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Cytochrom P450 oxidoreduktáza: Strukturálně funkční studie
Molekulární patologie Antley-Bixlerova syndromu

Cytochrome P450 oxidoreductase: Structurally functional study
Molecular pathology of Antley-Bixler syndrome

PhD thesis

Supervisor: Prof. MUDr. Pavel Martásek, DrSc.

Prague, 2015

STATUTORY DECLARATION

I hereby declare that I wrote this dissertation myself and I properly cited all sources and literature that I used. I also declare that my dissertation was not used to obtain any other academic degree.

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In Prague, 10.3.2015 Mária Tomková

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ABSTRAKT

NADPH-P450 oxidoreduktáza (POR) je membránový flavoprotein, který transportuje elektrony na široké spektrum hemoproteinů, řada z nich hraje podstatnou úlohu v metabolizmu xenobiotik a steroidů. Vzhledem k nezastupitelné roli POR v metabolismu léků je v posledních letech pozorován zvýšený zájem o analýzu genu *POR,* zejména v oblasti farmakogenomiky. Mutace v genu *POR* způsobují onemocnění, které souborně nazýváme POR deficience. Toto onemocnění se projevuje širokým spektrem fenotypových projevů sahajících od poruch steroidogenéze až po kosterní malformace známé jako Antley-Bixlerův syndrom (ABS). Cílem předložené práce je analýza genu *POR* u pacientů s podezřením na POR deficienci a poprvé v běžné české a židovské populaci. Analyzovali jsme 644 alel nepříbuzných českých jedinců a 1128 alel v populaci židovské, 330 alel Aškenázů, 798 alel Sefardů. Následně byl studován vliv vybraných nových genetických variant na aktivitu POR proteinu. Definovali jsme frekvence alel genu *POR* v obou populacích, popsali jsme 14 nových variant vedoucích k aminokyselinovým záměnám POR proteinu a prozkoumali jsme dvě z nich s cílem určit jejich vliv na aktivitu proteinu. Dostupnost struktury lidské POR umožnila modelování nově popsaných variant a popis defektu na molekulární úrovni. Analýza genu *POR* byla následně v laboratoři zavedena do rutinního vyšetření a jsme v České republice jedinou laboratoří, která v současné době poskytuje diagnostiku POR deficience.

Klíčová slova: CYP oxidoreduktáza; frekvence alel; Česká populace; Židovská populace, POR; farmakogenetika; syndrom Antley-Bixler

ABSTRACT

NADPH–P450 oxidoreductase (POR) is a membrane bound flavoprotein that donates electrons to a wide spectrum of heme-containing proteins, among which are several steroidogenic and many xenobiotics-metabolizing enzymes. Given the important role of POR protein in drug metabolism and pharmacogenomics, there is a particular need to understand the contributions of POR genetic variants to these processes. Mutations in *POR* gene cause a disorder called POR deficiency, which manifests with a wide phenotypic spectrum ranging from disordered steroidogenesis to skeletal malformation, namely, Antley-Bixler syndrome (ABS). The aim of the present work was to investigate the *POR* gene in patients suspected to have POR deficiency syndrome from Czech Republic and to perform genotyping in Czech and Jewish control populations. We analyzed 644 alleles in unrelated individuals from the general Czech population and 1128 alleles in Jewish population, where 330 alleles were of Askhenazi and 798 of Sephardic Jews. We have also studied the impact of selected new genetic variants on POR activity and identified fourteen amino acid variations, two of which we have studied in detail to establish their influence on POR activity. Using the available human POR three-dimensional structure, we then modelled the newly identified variants to describe these defects at the molecular level. Through this study, notably, we have systematically performed analysis of the *POR* gene and are providing POR deficiency diagnostics - the only laboratory in Czech Republic to provide this service. In conclusion, we have, for the first time, defined *POR* allele frequencies in the Czech and Jewish populations that have 14 novel amino acid variations of which two variations studied in detail impinge upon POR activity.

KEYWORDS: CYP oxidoreductase; allele frequencies; Czech Slavic population; Jewish population, *POR*; pharmacogenetics; Antley-Bixler syndrome

TABLE OF CONTENT

ABBREVIATION

ABS	Antley-Bixler syndrome
ACTH	adrenocrticotropic hormone
ADP	adenosine diphosphate
AJ	Ashkenazi Jews
AP2	transcription factor APETALA2
ATP	adenosine triphosphate
BJ	Bulgarian Jews
bp	base pare
BSA	bovine serum albumin
c.	coding
cDNA	coding DNA
CYP	cytochrome P450
CYP1A2	cytochrome P450 1A2
CYP2C19	cytochrome P450 2C19
CYP2D6	cytochrome P450 2D6
CYP3A4	cytochrome P450 3A4
CYP17A1	cytochrome P450 17A1 (17-alpha-hydroxylase/17,20lyase)
CYP19A1	cytochrome P450 19A1 (aromatase)
CYP21A2	cytochrome P450 21A2 (21-hydroxylase)
CYP51A1	cytochrome P450 51A1 (lanosterol 14-alpha-demethylase)
CYPOR	NADPH-cytochrome P450 oxidoreductase
CZ	Czech population
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
DOC	11-deoxycorticosterone
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EJ	Ethiopian Jews
EOMCC	2H-1-benzopyran-3-carbonitrile,7-(ethoxy-methoxy)-2-oxo-(9Cl)
ERα	estrogen receptor α

EXOI	exonuclease I
FAD^+ and $FADH_2$	flavinadenine dinucleotide (reduced and oxidized forms)
FGFR2	fibroblast growth factor receptor 2
FMN	flavin mononucleotide
g.	genomic
gDNA	genomic DNA
HO	heme oxygenase
HPLC	high performance liquid chromatography
MJ	Moroccan Jews
$NADP^+$ and NADPH	nicotinamid adenine dinucleotide (reduced and oxidized forms)
NR1	novel reductase 1
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PGRMC1	progesterone receptor membrane component 1
POR	NADPH - cytochrome P450 oxidoreductase
POR	gen for NADPH - cytochrome P450 oxidoreductase
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
Smad 3	mothers against decapentaplegic homolog 3
Smad 4	mothers against decapentaplegic homolog 4
SP1	transcription factor Sp1
TB	terrific broth
TF	transcription factor
TJ	Turkey Jews
TRβ	thyroid hormone receptor β
WT	wild type
YJ	Yemenite Jews

INTRODUCTION

NADPH – cytochrome P450 oxidoreductase

Human NADPH-cytochrome P450 oxidoreductase (POR) is a membrane bound flavoprotein that donates electrons to a wide spectrum of heme-containing enzymes (figure 1). It belongs to a small group of proteins containing two flavins, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) and it is located on the outer cytosolic side of endoplasmic reticulum. Flavin cofactors are essential for POR function and they serve as a bridge trough which electrons channel from nicotinamide adenine dinucleotide phosphate (NADPH) to metal ion centers of electron acceptors. Electron flow within POR protein starts at NADPH, goes to FAD and FMN and then to heme-containing electron acceptors (Kurzban and Strobel, 1986) (figure 1). Besides POR, the group of diflavin enzymes encompasses also nitric oxide synthase (Griffith and Stuehr, 1995), bacterial cytochrome P450 BM3 (Sevrioukova et al., 1999), methionine synthase reductase (Leclerc et al., 1998), sulfite reductase (Gruez et al., 2000) and protein NR1 (Paine et al., 2000).

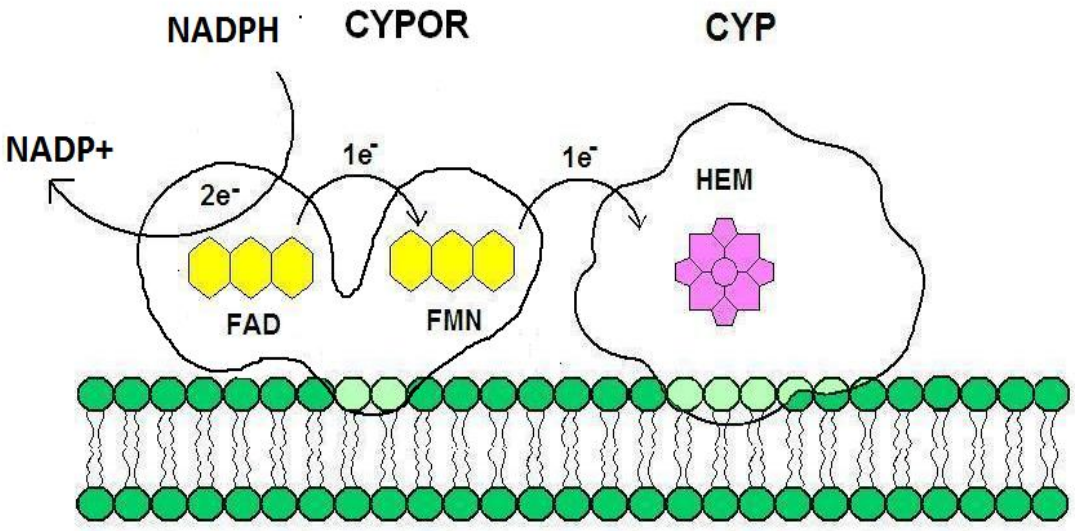


Figure 1. Scheme of the electron transport from NADPH-cytochrome P450 oxidoreductase to microsomal cytochrome P450 [modified according to (Miller, 2004)].

As inferred from its name, main POR protein partner constitute a group of microsomal cytochrome P450 enzymes (CYPs). In addition to CYPs, POR donates reducing equivalents also to some non-P450 proteins such as heme oxygenase

(Schacter et al., 1972), fatty acid elongase (Ilan et al., 1981), squalene epoxidase (Ono and Bloch, 1975) or cytochrome b5 (Enoch and Strittmatter, 1979). Moreover, in *in vitro* experimental assays it can transfer electrons also to the nonphysiological electron acceptor cytochrome c (Williams and Kamin, 1962) and to a variety of small-molecule dyes. In metabolism, POR plays an important role in oxidative clearance of endogenous and exogenous compounds. By interacting with microsomally located cytochromes, POR participates in xenobiotic and drug metabolism and steroidogenesis. By other biochemical pathways, it takes part in metabolism of prostaglandins and fatty acids. Additionally, it was shown that POR also directly activates the anticancer prodrug Mitomycin C (Wang et al., 2007).

History

The enzyme NADPH-cytochrome P450 oxidoreductase was originally isolated and purified by Horecker in 1950 (Horecker, 1950). At the beginning it was termed as cytochrome c reductase based on its ability to reduce cytochrome c. The new enzyme was not localized to any subcellular compartment. Later, Strittmatter and Velick (Strittmatter and Velick, 1956) discovered a separate microsomal fraction that catalyzed cytochrome c reduction 20 times faster with NADPH than with NADH. They proposed it could be the enzyme described previously by Horecker (Horecker, 1950). Few years later, microsomal localization of NADPH-cytochrome c reductase was confirmed at the same time by Williams and Kamin (Williams and Kamin, 1962) and by Phillips and Langdon (Phillips and Langdon, 1962). Both groups came to the same conclusion, namely that the enzyme comprises a flavin prosthetic group, identified by Horecker (Horecker, 1950) as FAD, moreover, Phillips and Langdon (Phillips and Langdon, 1962) suggested that NADPH-cytochrome c reductase participates in microsomal electron transport but not employing cytochrome c. Identification of cytochromes P450 as the physiological electron acceptors of the reductase was postponed because of the proteolytic solubilization used in early studies which resulted in protein unable to reduce cytochromes P450. As highly purifies detergents became available, the physiological redox partners of NADPH-cytochrome c, microsomal cytochromes P450, were identified (Lu and Coon, 1968). Finally, in 1973, Iyanagi and Mason (Iyanagi and Mason, 1973) reported that NADPH-cytochrome c (cytochrome P450) reductase contains equimolar quantities of

FAD and FMN. This was subsequently confirmed by Yasukochi and Masters (Yasukochi and Masters, 1976).

Crystal structures of POR

Human POR is a 78 kDalton, 680 amino acid protein. It has evolved as a fusion of two ancestral proteins, a flavodoxine and a ferredoxin reductase (Smith et al., 1994). The POR tertiary structure is well conserved throughout the different species indicating its catalytic importance (Jensen and Moller, 2010). Sequence of 25 amino acids in the N-terminal part of the protein determined the microsomal localization of the protein and its linkage to the membrane (Wang et al., 1997).

The first three dimensional structure of rat POR molecule was published by Wang *et al.* (Wang et al., 1997). The enzyme was crystalized lacking the membrane binding N-terminal 70 residues. The structure revealed that the molecule is composed of four structural domains (figure 2). From N to C terminus, they are the FMN-binding domain, the connecting domain, and the FAD- and NADPH-binding domains. The isoalloxazine rings of flavin cofactors are oriented 3.85Å apart at an angle of 140° and they form almost continuous ribbon with phenyl rings facing each other. As seen in the picture (figure 2B), the FMN-binding domain consists of five parallel β-sheets flanked by five α-helices. The connecting domain, situated between the two flavin domains, is comprised mainly of α -helices. The FAD-binding domain consists basically from the antiparallel β-sheets and the core of the fourth, NADPH-binding domain, is composed of another five parallel β-sheets folded by α-helices. Domains are arranged into two lobes linked up by the α-helical connecting (linker) domain. The overall shape of the molecule is an oval-shaped bowl with cofactors situated in the middle of the bowl (figure 1). Two important features were revealed in this study. Firstly, the existence of the flexible random coil "hinge" region between the FMN-binding domain and the connecting domain (figure 2A, in dots), that was deduced to be responsible for the pivoting of the FMN-binding domain with respect to the rest of the protein. Secondly, it was pointed out that the connecting domain situated between the two flavin domains might have an important function in bringing two flavin domains in close proximity to enable internal electron transfer within the molecule and also to facilitate POR to interact with its partner by bringing the FMN close to the cytochromes P450. The crystal structure of the rat POR described by Wang *et al.* (Wang 1997) was the first diflavin reductase for which a three

dimensional structure was solved. Therefore it represented a prototype for the spatial arrangement of the two flavins in the POR and provided insights into the mechanism of electron-transfer for other diflavin enzymes (Wang et al., 1997).

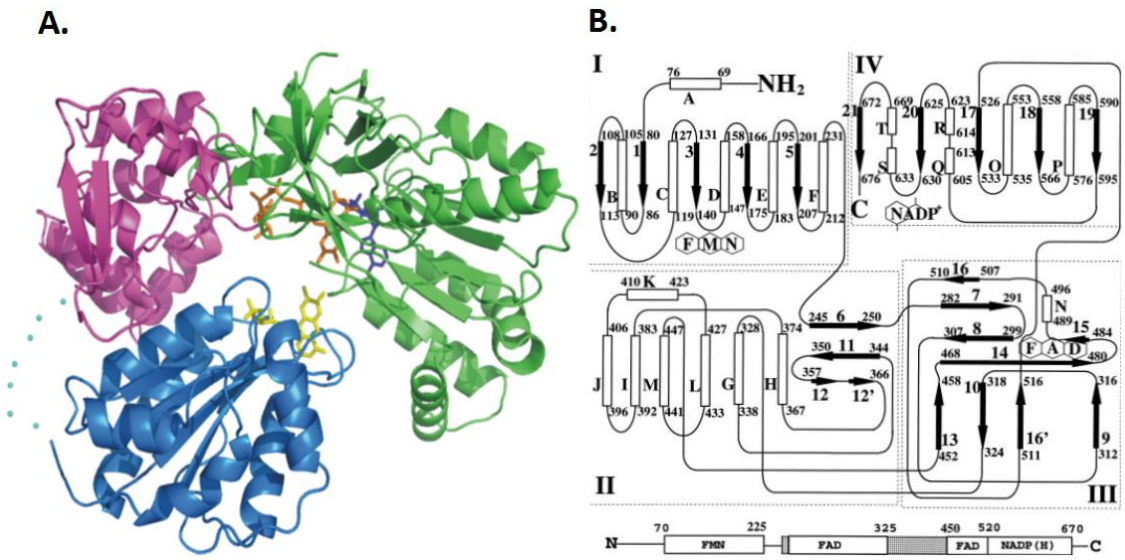


Figure 2. A crystal structure and a topology diagram of the rat POR.

A. The crystal structure of the rat POR (Protein Data Bank code 1AMO). The FMN-binding domain shown in blue, the connecting domain in purple and the FAD- and NADPH-binding domains are represented in green. The cofactors are shown as *stick structures*: *yellow* for FMN, *orange* for FAD, and *blue* for NADPH. **B.** The topology diagram of the rat POR (Wang et al., 1997). The FMN domain (I), connecting domain (II), FAD domain (III), and NADPH domain (IV) are identified by boxes. Beta-strands (filled arrows) are numbered sequentially from N to C terminus; α-helices (open cylinders) are lettered. Corresponding residue numbers are indicated.

In 2006, a crystal structure of yeast truncated POR was published (Lamb et al., 2006). The most striking feature of yeast POR compared to the rat POR was the presence of a second FMN-binding site in the interface of the connecting domain and the FMN-binding domain. Since only one FMN molecule was found to be required for POR function, authors hypothesized that FMN might shuttle between the two sites. Structure of both rat and yeast POR showed that two flavins are in a close proximity to one another (in closed conformation) (Lamb et al., 2006). Such flavins' configuration creates a suitable position for interflavin electron transfer. However, this conformation did not seem to be adequate for electron transfer from FMN to CYPs.

Hamdane *et al*. (Hamdane et al., 2009) measured the activity of several POR mutants affected in the hinge region. Dramatic reduction in the activity of the mutant ΔTGEE (four amino acids were deleted from the hinge region) prompted them to determine the crystal structure of this mutant. The deletion of several amino acids provided an enzyme crystallized in intermediate conformations between the fully opened and closed one (Hamdane et al., 2009).

Ambition to understand the role and properties of individual POR domains in the catalytic cycle eventuated in crystallization of a chimeric, yeast-human (YH) POR (figure 3) (Aigrain et al., 2009). The enzyme was composed of the yeast FMN domain and the human connecting and FAD domain. The YH structure revealed one FMN and one FAD molecule, which was consistent with the findings of Wang *et al.* (Wang et al., 1997) described in the structure of rat POR. In contrast to previous findings, the crystalized YH mutant protein showed flavin cofactors completely exposed to the solvent, it means in open conformation. The structure adopted an extraordinary extended conformation in which flavin coenzymes were separated by 86Å (Aigrain et al., 2009) compared to 4Å described in the rat crystal structure (Wang et al., 1997).

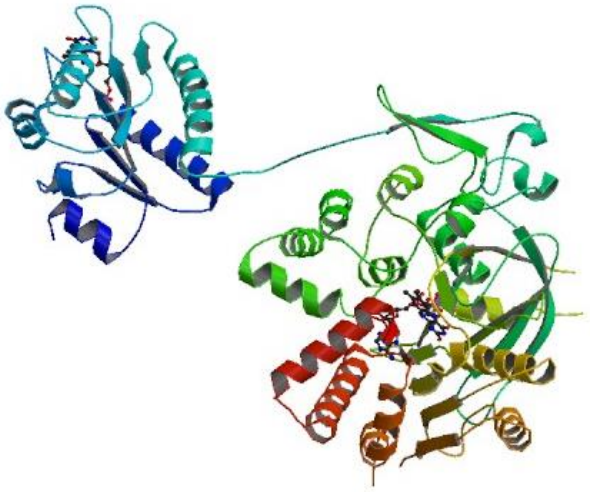


Figure 3. The crystal structure of the chimeric yeast-human POR (Protein Data Bank code 3FJO).

