´´
5.	32 repetition cycle 2 to 4		
6.	final extension	72°C	10´

9.D. PCR conditions for fragment 4.

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´30´´
2.	denaturation	94°C	30´´
3.	annealing	60°C	40´´
4.	extension	72°C	50´´
5.	32 repetition cycle 2 to 4		
6.	final extension	72°C	10´

9.E. PCR conditions for fragments 5, 6 and10.

	Step	*Temperature*	*Time*
1.	initial denaturation	95°C	2´30´´
2.	denaturation	94°C	30´´
3.	annealing	60°C	40´´
4.	extension	72°C	40´´
5.	32 repetition cycle 2 to 4		
6.	final extension	72°C	10´

9.F. PCR conditions for fragment 7.

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	30´´
3.	annealing	62°C	30´´
4.	extension	72°C	40´´
5.	32 repetition cycle 2 to 4		
6.	final extension	72°C	10´

7.5. Purification of the PCR products

Some PCR products were directly purified after the amplification reactions while other PCR products were extracted using electrophoresis from the agarose gel (sigma). The purification of the PCR products is done whether using QIAquick gel extraction kit (QIAGEN) or Wizard SV gel and PCR clean-up system (Promega). The purification procedures were done according to the manufacturer's protocol.

7.6. 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).

The chromatographic results were analyzed by the SeqScape Software v2.5 (Applied Biosystems) or Chromas Pro v1.5 (Technelysium, Tewantin, Australia).

7.7. Site-directed mutagenesis

The cDNA sequence of some patients with UROD disorders showed mutations which have been previously reported on the following web page http://www.hgmd.cf.ac.uk/ while other patients revealed a novel mutation. For the newly found mutation, we had to confirm that it is responsible for the development of the disease. This was performed by the site directed mutagenesis technique and the enzyme assay.

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 with the mutagenic primers listed in table 10. Complementary mutagenic primers were prepared in compliance with the web-based Primer Generator (http://www.stratagene.com/qcprimerdesign).

The integrity of the mutated inserts was therefore checked by sequencing to confirm the success of the process of the mutagenesis.

Table 10. The primers used for site directed mutagenesis

SNP (p.)	*Primer F (5'→3')*	*Primer R (5'→3')*
Phe55Ile	gggctgcccaggacattttcagcacgtgt	acacgtgctgaaaatgtcctgggcagccc

7.8. Protein expression and purification

7.8.1. 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.

7.8.2. Protein purification

The procedures for the protein purification were always done at temperature 4°C. The cell pellet was resuspended in buffer [300mM NaCl, 50mM (NaH_2PO_4)$2H_2O$, 10% glycerol and 5 mM DTT; pH 6.8] that contains the protease inhibitors [100 mM phenylmethysulfonil fluoride; 0.1 mM aprotinin; 1 mM pepstatin and 1 mM leupeptin (all by Sigma-Aldrich)]. The bacterial cell pellet was proteolyzed using lysozyme (Sigma-Aldrich) with final concentration 20 µg/ml, incubating on ice for 20 minutes and then sonicated three times for five minutes at amplitude 70 % with a five minutes rest between each sonication cycle. Ultra-centrifugation is then 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 Glutathione Sepharose 4B (GE Healthcare) is applied to the supernatant soluble protein after being purified from the cell debris for 1 hour at 4°C. Later centrifugation is done to precipitate the desired soluble protein which is bound to the sepharose marker for 5 minutes at 4°C at 500 x g. The precipitated protein is subsequently washed 10 times with the same buffer used for homogenization. Elution of the desired protein with the same buffer containing in addition Glutathione is done up to 5 times to obtain a highly purified fraction of the desired protein. The purity of the generated protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and the fractions showing a single band on the gel were extracted and concentrated (Centriprep YM-30, Millipore Corporation, Billerica, MA, USA). The activity measurements of UROD proteins were performed in collaboration with laboratory of Prof. Hubert de Verneuil in Bordeaux, France.

7.9. Multiplex Ligation-dependent Probe Amplification (MLPA)

DNA sequencing is not suitable to detect the big deletions, duplications and copy number variations (CNV) in the genes. Here comes the importance of MLPA assay in the molecular diagnosis of the genetic diseases because of these DNA abnormalities (Rusu, et al., 2007; Stupia, et al., 2012).

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 a 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. In this method, the gDNA is denaturated in TE buffer at 98°C for 5 minutes. Hybridization is carried out using 1.5 µl of MLPA buffer and 1.5 µl the SALSA probe-mix for each reaction. Hybridization is done at 95°C for 1 minute followed by incubation at 60°C for 1 hour. Subsequently, ligation with the probes is carried out by adding 3 µl ligase-65 buffer A, 3 µl ligase-65 buffer B, 1 µl Ligase-65 to 25 µl distilled water. The ligation is done at 54°C for 15 minutes followed by incubation at 98°C for 5 minutes. Finally, a PCR is prepared by adding 2 µl of the SALSA PCR primers to 0.5 µl of polymerase in 7.5 µl distilled water. All the previous steps were carried out in the genetic analyzer (Applied Biosystems3500xL, USA). The analysis of the fragments was carried out by the ABI PRISM 3100/3100-Avan Genetic analyze (Applied Biosystems3500xL, Foster city, CA, USA). The obtained data were checked by the softwares Peak Scanner v1.0 and Gene Mapper v4.0 (both Applied Biosystems, Foster city, CA, USA).

7.10. 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).

7.11. 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. The study was carried out on a set of 312 healthy control samples (149 males and 163 females) from the Czech population. The gDNA samples are broken down into fragments by the restriction enzymes that are separated by the gel electrophoresis according to their length. The RFLP reaction was carried out at 37°C for 5 to 10 minutes using the enzyme Bacillus brevis (BbvI) (Fermentas Biotech, Czech republic). The restriction primers used in this study are listed in table 11.

Table 11. RFLP primers

SNP (p.)	***Primer F (5'→3')***	***Primer R (5'→3')***
IVS3-48c	GTGGAGCACAGCTGGGTATT	ATCCTGCGGTACTGCTCTTG

The amplified PCR products were prepared in a total volume of 12 µl according to the protocol shown in tables 12 and 13.

Table 12. PCR reaction blend (all fragments)

Chemicals	***Stock concentration***	***Final concentration in 1 reaction***
PP master mix	2x	1x
Forward primer	3.2 pmol/µl	0.4 mM
Reverse primer	3.2 pmol/µl	0.4 mM
gDNA	50-100 ng/µl	50-100 ng

Table 13. PCR conditions for the RFLP.

	Step	***Temperature***	***Time***
1.	initial denaturation	95°C	2´
2.	denaturation	95°C	30´´
3.	annealing	56°C	30´´
4.	extension	72°C	30´´
5.	30 times repetition cycle 2 to 4		
6.	final extension	72°C	10´

8.

RESULTS

8.1. Molecular analysis of the *UROD* gene in patients with f-PCT and HEP

We analyzed the promoter and the 10 protein-coding exons with their flanking intron regions of the *UROD* gene in many cases in different populations suspected of whether f-PCT or HEP.