Flück *et al*. (Fluck et al., 2009) modeled the structure of human POR by *in silico* amino acid replacements in the rat POR crystal structure. They replaced 38 POR residues to make its sequence identical to human POR. The model was of high

quality and until human POR structure was published it had served for analyzing the structural implications of genomic sequence variants in human POR (Fluck et al., 2009).

Lastly, the atomic structures of the entire soluble human wild-type POR and two mutants were reported (figure 4) (Xia et al., 2011). According to the authors, the overall fold of human wild-type POR is essentially the same as that of the rat enzyme (Wang et al., 1997), except the residue H621, which is missing in the human enzyme (Xia et al., 2011).

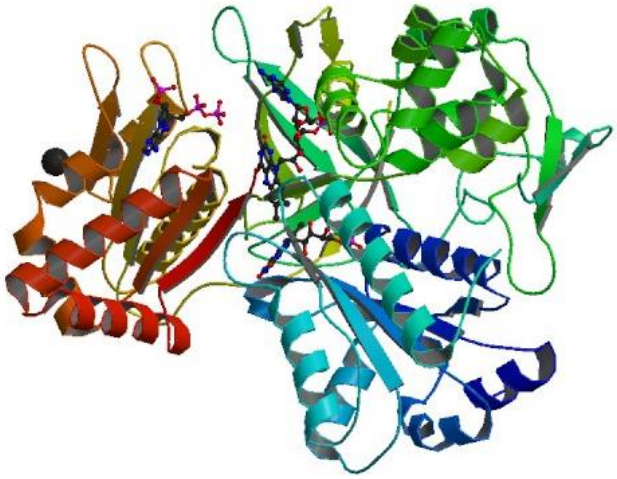


Figure 4. The crystal structure of the human POR (Protein Data Bank code 3QE2).

It should be noted that currently, all available POR structures are of soluble enzyme lacking the membrane anchor. In general, it is very difficult to obtain the crystal structure of the membrane proteins, and POR has been no exception. Removal of the N-terminal anchor was essential for protein crystallization. Truncated protein is still able of passing electrons to cytochrome c and other artificial partners, however, it was demonstrated that it is not capable to reduce its physiological redox partners and membrane spanning domain is essential for efficient electron transfer to microsomal cytochrome P450s (Yasukochi and Masters, 1976).

Domain motion in POR

Crystallographic studies of POR provided evidence that POR exists in solution in several alternative conformations. Spatial arrangement of the cofactors seen in the

rat (Wang et al., 1997) and yeast (Lamb et al., 2006) "compact" POR crystal structure appears to be ideally suited for the electron flow from one flavin to another, though, not for the further transfer to its acceptor. As a result, movement and reposition of the FMN-binding domain required for facilitating the protein interactions was largely anticipated.

The first structural insights into how CYPs might interact with FMN domain of POR were provided by the crystal structure of a complex between the heme- and FMN-containing domains of bacterial cytochrome P450 BM3 (Sevrioukova et al., 1999). The heme and the reductase domains of P450BM3 share significant homology with P450 4 family and POR (Porter, 1991). In the cytochrome P450 BM3 model, the methyl group of the FMN is pointing toward the heme-binding loop (Sevrioukova et al., 1999). This configuration of the methyl group represents the most efficient pathway for electron transport to the heme iron. In contrary, methyl group of FMN in oxidized POR is oriented toward FAD (Wang et al., 1997). Taking into account these facts, the authors concluded that POR with its protein partner is likely to form the complex similar to what they observed in P450 BM3, but because of the orientation of the FMN methyl group, it has to undergo some structural rearrangement to make the FMN accessible for CYP (Sevrioukova et al., 1999). Hubbard *et al.* (Hubbard et al., 2001) investigated the nature of interactions between the NADPH and C-terminal aromatic tryptophan residue of FAD-binding domain. They crystalized three mutant rat reductases with deleted or substituted Trp 677. The indol ring of the residue Trp 677 lies in the crystal structure of the rat POR stacked against the isoalloxazine ring of the FAD (Wang et al., 1997) and it is thought to be important for proper electron flux. Superimposition of the mutant and wild-type structures showed significant mobility between flavin domains suggesting conformational changes of POR during catalysis (Hubbard et al., 2001). Further experimental evidence supporting the relevance of domain movements during the catalytic cycle came from the observation that the deletion of several amino acids from the flexible hinge region leads to a dramatic decrease in the rate of interflavin electron fluxion (Hamdane et al., 2009). The abolition of four residues resulted in a marked decrease in the ability of the enzyme to catalyze reduction of cytochrome c or cytochrome P450 2B4 due to a defect in interflavin electron flow. The mutant protein transported electrons from the FMN to the cytochrome P450 at the same rate as wild-type protein, but was not able to transfer them from FAD to FMN (Hamdane et al.,

2009). These results supported the hypotheses of Wang *et al.* (Wang et al., 1997) about the importance of the hinge region in the FMN-binding domain motion. Ellis *et al*. (Ellis et al., 2009) conducted nuclear magnetic resonance (NMR) and small-angle X-ray scattering (SAXS) experiments addressing directly the question of wild-type human POR domain arrangement. The experimental data provided the clear evidence for the existence of a two-state conformational equilibrium of POR in the solution (figure 5).

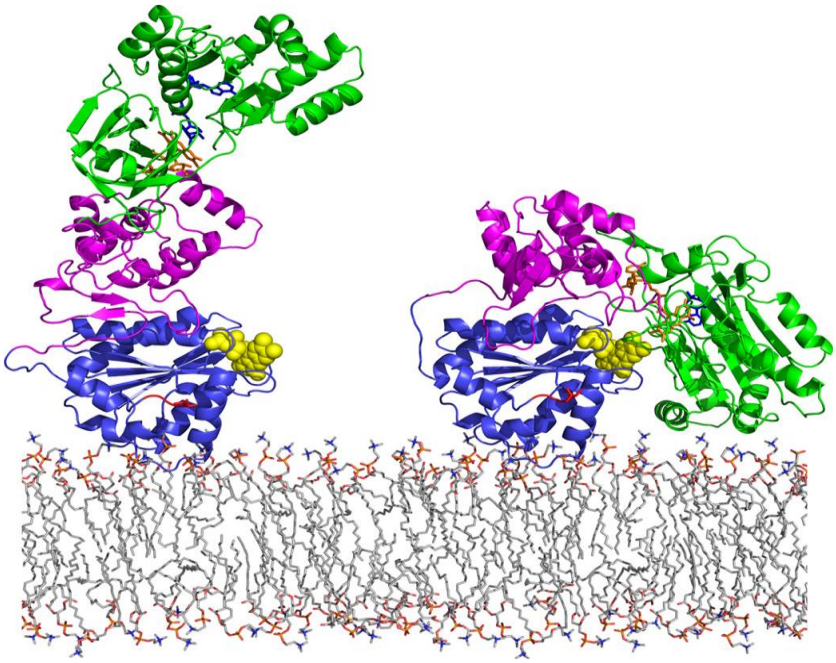


Figure 5. Model of the conformational equilibrium in the POR.
Left, the extended state, adequate for electron transfer from FMN to P450s. Right, the compact state, adequate for electron transfer from FMN to P450s (Ellis et al., 2009).

The SAXS data showed that the enzyme exists in one compact and one more extended (open) conformation. The compact state corresponded to the crystal structure favoring efficient interflavin electron transfer, while the open conformation appeared to be adequate for proper electron transport from FMN to CYPs. The equilibrium between these two states depends on the redox state and NADPH coenzyme binding (Ellis et al., 2009). The open structure found by Ellis *et al.* (Ellis et al., 2009) is not consistent with the open conformation of YH chimeric POR (Aigrain et al., 2009), which probably not even naturally occur. The divergent structures of the YH POR served to reflect the potential of enzyme structural changes during the catalytic cycle.

In contrast to previous study, investigation of the domain arrangement in the human POR by Vincent *et al*. (Vincent et al., 2012) showed that FMN and FAD domains adopt a rigid and closed conformation resembling crystal structure in solution in oxidized state and not in reduced state as referred by Ellis *et al.* (Ellis et al., 2009).

Based on these findings, it is conceivable that rearrangement of the FMN containing domain is required to enable the electron flow from POR to its protein partner and the dynamic nature of POR is now beyond doubt. However, the exact role of the connecting domain is still uncertain because it stays structurally unchanged between the open and closed conformation (Aigrain et al., 2009; Hamdane et al., 2009).

POR protein partners

Cytochrome P450 enzymes

As mentioned before, microsomal cytochrome P450 enzymes (CYPs) are main electron acceptors of the POR. Cytochromes P450 are so-called because of their characteristic spectral shift at 450 nm. The classic catalytic cycle of CYPs is based on activation of atmospheric dioxygen and insertions of one oxygen atom into an organic substrate while the other is reduced to water. In humans, the cytochromes P450 constitute one of the most important families of the proteins involved in the biosynthesis and degradation of a vast number of drugs and foreign lipophilic compounds. The human genome contains 57 genes encoding cytochromes P450. Seven of them comprise a group of mitochondrial enzymes (Type I) and 50 create a group of microsomal CYPs (Type II). Mitochondrial CYPs receive electrons from NADPH through ferredoxin reductase, whereas, microsomal CYPs accept electrons from NADPH through a single protein, NADPH-cytochrome P450 oxidoreductase (Devlin, 2006). Interaction between POR and CYPs is thought to be based on electrostatic forces between a negatively charged surface of the FMN-connecting domain and the positively charged surface of cytochrome P450 enzymes (Zhao et al., 1999). In the human body, CYPs are present in large excess over reductase, with the ratios ranging from 10:1-20:1 (Devlin, 2006).

Mechanism of electron transfer

The electron flow through the POR electron transport assembly is based on the redox properties of the flavin cofactors (Munro et al., 2001). Redox reactions involve the transfer of electrons from one chemical species to another. Direction of the electron transport chain in such reactions depends on the redox potentials of electron donor and acceptor. Electrons move spontaneously from molecules with more negative redox potentials to molecules with more positive reduction potentials. Munro *et al.* (Munro et al., 2001) reported the first determination of redox potentials of the flavin couples in human POR.

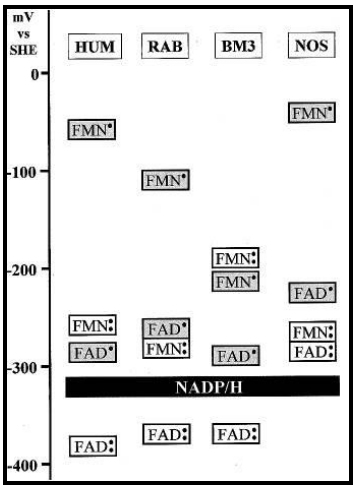


Figure 6. Flavin reduction potentials in various members of the POR family.
HUM – human, RAB – rabbit, BM3 – cytochrome P450 BM3, NOS – nitric oxide synthase. The relative reduction potentials of flavins: semiquinone/hydroquinone couple (white box), oxidized/semiquinone couple (gray box). Dots indicate number of electrons (Munro et al., 2001).

The study showed that the redox properties of human POR are similar to those of membrane bound rat and rabbit PORs and the reductase domain of neuronal nitric oxide synthase (figure 6). However, they were markedly different from those of the yeast POR and from the bacterial cytochrome P450 BM3, which they proposed is due to an important evolutionary difference in electronic regulation of the enzyme. The electron transfer pathway was thus clearly established (Munro et al., 2001).

Cytochrome P450s require the delivery of two electrons for catalytic function. The role of POR is to split this two electrons obtained from NADPH and transfer them

to its acceptor in two one-electron transfer steps. Reductase serves to couple a two-electron donor (NADPH) with one-electron acceptors. In POR, FAD serves as an electron acceptor from NADPH, whereas the FMN moiety interacts with the protein partner. The overall mechanism by which cytochrome P450 catalyzes the monooxygenation of their substrate is shown in figure 7. The catalytic cycle begins with binding of substrate to the resting state of the low spin, hexa-coordinate, ferric enzyme (1) converting it to high-spin, penta-coordinate substrate bound complex. Upon substrate binding, there is a conformational change of the POR structure. This enables the first electron to pass from NADPH to FAD and from a ferrous cytochrome P450-dioxygen complex (2). After that, dioxygen can be bound to substrate (3) followed by a second electron transfer from POR (4). Subsequently, one atom of the molecular oxygen reacts with two protons and two electrons and formats molecule of H_2O (5) while other is incorporated to the substrate (6). Product dissociation completes the cycle (Devlin, 2006).

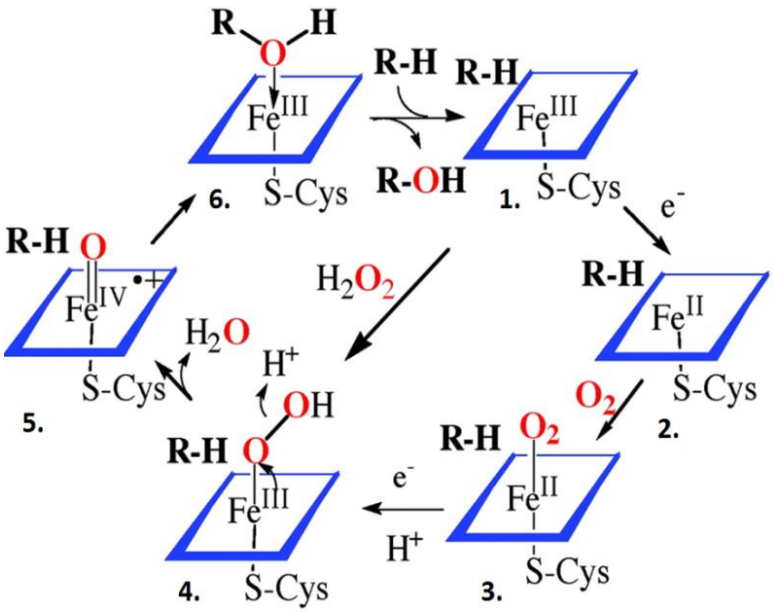


Figure 7. Cytochrome P450 catalytic cycle (Groves, 2003).

The rate of electron transport within the POR molecule and between two molecules can be modulated by different factors. Gutierrez *et al.* (Gutierrez et al., 2003) investigated the role of the NADPH binding to reductase. They demonstrated that occupation of the 2´-phosphate binding site by NADPH leads to conformational changes of the enzyme and so it optimizes electron transfer between the flavin

cofactors. Reduction of the enzyme with NADH led to a slower rate of internal electron flow due to the missing phosphate group in the 2´-phosphate–binding site. The 2´-phosphate group of NADPH is therefore considered critical for optimal electron transfer (Gutierrez et al., 2003). Proper electron flow can be severely affected by disrupted binding of prosthetic groups. It was proved that some *POR* mutations cause diminished affinity of affected proteins for the essential FAD cofactor (Marohnic et al., 2006). In this situation, the important coenzyme-cofactor relationship is impaired such that POR cannot transfer electrons to its acceptors (Marohnic et al., 2006). The role of the POR membrane anchoring has also been investigated. Das *et al.* (Das and Sligar, 2009) demonstrated that the lipid bilayer take part in defining the redox potential of the POR. The redox potential of full length POR was found to be similar to the soluble one (Brenner et al., 2008), but shifted to more positive values when the protein was incorporated in the membrane bilayer as compared to a solubilized state. It seems that membrane lipid change the redox potential of POR to make several processes in the electron transfer more thermodynamically feasible (Brenner et al., 2008). Another interesting observation was that electron transfer rate is diminished by inclusion of glycerol, probably due to altered equilibrium distribution of the different conformational states of POR (Gutierrez et al., 2002).

Heme oxygenase

Heme oxygenase (HO 1) is the POR dependent, stress inducible enzyme that metabolizes heme to biliverdin, carbon monoxide (CO) and ferrous iron (Fe^{2+}). Marohnic *et al*. (Marohnic et al., 2011) first examined the relative affinities among POR mutants and HO 1 and they demonstrated that HO 1 activity is diminished by several naturally occurring *POR* mutations, where the greatest inhibition generated mutants affecting the FAD or FMN-binding. Furthermore, titration of the POR-HO1 complex showed that the best POR: HO ratio for their activity is 1:2, suggesting the formation of HO 1 complexes (Marohnic et al., 2011).

Progesterone receptor membrane component 1

In 2007, another protein occurred to play a role in POR catalytic function. Hughes *et al.* (Hughes et al., 2007) reported on a small microsomally localized hemoprotein progesterone receptor membrane component 1 (PGRMC1) that forms a stable complex with several members of the cytochrome P450 family of enzymes and regulates their activities. Beyond POR and cytochrome b5, PGRMC1 is the third

protein known to interact with microsomal cytochromes P450 (Hughes et al., 2007). A gene for PGMRC1 (*PGRMC1* gene) is localized on X chromosome and it contains three exons. It has been showed that PGRMC1 positively regulates CYPs involved in sterol biosynthesis (Hughes et al., 2007), but it decreases the activities of some drug-metabolizing CYPs (Szczesna-Skorupa and Kemper, 2011), although it is probably isoform dependant (Oda et al., 2011). Moreover, the study showed that PGRMC1 binds also to POR, which may influence CYPs activity too (Szczesna-Skorupa and Kemper, 2011).

POR deficiency syndrome

P450 oxidoreductase deficiency (POR deficiency) is a recently described disorder caused by mutations in the *POR* gene (Miller, 2004). It comprises wide spectrum of clinical manifestations including skeletal malformations referred to as Antley-Bixler syndrome (ABS), disorders of sex development, pubertal failure, adrenal dysfunction, and maternal virilization during pregnancy. Some authors consider POR deficiency as a new form of congenital adrenal hyperplasia (CAH), where distinctive characteristics from classical CAH forms are the presence of craniofacial and skeletal deformities, disorders of sexual development in both sexes and glucocorticoid deficiency (Miller, 2004). Phenotype manifestations of different *POR* mutations vary. Genetic changes in POR modulate activity of its partners, microsomal cytochrome P450s, to different degrees (Sandee et al., 2010), apparently explaining the great variability in the clinical findings of POR deficiency patients. Clinical spectrum of PORD phenotypes ranges from mildly affected individuals with polycystic ovary to severely affected children with ambiguous genitalia and Antley Bixler syndrome (Miller, 2004).

Disordered steroidogenesis

Mutations in POR cause apparent decreased activity of 17-α-hydroxylase (CYP17A1) and 21-hydroxylase (CYP21A2). First mention of a disordered steroidogenesis that resembles combined 17-α-hydroxylase and 21-hydroxylase deficiency (so called mixed oxidase deficiency) was reported already in 1985 (Peterson et al., 1985). In 1986, Miller proposed that the mixed oxidase deficiency described by Peterson *et al.* (Peterson et al., 1985) was caused by mutations in P450 oxidoreductase (Miller, 1986). Nevertheless, this suggestion was not accepted that time and for several years it had seemed unlikely, prompted by two main reasons.

First, it is known, that POR is the obligate electron donor to all microsomal CYPs that participates in the metabolism of most commercially available drugs, but none of the patients with partial combined deficiencies of 17-α-hydroxylase and 21-hydroxylase activities were described as having associated disorders in drug metabolism (Evans and Relling, 1999). Second, functional inactivation of the *Por* gene in mice led to widespread developmental defects and it caused early embryonic lethality (Otto et al., 2003; Shen et al., 2002), therefore *POR* mutations in humans had been unexpected. Regardless, in 2004 three different authors reported genetic changes in *POR* in patients with Antley-Bixler syndrome and disordered steroidogenesis (Adachi et al., 2004a; Arlt et al., 2004; Fluck et al., 2004).

Disordered sex development in the patients with POR deficiency is primarily consistent with a disruption of POR dependent CYP17A1 enzyme activity (17-α-hydoxylase and 17, 20-lyase). Another effected enzymes are CYP21A2 (21-hydroxylase), CYP19A1 (aromatase) and CYP51A1 (lanosterol-14-α-demethylase) (Adachi et al., 2004a; Arlt et al., 2004; Fluck et al., 2004; Pandey et al., 2007). Since multiple enzymes are defected, steroid profiles in patients with POR deficiency are variable. As shown in figure 8, several steps in steroidogenesis pathway might be impaired by depressed activity of these enzymes.

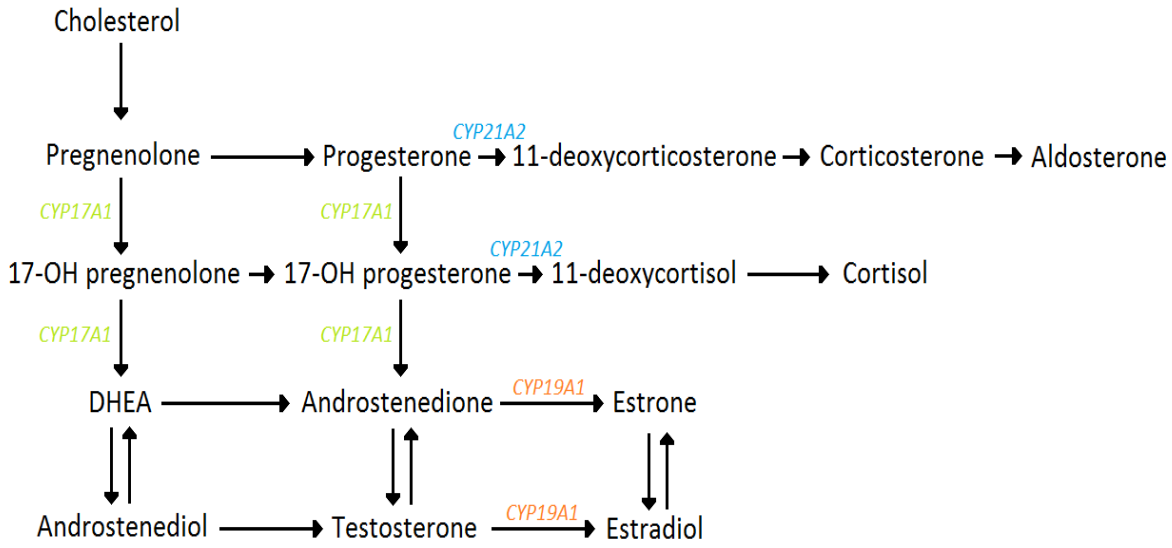


Figure 8. Steroidogenesis [modified according to (Arlt et al., 2004)].

Mutations in POR result in clinical presentation with disordered sex development (DSD) with/or without ambiguous genitalia in both sexes and glucocorticoid deficiency (Arlt et al., 2004; Fluck et al., 2004). Aldosterone deficiency with salt wasting has not yet been described in the cases of POR deficiency. Females can present with virilized genitalia at birth, while males are typically underdeveloped (Fukami et al., 2009; Homma et al., 2006). While the undervirilization of affected males is easily explained by insufficient sex steroid synthesis due to defects in 17, 20-lyase activity (Huang et al., 2008), females' virilization is explained by alternative backdoor pathway of androgen biosynthesis (Arlt et al., 2004; Homma et al., 2006) (figure 9). In this pathway, the conversion of 21 carbon steroid precursors are mediated by 5α-reductase and converted directly to 5α-dihydrotestosterone, bypassing the common precursors androstenedione and testosterone.

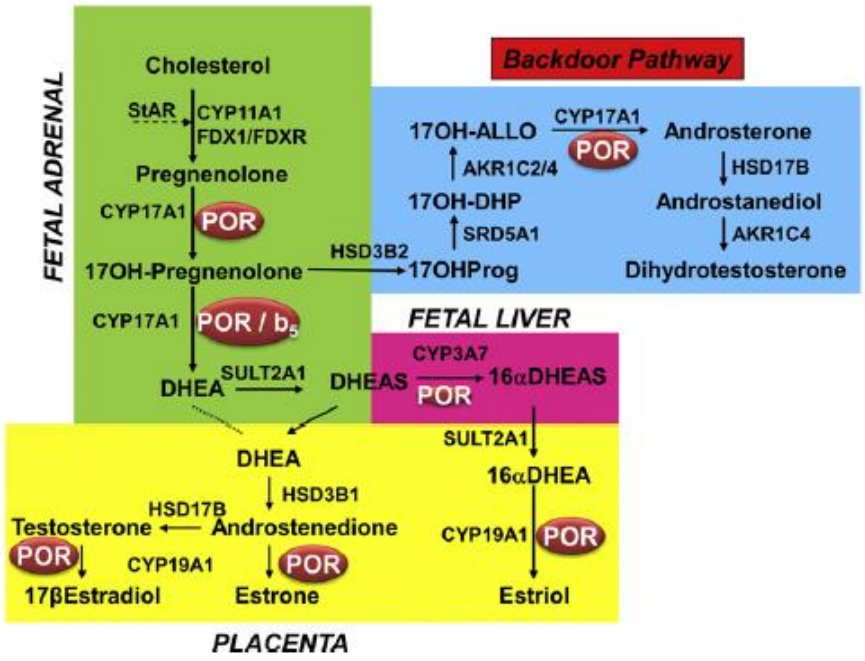


Figure 9. The alternative backdoor pathway of androgen biosynthesis (Pandey and Fluck, 2013).

Interestingly, the virilization may occur also to a mother of effected child (Fluck et al., 2004; Fukami et al., 2009). This outcome is typical for children carrying the mutation p.Arg457His prevalent in Japan (Fukami et al., 2009), but not with the p.Ala287Pro mutation prevalent in Europe (Fluck et al., 2004; Huang et al., 2005). Consistent with this, the p.Ala287Pro mutant supports 100% of aromatase activity,

whereas protein with the mutation p.Arg457His supports only 1% of aromatase activity (Pandey et al., 2007). Therefore, in mothers carrying children with mutation p.Arg457His, diminished activity of aromatase causes accumulation of androgens resulting in virilization.

Antley-Bixler syndrome

As mentioned before, another feature of POR deficiency is the presence of skeletal malformations resembling Antley-Bixler syndrome. Antley-Bixler syndrome (ABS, OMIM 207410) is a multiple congenital anomaly syndrome characterized by numerous craniofacial and skeletal abnormalities (McGlaughlin et al., 2010). The first case of ABS was described in 1975 by Antley and Bixler (Antley and Bixler, 1975). The authors reported a patient with craniosynostosis, radiohumeral synostosis, and femoral bowing. With an increasing number of new ABS cases, it was demonstrated that prominent characteristics of the syndrome include brachycephali, sever mid-face hypoplasia, radiohumeral and radioulnar synostosis, craniosynostosis, femoral bowing and spontaneous long-bone fractures (Hassell and Butler, 1994). These malformations appear in the majority of ABS cases (Hassell and Butler, 1994). Besides the main symptoms, other features may occur. Loss of hearing, choanal atresia/stenosis, frontal bossing, dysplastic ears, "pear shaped nose", proptosis, arachnodactiliaand occasionallycardiac defects were associated with ABS (Hassell and Butler, 1994). Anorectal and urinary anomalies are also occasionally seen in the patients, probably due to decreased activity of CYP26 important for retinoic acid metabolism (Fukami et al., 2010). Many patients suffer early death, which was reported in approximately half of known cases, usually due to respiratory complications (Hassell and Butler, 1994). Intelligence of affected individuals is retarded and prognosis of mental development probably dependent on two main factors, which are craniosynostosis and obstruction of upper airways (Bottero et al., 1997). It seems that early and efficient management of these complications are prerequest for good mental condition of the patient.

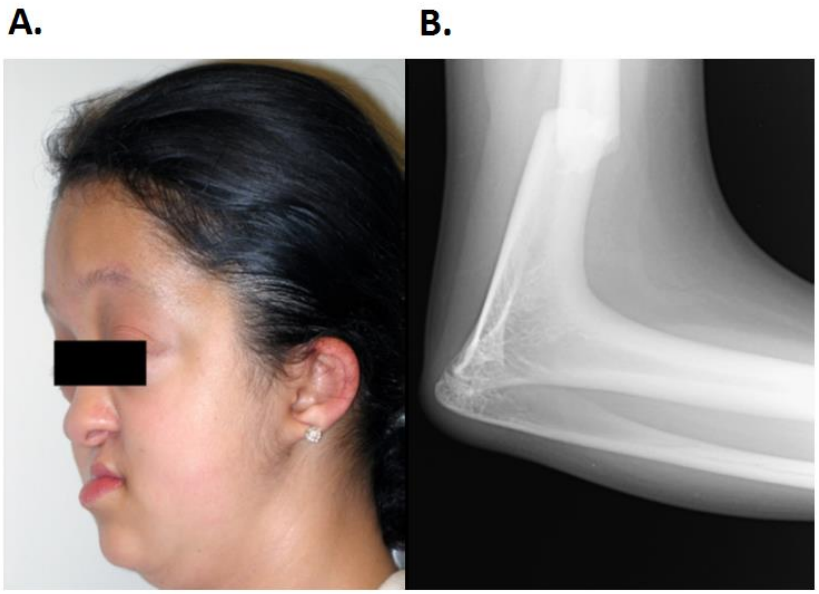


Figure 10. A female with Antley-Bixler syndrome and a radiograph showing radiohumeral synostosis. A. Photograph of 12-year-old female with Antley-Bixler syndrome. Typical low set ears with poorly formed antihelix and pear-shaped nose are presented. B. Right arm radiograph demonstrating radiohumeral synostosis and humeral fracture (McCall et al., 2007).

The etiology of ABS was at the beginning controversial, autosomal recessive as well as digenic inheritance seemed to be possible. Initially, ABS was considered to be a disorder with an autosomal recessive pattern of inheritance. This assumption was based on two cases where children with ABS were born to consanguineous couples (Feigin et al., 1995; Yasui et al., 1983) and three cases of ABS which occurred in siblings (LeHeup et al., 1995; Schinzel et al., 1983; Suzuki et al., 1987). However, all other ABS cases occurred individually, not within a close family of the patient.

In 1998 Chun *et al.* (Chun et al., 1998) reported a child with abnormalities characteristic for ABS. The child carried dominant de novo mutation in the fibroblast growth factor receptor 2 (*FGFR2*) gene. It was proposed that ABS is an autosomal dominant disease.

Further insights into the etiology of ABS were provided by the reports where an ABS-like phenotype occurred in offspring of mothers on high doses of fluconazole in pregnancy (Aleck and Bartley, 1997; Pursley et al., 1996). Fluconazole is an antibiotic used for the treatment and prevention of superficial and systemic fungal infections. In bacteria, it inhibits lanosterol-14-alpha-demethylase (CYP51A1), which

blocks lanosterol demethylation and thus causes depletion of ergosterol in the cell-wall. In mammalian cells CYP51A1 participates in cholesterol synthesis. In further studies, teratogenic effects of fluconazole have not been proved (Jick, 1999), but this discussion and abnormal growth of certain patients´ genitals impel researchers to study steroid metabolism in affected individuals.

Important findings regarding ABS were reported by Reardon *et al.* (Reardon et al., 2000). They described alteration of steroid biosynthesis in 7 of 16 patients with ABS and only one of them carried *FGFR2* mutation. Disruptions of steroidogenesis in examined patients were not so severe and five of the females had ambiguous genitalia. The report on abnormalities in steroidogenesis indicated insufficiency of the enzyme 21-hydroxylase (CYP21A2), but DNA analysis of *CYP21A2* gene did not reveal any mutation (Reardon et al., 2000).

Nevertheless, in 2004, Flück *et al.* (Fluck et al., 2004) described *POR* mutations in three children with Antley-Bixler syndrome and a phenotypically normal adult woman with primary amenorrhea, ovarian cysts and normal pubertal development. All four patients had recessive, loss of function amino acid replacement mutation in *POR.* This report first referred to the fact that cases of ABS are outcomes of two distinct genetic events, concretely, some are caused by mutations in the *FGFR2* gene and some by mutations in *POR* gene (Fluck et al., 2004).

ABS is generally considered as a very rare syndrome, but it is quite difficult to estimate the prevalence of ABS patients. Approximately 100 individuals have been reported since the syndrome was described by Antley & Bixler (Antley and Bixler, 1975); however, both under-reporting and reporting of *FGFR2*-related craniosynostosis as ABS, influence this number.

Investigation of a *POR* gene brought breakthrough in the understanding of molecular pathogenesis of two previously poorly understood conditions, mixed oxidase deficiency and Antley-Bixler syndrome. It revealed that they share the same genetic origin, mutations in the *POR* gene.

Congenital malformations

Majority of POR deficiency patients described up to day have had skeletal deformities (Scott and Miller, 2008), but there are several reports of phenotypically normal adults with infertility (Fluck et al., 2004; Huang et al., 2005). Since skeletal malformations are observed in embryonic lethal *Por* knockout mice (Shen et al.,

2002), it is reasonable to conclude that disruption of POR function may be responsible for skeletal abnormalities in POR deficiency patients. However, the effects of *POR* mutations on the development of skeletal anomalies remain unclear. It has been shown that mouse embryos with disrupted cytochrome CYP26A1 (a POR-dependent enzyme) undergo defects in vasculogenesis and head development (Ribes et al., 2007). CYP26A1 plays a role in retinoic acid (RA) metabolism and it is required to prevent teratological effects that may result from RA signaling. Another study showed that expression of connexin 43, gap junction protein participating in bone formation and hydroxylated by CYP26, is dramatically decreased in *Por* knocked-down mice (Polusani et al., 2011). Conditional deletion of *Por* in osteoprogenitor cells of mice led to abnormal FGFR signaling resulting into a pour skull and long bone development, as well as smaller body size in mice (Panda et al., 2013). The focus is also on cholesterol metabolism. Synthesis of cholesterol relies on two POR-dependent enzymes, squalene monoxygenase and CYP51A1 and isorders involving enzyme defects in cholesterol biosynthesis include skeletal malformation phenotypes (Herman, 2003). It is hypothesized that anomalies may develop due to reduced activity of CYP51A1 (Debeljak et al., 2003) but the main mechanism remains to be determined.

Diagnosis and treatment

Because of a broad range of clinical manifestations, the diagnosis of POR deficiency is not straightforward and it is assumed that there is large number of underdiagnosed patients. The POR deficiency diagnosis may be considered from clinical and biochemical findings, but always requires confirmation by genetic analysis.

Prenatal diagnosis is possible from ultrasound findings, amniotic fluid or urine of a mother (Shackleton et al., 2004). The urine steroid profile of the mother carrying a POR deficiency fetus is characteristic for low estriol, increased epiallopregnanediol disulfate and increased aldosterone (Reisch et al., 2013). Pregnancy complications may include also maternal virilization with an onset in second trimester (Reisch et al., 2013). Patients with POR deficiency can be picked up at neonatal screening or at baseline biochemical work-up for mild to moderately increased serum 17-OH progesterone (Pandey and Fluck, 2013). Patients` baseline serum adrenocrticotropic hormone (ACTH) and cortisol are mostly in the normal range and plasma sex

steroids and precursors are typically low (Pandey and Fluck, 2013). On the contrary, serum progesterone, 11-deoxycorticosterone (DOC), corticosterone, 18-OH corticosterone and 21-deoxycortisol are mostly elevated (Fluck et al., 2004). Basal aldosterone grossly remains within the normal range (Fukami et al., 2009). To assess the complex steroid profile of patients, a panel of 6 diagnostic ratios calculated from specific urine metabolites has been recently suggested for diagnosing POR deficiency by a quantitative gas chromatography/mass spectrometry (Pandey and Fluck, 2013).

The characteristic association of craniofacial deformity and radiohumeral synostosis typical for the ABS can be confirmed at birth (Adolphs et al., 2011). Krone *et al*. (Krone et al., 2012) developed a novel scoring system to standardize malformation assessment and to provide the basis for an improved detection and more accurate assessment of POR deficiency-associated malformations.

The complexity of complications in POR deficiency patients requires an interdisciplinary treatment approach. Bottero *et al.* (Bottero et al., 1997) observed that majority of patients who died in early infancy had choanal obstruction. Therefore, airway management is often a primary concern in individuals with ABS. In cases of undervirilization in males, testosterone injections have been successful in some patients (Fukami et al., 2005). Testosterone replacement has been initiated in males in whom testosterone levels remained relatively low after onset of puberty (Hershkovitz et al., 2008). Virilization in girls does not progress postnatally (Fukami et al., 2005); therefore it does not require any treatment. Insufficient cortisol production, especially during the periods of stress is possible. All patients should therefore receive hydrocortisone treatment to prevent life-threatening adrenal crisis (Idkowiak et al., 2010; Iijima et al., 2009). The potential of using riboflavin therapy to correct POR deficiencies caused by defects in binding flavin cofactors was suggested in some studies (Marohnic et al., 2011). Another potentially important clinical consideration is the effect of *POR* mutations on drug metabolizing CYPs and individually tailored dosing of medications are suggested in POR deficiency patients (Tomalik-Scharte et al., 2010).

POR genetics

The human *POR* gene (gene encoding POR) was identified by Shephard *et al*. and it was localized to 7q11.2 (Shephard et al., 1989). The *POR* gene contains 15

coding exons. In 2007, a first untranslated exon that lies 38.8 kb upstream the first exon, was described (Scott et al., 2007). Thus the human *POR* gene contains 16 exons spanning 73.2 kb.