8.1.1. Slavic Czech population

8.1.1.1. Patients fPCT1 and fPCT2

To date, more than 60 mis/nonsense mutations have been identified in the *UROD* gene. (Human Gene Mutation Database, accessed in February 2015, http://www.hgmd.org/)
We found a previously reported non-sense mutation in the *UROD* gene in two unrelated Czech patients with f-PCT. The heterozygous mutation was found to be exactly the same in both cases: in (c.616) in exon 6 of the *UROD* gene. The *UROD* sequence of these patients showed C→A transition at position 206 in exon 6 leading to TAA instead of CAA (UAA is a stop codon) as shown in figure 8. The point mutation leads from Glutamine to a stop codon substitution p.[Gln206*];[=]. Accession number on Biobase HM971362.

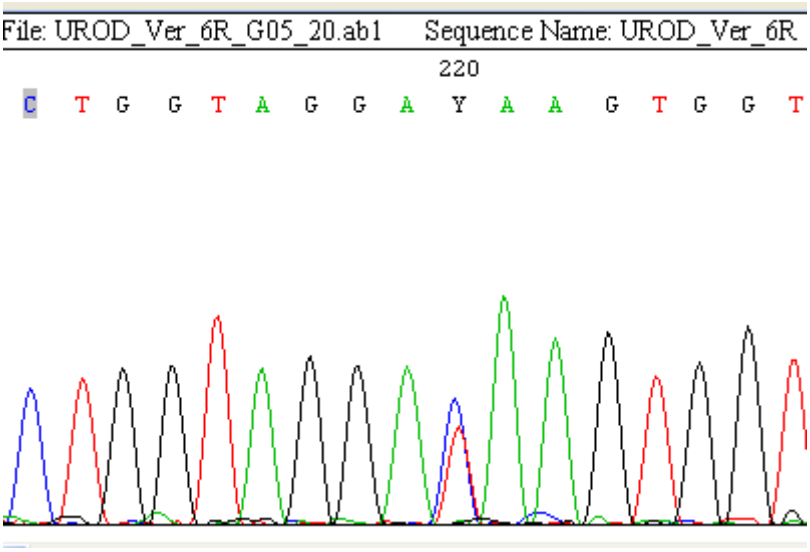


Figure 8. The sequence of the heterozygous mutation [Gln206*];[=] in in the *UROD* gene in the patients with fPCT.

8.1.1.2. Patient fPCT3

The patient started to suffer from dark urine about 5 years ago. Then later on (about 3 years ago) the patient started to complain of a skin inflammation and erosions on the dorsum of the hand. Hyperpigments were also noticed on the hands. Urinary uroporphyrinogen was found to be elevated. The patient was diagnosed based on clinical and biochemical findings as f-PCT and a request for *UROD* gene analysis was sent to our laboratory. The *UROD* molecular analysis showed heterozygous point mutations found in c. 399, c.400, c.401in Exon 5 of the *UROD* gene as shown in figure 9. The heterozygous T to C transition at c.399 doesn't lead to any change in the amino acid sequence while the missense mutations G to C transition at c.400 and the T to A transition at the c.401 leads to the substitution from valine to a glutamine (polar amino acid). (c.399-401 delins CCA) (V134Q). The substitution from a non-polar amino acid (Valine) to the polar amino acid (Glutamine) leads to change in the helical structure of the protein. (134 position lies in a helix). This suggests the reason of the decrease in the UROD enzyme activity. The inserted deletion was reported before associated with another point mutation by (Meguro, et al., 1994). So based on the previous report, we assume that the found mutation leads to decrease in the UROD catalytic activity resulting in the occurance of the disease in the female patient. Later, the DNA sample of the sister's patient was sent to our laboratory and the same mutation was found in the *UROD* gene.

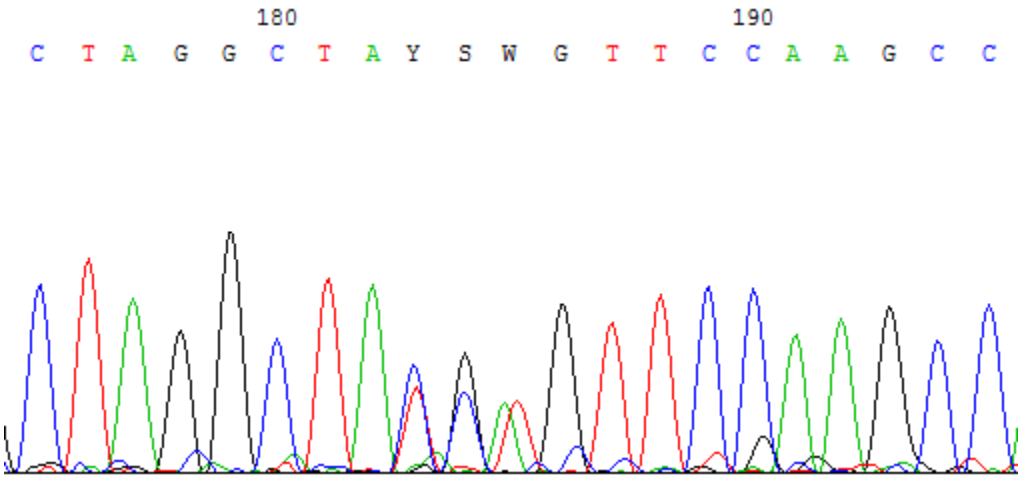


Figure 9. The sequence of the heterozygous point mutations found in one of the female sisters with f-PCT.

8.1.1.3. Patients fPCT4 and fPCT5 (Mother and son)

The mother was suffering from morning red urine and recurrent skin inflammations. The urinary uroporphyrin was investigated and found to be slightly high. Her son was not complaining yet from any health problems. The *UROD* molecular analysis in both the mother and her son showed a splice site mutation in intron 9 heterozygous c. 942+1 G>A as shown in figure 10. We assume that the found mutation in the intron 9 will lead to unproper splicing of the mRNA resulting in an abnormal protein with diminished catalytic activity. The mutation was reported before by (Savino, et al., 2010).

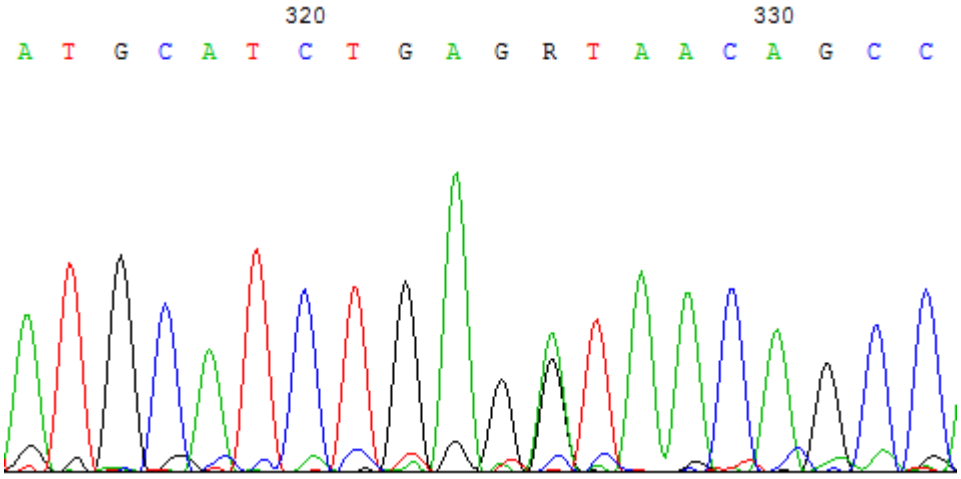


Figure 10. The sequence of the heterozygous c. 942+1 G>A point mutations found in intron 9 in the female patient with f-PCT.