Mutations in *POR* were for the first time reported in 2004. Miller´s group discovered six allelic variants of the *POR* gene (p.Arg457His, p.Val492Glu, p.Ala287Pro, p.Cys569Tyr, p.Val608Phe and an intron 6 splice variant in a premature stop codon) in four unrelated patients with disordered steroidogenesis (Fluck et al., 2004). Arlt *et al.* (Arlt et al., 2004) identified another *POR* mutation (p.Tyr181Asp) by analysis of the *POR* gene in patients with congenital adrenal hypoplasia. Several new *POR* variants were characterized among Japanese patients. They included frame shift mutations (I444fsX449 and L565fsX574) (Adachi et al., 2004b), a silent mutation (p.5Gly=), a deletion (L612_W620delinsR), and a missense mutation (p.Tyr578Cys) (Fukami et al., 2005). All patients had disordered steroidogenesis. Subsequently, 11 new mutations (p.Ala115Val, p.Tyr142Ala, p.Gln153Arg, p.Met263Val, p.Tyr459His, p.Ala503Val, p.Gly539Arg, p.Leu565Pro, p.Arg616*, p.Val631Ile and p.Phe646del) were discovered in a study in which 32 ABS patients were queried to address the distribution of *POR* and FGFR2 variants (Huang et al., 2005). Homma *et al.* (Homma et al., 2006) described three new *POR* variants (p.Gln201*, A462_S463insIA and p.Glu580Gln) in a large study that included 22 Japanese patients with POR deficiency and 1763 controls. Nine undescribed mutations reported Hart *et al.* (Hart et al., 2007), including three missense mutations p.Lys49Asn, p.Leu420Met and p.Leu577Pro. Another study by Miller´s group investigated *POR* gene in four patients with POR deficiency (Sahakitrungruang et al., 2009). All four were compound heterozygotes for *POR* mutations, including five new mutations: p.Leu577Arg, p.Asn185Lys, deletion and two frameshift mutations (Sahakitrungruang et al., 2009). The novel *POR* frameshift mutation E601fsX12 described Idkowiak*et al*. (Idkowiak et al., 2010) in patient with disordered steroidogenesis. Most recently, a novel 3 amino acid deletion (P399_E401del) identified in two unrelated patients with POR deficiency were reported (Fluck et al., 2011).

More than 40 different *POR* mutations and polymorphisms have been identified to date, including missense mutations, frameshift mutations, deletions and splicing errors. Studies on *POR* gene revealed several conclusions. All patients

described up to date carried at least one more or less functional allele (no patient with two null alleles were described), suggesting that a low level of residual activity may be required for embryonic viability. The most common mutation in the European population is p.Ala287Pro, while in Japanese population it is p.Arg457His (Adachi et al., 2004b; Arlt et al., 2004; Fukami et al., 2005; Huang et al., 2005) and these mutations are by far the most frequent mutations in POR deficiency patients. Fukami *et al.* (Fukami et al., 2009) found p.Arg457His mutation on 42 alleles in 35 Japanese patients and provided evidence for the founder effect of p.Arg457His in Japanese population (Fukami et al., 2009). Mutation p.Ala287Pro was so far described on 13 alleles from 12 subjects described as Caucasian or European, but never in Japanese patients (Adachi et al., 2004b; Arlt et al., 2004; Fukami et al., 2005; Huang et al., 2005). Most of the other *POR* genetic variants were found only once. Mutations have been found in all four POR domains, but majority of them lie in the close proximity to the central electron transfer area (Adachi et al., 2006; Fukami et al., 2005). According to Scott and Miller (Scott and Miller, 2008), 12% of POR deficiency patients have only one identified mutation, suggesting that the second mutation must be situated within the unknown region. The most common polymorphism resulting in the amino acid change is p.Ala503Val (Huang et al., 2008).

It seems that the human *POR* gene is highly polymorphic. Since first genetic variations in *POR* were identified, several comprehensive studies of *POR* gene polymorphisms have been published. Huang *et al*. (Huang et al., 2008) sequenced 5,655 bp of the *POR* gene in 842 healthy unrelated individuals from four ethnic groups. They found 140 SNPs, of which 43 were found in >1% of alleles. Other studies followed and at presence more than 800 *POR* SNPs have been described (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011; Tomkova et al., 2012).

POR promoter

Promoter is a regulatory region of DNA typically located upstream of a gene. Eukaryotic promoters are extremely diverse and are difficult to characterize. The most common promoter element in eukaryotes is TATA box (sequence TATAAA); further elements typically presented in promoter regions are CAAT box or GC rich box. The untranslated first exon of the rat *Por* gene was described in 1994 (O'Leary et al., 1994). It was located 30.5 kb upstream the first coding exon. Similar to many

housekeeping genes, the 5' flanking region of rat *Por* gene possesses neither a TATA nor a CCAAT box, but it contained five GC-rich consensus sequences for the binding of transcription factor Sp1 (O'Leary et al., 1994). To determine the organization of the human *POR* gene, Scott *et al.* (Scott et al., 2007) probed the human genome database with the 56-nucleotide sequence of the rat untranslated first exon and they discovered the same DNA region approximately 38.8 kb upstream the first coding exon. They termed this exon 1U. Identification of the first untranslated exon provided the possibility for examination the transcriptional regulation of the human *POR* gene.

Tee *et al.* (Tee et al., 2011) studied 3193 bp upstream exon 1U in human adrenal and liver cells and located the proximal promoter at -325/-1 bp from the untranslated exon. They identified several transcription factors (TF) essential for the regulation of *POR* gene expression (figure 11). A heterotrimer of two Smad3 and one Smad4 TF molecules binds to the region between -263 and -249. Thyroid hormone receptor β (TRβ), another factor found to be essential for human *POR* gene transcription, binds at -240 and -245. Functional data showed that also and estrogen receptor α (ERα) coupled with the nuclear receptor, but the principal topic regulation is TRβ. Moreover, common polymorphism at -152 (rs72553972) was found to play a role in genetic variation in steroid biosynthesis and drug metabolism (Tee et al., 2011).

Soneda *et al.* (Soneda et al., 2011) studied promoter region in three patients with POR deficiency. All three were compound heterozygotes with a p.Arg457His mutation and one apparently null mutation. They identified two types of cryptic deletions, one involving exon 1U alone and one encompassing exon 1U and also exon 1. By further analysis they showed a critical role of the evolutionally conserved SP1 binding sites just upstream of exon 1U, especially the binding site at the position -26/-17 (figure 11) (Soneda et al., 2011).

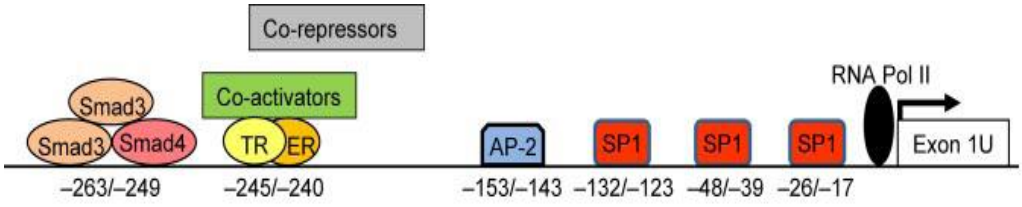


Figure 11. Schematic representation of the proximal *POR* promoter showing the binding sites for various transcription factors (Soneda et al., 2011).

Expression

Consistent with its importance in many metabolic pathways, POR is a widely expressed protein, found at some level in all tissues examined. In rat tissues, POR showed the highest activity in adrenal gland (Benedetto et al., 1976). Lower activity was detected in intestine (89% of adrenal activity), liver (70% of adrenal activity), kidney (47% of adrenal activity), and lung (31% of adrenal activity) (Benedetto et al., 1976). Expression of POR in humans has not been determined so well. Based on immunological staining, the reductase showed the differential distribution of expression within variant tissues (Hall et al., 1989). The intensive staining was observed in different segments of gastrointestinal tract, particularly in liver, small intestine and pancreas. Some staining was seen also in the kidney and lung. On the contrary, the intensity of staining in the stomach and rectum was noticeably lower (Hall et al., 1989).

Cytochrome P450 reductase activity was detected also in human tumor cells. Yu *et al.* (Yu et al., 2001) detected POR in a variety of tumor cell lines, including cells of central nervous system, breast, colon, lung, ovarian, prostate, renal tumors, melanoma and leukemia. The level of POR activity in the affected tissues is generally diminished comparing to normal cells (Forkert et al., 1996). However, latter study shows that specific activity of POR may rise up to 1.8-fold in human liver cancer tissue in comparison to health tissue (Plewka et al., 2000).

Catalytic studies

Almost all known *POR* missense mutations were artificially created by site directed mutagenesis, expressed in E. coli, purified and assayed for activity. Rat and human PORs share approximately 95% amino acid sequence identity, therefore, human *POR* mutations were interpreted using the crystal structure of the soluble rat POR. Initial studies focused predominantly on assays with cytochrome c and steroid metabolizing CYPs (Arlt et al., 2004; Huang et al., 2008; Huang et al., 2005; Pandey et al., 2007). Recently, several studies focusing on drug metabolizing CYPs were reported (Agrawal et al., 2008; Fluck et al., 2010; Hart et al., 2008; Kranendonk et al., 2008; Sandee et al., 2010; Subramanian et al., 2012) and some *in vivo* analysis were conducted (Dobrinas et al., 2012; Oneda et al., 2009; Zhang et al., 2011). All these studies showed that the activity of one POR genetic variant with specific cytochrome P450 cannot predict its ability to interact with another CYP.

Amino acid change p.Ala503Val (POR*28) is a common *POR* genetic variation found in 19.1% of African-Americans, 26.4% of Caucasians, 31% of Mexican-Americans and 36.7% of Chinese-Americans (Huang et al., 2008). Assays conducted with p.Ala503Val variant showed that in general this polymorphism does not alter the protein function dramatically and function modifications differ with the CYP and substrate used in the assay. For example, it retains only 68% of wild type in the 17-α-hydroxylase assay, 58% in the 17, 20-lyase assay and 67% in cytochrome c assay (Huang et al., 2008), but support normal activity with CYP1A2, CYP2C19 (Agrawal et al., 2008) or CYP3A4 (Fluck et al., 2010). Another study showed that p.Ala503Val impaired CYP2D6 activities with two commercially available drugs, dextromethorphan and bufuralol, by 62% and 53%, whereas reducing the metabolism of two different commonly used drugs only to 85% of wild type protein activity (Sandee et al., 2010). Recent studies on p.Ala503Val polymorphism have provided another evidence for CYP dependant variability of POR catalytic function. The p.Ala503Val POR variant showed modest enhancement of enzyme activity with three CYP2C9 isoforms and three different substrates (Subramanian et al., 2012), but was found to have decreased activity to support CYP1A2 while smoking (Dobrinas et al., 2012) .

POR variant p.Ala287Pro is the most common disease-causing mutation in the European population (Huang et al., 2005). The protein with p.Ala287Pro mutation shows normal activity in CYP19A1 assays (Pandey et al., 2007), but has diminished activity in assays based on CYP17A1 (Huang et al., 2005) or CYP3A4 (Fluck et al., 2010) and not detectable activity with CYP1A2 or CYP2C19 (Agrawal et al., 2008). In the assay with CYP2D6 p.Ala287Pro retains only 27% of wild type activity to support the metabolism of dextromethorphan and bufuralol and has not detectable activity with EOMCC (Sandee et al., 2010). By contrast, p.Arg457His, the most frequent mutation in people of Japanese descent, has barely detectable activity in all these analysis (Agrawal et al., 2008; Fluck et al., 2010; Huang et al., 2005; Pandey et al., 2007), except the assay with CYP2D6, in which it has 24-27% of wild type activity. Thus each *POR* variant/cytochrome P450 couple requires separate study.

POR	*Cyt c reduction*	*17OH-ase*	*17, 20 lyase*	*19A1*	*1A2*	*2C19*	*3A4*
WT	100	100	100	100	100	100	100
Ala287Pro	9	40	21	104	ND	ND	26
Arg457His	1	3	ND	1	ND	ND	ND
Ala503Val	67	68	58	NM	85	113	107

Table 1. Wild-type (WT) and mutant POR activities in different assays.

Data depict Vmax/Km as a percent of WT control. Data compiled from Huang *et al. (Huang et al., 2008; Huang et al., 2005)*, Pandey *et al.* (Pandey et al., 2007), Agrawal *et al.* (Agrawal et al., 2008) and Flück *et al.* (Fluck et al., 2010). ND- activity not detectable, NM – activity not measured.

Moreover, Sandee *et al.* (Sandee et al., 2010) showed that the catalytic activity of the POR is not only dependent on the specific CYP but also on the substrate metabolized. Therefore, it is not surprising that with so many molecular interactions required for POR functionality, the various mutations/polymorphisms in the *POR* gene result in loss of function via different mechanisms.

Also cytochrome b5 plays an important role in POR activity, addition of cytochrome b5 to POR CYP1A1 expressed in the eukaryotic system led to a more than 2-fold increase of specific CYP1A1 dependant drug metabolism (Indra et al., 2014).

The differences between the activities of particular mutant proteins and their interactions with different CYPs might be explained by the localization of the affected residue. For example, modeling of human P450 oxidoreductase by *in silico* mutagenesis and molecular dynamic simulation shows that Ala503 is a solvent-exposed residue located in the FAD binding domain (Fluck et al., 2009). The change of alanine to valine is very conservative, thus it does not affect the activity of the protein dramatically. It is, however, not determine how this common polymorphism influences POR structure and crystallographic studies of human p.Ala503Val will be needed to do so. By contrast, in the crystal structure of the rat POR residue Ala287 lies below the FAD-binding region and severely influences interactions with several CYPs (Fluck et al., 2007). Amino acid alanine 287 is located at the start of a loop in POR that is interacting with C-terminus loop of CYP17A1 (Pandey 2007). A change from alanine to proline breaks the loop in POR at the beginning, and as a consequence the interacting loop in CYP17A1 will no longer be in close proximity with POR (Pandey et al., 2007). In contrast, interaction of POR with CYP19A1 does

not seem to involve residue Ala287, therefore the activity of the CYP19A1 is not affected by the p.Ala287Pro substitution (Pandey et al., 2007). Residue Arg457 is located in a highly conserved FAD-binding domain and it stabilizes the pyrophosphate of FAD by hydrogen bond (Wang et al., 1997). Substitution of histidine for arginine at position 457 disrupts the binding of FAD cofactor and activities of all investigated CYPs are therefore dramatically decreased.

POR and pharmacogenetics

Pharmacogenetics is the study of the genetic factors that influence drug metabolism and toxicity. Microsomal cytochrome P450s of families 1, 2 and 3 catalyze biosynthesis of up to 80-90% of commonly used drugs (Evans and Relling, 1999; Rendic and Guengerich, 2014). Since POR is the exclusive electron donor to all microsomal CYPs, it is reasonable to assume, that disruption of electron flow in the POR would have adverse effect on drugs oxidation. In animal model, inactivation of hepatic POR resulted in essentially complete ablation of hepatic microsomal P450 activity (Henderson et al., 2003). Although POR null mice were viable and healthy, they had a severely compromised ability to metabolize the narcotic drug pentobarbital or the analgesic acetaminophen, indicating the major role of the hepatic CYP enzymes in the pharmacology and toxicology (Henderson et al., 2003).

Several *in vitro* studies addressed the question of the importance of *POR* genetic variants in the drug metabolism (Agrawal et al., 2008; Hart et al., 2008; Sandee et al., 2010). Hart *et al.* (Hart et al., 2008) genotyped *POR* gene in a set of 99 human liver samples. He identified several known and novel polymorphisms which, in biochemical assays, decreased POR activity and drug metabolism, but were not associated with POR deficiency disease. Agrawal *et al.* (Agrawal et al., 2008) examined the effects of already described *POR* sequence variants on two drug-metabolizing CYP enzymes, CYP1A2 and CYP2C19, and they also came to the conclusion that genetic changes in POR protein can affect the ability of different CYPs to metabolize drugs. Another *in vitro* study assessed the capacity of known POR variants to support the activities of human CYP2D6 with few different substrates (Sandee et al., 2010). They found out that the POR protein activity depends not only on CYP assayed but also on the substrate metabolized and p.Ala503Val *POR* common polymorphism showed moderately impaired activity indicating its possible role in drug oxidation (Sandee et al., 2010). Recently conducted studies addressing

POR pharmacogenetics revealed that p.Ala503Val variant does not affect pharmacokinetics of an immunosuppressant used to prevent rejection in organ transplantation (sirolimus) (Woillard et al., 2013) and that p.Ala503Val and p.Gln153Arg variants increase their activities with three drug metabolizing CYP2C9 isofroms and three different substrates (diclofenac, flurbiprofen, and tolbutamide) (Subramanian et al., 2012).

A functional impact of sequence changes in *POR* on hepatic drug metabolism has been investigated also in some *in vivo* studies (Gomes et al., 2009; Oneda et al., 2009; Tomalik-Scharte et al., 2010). First such study was performed in human liver microsomes obtained from general population, where genetic and nongenetic *POR* variability and its impact on drug oxidation were investigated (Gomes et al., 2009). According to the authors, investigated common SNPs did not have a pronounced influence on POR function, thus neither on drug metabolism and commonly found *POR* variant p.Ala503Val was not associated with any activity or expression changes (Gomes et al., 2009). Different results were obtained by Oneda's group while examining the genetic bases that contribute to the variation of CYP3A activity (Oneda et al., 2009). They genotyped 251 individuals for different CYP3A variants and p.Ala503Val *POR* polymorphism and phenotyped them for CYP3A activity. Results showed that *POR* polymorphism p.Ala503Val seems to be a better genetic marker of the variability of total CYP3A activity *in vivo* than all other CYP3A genetic variants (Oneda et al., 2009). Finally, recent study investigating an adult patient with POR deficiency provided clear *in vivo* evidence for an important role of POR in regulating drug metabolism and detoxification *(Tomalik-Scharte et al., 2010).*

It is obvious now that POR represent an important factors influencing drug metabolism, but further studies on functional characterization of the *POR* genetic variants and their impact on the protein are needed so as it can be used for therapeutic purposes.

AIMS OF THE STUDY

General aim of the present work was to investigate gene *POR* in selected patients and in Czech and Jewish general population and to study the impact of described polymorphisms on POR protein.

Specific aims of the study:

1. To establish a molecular analysis of a *POR* gene in our laboratory and to provide a possibility of molecular genetic diagnostic for patients suspected to have POR deficiency

2. To look for genetic variations in *POR* gene in Czech and Jewish general population and to establish *POR* allele frequencies in these populations

3. To suggest *POR* genetic variants that could play a role as a bio-markers in pharmacogenetics

4. To investigate *POR* gene in patients suspected to have POR deficiency

5. To address the pathology of found mutations

6. To establish the molecular analysis of a *PGRCM1* gene in our laboratory and to study *PGRMC1* gene in selected patients

METHODS

Ethics

The present study was carried out in accordance with 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, in accordance with guidelines of participating institutions (First Faculty of Medicine, Charles University at Prague and The National Laboratory for Genetics of Israeli Populations at Tel Aviv University), was obtained from all participated adults and from parents or legal guardians of underage individuals.

DNA Samples

Patients

Ten patients (7 males and 3 females) with skeletal deformities were investigated for the mutations in the *POR* gene.

Healthy controls

The study enrolled a total of 886 healthy controls, including 564 individuals from Jewish population and 322 individuals from Czech population. DNA samples from Jewish population were obtained from the collection of The National Laboratory for Genetics of Israeli Populations at Tel Aviv University (Tel Aviv, Israel). All samples in the study group are from healthy unrelated adult donors who have self-identified their ethnicity as Ashkenazi (AJ, 35 males and 130 females), Bulgarian (BJ, 59), Ethiopian (EJ, 19), Moroccan (MJ, 57 males and 79 females), Turkey (TJ, 68) and Yemenite Jews (YJ, 117). In BJ, EJ, TJ and YJ groups a gender was not provided. DNA samples from Czech population (CZ, 144 males and 178 females) were obtained from both adults and children. 227 DNA samples of adults were acquired during the longitudinal collection of control samples of healthy individuals from Czech population in our laboratory. DNA of 95 healthy neonates was extracted from cord blood.

DNA extraction

Genomic DNA was extracted from peripheral blood sample anticoagulated with EDTA according to a standard protocol. Alternatively, when only a small amount

of blood (1-2 ml) was available, QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) was used according to manufacturer´s protocol. Cord blood samples were collected from the placental part of the umbilical cord immediately after the delivery of the child. Neonates were born at the gestational age between 25-41 weeks of gestation and all pregnancies were uneventful till birth. Cord blood DNA was extracted according to the previously described techniques (Pejznochova et al., 2008). Concentration of gDNA was evaluated using spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA).

Ad 1 and 5) Molecular genetic methods

For *POR* gene analysis, two approaches were used. Sequencing and a new generation amplicon high-resolution melting (HRM) analysis were implemented. The HRM method was optimized only for the *POR* exons 1, 2, 5, and 6. The rest of the *POR* exons harbor a lot of intronic and exonic SNPs and it is difficult to analyze them by HRM method. Therefore *POR* exons 1U, 3, 4, and 7 to 15 were sequenced directly with a forward primer. Identified DNA variations were confirmed by independent sequencing of the PCR product from both directions. Using the HRM method, amplicons that resulted in an altered melting curve compared to controls were subjected to direct sequencing from both directions.

PGRMC1 gene was analyzed by direct sequencing.

Polymerase chain reaction

POR gene

Specific primers in intronic regions flanking 16 exons were designed using GeneBank reference sequence NG_008930.1 to avoid the known polymorphisms and minimize undesirable base pairing interactions. Exon pairs 8 and 9, 12 and 13, 14 and 15 were amplified as single PCR products, named E89, E1213 and E1415, respectively. At the beginning, we analyzed 15 coding exons in all samples. Afterward, we decided to sequence also first untranslated exon 1U. Initially, we sequenced only the region of exon 1U and its intron/exon boundaries (with primers E1U). Later on, we extended the analyzed sequence for the promoter region harboring three SP1 binding sites showed to be critical for *POR* transcription (Soneda et al., 2011) and we called the used primers E1UPP. All 14 sets of primers and the sizes of the specific PCR products are listed in table 2.

Table 2. List of primers and corresponding length of PCR products for *POR* gene.

Fragment	*Primer F (5'→3')*	*Primer R (5'→3')*	*Length of the PCR product*
E1U	cgtaccaagagcgcaaat	gagataccgagccctaacc	493
E1UPP	cgaaggaggaggctagaccg	aagctgtggaaaagtcgaccc	615
E1	accctctgctgacatctgct	caccccaaaatgctacaagg	347
E2	ctgtaggggaaatgggaagg	acatcctctatgcggtgacg	385
E3	agaagggactcaaagccaggaa	aaggcaacttccgaggacg	396
E4	tcccacgacactcagacatcc	attctcgtagtgctggggtctg	453
E5	gcccagtgttccttgcagtg	ctctgtgttggaggtgcgtgt	407
E6	ccttcctgatgctctgggttt	gtggcagagtgagtccttggct	407
E7	gctcccctgcttcttgtcgt	ctcagtacaaactgggcgagtg	428
E89	gagattccctgtgctttgtgc	cctaagcagaagctcaaccca	524
E10	gccttgtttccagcaccag	tcctaagagacacgggggtga	415
E11	cgcaagatggcctcctcct	ccttgcactctgcctgctgt	354
E1213	tgcagaacgggacttggg	agcctcatgcccaccttcgt	605
E1415	gagcagtcccacaaggtgaga	gccaaacacacccaggagactac	515

The PCR amplifications were optimized and carried out in a total volume of 12.5 µl according to following protocols (tables 3 and 4).

Table 3. PCR mix (all fragments) for *POR* gene.

Chemicals	*Stock concentration*	*Final concentration in 1 reaction*
[a] PP mix	2x	1x
[b] 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

[a] Top Bio, Prague, Czech Republic; [b] Sigma-Aldrich, St. Louis, MO, USA,*4% for fragments 4, 5, 6, 7, 89, 1213 and 1415 and 8% for fragments 1U, 1UPP, 1, 2, 3, 10 and 11

Table 4. PCR conditions for *POR* gene.

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	30´´
3.	annealing	*	30´´
4.	extension	72°C	40´´
5.	32 times repeating steps 2 to 4		
6.	final extension	72°C	5´

* 55°C for fragments 1 and 2, 58°C for fragment 1U, 64°C for fragments 3 to 15 and 65°C for fragment 1UP

PCR reactions were performed using DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Waltham, MA, USA).

PGRMC1 gene

The 3 coding exons of the *PGMRC1* gene, along with 50 bp of their flanking regions, were amplified using specific primers (table 5) designed according to the GeneBank reference sequence NG_016756.1.

Table 5. List of primers and corresponding length of PCR products for *PGRMC1* gene.

Fragment	*Primer F (5'→3')*	*Primer R (5'→3')*	Length of the PCR product
E1	ggaggagaaagtggcgagtt	ctacccgcctaccctagctc	527
E2	agggaagccattttctgtgt	caaaatgcacccaagtttcag	341
E3	ggcaaggacggtggtataaa	tgacacacaaacacatcatgc	451

The PCR amplifications were performed on DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories) in a total volume of 12.5 µl according to following protocols (tables 6 and 7).

Table 6. PCR mix (all fragments) for *PGRMC1* gene.

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

Table 7. PCR conditions for *PGRMC1* gene.

	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 times repeating steps 2 to 4		
6.	final extension	72°C	5´

Long Range PCR

To identify, whether two mutations (p.Val164Met and p.Glu398Ala) found in the one DNA samples are localized on the one allele or two different alleles, we needed to amplify a fragment containing both mutations spanning from exon 4 to exon 10. To do so, we used Expand Long Template (LT) PCR system (Roche, Mannheim, Germany). Primers E4F and E10R (table 2) were used to amplify a single 4900 bp PCR product. PCR reaction was carried out in a total volume 50 µl according to following protocols (tables 8 and 9).

Table 8. PCR mix for Long Range PCR.

Chemicals	*Stock concentration*	*Final concentration in 1 reaction*
[a]**Expand LTbuffer 1**	10x	1x
[b] **dATP**	10 mM	0.35 mM
[c] **dCTP**	10 mM	0.35 mM
[d] **dGTP**	10 mM	0.35 mM
[e] **dTTP**	10 mM	0.35 mM
[f] **DMSO**	100%	4%
Forward primer E4F	10 pmol/µl	0.3 mM
Reverse primer E10R	10 pmol/µl	0.3 mM
[g] **Expand LTEnzyme Mix**	5 U/ µl	4 U
gDNA	50-100 ng/µl	500 ng

[a, g] Roche; [b, c, d, e, f] Sigma-Aldrich

Table 9. PCR conditions for Long Range PCR.

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	10´´
3.	annealing	60°C	30´´
4.	extension	68°C	4´
5.	10 times repeating steps 2 to 4		
6.	denaturation	94°C	15´´
7.	annealing	60°C	30´´
8.	extension	68°C	*4´
9.	20 times repeating steps 6 to 8		
10.	final extension	68°C	7´

*+20 s cycle elongation for each successive cycle

Purification of PCR products

Post-PCR clean-up for sequencing was performed by ExoSap cleanup method, where 0.5 U of shrimp alkaline phosphatase (*SAP*) and 10 U of exonuclease I (*ExoI*) (both from Fermentas, Burlington, Canada) were mixed with 2 µl of the PCR product, adjusted with PCR-grade water to 10 µl and incubated for 15 min at 37°C followed by 20 min incubation at 80°C.

DNA sequencing

The first part of samples (AJ and MJ group) was sequenced on the automatic sequencer ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in a commercial laboratory of UT Health Science Center San Antonio (Texas). Later, an automatic sequencer ABI PRISM 3100-Avant Genetic analyzer (Applied Biosystems) became available in the laboratory and sequencing process was optimized for the new instrument. The Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,) was used for the PCR sequencing reaction according to manufacturer´s protocol. 3.2 mM primers and 20-30 ng of purified PCR product were used for sequencing PCR reaction.

Table 10. PCR conditions for sequencing.

	Step	***Temperature***	***Time***
1.	initial denaturation	95°C	5´
2.	denaturation	95°C	5´´
3.	annealing	50°C	25´´
4.	extension	68°C	50´´
5.	35 times repeating steps 2 to 4		

The products of the sequencing PCR were cleaned using the common ethanol precipitation protocol. Obtained chromatograms were analyzed manually using the SeqScape Software v2.5 (Applied Biosystems) or Chromas Pro v1.5 (Technelysium, Tewantin, Australia).

HRM analysis

PCR reactions for exons 1, 2, 5 and 6 were performed using the same primers as for standard PCR (table 2) with addition of the DNA saturating fluorescent dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA). The PCR amplifications were optimized and carried out in a total volume of 10 µl. All PCR reactions were

performed in duplicate in a 96-well plate using a DNA Engine Dyad Cycler (Bio-Rad Laboratories). The reaction mix was overlaid with 15 µl of mineral oil (Sigma-Aldrich). Chemicals and PCR conditions are summarized in tables11 to 14.

Table 11. PCR mix (E1 and E2) for HRM analysis.

Chemicals	*Stock concentration*	*Final concentration in 1 reaction*
[a] **Thermo-Start reaction buffer**	10x	1x
[b] **dNTP**	2 mM	0.2 mM
[c] **$MgCl_2$**	25 mM	1.175 mM
[d] **DMSO**	100%	4%
Forward primer	10 pmol/µl	0.4 mM
Reverse primer	10 pmol/µl	0.4 mM
[e] **LC Green Plus**	10x	1x
[f] **Thermo –Startpolymerase**	5 U/ µl	1 U
gDNA	50-100 ng/µl	50-100 ng

[a, f]ABgene House, Epson, UK; [b]Deoxynucleotide Mix, Sigma-Aldrich; [c]Sigma-Aldrich; [d] Top Bio; [e]Idaho Technology

Table 12. PCR mix (E5 and E6) for HRM analysis.

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
[c] **LC Green Plus**	10x	1x
gDNA	50-100 ng/µl	50-100 ng

[a]Top Bio; [b] Sigma-Aldrich; [c] Idaho Technology

Table 13. PCR conditions (E1 and E2) for HRM analysis.

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	15´
2.	denaturation	94°C	30´´
3.	annealing	58°C	30´´
4.	extension	72°C	40´´
5.	40 times repeating steps 2 to 4		
6.	final extension	72°C	5´

Table 14. PCR conditions (E5 and E6) for HRM analysis.

	Step	*Temperature*	*Time*
1.	initial denaturation	94°C	2´
2.	denaturation	94°C	30´´
3.	annealing	62.5°C	30´´
4.	extension	72°C	40´´
5.	40 times repeating steps 2 to 4		
6.	final extension	72°C	5´

After cycling, the PCR products were incubated for an additional 30 s at 94°C followed by a rapid cooling down to 20°C and centrifuged briefly at 2000 g. Melting acquisition was performed on the Light Scanner (Idaho Technology), in which DNA samples were melted by gradually increasing the temperature from 55°C to 98°C at a rate of 0.1 °C/s. Upon completion of the run, data were analyzed using the LightScanner Software version 1.5 (Idaho Technology). The melting curves were normalized and the temperatures shifted (80 - 90°C for the lower temperature range and 85 - 95°C for the upper temperature range) to allow samples to be directly compared. Samples were then automatically clustered into groups and the melting curves and the plots were inspected. The sensitivity level was set on 1. Differences were considered as significant if the replicates fell outside the range of the variations seen in the wild type samples.

Cloning

To identify allelic localization of two mutations, used molecular cloning techniques were implored. After long range PCR, the fragment with mutations was ligated into the pCR4-TOPO vector from the TOPO TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA, USA) and then transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Cloning, transformation, and plasmid amplification were performed according to manufacturer´s protocol. Amplified plasmids from ten different colonies were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using the universal T7 and T3 primers included in the TOPO TA Cloning Kit for Sequencing.

Site directed mutagenesis

Vector for mutagenesis and further expression of the full-length POR were kindly provided by prof. Bettie Sue Masters from UT Health Science Centrum at San Antonio (Texas). Vector pOR263 (Shen 1989-supplement) was used to construct the plasmid pPORh for expression of membrane-bound human POR. The exact technique of construction of bacterial expression plasmid encoding human POR was described previously (Marohnic et al., 2006). Site-directed mutagenesis was performed to generate desired mutations using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol with the mutagenic primers listed in table 15. Complementary mutagenic oligonucleotide primers were designed using the web-based Primer Generator

(*http://www.stratagene.com/qcprimerdesign*). Mutated inserts were subsequently submitted to sequencing to confirm the result of mutagenesis.

Table 15. List of primers for site directed mutagenesis.

SNP (p.)	*Primer F (5'→3')*	*Primer R (5'→3')*
Thr29Ser	aagtatctcttttcagcatgtcggacatgattctgttttcg	cgaaaacagaatcatgtccgacatgctgaaaagagatactt
Thr64Ile	ccaaaattcagacattgatctcctctgtcagagagag	ctctctctgacagaggagatcaatgtctgaattttgg
Val85Met	gggaggaacatcatcatgttctacggctccc	gggagccgtagaacatgatgatgttcctccc
Ser102Pro	gccaaccgcctgcccaaggacgccc	gggcgtccttgggcabbcggttggc
Val164Met	ctgcaggagacagacatggatctctctgggg	ccccagagagatccatgtctgtctcctgcag
Val191Met	ccatgggcaagtacatggacaagcggctg	cagccgcttgtccatgtacttgcccatgg
Asp344Asn	aaatcctgggtgccaacctggacgtcgtc	gacgacgtccaggttggcacccaggattt
Arg371His	cctacgtcctaccacacggccctcacc	ggtgagggccgtgtggtaggacgtagg
Pro384Leu	catcaccaacccgctgcgtaccaacgtgc	gcacgttggtacgcagcgggttggtgatg
Glu398Ala	gtacgcctcggcgccctcggagc	gctccgagggcgccgaggcgtac
Val472Leu	caaggtccacccccaactctctgcacatctg	cagatgtgcagagagttggggtggaccttg
Thr529Met	cccttcaaggccaccatgcctgtcatcatg	catgatgacaggcatggtggccttgaaggg
Arg550Gln	tcatccaggagcaggcctggctgcg	cgcagccaggcctgctcctggatga
Arg570His	tactacggctgccaccgctcggatgag	ctcatccgagcggtggcagccgtagta
Asp648Asn	gcagaacaccttctacaacatcgtggctgagct	agctcagccacgatgttgtagaaggtgttctgc

Proteomics

Protein expression

Wild type POR protein and two POR variants (p.Val164Met and p.Pro384Leu) were expressed in *E. coli* strain BL21 (Stratagene). Cells were cultured overnight at 37°C with 210 rpm shaking in Terrific Broth (TB) media containing 125 μM ampicillin (Sigma-Aldrich). Afterwards, cells were induced by addition of 0.4 isopropyl 1-thio-β-D-galactopyranoside (Fisher HC, Houston, TX, USA) followed by further overnight incubation in TB media containing 100 μM riboflavin and 125 μM ampicillin at 28°C with 160 rpm shaking. Cells were harvested by centrifugation at 4°C for 15 minutes at 6000 g.

Protein purification

Cell pellets were resuspended in buffer 1 [50mM Tris-HCl; 0.5 mM EDTA, 10% glycerol and 1 mM DTT; pH 8] containing the protease inhibitors [100 mM phenylmethysulfonil fluoride; 0.1 mM aprotinin; 1 mM pepstatin and 1 mM leupeptin

(all by Sigma-Aldrich)]. To obtain the protein, cells had to be disrupted by adding lysozyme (Sigma-Aldrich) at concentration 20 µg/ml (incubated 30 minutes on ice) and by sonification of such treated cells, five times for two minutes. Soluble and solid fractions were then separated by ultracentrifugation (Beckman J25, Brea, CA, USA) for 1 h at 100 000 x g. Pellets acquired after centrifugation were homogenized using dounce homogenizer in buffer 2 [50 mM Tris-HCl; 0.1 mM EDTA; 10% glycerol, 0.05 mM DTT; pH 7.7]. For protein solubilization, 0.1% Triton X-100 (MP Biomedicals, Cleveland, OH, USA) was added to the homogenate and the homogenate was then incubated at 4°C for 12 h. The detergent-solubilized protein fraction was cleared from cellular debris by ultracentrifugation for 1 h at 100 000 x g and the supernatant was applied to 2', 5'- ADP – Sepharose 4B (GE Healthcare). The column was washed with buffer 2 and then eluted with the same buffer supplemented with 5 mM 2', 3'-AMP mixed isomers (Sigma-Aldrich). All purification steps were carried out at 4°C. Protein purity was determined by SDS-polyacrylamide gel electrophoresis (PAGE). Fractions exhibiting a single band were pooled and concentrated (Centriprep YM-30, Millipore Corporation, Billerica, MA, USA). Proteins were quantified by Micro BSA assay (Pierce, Rockford, IL, USA) according to the standard protocol and protein aliquots were stored under liquid nitrogen.

Biochemical methods

Cytochrome c assay

The ability of p.Val164Met and p.Pro384Leu POR variants to reduce cytochrome c was measured by determining the absorption rate at 550 nm using the extinction coefficient, $\varepsilon = 21\ mM^{-1}cm^{-1}$ (Massey, 1959). All measurements were carried out in triplicate in 96-well plate using VERSA maxmicroplate reader (Molecular Devices, Sunnyvale, CA, USA). The reaction was performed in 50 mM KPi buffer [50 mM Tris-HCl, 100 mM NaCl, 50 mM K_2HPO_4; pH 7.4]. The cytochrome c concentration was 80 µM when enzyme was the variable substrate ranging from 5 nM to 20 nM. NADPH concentration varied within a range from 0.1 to 200 µM. In order to maintain constant NADPH concentration, a NADPH-regenerating system was used, consisting of 10 mM isocitrate, 0.5 units of isocitrate dehydrogenase, and 5 mM MgCl2 (Sigma-Aldrich). Reactions were monitored over 5 minutes and rates were extrapolated from the linear range of the kinetic traces. Plots of rate versus NADPH concentration were fitted to the Michaelis-Menten equation.

Flavin content analysis

Flavin separation and quantification was performed by high-performance liquid chromatography (HPLC). Samples were diluted in water (10 µM final concetration) and denatured by boiling for 5 min in order to release protein-bound flavins. Afterward, samples were chilled on ice and aggregated proteins were harvested by centrifugation at 14.000 x g for 10 min. Diluted flavins were separated by HPLC using a Waters Corporation (Milford, MA, USA) analytical HPLC system, equipped with a 2487 absorbance detector. 20 µl of sample supernatant were loaded to a Nova-Pak C_{18}, 60-Å, 4-µm (3.9 x 150mm) column, fitted to a guard column packed with Nova-Pak C18 Guard-Pak inserts. For the column equilibration, the mobile phase buffer [10mM $(NH_4)_2HPO_4$, 12% acetonitrile, pH 5.5] was used. The solvent flow rate was 1.00 ml/min. Eluent peaks were monitored at 473nm with ϵ_{FAD}= 10.1 $mM^{-1}cm^{-1}$ and ϵ_{FMN} = 8.0 $mM^{-1}cm^{-1}$. Standards contained in the mobile phase buffer were measured in order to estimate extinction coefficients. Integration and analysis was determined using Waters Millennium 32 Chromatography Manager.