8.1.2. Arabic patients

In this study, the *UROD* gene was analyzed on the molecular level in three cases from the Egyptian population (brother, sister and their mother). The two siblings were diagnosed clinically as having cutaneous porphyria at the Department of dermatology at Cairo university hospital. They were presented by red urine after birth. Figure 11 shows hypertrichosis on the cheecks of the young boy and figure 12 shows hypertrichosis as well in the cheeks and increase hair growth on the dorsum of the forearms in the girl. Biochemical investigations were carried out for the siblings and showed increased levels of polycarboxylated porphyrin in the urine. Biochemical findings were not available for us. The mother neither suffered from any symptoms nor showed any signs of porphyria. The molecular sequence of the 10 exons of the *UROD* gene was done in our laboratory for all the cases. Unfortunately, the father was not available for DNA analysis. The results revealed a novel missense homozygous mutation in the siblings and thus were diagnosed as HEP. Their mother had shown to be a healthy heterozygous carrier of the mutation as shown in figure 13. The *UROD* gene sequence in the Egyptian family showed T→A transition at position 163 in exon 3 (c.163T>A), that leads to the substitution of phenylalanine to isoleucine at the codon 55 (F55I). According to the crystal structure of the UROD protein, position 55 in the amino acid chain is demonstrated in the substrate binding site (Whitby, et al., 1998). A cartoon representation of UROD structure (PDB 1r3y, only one monomeric unit is displayed) with coproporphyrinogen product in space-filling rendering with the 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 as shown in figure 14. Alignment of a segment surrounding F55 (arrow) in human UROD with corresponding orthlobous sequences identified in selected metazoa, plants, fungi, bacteria and archea is presented in figure 15.

To prove the above results, we were in need to determine the activity of the UROD enzyme in the probands. The identified homozygous missense mutation in the siblings was created into the pGEX-UD vector by site directed mutagenesis. Both the recombinant mutant UROD protein and the recombinant wild-type UROD were purified in our laboratory and sent to Prof. H. De Verneuil at the University of Bordeaux, France for measurement of the enzyme activity. Figure 16 shows both the purified UROD wild type protein and the mutated UROD protein on an SDS-PAGE. The specific activity of the mutated UROD protein towards pentacarboxyl porphyrin I as a substrate was determined and compared with the recombinant

wild-type UROD. The activity measurement of the F55I mutant UROD was found to be 19 % of the wild-type towards pentaporphyrinogen I as shown in table 14. The marked decrease in the enzymatic activity of the mutated UROD confirms the hypothesis that the newly found mutation in the probands is responsible for the disease.

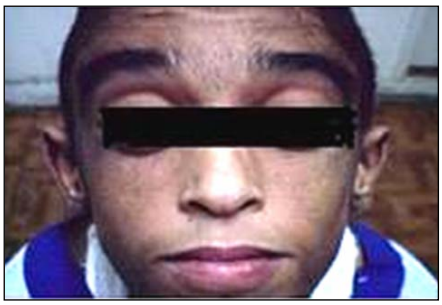


Figure 11. Hypertrichosis on the face.

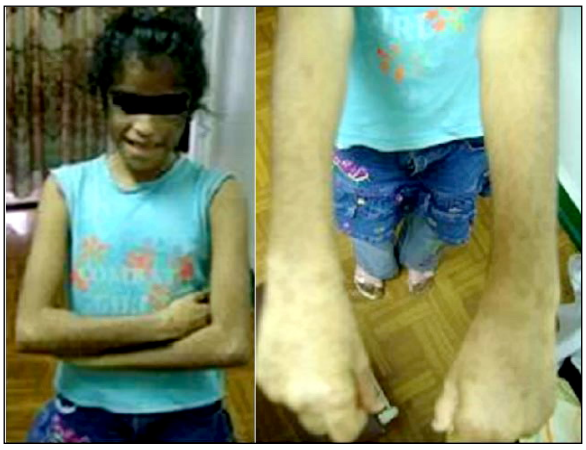
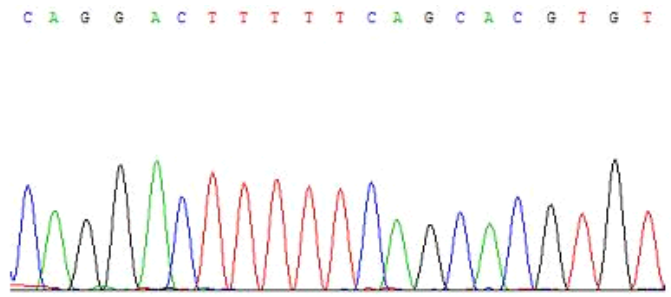
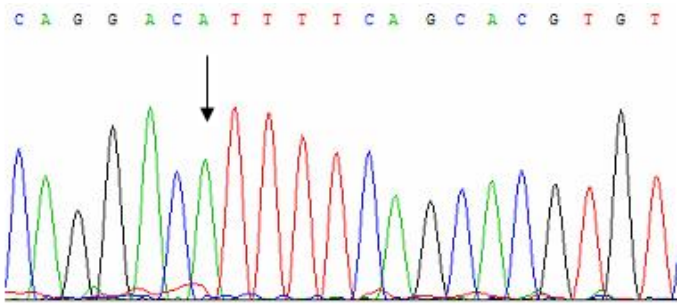


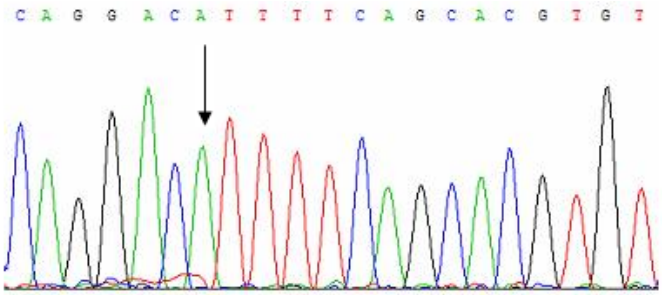
Figure 12. Hypertrichosis on the cheeks & increase hair growth over the dorsal aspects of the forearms.



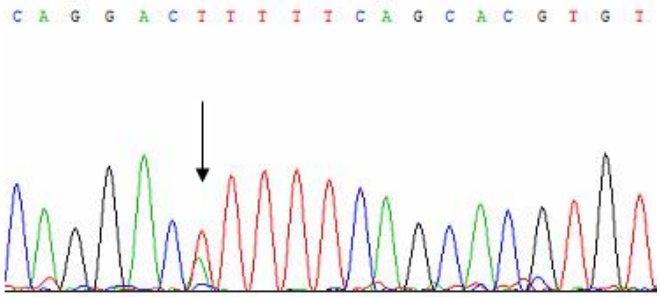
Control



Son



Daughter



Mother

Figure 13. 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.