Statistical methods

Statistical analysis was performed by using software STATISTICA 10 (StatSoft, Prague, Czech Republic) and Pearson's chi-squared test.

RESULTS

Ad 2) Genetic variations in the *POR* gene in general population

To identify POR amino acid sequence variations in common population, we have analyzed the 15 protein-coding exons and approximately 20 bp of both their flanking intron regions in 886 individuals from Czech (322) and Jewish (564) population. With the view of identifying potential regulatory variants, we sequenced also exon 1U. Initially, we analyzed exon 1U and its intron/exon boundaries (AJ and MJ groups) without promoter regions. Later on, we have extended the analyzed sequence for the promoter region harboring three SP1 binding sites showed to be critical for *POR* transcription (Soneda et al., 2011). We sequenced extended exon U1 in Czech population (CZ). Analysis has not covered whole proximal promoter, but since previous reports did not reveal any important SNPs in proximal promoter in the common population (Huang et al., 2008; Tee et al., 2011), we did not consider sequencing of the whole proximal promoter as a pivotal issue.

Czech population

In the Czech population, a total of 25 *POR* SNP genetic variations (table 17) were identified. Four of them were found in 5´flanking region of the gene, 7 in introns and 14 in the protein coding regions (exons). From the 14 variations found in exons, 7 were synonymous, 6 were missense SNPs and one substitution was localized in the first untranslated exon not coding for any amino acid. Of the 25 found variants, 7 were new SNPs, not described previously, including two SNPs in the 5´flanking region (g.4965 C>T and g.4994 G>T), one intronic variant (c.1899 -20C>T), one synonymous SNP (p. 20Ala=) and three nonsynonymous SNPs (p.Thr29Ser, p.Pro384Leu and p.Thr529Met). All 7 SNPs were found as individual heterozygotes at allele frequencies 0. 002. Described SNPs are summarized in table 17.

Common variants: Nine of the 25 identified genetic variants had allele frequencies greater than 10% (rs72553972, rs3823884, rs1135612, rs4732516, rs2286822, rs2286823, rs2228104, rs1057868, rs1057870) and are considered as common *POR* SNPs. The most common amino acid changing SNP, rs1057868 (p.Ala503Val), was present at the allele frequency of 26.9%. We did not see any significant difference in the p.Ala503Val allele frequency described in Czech cohort and p.Ala503Val allele frequencies observed in different population in previous

studies. Similarly, no significant differences were observed in allele frequencies of other common SNPs. Comparing male and female group, no statistically significant differences ($p>0.05$) in allele frequencies of the common SNPs were found (table 16). The only considerable deviation in allele distribution between man and woman group was observed in uncommon polymorphism rs72557941 ($p<0.05$).

Uncommon variants: All but one uncommon variants occurred with the frequency lower than 1%. We reported relatively high frequency of rs72557941 (more than 1%). In previous studies only Gomes *et al.*(Gomes et al., 2009) reported this SNP and with much lower frequency (0.007). We have not detected any rs10262966, which has been described in Caucasian population in all previous studies, with allele frequencies varied from 0.007 to 0.045 (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008).

Table 16. Comparison of *POR* sequence variants in Czech males and females.

Exon	c.	p.	SNP ID	F	M	P value
5´-Flanking	4849 C>A		rs72553972	0,197	0,149	0,1165
5´-Flanking	4883 C>T		rs139824475	0,008	0,000	0,1184
5´-Flanking	4965 C>T			0,003	0,000	0,3680
5´-Flanking	4994 G>T			0,000	0,003	0,2659
Exon 1U	5036 A>C	-47 A>C	rs3823884	0,264	0,226	0,2618
Intron 1U	5099 C>T	-5 +21 C>T	rs72553978	0,003	0,014	0,1112
Exon 1	60 C>T	Ala20=		0,000	0,003	0,2659
Exon 1	85 A>T	Thr29Ser		0,003	0	0,3680
Exon 4	387 A>G	Pro129=	rs1135612	0,284	0,285	0,9774
Intron 4	517 -4 G>A		rs41299496	0,003	0	0,3680
Exon 9	984 C>T	Ala328=	rs72557941	0,003	0,021	0,0283
Intron 9	1067 -13C>G		rs4732516	0,989	0,979	0,3274
Exon 10	1112 G/A	Arg371His		0	0,003	0,2659
Exon 10	1151 C>T	Pro384Leu		0,003	0	0,3680
Exon 10	1194 G>A	Ser397=	rs72557928	0,003	0	0,3680
Exon 10	1200 G>A	Ser400=		0,003	0	0,3680
Intron 10	1248 +12C>T		rs2286822	0,346	0,344	0,9628
Intron 10	1248 +20G>A		rs2286823	0,343	0,344	0,9777
Exon 12	1455 T>C	Ala485=	rs2228104	0,983	0,979	0,7104
Exon 12	1508 C>T	Ala503Val	rs1057868	0,258	0,281	0,5159
Exon 12	1586 C>T	Thr529Met		0	0,003	0,2659
Exon 13	1716 G>A	Ser572=	rs1057870	0,362	0,323	0,2950
Exon 14	1891 G>A	Val631Ile	rs145782750	0	0,003	0,2659
Intron 14	1898 +13 G>T		rs72557956	0,003	0	0,3680
Intron 14	1899 -20 C>T			0	0,003	0,2659

g. – genomic position NG_008930.1, c. – coding position NM_000941.2, p. – amino acid position NP_000932.3, F – female, M – male. In red – new *POR* gene variants found in Czech cohort

U1 variants: Sequencing of the first untranslated exon and the proximal promoter revealed five genetic changes. Three of them are already known *POR* genetic polymorphisms (rs72553972, rs139824475 and rs3823884) and two are to our knowledge so far undescribed genetic chaneges (g.4965 C>T and g.4994 G>T). Allele frequency of the common SNP rs72553972 correspond to the data found previously (Huang et al., 2008).

Jewish population

Analyzing of the *POR* gene in the Jewish population showed a total of 38 different *POR* genetic variations. We described 4 genetic changes in 5´-flanking region, 7 intronic and 27 exonic variants. Sixteen of the 27 exonic genetic variations changed the encoded amino acid. We detected 17 previously uncharacterized genetic changes, two in the 5´-flanking region (g.5003A>C and g.5078G>A), one in the intronic region (c.189 -10T>C), three silent changes (p.143Tyr=, p.248Tyr= and p.Ser400=) and 11 missense genetic changes (p.Thr64Ile, p.Val85Met, p.Ser102Pro, p.Val164Met, p.Val191Met, p.Asp344Asn, p.Glu398Ala, p.Val472Leu, p.Arg550Gln, p.Arg570His and p.Asp648Asn). Variants found in the Jewish cohort are summarized in the table 17.

Common variants: Six of the 38 identified SNPs had allele frequencies greater than 10% in all investigated groups (rs1135612, rs4732516, rs2286822, rs2286823, rs2228104, rs1057868). Variant rs1057870 exhibited an allele frequency greater than 10% in all groups except EJ and interestingly, variant rs10262966 exhibited higher than 10% frequency only in EJ group. The common variant rs1057868 (p.Ala503Val) was present at an allele frequencies of 29.4% (AJ), 38.2% (BJ), 13.2% (EJ), 20.6% (MJ), 34.6% (TJ) and 28.6% (YJ). The differences in the p.Ala503Val allele frequencies among the groups vary significantly ($p<0,001$), but their values correspond with values found elsewhere (http://www.ncbi.nlm.nih.gov/gene/5447; Huang et al., 2008). The lowest frequency of the p.Ala503Val allele was found in the EJ group and it tends to exhibit allele frequencies as found in Sub-Saharan Africans according to the (http://www.ncbi.nlm.nih.gov/gene/5447). In the EJ group, we have identified several variants which frequencies differed significantly ($p<0.05$) from the frequencies described in the other Jewish subgroups. Differences were observed in the common

SNPs (rs4732516, rs1057868 and rs1057870) as well as uncommon variants (rs10262966, rs41295381, rs41299490, rs41299496 and p.Ala115Va) (see table 17).

Uncommon and new variants: From the uncommon SNPs, none of them exceed 1% allele frequencies in all groups. We have detected four known non-synonymous amino acid variants p.Ala115Val, p.Pro228Leu, p.Glu300Lys and p.Val631Ile. Two of them were found only on the one allele in the given groups and two variants were detected more than once; variant p.Glu300Lys was reported in two AJ samples and variant p.Ala115Val was reported in 3 EJ samples which brings it to allele frequency of 7.9%. In the AJ sample with the p.Pro228Leu variant, the heterozygous variant p.Ala229= was also found.

From the previously uncharacterized silent mutations, mutation p.Tyr143=, was present in four heterozygous MJ individuals, but were not found in any other Jewish subgroup. Silent mutation p.Tyr284= were present in one YJ and one MJ individual, and the third, p.Ser400= silent mutation, were present in one YJ, one BJ and one MJ individual. None of the three silent mutations were present in the Czech population and all were found as heterozygous substitutions.

Eleven of the 14 newly described *POR* variants changed the encoded amino acid. All of them are missense SNPs found on a single allele; p.Ser102Pro, p.Val191Met and p.Asp648Asn in the AJ population; p.Val85Met in EJ population; p.Val164Met, p.Asp344Asn and p.Glu398Ala in the MJ population; p.Arg570His in TJ population and p.Arg550Gln, p.Val472Leu and p.Thr64Ile in YJ population. In one MJ sample, the double mutation (p.Val164Met and p.Glu398Ala) has been identified.

U1 variants: In the MJ and AJ group, exon 1U has been analyzed. Other groups were not analyzed because of the time deficiency, but we are planning to complete the analysis also in the remaining ethnic groups. In the first untranslated exon, we have described four genetic variations (table 17). Two of them (rs3823884 and rs72553977) are known polymorphisms reported in the previous study (Huang et al., 2008) and two are new (g.5003 A>C and g.5078 G>T), not described previously. Both undescribed genetic variations were found only in the Ashkenazi population, while polymorphism rs72553977 was described only in the Moroccan Jewish population. Distribution of the allele rs3823884 is similar to the frequency of this allele in the Caucasian population (Huang et al., 2008).

Comparison of Czech and Jews populations

Twenty-three of the polymorphisms described in the investigated cohorts were population-specific, found in only one ethnic group (for this statement we excluded SNPs found in the proximal promoter and exon 1U since they were not analyzed in all studied groups). Most of them (18) were previously undescribed SNPs. None of the known *POR* amino acid variants causing disordered steroidogenesis or ABS (p.Thr142Ala, p.Gln153Arg, p.Tyr181Asp, p.Asn185Lys, p.Met263Val, p.Ala287Pro, p.Arg457H, p.Tyr459His, p.Val492Glu, p.Gly539Arg, p.Leu565Pro, p.Cys569Tyr, p.Leu577Arg, p.Tyr578Cys, p.Val608Phe, p.Arg616*, delF646, delE217, I444fsX449, L612W620delinsR, 1363delC, 697-698insGAAC and delP399_E401) were found in our cohort (Arlt et al., 2004; Fluck et al., 2011; Fluck et al., 2004; Fukami et al., 2005; Huang et al., 2005; Sahakitrungruang et al., 2009).

Described allele frequencies of common SNPs were basically similar to frequencies found in previous studies (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011). Comparison of allele frequencies between Czech and Jewish populations for seven common SNPs (rs1135612, rs4732516, rs2286822, rs2286823, rs2228104, rs1057868, rs1057870) showed statistically significant differences ($p<0.05$) in two cases (rs4732516 and rs1057870). Another relatively frequent SNP, rs10262966, was surprisingly found in 32 samples from Jewish population, but was not described in any sample from Czech population. The statistical comparison of the allele frequencies of this SNPs between observed populations showed high statistically significant difference ($p<0.001$).

Table 17. Allele frequencies of the *POR* gene in Czech and Jewish populations.

EXON	g.	c.	p.	SNP ID	CZ (644)	AJ (330)	BJ (118)	EJ (38)	MJ (272)	TJ (136)	YJ (234)
5´-Flanking	4849 C>A			rs72553972	0,175	-	-	-	-	-	-
5´-Flanking	4883 C>T			rs139824475	0,005	-	-	-	-	-	-
5´-Flanking	4965 C>T				0,002	-	-	-	-	-	-
5´-Flanking	4994 G>T				0,002	-	-	-	-	-	-
Exon 1U	5003 A>C				0	0,003	-	-	0	-	-
Exon 1U	5036 A>C			rs3823884	0,247	0,224	-	-	0,221	-	-
Exon 1U	5050 G>T			rs72553977	0	0	-	-	0,018	-	-
Exon 1U	5078 G>A				0	0,006	-	-	0	-	-
Intron 1U	5099 C>T			rs72553978	0,008	-	-	-	-	-	-
Exon 1	43906 A>G	15 A>G	Gly5=	rs10262966	0	0,012	0	0,158	0,037	0,044	0,026

EXON	g.	c.	p.	SNP ID	CZ (644)	AJ (330)	BJ (118)	EJ (38)	MJ (272)	TJ (136)	YJ (234)
Exon 1	43976 A>T	85 A>T	Thr29Ser		0,002	0	0	0	0	0	0
Exon 1	43978 G>A	87 G>A	Thr29=	rs41295381	0	0,003	0	0,026	0,011	0,015	0
Intron 1	62302 T>C	189 -10 T>C			0	0,003	0	0	0,004	0	0
Exon 2	62314 C>T	191 C>T	Thr64Ile		0	0	0	0	0	0	0,004
Exon 3	69365 G>A	253 G>A	Val85Met		0	0	0	0,026	0	0	0
Exon 3	69416 T>C	304 T>C	Ser102Pro		0	0,003	0	0	0	0	0
Exon 4	69456 C>T	344 C>T	Ala115Val		0	0	0,008	0,079	0,004	0	0,004
Exon 4	70240 C>T	369 C>T	Ala123=	rs41299490	0	0	0,008	0,079	0	0	0,004
Exon 4	70258 A>G	387 A>G	Pro129=	rs1135612	0,284	0,318	0,246	0,237	0,360	0,184	0,432
Exon 4	70300 C>T	429 C>T	Tyr143=		0	0	0	0	0,015	0	0
Exon 4	70361 G>A	490 G>A	Val164Met		0	0	0	0	0,004	0	0
Intron 4	70943 G>A	517 -4 G>A		rs41299496	0,002	0	0	0,053	0,000	0	0
Exon 5	71001 G>A	571 G>A	Val191Met		0	0,003	0	0	0	0	0
Intron 5	71411 C>G	642 -5C>G		rs72555509	0	0,009	0	0,026	0,007	0,015	0,013
Exon 6	71457 C>T	683 C>T	Pro228Leu	rs17853284	0	0,003	0	0	0	0,007	0
Exon 6	71461 C>T	687 C>T	Ala229=	rs72557906	0	0,003	0	0	0	0,007	0
Exon 7	72135 C>T	744 C>T	Tyr248=		0	0	0	0	0,004	0	0,004
Exon 8	73486 G>A	898 G>A	Glu300Lys	rs11540674	0	0,006	0,008	0	0	0	0
Exon 9	73673 C>T	984 C>T	Ala328=	rs72557941	0,011	0	0	0	0	0	0
Exon 9	73718 C>T	1029 C>T	Ala343=	rs72557942	0	0	0	0	0,004	0	0
Exon 9	73719 G>A	1030 G>A	Asp344Asn		0	0	0	0	0,004	0	0
Intron 9	74663 C>G	1067 -13C>G		rs4732516	0,984	0,991	0,992	0,737	0,952	0,956	0,970
Exon 10	74721 G>A	1112 G/A	Arg371His		0,002	0	0	0	0	0	0
Exon 10	74760 C>T	1151 C>T	Pro384Leu		0,002	0	0	0	0	0	0
Exon 10	74800 G>A	1191 G>A	Ser397=	rs72557928	0,002	0	0	0	0	0	0
Exon 10	74802 A>C	1193 A>C	Glu398Ala		0	0	0	0	0,004	0	0
Exon 10	74809 G>A	1200 G>A	Ser400=		0,002	0	0,008	0	0,004	0	0,004
Intron 10	74869 C>T	1248 +12C>T		rs2286822	0,345	0,385	0,339	0,421	0,393	0,257	0,483
Intron 10	74877 G>A	1248 +20G>A		rs2286823	0,343	0,382	0,331	0,421	0,393	0,257	0,483
Intron 10	74946 G>A	1249 -11G>A			0	0	0,008	0	0	0	0
Exon 12	75493 G>C	1414 G>C	Val472Leu		0	0	0	0	0	0	0,004
Exon 12	75534 T>C	1455 T>C	Ala485=	rs2228104	0,981	0,991	1,000	0,868	0,952	0,956	0,974
Exon 12	75587 C>T	1508 C>T	Ala503Val	rs1057868	0,269	0,294	0,381	0,132	0,206	0,346	0,286
Exon 12	75665 C>T	1586 C>T	Thr529Met		0,002	0	0	0	0	0	0
Exon 12	75728 G>A	1649 G>A	Arg550Gln		0	0	0	0	0	0	0,004
Exon 13	75861 G>A	1709 G>A	Arg570His		0	0	0	0	0	0,007	0
Exon 13	75868 G>A	1716 G>A	Ser572=	rs1057870	0,345	0,306	0,271	0,079	0,335	0,353	0,188
Exon 14	76133 G>A	1891 G>A	Val631Ile	rs145782750	0,002	0	0,008	0	0,004	0,007	0
Intron 14	76153 G>T	1898 +13G>T		rs72557956	0,002	0	0	0	0	0	0
Intron 14	76216 C>T	1899 -20C>T			0,002	0	0	0	0	0	0
Exon 15	76279 G>A	1942 G>A	Asp648Asn		0	0,003	0	0	0	0	0

CZ - Czech population, AJ – Ashkenazi Jews, BJ – Bulgarian Jews, EJ – Ethiopian Jews, MJ – Moroccan Jews, TJ – Turkey Jews, YJ – Yemenite Jews, g. – genomic position NG_008930.1, c. – coding position NM_000941.2, p. – amino acid position NP_000932.3. In red – new *POR* gene variants found in our cohort

Ad 4) Investigation of *POR* gene in patients suspected of POR deficiency

Direct sequencing of the *POR* gene in patients with skeletal deformities did not reveal any molecular genetic defect.

Table 18. Single nucleotide polymorphisms found in patients suspected of POR deficiency.

Exon	g.	c.	p.	SNP ID	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
5'-Flanking	4849 C>A			rs72553972	-	-	-	-	-	-	ht.	ht.	-	-
Exon 1U	5036 A>C			rs3823884	-	-	-	ht.	-	ht.	-	ht.	-	ht.
Exon 2	43906 A>G	15 A>G	Gly=	rs10262966	-	-	-	-	-	ht.	-	-	-	-
Exon 5	70258 A>G	387 A>G	Pro129=	rs1135612	-	-	-	-	ht.	-	ht.	-	-	ht.
Intron 10	74663 C>G	1067 -13C>G		rs4732516	hm.	hm.	hm.	hm.	hm.	ht.	hm.	hm.	hm.	hm.
Intron 11	74869 C>T	1248 +12C>T		rs2286822	-	-	-	-	ht.	ht.	ht.	-	-	ht.
Intron 11	74877 G>A	1248 +20G>A		rs2286823	-	-	-	-	ht.	ht.	ht.	-	-	ht.
Exon 13	75534 T>C	1455 T>C	Ala485=	rs2228104	hm.	hm.	hm.	hm.	hm.	ht.	hm.	hm.	hm.	hm.
Exon 13	75587 C>T	1508 C>T	Ala503Val	rs1057868	-	hm.	hm.	-	-	-	ht.	ht.	-	ht.
Exon 14	75868 G>A	1716 G>A	Ser572=	rs1057870	hm.	-	-	hm.	ht.	-	-	ht.	hm.	-

g. – genomic position NG_008930.1, c. – coding position NM_000941.2, p. – amino acid position NP_000932.3, P – patient, ht. – heterozygote, hm. - homozygote

Ad 5) Addressing the pathology of the found mutations

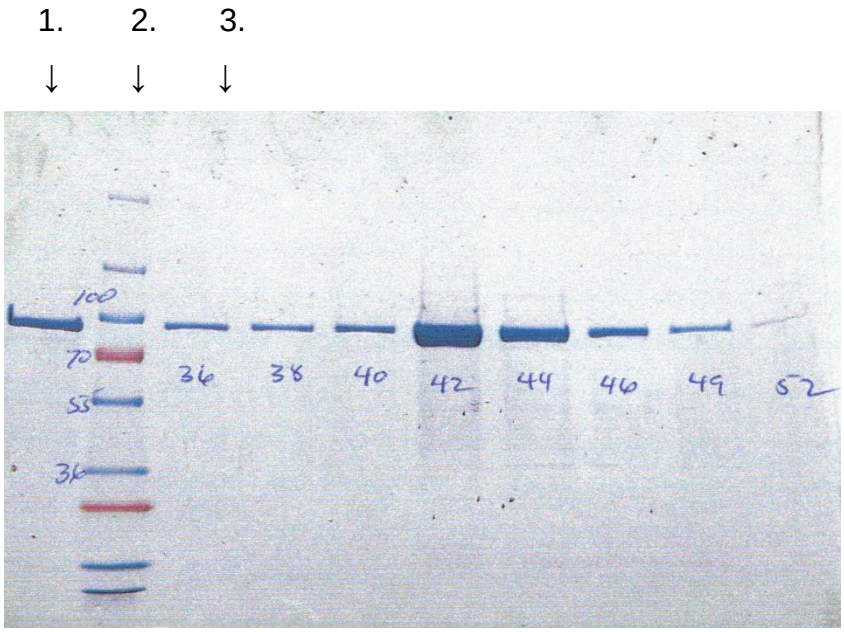
Allele localization

In one DNA sample, we have identified two genetic variations, p.Val164Met and p.Glu398Ala. We subcloned a long PCR product containing both genetic variants into the pCR4-TOPO vector (Invitrogen) and we transformed this vector into *E. coli* Top10 chemically competent cells (Invitrogen). Further multiplication of the plasmid in the cells led to segregation of the two *POR* gene alleles so we could sequence the two *POR* alleles separately. In that manner we have identified that identified SNPs were located on two different alleles, thus the individual is composed heterozygote for the two *POR* SNPs.

Full length human POR expression and purification

Wild-type, p.Val164Met and p.Pro384Leu human POR molecules were bacterially expressed and purified as membrane anchor-containing (holo) proteins. For the wild-type enzyme, 11 mg of purified protein was produced per liter of culture. The purified protein had greenish-brown color. This coloration originate in spectral contribution of oxidized FAD (*yellow*) and air-stable FMN semiquinone (*blue-gray*). The purified enzyme exhibited the molecular mass of expected ~77 kDa. The same coloration and molecular mass had p.Val164Met and p.Pro384Leu variant enzymes. For p.Val164Met 11.25 mg of purified protein per liter of culture was produced and for the p.Pro384Leu 7.25 mg of purified protein per liter of culture. SDS/PAGE analysis of the proteins showed that p.Val164Met and p.Pro384Leu variants expression were indistinguishable from the wild type protein.

Figure 12. Fractions of p.Pro384Leu holo enzyme preparation.

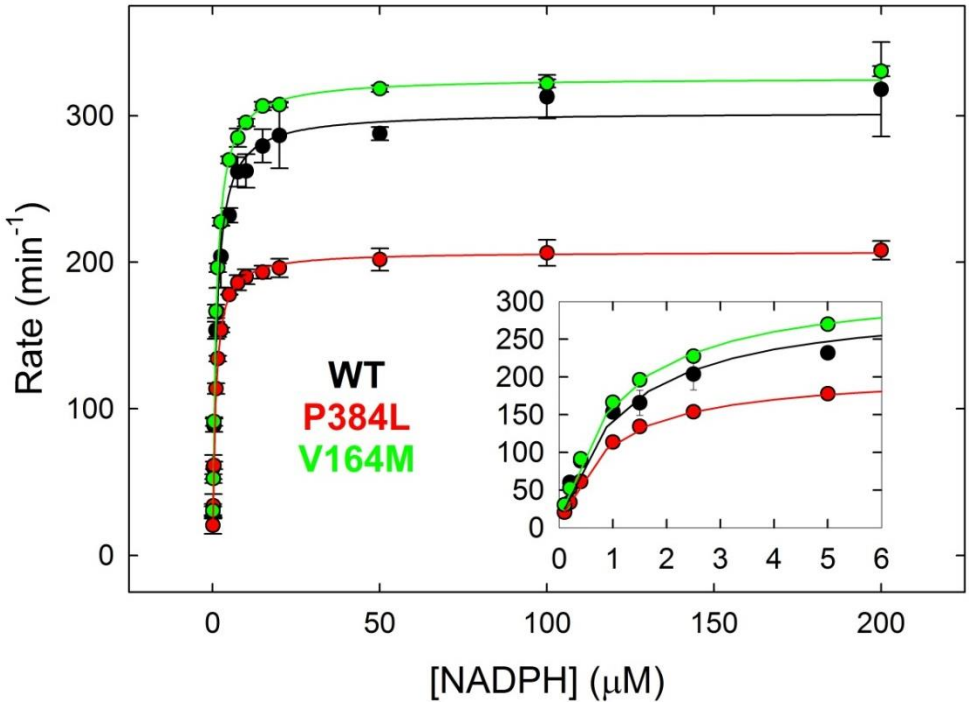


1. POR control, 2. PageRulerPrestained Protein Ladder (Fermentas), 3. Full-length p.Pro384Leu POR preparation fractions

Kinetic analysis of purified full length variants

To understand the enzymatic consequences of p.Val164Met and p.Pro384Leu amino acid replacement mutations, we assayed wild-type; p.Val164Met and p.Pro384Leu full length proteins for the ability to catalyze NADPH-cytochrome c reduction (figure 13). The p.Pro384Leu variant retained ~65% of WT turnover with no significant effect on K_m^{NADPH} (WT = 1 μM and p.Pro384Leu = 0.9 μM), while the p.Val164Met variant showed modest increase in its activity (~110% of WT turnover) in the cytochrome *c* reductase assay.

Figure 13. Cytochrome c assay.



Full length POR flavin content

Protein and flavin quantitative analyses were performed on the full lenth proteins. The HPLC analysis showed that the wild-type enzyme and both new variant proteins had the full complement of FAD and FMN and FAD:FMN:protein ratio is 1:1:1 (figure 14).

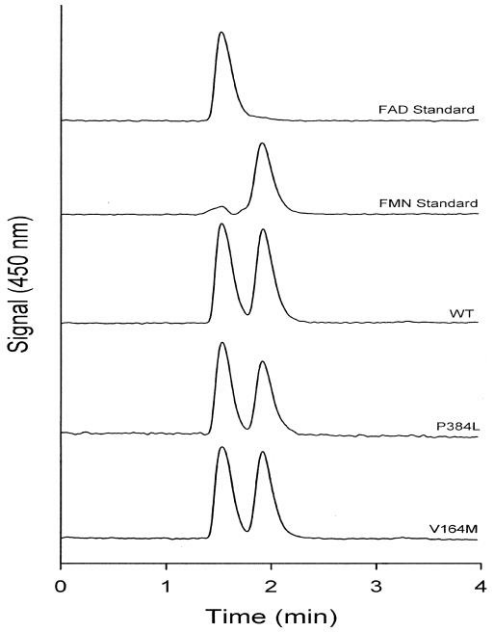
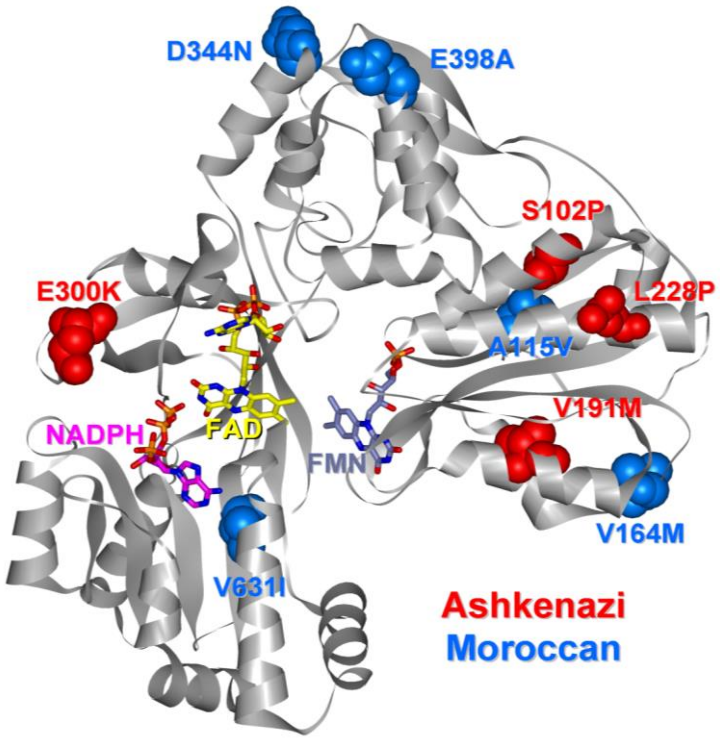


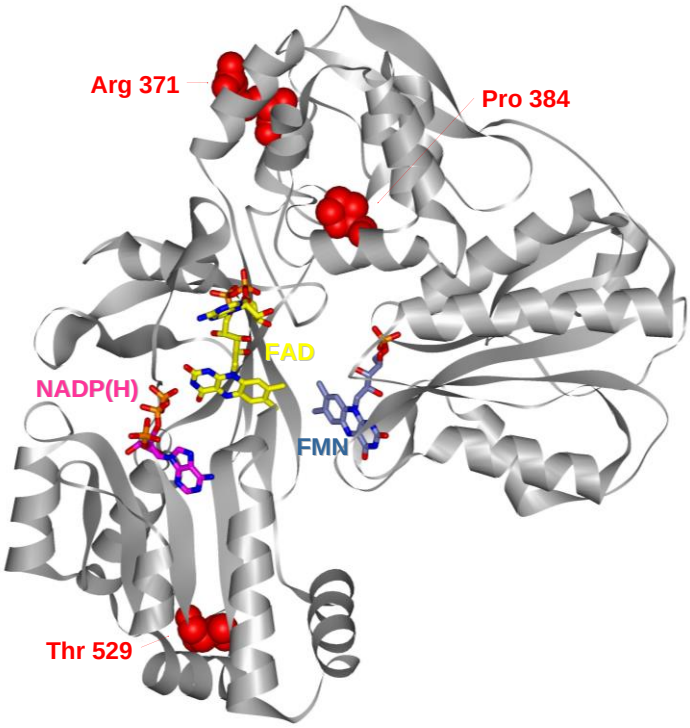
Figure 14. Flavin analysis. The relative flavin content of each protein preparation was analyzed using HPLC. The top part shows the elution profiles of FAD and FMN standards. The panel shows flavin content of wild-type, p.Val164Met and p.Pro384Leu holo proteins purified.

Figure 15. POR missense variants found in Jewish (A) and Czech Slavic (B) population.

The human POR structure (Xia et al., 2011) is depicted, gray ribbons indicate the peptide backbone. FAD (yellow) and FMN (blue–gray) cofactors and NADP(H) coenzyme (only 2´,5´-ADP of NADP+ was structurally resolved, pink) are shown in stick configuration. Amino acid residues corresponding to new variants are shown in space-filling configuration (p.Thr29Ser and p.Thr64Ile are not a part of the model, which is based on delta66 truncation wild-type POR).



A.



B.

Ad 6) Investigation of *PGRMC1* gene in patients suspected of POR deficiency

We have analyzed the *PGRMC1* gene in 7 of 10 patients with skeletal deformities. None of them showed to harbor any *PGRMC1* genetic variations.

DISCUSSION

Ad 2) and 3) Genetic variations in the *POR* gene in general population

The role of POR in pharmacogenetics

Research in pharmacogenetics investigates how differences in our genes can affect our response to drug therapy. It was already said that CYPs from families 1 to 3 mediate 80-90% of all phase I-dependent metabolism of the most important clinical drugs and other xenobiothics in human liver (Bertz and Granneman, 1997; Evans and Relling, 1999; Rendic and Guengerich, 2014). Since POR is the unique electron donor to all microsomal CYPs, the contribution of common *POR* variants to inter- and intra-individual variability is of great pharmacogenetic interest. Pharmacogenetics is often mentioned in connection with adverse drug reactions (ADRs), which represent a major public health concern (Budnitz et al., 2006). There is evidence that drug therapy based on an individual's genetic makeup may result in a clinically significant reduction in adverse outcomes (Woodcock and Lesko, 2009). Knowledge of the effects of *POR* variation on CYPs activity has a potential for a better understanding of individual differences in the response to medicines and this information could increase benefit and reduce harm in people whose drug responses are not "average."

Population genetics

Important role of POR in drug metabolism led to hypothesis that genetic variations in *POR* would account for some of the observed pharmacokinetics variations. Several studies have addressed the question of distribution of *POR* genetic differences within various populations (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011). Their results provided an important basis for the *POR* pharmacogenetic research. First of all they showed that the human *POR* gene is highly polymorphic (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008; Saito et al., 2011). Huang *et al.* (Huang et al., 2008) identified in 824 individuals 140 distinct *POR* nucleotide variations, with a frequency of approximately 3.1 SNPs per kb of sequence. Secondly, they revealed several common polymorphisms, which were afterward shown to alter POR function (Fluck et al., 2010; Gomes et al., 2009;

Hart et al., 2008; Zhang et al., 2011) and they provided population allele frequencies of these SNPs.

Previous works have been conducted mostly in Caucasian population (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008). Other less studied population groups consisted from African Americans, Chinese Americans, Mexican Americans and Japanese population. Our research was undertaken to further define *POR* allele frequencies in Caucasian subpopulation, namely in the Czech population and in unstudied Jewish population. We decided to study all six Jewish ethnic groups separately. It was shown lately that the Jewish people differ genetically among themselves according to their ethnicity and that both Ashkenazi and non-Ashkenazi Jews differ from non-Jewish Caucasians (Klitz et al., 2010). Furthermore, each Jewish group shows evidence of gene flow from local populations (Behar et al., 2008). Thus, it was of interest to study *POR* mutations in all six Jewish groups separately although these ethnics have been separated from one another for only about two millennia.

A total of 51 SNPs were identified in 886 individuals. The number of identified SNPs is by far lower than in Huang`s study (Huang et al., 2008), in which 140 SNPs were identified in 824 subjects. The discrepancy is caused due to the size of the *POR* gene examined, which was approximately 3330 bp in our study compared to 5655 bp in Huang *et al.* study (Huang et al., 2008). Moreover, we have examined mostly exons while Huang *et al.* (Huang et al., 2008) examined extended intronic regions, which are more probable to gather genetic variations. It is known that in comparison with exons, introns are more variable because in general selective pressure in intronic regions is much less than exons. When we compared only the exonic SNPs in both studies, we got to almost the same results, 33 SNPs in the Huang *et al.* study (Huang et al., 2008) and 34 SNPs described in our study.

The present work confirmed several of the already reported *POR* SNPs in both Czech and Jewish populations. Allele frequencies of the observed variants were generally similar to published data where available. We observed the striking difference in allele frequency only in two cases. We have not detected any rs10262966 in Czech population, but it has been described in Caucasian population in all previous studies with frequencies between 0.007-0.045 (Gomes et al., 2009; Hart et al., 2008; Huang et al., 2008). Further, present also in Czech population, we

have reported relatively high frequency of rs72557941 (more than 1%), which was described in previous studies only by Gomes *et al.* (Gomes et al., 2009) with the frequency of 0.007.

We have identified a total of 20 genetic changes causing amino acid substitution, six of which were known genetic variants and 14 were newly described. All of them were found as individual heterozygotes. By far the most common polymorphism changing amino acid was the variant p.Ala503Val. It was found in 26.8% of Czech alleles and in 13.2 – 38.4% of Jewish alleles. Although this variant results into an amino acid change in a highly conservative region of the protein (Fluck et al., 2007), it is not associated with any pathological phenotype manifestation. The activity of this variant have been measured in numerous *in vitro* and *in vivo* assays. In the classic, non-physiologic cytochrome c test, the ability of p.Ala503Val variant to reduce cytochrome c was 67% of WT (Huang et al., 2008). Measurement of the p.Ala503Val variant activity with steroidogenic enzymes showed only moderately decreased function for 17α-hydroxylase (Fluck et al., 2007) or 21-hydroxylase enzymatic activities (Gomes et al., 2009). In two microsomal studies by Hart *et al.* (Hart et al., 2008) and by Gomes *et al.* (Gomes et al., 2009) investigating the impact of POR variants on drug-oxidation activities, the p.Ala503Val was not associated with any activity or expression changes. On the contrary, in two latter works p.Ala503Val had 61–77% of wild type activity to support CYP3A4 hydroxylation of midazolam and testosterone (Gomes et al., 2009) and supported only 62% and 53% of WT CYP2D6 activity with dextromethorphan and bufuralol, respectively (Sandee et al., 2010). Oneda *et al*. (Oneda et al., 2009) explored the activity of the p.Ala503Val in patients on clozapine treatment and in heroin-dependent individuals. He observed a 1.6-fold enhancement of drug clearance in p.Ala503Val carriers over that observed in wild type carriers. This result differed from outcomes observed in *in vitro assays* (Agrawal et al., 2008; Fluck et al., 2010; Gomes et al., 2009). The discrepancy could be explained by knowing a biochemical catabolism of midazolam. Both, CYP3A4 and CYP3A5 contribute to midazolam biotransformation (Patki et al., 2003), thus the rate of CYP3A4 participation in midazolam metabolism cannot be clearly determined *in vivo*. Another study investigated p.Ala503Val variant in a cohort of renal allograft recipients under tacrolimus-based immunosuppression therapy (de Jonge et al., 2011). They observed that patients who were expressing *CYP3A5*1 and* carrying at

least one p.Ala503Val allele required elevated tacrolimus doses especially in first days after the transplantation. To summarize, it remains unclear whether p.Ala503Val POR variant participates in modification of phenotypic features in POR deficiency, but as indicated, in particular cases it could be used to explain differences in drug metabolism.