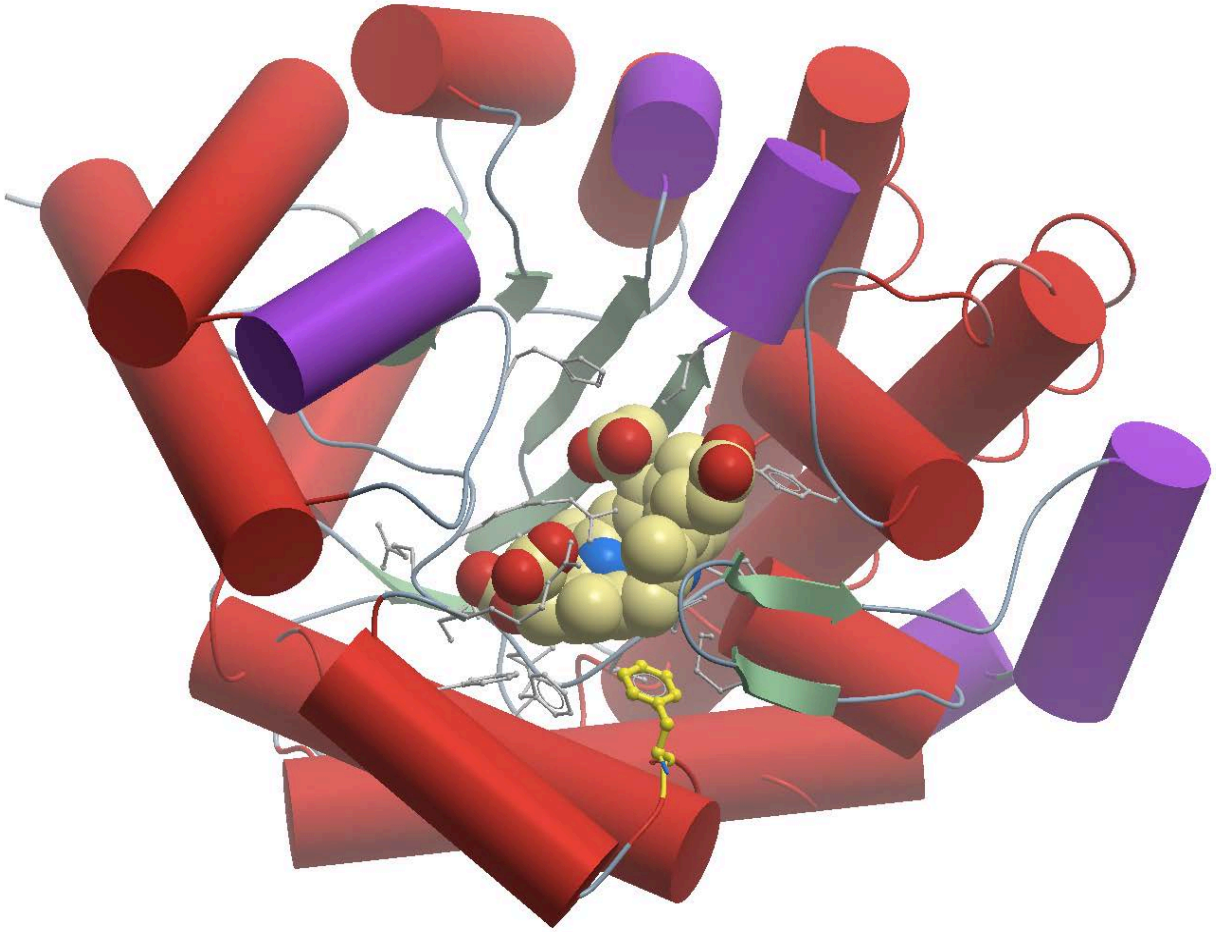


Figure 14. Graphical representation of UROD structure (PDB 1r3y, only one monomeric unit is displayed) with coproporphyrinogen product in space-filling rendering. Sidechains participating in the direct binding of the product are displayed in ball and stick.
Phe 55 (mutated in the probands to Ile) is highlighted in bold and yellow.

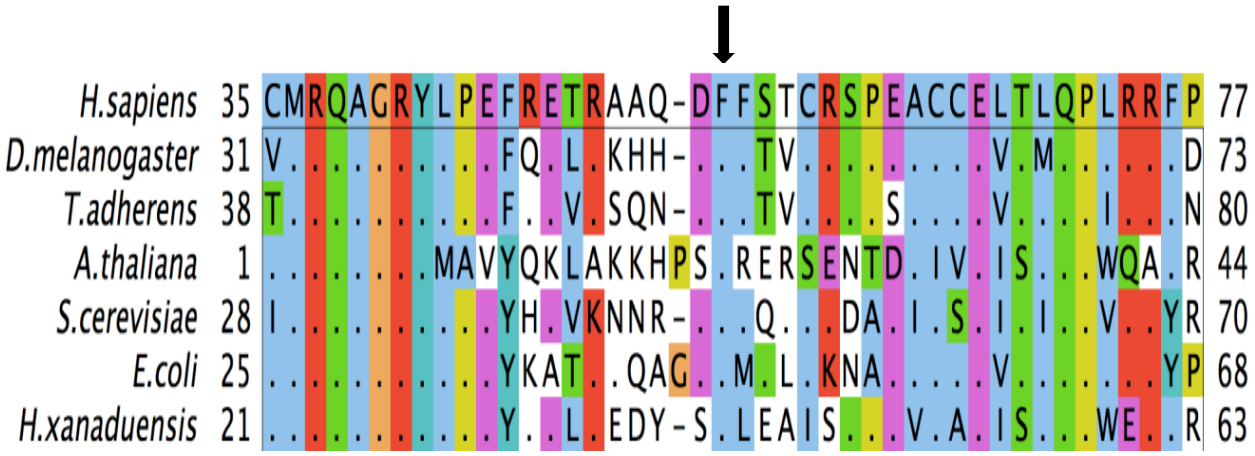


Figure 15. Alignment of a segment surrounding F55 (arrow) in human UROD with corresponding orthlobous sequences identified in selected metazoa, plants, fungi, bacteria and archea. Species and UniProt sequence identifiers from top to bottom: *Homo sapiens*, P06132; *Drosophila melanogaster*, Q9V595; ***Trichoplax 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).

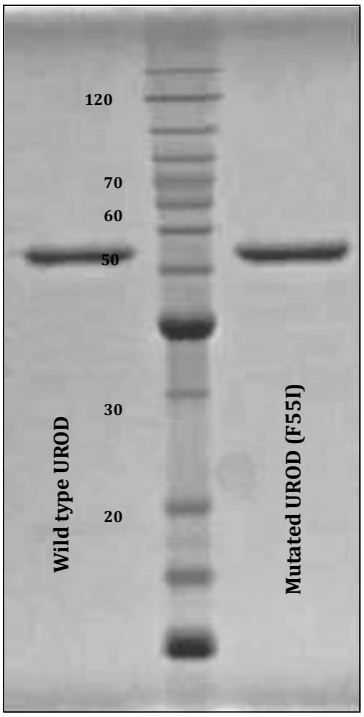


Figure 16. The purified wild-type UROD protein on the right hand side and the purified mutated UROD F55I on the left hand side on SDS-PAGE.

Table 14. Expression of the normal and mutated GST-UROD fusion proteins

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

Data are given as picomoles of coproporphyrinogen I formed per hour and per milligram of protein.

8.2. Molecular analysis of the FECH gene in the patients of Czech, Slovak Slavic origin and normal Czech control

8.2.1. Screening the frequency of the low expression allele IVS3-48C in the FECH gene in the Czech control cohort

EPP is a rare autosomal dominat disorder that requires the coinheritance of a *FECH* gene abnormality together with a wild-type low expression allele IVS3-48c to produce the marked decrease in the FECH catalytic activity and to develop the clinical manifestations of the disease (Gouya, et al., 1999).

The frequency of the low expression allele was determined in many populations world-wide. The frequency of the IVS3-48c allele in the Japanese population was found to be 45 % (Nakano, 2006),in the Chinese population (Han) 41 % while being 31 % in the southeast Asians (Kong, et al., 2008)

The frequency in the white French was estimated around 5,5 % (Gouya, et al., 2006) and in the British population was 13 % (Berroeta, et al., 2007).

Morever the frequency in the Ashkenazi Jews is 8 % (Schneider-Yin, 2008), in the Swiss population 7 % (Schneider-Yin, et al., 2009) , in the Spanish population 5.2 % (Herrero, et al., 2007), in the north African population 2.7 %, in the black west African populations is less than 1 % (Gouya, et al., 2006) and in the Italians is 1 % (Aurizi, et al., 2007).

Till the time being we have no figure about the frequency of the low expression allele in the Czech population. The study was carried out in compliance with the principles of Helsinki declaration on normal candidates from Czech west Slavic origin of random ages.

We screened the frequency of the splice site modulator in 624 alleles in 312 controls,149 controls were males and 163 controls were females. The Czech healthy control identified 277 homozygotes IVS3-48t/t and 35 heterozygotes IVS3-48c/t. Therefore, the frequency was estimated as 5.5 % in the Czech population, 5 % among males and 6 % among females as shown in table 15.

Table 15. showing the Incidence of distinct genotypes of the *FECH* IVS3-48T/C polymorphisms in males, females and 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

8.2.2. Molecular analysis of *FECH* gene in patients with EPP

We have analyzed the 11 protein-coding exons of the FECH gene together with their flanking intron regions in patients with EPP of Czech and Slovak origin. The promoter and the low expression allele of the FECH gene were also analyzed in all these patients. In this study, we identified mutations in the *FECH* gene in three individual Czech patients and one Slovak patient.

8.2.2.1. An analysis of extended Czech family with EPP

A Czech family of eight members in four generations was studied as illustrated in the pedigree shown in figure17. The probands 40 years old male and his mother 67 years old, have suffered from dermatological problems on exposure to sun light since early childhood. They were diagnosed as EPP based on clinical findings and biochemical investigations; a distinct peak in the plasma emission scan at 634 nm and high erythrocyte protoporphyrin content.

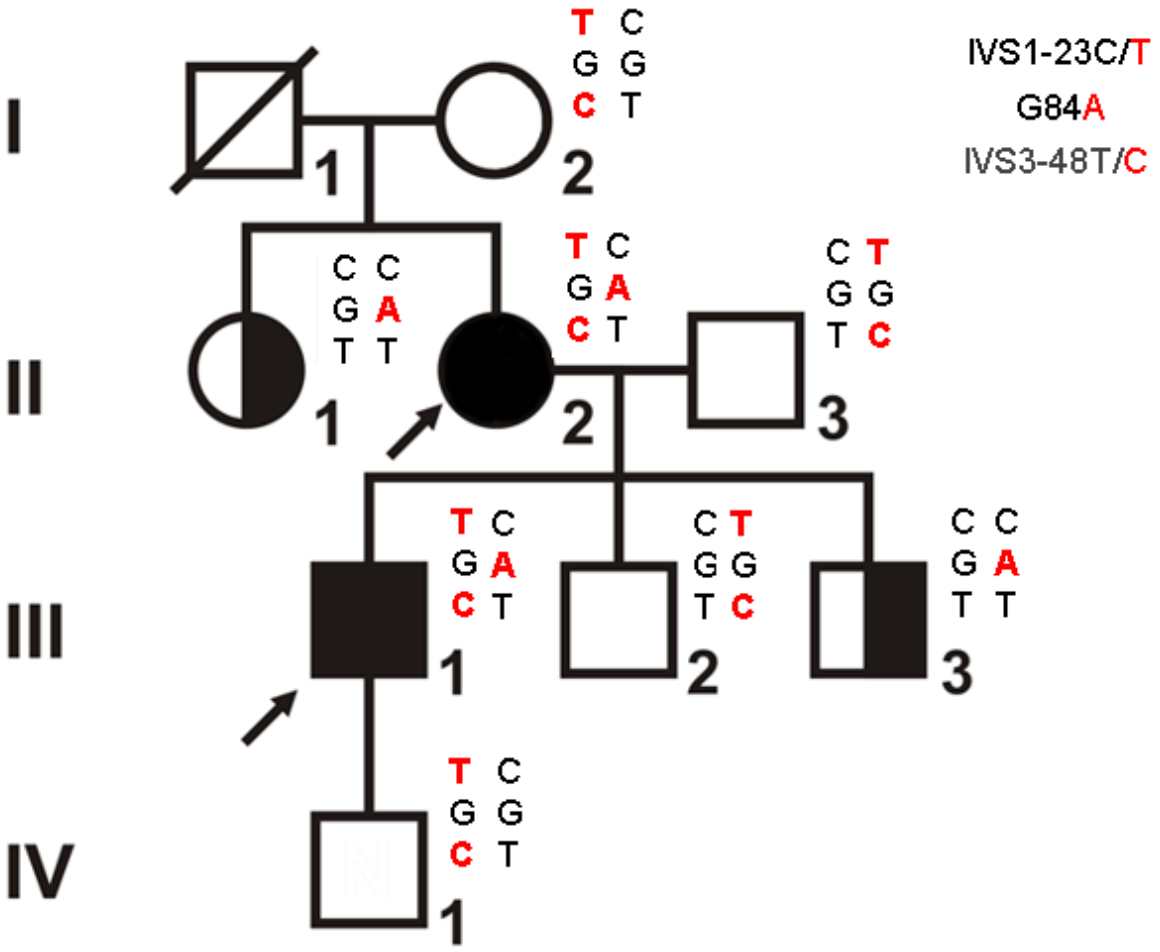


Figure 17. Genealogical tree of the EPP family with p.W28* mutation. A pedigree of the proband´s family. ○ - female, □ - male, full filled - clinically manifest porphyria, half-filled - clinically silent.

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.

8.2.2.1.1. Biochemical results

We measured the free protoporphyrin content in both probands with EPP (individuals II/2 and III/1 in figure 17) as well as in the clinically normal grandson (individual IV/1 in figure 17), finding it high in the probands (common in patients with EPP) while normal protoporphyrin concentrations were found in the grandson shown in table 16.

Table 16. Showing Protoporphyrin (PP) content in erythrocytes, PP - protoporphyrin (Normal values: Total PP ≤2.00 µmol/µl)

Subject	**Total PP (umol/)**	**Zinc PP %**	**Free PP %**
Proband I (son)	24.5	11	89
Proband II (mother)	23,64	7	93
Grandson (IV/1)	1,16	-	-

8.2.2.1.2. Molecular results

Molecular genetic analyses of the ferrochelatase gene revealed a novel heterozygous G84A transition in exon 2 as shown in figure 18. 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 17. 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.

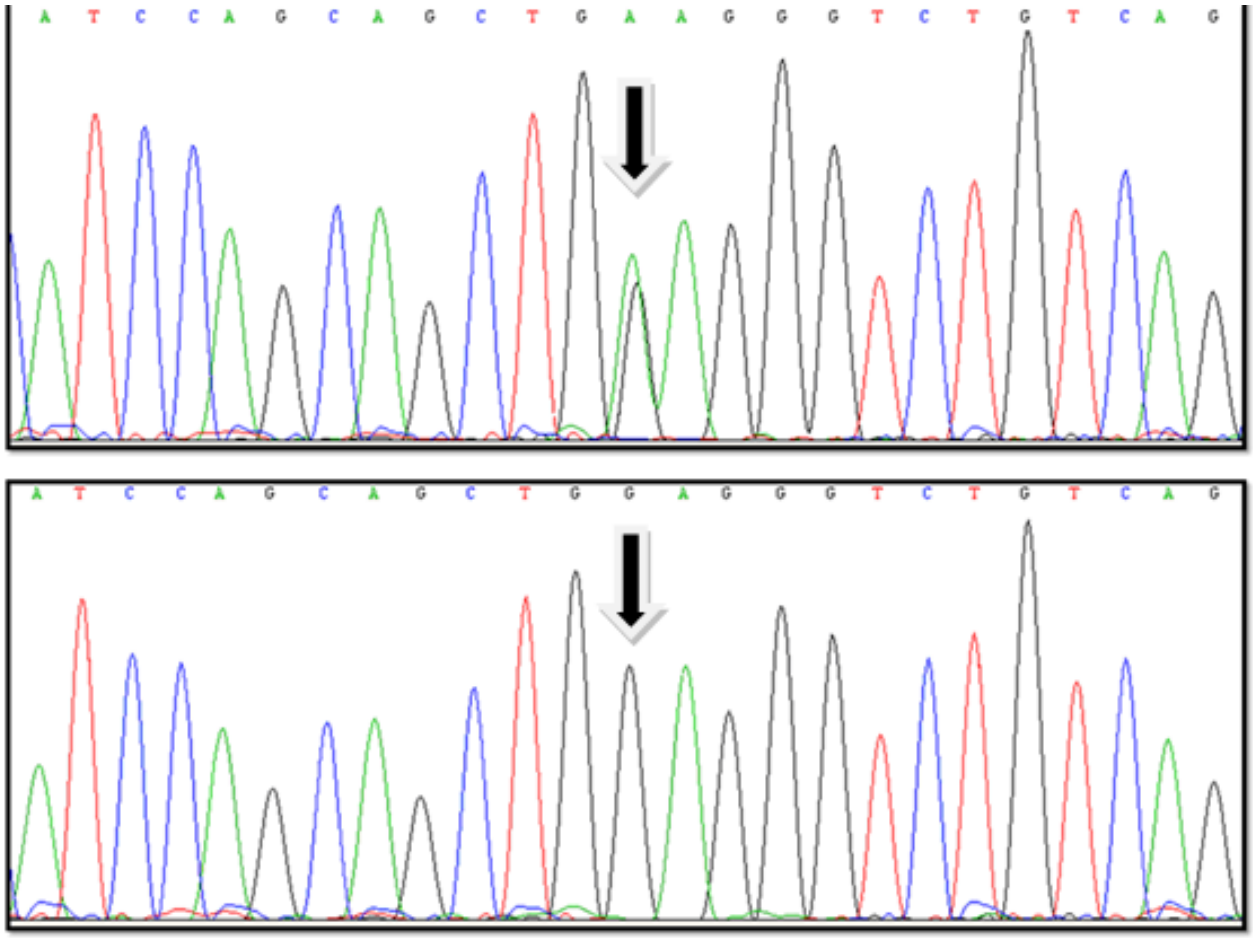
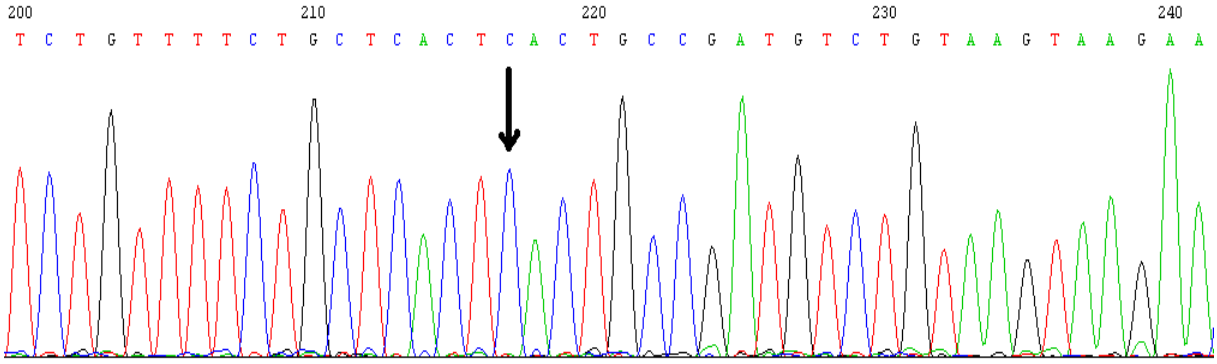


Figure 18. G84A mutation in exon 2 in the *FECH* gene with comparison to a control sequence below.

8.2.2.2. Results of EPP5 patient

A female Slovak patient (EPP5) 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 19. Unfortunately, no relatives of the proband were available for the molecular study of the *FECH* gene.The low expression allele IVS3-48 T>C was found to be heterozygous. SNPs that were found on analysis of the *FECH* gene are listed in table 17. A missense mutation S264L, affecting the same codon has been previously reported, in an Italian patient (Aurizi, et al., 2007).

Control



Mutation:

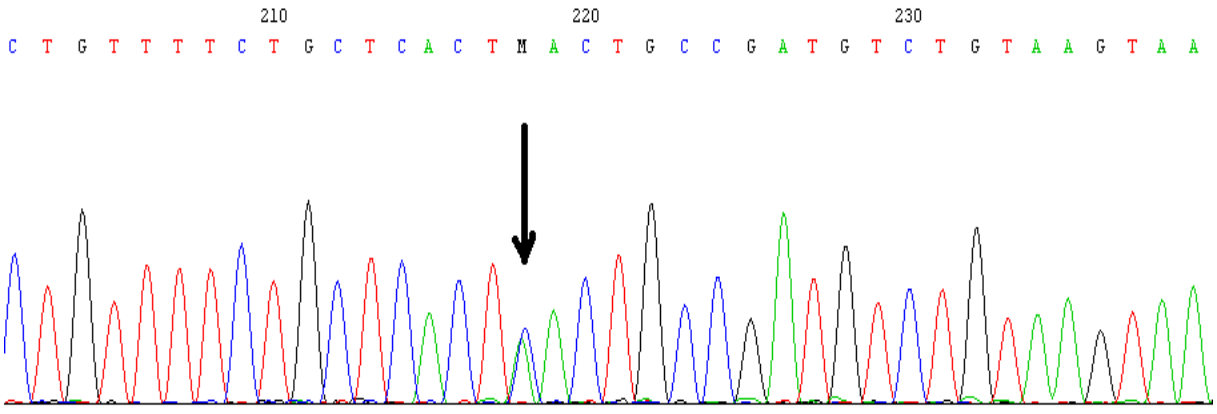


Figure 19. The S264X mutation in exon 7 of the *FECH* gene with comparison to a control sequence above.

Table 17. SNPs found in the *FECH* gene in the EPP5 patient.

Position	***Polymorphism***	***Polymorphism***
Intron 3	***IVS3-48 T>C***	**T/C**
Exon 9	**Homozygous polymorphism in bp 32957**	**G/G**
Exon 11	**homozygous polymorphism in bp 36778**	**C/C**

8.2.2.3. Findings in patient EPP6

A middle age male of Czech origin was diagnosed both clinically and biochemically as EPP. In this patient, the molecular analysis of the *FECH* gene was performed. PCR of all the exons with the flanking introns together with the promotor was done. The sequence of the gDNA didn't show any mutation. The low expression allele IVS3-48C was found to be heterozygous. Therefore, a MLPA was performed and we were able to identify a big heterozygous deletion in the promoter and exon 1 of the *FECH* gene. To double check the obtained data, a real time PCR copy number assay was performed for the FECH gene and confirmed the obtained results. The MLPA results that were found in the patient EPP6 are shown in figure 20. The SNPs that were identified are listed in table 18. Few SNPs that were identified were synonymous variants while other SNPs were of genetic importance.

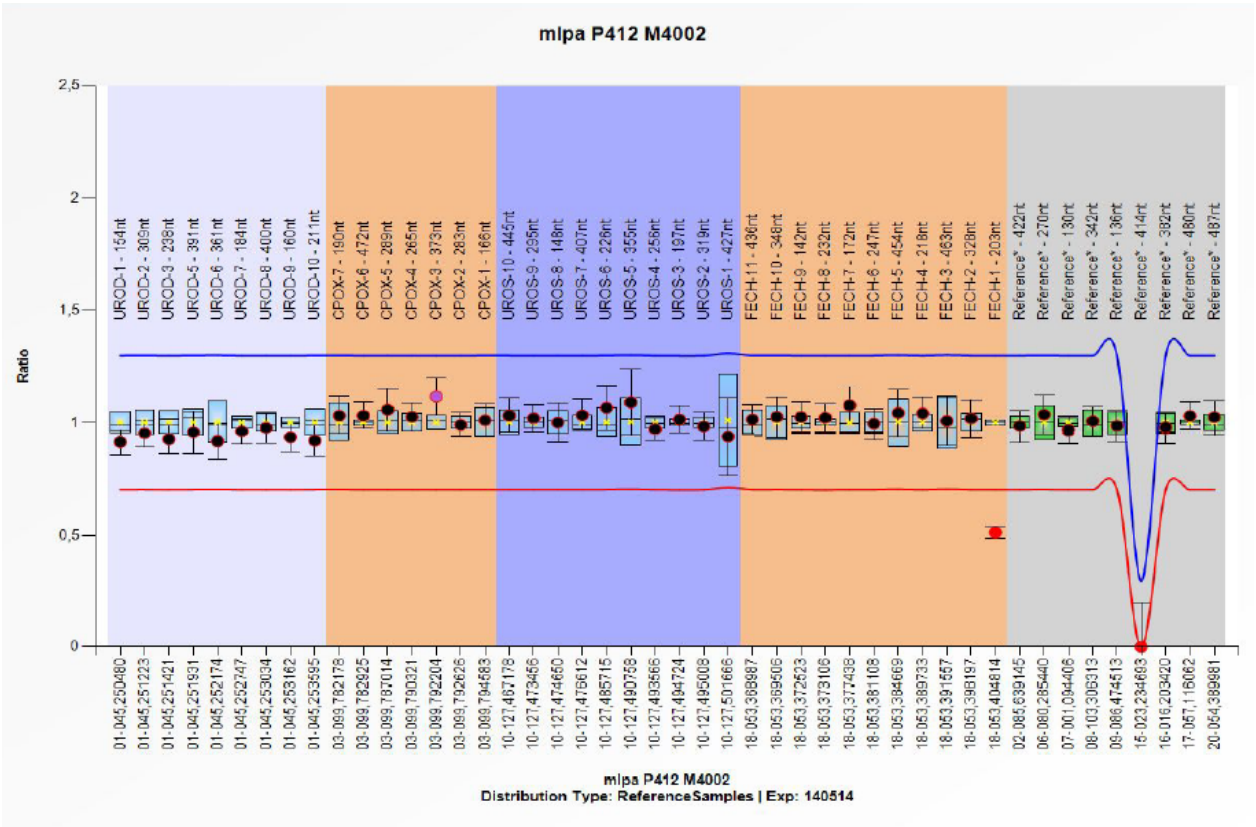


Figure 20. The deletion in exon 1 of the *FECH* gene in the young Czech patient with EPP.

Table 18. The SNPs found in the *FECH* gene of patient EPP6.

Position	Polymorphism	Inheritance
promotor	c. 252 A>G c. 67+76 C>T	Homozygous Homozygous
Intron 1	c.68-23 C>T	Heterozygous
Intron 2	c. 314 +23 A> G	Homozygous
Intron 3	c. 315- 48 T>C	Heterozygous
Exon 7	c. 798 C>G	Homozygous
Intron 9 ***Exon 9***	c.913-62_913-61 del AG c.921 A> G	Heterozygous Homozygous
Exon 11	c.*248c> T	Heterozygous

We assume that the deletion in exon 1 of the *FECH* gene affects the translation initiation codons. Therefore, the mutation together with the low expression allele IVS3-48C lead to a non-functioning protein. Moreover, few of the SNPs found in patient were synonymous variants while others were more important. For instance, the SNP variant c.252 A>G in the promoter reduces the gene transcription (Di Pierro, et al., 2005) and the SNP c.68-23 C>T in intron 1 most propably change splicing (Nakahashi, et al., 1992). Research is still going on in our laboratory to identify the exact part of the *FECH* gene which is deleted in patient EPP6.

9.

DISCUSSION

9.1. General part of discussion

Enzymes involved in the biosynthesis of heme and heme metabolism are critical for living organisms. Their conservation across biological taxa offers a wealth of data sheding light at basic mechanisms supporting life functions. Studies of mutations leading to phenotypic manifestation such as a disease, are directly leading to discoveries of functionally important primary structures of enzymes. Disease is in this respect a consequence of a direct disturbance of protein function and biological medicine is becoming an important contributor to general biology, in this case, biology of heme and its availability.

Nowadays, the molecular analysis of the DNA of the porphyric patients showed a significant importance in their diagnosis. Being an accurate technique, it provides us to confirm the clinical and the biochemical diagnosis and moreover to identify other family members of the patient's family that were not diagnosed before. Early identification of other family memebers with the disease by the genomic analysis enables them to recieve an early proper management even before the development of the disesase and to be less susceptible for acute attacks.

9.2. Study of the Czech cohort of patients with fPCT

In the present study, patients with f-PCT with mutations in the *UROD* gene were characterized at the molecular level. Following mutations were found, all of them were previously published:

fPCT1 and fPCT2 (non-related patients) = p.[Gln206*];[=].

fPCT3 = pV134Q

fPCT4 and fPCT5 (mother and son) = Heterozygous c. 942+1 G>A

Two unrelated patients with f-PCT of Czech origin (fPCT1 and fPCT2) showed exactly the same mutation which was previously reported in the literature for Italian patient (accession number on Biobase HM971362). We assume that the identified mutation could be a frequent mutation in the *UROD* gene in the Slavic population. In the near future, we plan to perform the molecular analysis of the *UROD* gene in more Czech patients to widen our knowledge about existing mutations.

9.3. Study of molecular pathology of new case of rare HEP in Arabic family from Egypt (See 11.1.2)

Enzymes involved in the biosynthesis of heme and heme metabolism are critical for living organisms. Their conservation across biological taxa offer a wealth of data sheding light at basic mechanisms supporting life functions. UROD is the fifth enzyme of the heme biosynthetic pathway (figure 1) which catalyzes the removal of four carboxymethyls from uroporphyrionogen reversing it to coproporhyrinogen thus affecting the sterical proportions of the substrate and tranforming it into further enzymatically manageable new substrate (Elder et al., 1978; Silva et al., 2005). Interestingly, this enzyme homodimerizes with a low monomer-dimer dissociation constant of 0.1 μmol/l (in case of human UROD) (Philips et al. 2007).
The dimerization is also found in the case of bacterial UROD, nevertheless it is not clear if the dimerization is necessary for UROD enzymatic function. Porphyring ring biosynthesis shares a common pathway for animals and plants including UROD enzyme (Heinemann et al., 2008)
HEP is a rare disease: fewer than 100 cases of HEP have been described in the literature (Liu et al, 2013). Table 3 depicts the frequency of the mutations of 21 unrelated families reported in the literature. A genetic homogeneity has been observed in Spain with the predominance of the G281E mutation. By contrast, a great heterogeneity of mutations has been found for other countries in Europe, Africa or America. Because of the heterogeneity of the mutations, the clinical outcome is very variable, with mild or moderate phenotypes difficult to distinguish with a familial PCT or a more severe phenotype similar to CEP or homozygous VP. For example, the genotype F46L found in two different families (Ged et al 2002, Armstrong et al 2004) is associated with a mild phenotype. By contrast, the genotype G281E is associated with a severe phenotype resembling CEP.
This report describes 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 14). Moreover, F55 is conserved in both eukaryota and prokaryota

(Figure15). 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 14). We do not have possibility to investigate biological samples of 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 (figure 11, 12; table 14).

The mutation identification helps us to better understand the disease and helps in counselling to affected families. We report the first cases of HEP in the Egyptian population based on molecular diagnosis of the *UROD* gene. Because of 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 (figure14) forming the bottom of the substrate-binding site is thus disrupted and the substrate binding becomes probably much looser. The absolute conservation (figure 15) 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.

9.4. Study in *FECH* gene

On the other aspect of our study, we performed molecular analysis of the *FECH* gene for many patients with EPP in our laboratory. The EPP patients from the Slavic origin that were analyzed for the *FECH* gene always inherited the heterozygous form of the low expression allele IVS3-48C. Therefore, we hereby confirm the importance of the co-inheritance of the low expression allele to produce the marked decrease in the FECH activity and to develop the obvious clinical manifestations as reported before by (Gouya, et al., 1999). Moroever, we identified two novel mutations in patients with EPP which broadens the molecular heterogeneity of the *FECH* gene.

9.4.1. Analysis of molecular pathology of EPP5 and EPP6 patients

We identified a novel mutation in EPP5 patient that broadens our knowekedge about the heterogeneity of the disease. Moreover, in EPP6 patient, we assume that the deletion in exon 1 of the *FECH* gene affects the translation initiation codons. Therefore, the mutation together with the low expression allele IVS3-48C lead to a non-functioning protein. Moreover, some of the SNPs found in patient were synonymous variants while others might be more important; the SNP variant c.252 A>G in the promotor reduces the gene transcription (Di Pierro, et al., 2005) and the SNP c.68-23 C>T in intron 1 propably change splicing (Nakahashi, et al., 1992). Research is still going on in our laboratory to identify the exact part of the *FECH* gene which is deleted in these patients.

9.4.2. EPP in extended Czech family (See 11.1.1)

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 (Meerman, 2000; 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 (Figure17), it is impossible to assess the distinct impact of IVS1-23T in combination with G84A mutation.

9.4.3. 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 a good availability of a laboratory diagnosis of this disorder. According to our knowledge, there were 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 study was not performed in any Slavic

population yet. 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).

We screened the frequency of the IVS-48C allele in 624 alleles in 312 Czech control individuals, 149 subjects were males and 163 subjects were females. The Czech control subjects identified 277 homozygotes IVS3-48T/T and 35 heterozygotes IVS3-48C/T. Therefore, the frequency of IVS-48C allele was estimated as 5.5 % in the Czech population, 5% among males and 6 % among females as shown in table 15.

These results invoke the attention of the health care professionals in the Czech Republic with the diagnosis of EPP and to be aware to investigate patients with intolerance to sun-exposure to the diagnosis of the disease. Nevertheless, we highlight the importance of the molecular analysis in the diagnosis of EPP as it gives a full picture about the pathology for better understanding of the disease and help physicians to identify asymptomatic carriers and therefore avoid further propagation of the disease.

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 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.

10.

CONCLUSION

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

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

10.3 We report the first cases of HEP in the Egyptian population based on molecular diagnosis of the *UROD* gene. Because of 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

10.4 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 extended Czech EPP family as well as in one single case.

10.5 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.

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

11.

LIST OF ORIGINAL PUBLICATIONS

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

11.1.1. **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 (Praha), 2015; *in press*
IF = 1,000

11.1.2. **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 (Praha), 2015; *in press*
IF = 1,000

11.2. 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)

11.3. 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, pp: 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

12.

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