The other five known amino acid changes (p.Ala115Val, p.Pro228Leu, p.Glu300Lys, p.Arg371His and p.Val631Ile) were found much less frequently. The variant p.Ala115Val was first described by Huang *et al.* (Huang et al., 2005). Substitution of alanine to valine on position 115 is a conservative change in a region without apparent function (Fluck et al., 2007). Recombinant expression and analysis of the p.Ala115Val mutant activity showed complete loss of function with CYP1A2 and CYP2C19 drug-metabolizing enzymes (Agrawal et al., 2008), whereas the activities for the steroid-metabolizing CYPs and for drug-metabolizing CYP3A4 were not affected dramatically (Fluck et al., 2010). We found the p.Ala115Val variant in the Jewish population with the frequency distribution of 0.8-7.9%, but not in the Czech population. Likewise the variant p.Ala115Val also the *POR* variant p.Pro228Leu (first described on the BioVentures Website) exhibits significantly decreased support of CYP1A2- and CYP2C19-mediated activities, but do not appear to substantially affect steroid–metabolizing P450s either CYP3A4 (Agrawal et al., 2008; Fluck et al., 2010). In our study we found this genetic change in two Jewish individuals. The third variant p.Glu300Lys was described in three Jewish subjects, but again not in the Czech population. This variant has according to Agrawal *et al.* (Agrawal et al., 2008) practically no effect on CYP activity, but further biochemical essays should be done for better biochemical characterization. Amino acid variant p.Val631Ile, located near NADPH binding site, had in CYP17A1 and CYP3A4 assays relatively mild effect on POR activity (Fluck et al., 2010) and had low but measurable activity (6–41% of wild-type) with both CYP1A2 and CYP2C19 (Agrawal et al., 2008). All four described variants were not associated with POR deficiency, but as seen three of them (p.Ala115Val, p.Pro228Leu and p.Val631Ile) severely decrease the activity of some drug-metabolizing CYPs (Agrawal et al., 2008), thus we think these *POR* missense variants may be potential biomarkers for future POR pharmacogenetics screening. Amino acid change p.Arg371His has been for the first time described in the database of an Exome Variant Server (EVS) (http://evs.gs.washington.edu/EVS/) and its

impact on the POR function has not been studied yet. For further investigation, allele frequency data from a larger population will inevitably be required.

Some of the described intronic SNPs have been associated with changed POR activity. Hart *et al.* (Hart et al., 2008) has found that an intronic polymorphism rs41301427 diminish the function of POR protein. Another intronic variant, rs22868232, found in our study has been identified as an effector of P450 drug-oxidation activity; it has significantly increased microsomal activities of several CYPs (Gomes et al., 2009). Also other intronic SNPs not identified here have been associated with altered function of POR (Gomes et al., 2009; Zhang et al., 2011). Zhang *et al.* (Zhang et al., 2011) described in *in vivo* study that one common intronic *POR* polymorphisms contribute to the interindividual variability in the warfarin (drug used to prevent heart attacks and strokes) maintenance dose. It is likely that some common intronic variants may be good candidates for further research into their effects in drug oxidation activities. In our study, we have examined only exonic and flanking intronic regions of the *POR* gene, but the importance of screening *POR* noncoding regions is clear.

We have identified 14 novel amino acid changing variations. Localizations of the amino acid substitutions within the POR molecule are displayed in the picture (figure 15). It seems that some of the found variants are in the regions susceptible to mutations. Saito *et al.* (Saito et al., 2011) described two amino acid changes (p.Arg550Trp and p.Arg570Cys) in the same positions as we did, but substituted amino acids were due to different base substitutions distinct. Likewise, three amino acids replacing SNPs in the same position but with the different amino acid change as in our study (p.Val85Leu, p.Val191Leu, and p.Val472Met) are listed in the EVS database (http://evs.gs.washington.edu/EVS/). Two of the newly described variants were expressed and examined in the cytochrome c assay. Variants p.Pro384Leu and p.Val164Met were identified within a pilot research project. Especially an interesting nature of substitution of a cyclic amino acid proline for a hydrophobic amino acid leucine and an important location of Pro384 residue within the connecting domain, led us to express and purify this variant protein. Functional significance of the other novel variations should be clarified in the future.

All together, we have identified four genetic variants in the proximal promoter region. None of them lay in the critical SP1 binding sites, but one of the described

common polymorphism, rs72553972 (-152), was shown previously to significantly reduce *POR* transcription (Tee et al., 2011). Another four variants were identified within the region of the exon 1U; two of them were newly described. Because of the time pressure, analyzing of exon 1U and *POR* promoter region was not finished in all ethnic groups, but we are planning to do so in the future. The role of proximal promoter of the *POR* gene and exon 1U has been studied previously (Soneda et al., 2011; Tee et al., 2011). Tee *et al.* (Tee et al., 2011) located the proximal promoter at -325/-1 bp from the untranslated exon. Previous sequencing of this region revealed three common polymorphisms rs12537282 (-208), rs72553971 (-173) and rs72553972 (-152) (Huang et al., 2008). Further investigation showed that first two SNPs had little influence on transcription, but the polymorphism rs72553972 reduced *POR* transcription significantly (35-60% of WT activity), thus it was suggested that rs72553972 may play a certain role in the genetic variation of steroid biosynthesis and drug metabolism (Tee et al., 2011). Soneda *et al.* (Soneda et al., 2011) described two deletions encompassing exon 1U in two patients with POR deficiency and showed a pivotal role of the evolutionally conserved SP1 binding sites in the *POR* transcription. Taken together, it was shown that it is important to explore noncoding regions of the *POR* gene and that some of the *POR* common SNPs found in this region might alter drug metabolism.

Most DNA samples in our cohort were obtained from adult individuals, but in the Czech cohort, 95 DNA samples were obtained from cord blood of neonates. Clinical features present in POR deficiency can be readily assessed by prenatal diagnosis (Reisch et al., 2013) or manifest already in newborn (Krone et al., 2012). Therefore, we do not consider the age variations of the cohort to have impact on the *POR* allele frequency.

Ad 4) Investigation of *POR* gene in patients suspected to have POR deficiency

POR deficiency is a newly characterized disorder of adrenal and gonadal steroidogenesis combined with ABS malformation syndrome (Miller et al., 2004). In our cohort of patients with skeletal deformities, we have not identified any of the *POR* genetic defects. Negative results might be attributed to two main reasons. First, POR deficiency manifests with a wide range of clinical signs and symptoms including

glucocorticoid deficiency, disordered sex development (DSD) and mild to moderate skeletal deformities. Similar phenotype manifestations only without DSD occur also in patients with mutations in *FGFR2* gene (Chun et al., 1998) or in children born to mothers treated during the pregnancy with high doses of antifungal drug fluconazole (Aleck and Bartley, 1997). The wide spectrum of symptoms might sometimes cause a misdiagnosis of the condition. For example, based on clinical presentation and steroid measurements, some cases of POR deficiency have previously been mistakenly diagnosed as 17, 20-lyase deficiency (Hershkovitz et al., 2008), or 21-hydroxylase deficiency, aromatase deficiency (Fukami et al., 2006). Also, some patients identified with skeletal deformities resembling ABS or with classics clinical and biochemical POR deficiency presentation carried no *POR* mutations (Huang et al., 2005; Krone et al., 2012). The negative findings in our cohort might be the outcome of wrong diagnosis. Another possible reason for negative results could be so called "false negativity" when the disease is not determined with available laboratory methods. Krone *et al.* (Krone et al., 2012) recently identified a splice site mutation in intron 7 in two unrelated POR deficiency patients. POR deficiency relating genetic changes were associated also with mutations in exon 1U, sequencing of the long PCR products in POR deficiency patient revealed a microdeletion encompassing exon 1U (Soneda et al., 2011). Moreover, in some patients presenting with hallmark clinical and biochemical symptoms of POR deficiency, only one mutant allele was identified (Krone et al., 2012; Scott and Miller, 2008). Since in our study we analyzed only exons and adjacent intronic regions, it is not excluded that *POR* mutations are hidden somewhere in deep intronic regions.

Ad 5) Addressing the pathology of the found mutations

All protein expression and purification steps and also the biochemical assays were carried out in the laboratory of professor Bettie Sue Siler Masters at UT Health Science Centrum at San Antonio (Texas). In time of my staying in Texas, we had discovered only two of the 14 new *POR* SNPs, therefore, up to date we have had expressed only two POR variants. In the future we are planning to expresses and purify all the rest of the new *POR* polymorphisms.

To study the impact of the p.Val164Met and p.Pro384Leu genetic variants on protein structure and functional consequences, mutated proteins were expressed in *E. coli* and enzymatic properties were characterized. All the recombinant expressed

and purified proteins were inspected by SDS/PAGE. All three, WT, p.Val164Met and p.Pro384Leu, displayed homogeneous bands on SDS/PAGE. The classical assay of POR activity is the measuring of cytochrome c reduction. However this assay is non-physiological, cytochrome *c* is located in the mitochondria and it is not a biological substrate for POR, it still remains very useful tool in studying *POR* gene mutations. Cytochrome c assay revealed that the p.Pro384Leu substitution reduced the POR activity to ~65% of WT, while the p.Val164Met slightly increased its activity to ~110% of WT.

Ad 6) Investigation of *PGRMC1* gene in patients suspected of POR deficiency

In 2007, protein PGMRC1 has emerged as a new protein partner of several CYPs involved in steroid metabolism (Hughes et al., 2007) and recently it was showed that it also binds to POR protein (Szczesna-Skorupa and Kemper, 2011). It was, therefore, reasonable to address whether genetic changes in *PGMRC1* gene occur in individuals with symptoms of POR deficiency. We have done *PGRMC1* gene analysis in 7 patients with skeletal deformities, but none of them carried any *PGRMC1* genetic change. There can be several reasons for the negative results; some of them I already mentioned (Discussion, Ad 4). In spite of the negative results I consider it important to further investigate this gene in patients with disordered steroidogenesis.

CONCLUSION

Human NADPH-cytochrome P450 oxidoreductase is a protein of high importance since it affects wide spectrum of hem-containing proteins, including several steroidogenic and most drug metabolizing enzymes. In recent years, especially pharmacogenomics is raising its interest in POR genetic variants studies. Current work provides valuable data that may help to highlight some important questions regarding *POR* sequence differences among various populations, POR function and role of the POR protein in pharmacogenomics. Main achievements of present work are:

- We introduced the analysis of *POR and PGRMC1* genes. Currently we are the only laboratory in the Czech Republic that provides the POR deficiency diagnostics and speeches given at seminars and conferences helped to familiarize Czech doctors with this relatively new described disorder.

- We determined the allele frequencies of a *POR* gene in the, so far unstudied, Czech and Jewish populations.

- We described 14 novel *POR* amino acid variations, five of which are already reported on the official website of *POR* polymorphisms.

- We examined the effect on POR activity of two newly described *POR* variants. Reduction in the activity of p.Pro384Leu change provides evidence that some of the uncommon *POR* variants can potentially alter enzyme activity without causing POR deficiency.

- In the studied populations, we indicated *POR* variants that might play a role in altered drug metabolism.

For further investigation, it is important to investigate *POR* gene deeper intronic sequences, especially in patients suspected to have POR deficiency syndrome, and to augment the general population cohort.

TABLE OF FIGURES

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REFERENCES

Adachi, M. et al., 2006. POR R457H is a global founder mutation causing Antley-Bixler syndrome with autosomal recessive trait. Am J Med Genet A, 140(6): 633-5.

Adachi, M., Asakura, Y., Tachibana, K. and Shackleton, C., 2004a. Abnormal steroidogenesis in three patients with Antley-Bixler syndrome: apparent decreased activity of 17alpha-hydroxylase, 17,20-lyase and 21-hydroxylase. Pediatr Int, 46(5): 583-9.

Adachi, M. et al., 2004b. Compound heterozygous mutations of cytochrome P450 oxidoreductase gene (POR) in two patients with Antley-Bixler syndrome. Am J Med Genet A, 128A(4): 333-9.

Adolphs, N. et al., 2011. Antley-Bixler-syndrome--staged management of craniofacial malformations from birth to adolescence--a case report. J Craniomaxillofac Surg, 39(7): 487-95.

Agrawal, V., Huang, N. and Miller, W.L., 2008. Pharmacogenetics of P450 oxidoreductase: effect of sequence variants on activities of CYP1A2 and CYP2C19. Pharmacogenet Genomics, 18(7): 569-76.

Aigrain, L., Pompon, D., Morera, S. and Truan, G., 2009. Structure of the open conformation of a functional chimeric NADPH cytochrome P450 reductase. EMBO Rep, 10(7): 742-7.

Aleck, K.A. and Bartley, D.L., 1997. Multiple malformation syndrome following fluconazole use in pregnancy: Report of an additional patient. American Journal of Medical Genetics, 72(3): 253-256.

Antley, R. and Bixler, D., 1975. Trapezoidocephaly, midfacial hypoplasia and cartilage abnormalities with multiple synostoses and skeletal fractures. Birth Defects Orig Artic Ser, 11(2): 397-401.

Arlt, W. et al., 2004. Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. Lancet, 363(9427): 2128-35.

Behar, D.M. et al., 2008. Counting the founders: the matrilineal genetic ancestry of the Jewish Diaspora. PLoS One, 3(4): e2062.

Benedetto, C., Slater, T.F. and Dianzani, M.U., 1976. Nadph-Cytochrome C Reductase, Cytochrome-P-450 and Nadph-Linked Lipid Peroxidation in Microsomal Fractions Obtained from Rat Tissues. Biochemical Society Transactions, 4(6): 1094-1097.

Bertz, R.J. and Granneman, G.R., 1997. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. Clin Pharmacokinet, 32(3): 210-58.

Bottero, L., Cinalli, G., Labrune, P., Lajeunie, E. and Renier, D., 1997. Antley-Bixler syndrome. Description of two new cases and a review of the literature. Childs Nerv Syst, 13(5): 275-80; discussion 281.

Brenner, S., Hay, S., Munro, A.W. and Scrutton, N.S., 2008. Inter-flavin electron transfer in cytochrome P450 reductase - effects of solvent and pH identify hidden complexity in mechanism. FEBS J, 275(18): 4540-57.

Budnitz, D.S. et al., 2006. National surveillance of emergency department visits for outpatient adverse drug events. JAMA, 296(15): 1858-66.

Das, A. and Sligar, S.G., 2009. Modulation of the cytochrome P450 reductase redox potential by the phospholipid bilayer. Biochemistry, 48(51): 12104-12.

de Jonge, H., Metalidis, C., Naesens, M., Lambrechts, D. and Kuypers, D.R., 2011. The P450 oxidoreductase *28 SNP is associated with low initial tacrolimus exposure and increased dose requirements in CYP3A5-expressing renal recipients. Pharmacogenomics, 12(9): 1281-91.

Debeljak, N., Fink, M. and Rozman, D., 2003. Many facets of mammalian lanosterol 14alpha-demethylase from the evolutionarily conserved cytochrome P450 family CYP51. Arch Biochem Biophys, 409(1): 159-71.

Devlin, T.M., 2006. Textbook of Biochemistry with Clinical Correlations

Dobrinas, M., Cornuz, J., Pedrido, L. and Eap, C.B., 2012. Influence of cytochrome P450 oxidoreductase genetic polymorphisms on CYP1A2 activity and inducibility by smoking. Pharmacogenet Genomics, 22(2): 143-51.

Ellis, J. et al., 2009. Domain motion in cytochrome P450 reductase: conformational equilibria revealed by NMR and small-angle x-ray scattering. J Biol Chem, 284(52): 36628-37.

Enoch, H.G. and Strittmatter, P., 1979. Cytochrome b5 reduction by NADPH-cytochrome P-450 reductase. J Biol Chem, 254(18): 8976-81.

Evans, W.E. and Relling, M.V., 1999. Pharmacogenomics: translating functional genomics into rational therapeutics. Science, 286(5439): 487-91.

Feigin, E. et al., 1995. Antley-Bixler syndrome and esophageal atresia in a patient with trisomy 21. Clin Genet, 47(1): 53-5.

Fluck, C.E. et al., 2011. Deletion of P399_E401 in NADPH cytochrome P450 oxidoreductase results in partial mixed oxidase deficiency. Biochem Biophys Res Commun, 412(4): 572-7.

Fluck, C.E., Mullis, P.E. and Pandey, A.V., 2009. Modeling of human P450 oxidoreductase structure by in silico mutagenesis and MD simulation. Mol Cell Endocrinol, 313(1-2): 17-22.

Fluck, C.E., Mullis, P.E. and Pandey, A.V., 2010. Reduction in hepatic drug metabolizing CYP3A4 activities caused by P450 oxidoreductase mutations identified in patients with disordered steroid metabolism. Biochem Biophys Res Commun, 401(1): 149-53.

Fluck, C.E., Nicolo, C. and Pandey, A.V., 2007. Clinical, structural and functional implications of mutations and polymorphisms in human NADPH P450 oxidoreductase. Fundam Clin Pharmacol, 21(4): 399-410.

Fluck, C.E. et al., 2004. Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. Nat Genet, 36(3): 228-30.

Forkert, P.G., Lord, J.A. and Parkinson, A., 1996. Alterations in expression of CYP1A1 and NADPH-cytochrome P450 reductase during lung tumor development in SWR/J mice. Carcinogenesis, 17(1): 127-32.

Fukami, M. et al., 2006. Cytochrome P450 oxidoreductase deficiency in three patients initially regarded as having 21-hydroxylase deficiency and/or aromatase deficiency: diagnostic value of urine steroid hormone analysis. Pediatr Res, 59(2): 276-80.

Fukami, M. et al., 2005. Cytochrome P450 oxidoreductase gene mutations and Antley-Bixler syndrome with abnormal genitalia and/or impaired steroidogenesis: molecular and clinical studies in 10 patients. J Clin Endocrinol Metab, 90(1): 414-26.

Fukami, M. et al., 2010. Anorectal and urinary anomalies and aberrant retinoic acid metabolism in cytochrome P450 oxidoreductase deficiency. Mol Genet Metab, 100(3): 269-73.

Fukami, M. et al., 2009. Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype-phenotype correlations in 35 Japanese patients. J Clin Endocrinol Metab, 94(5): 1723-31.
Gomes, A.M. et al., 2009. Pharmacogenomics of human liver cytochrome P450 oxidoreductase: multifactorial analysis and impact on microsomal drug oxidation. Pharmacogenomics, 10(4): 579-99.
Griffith, O.W. and Stuehr, D.J., 1995. Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol, 57: 707-36.
Groves, J.T., 2003. The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies. Proc Natl Acad Sci U S A, 100(7): 3569-74.
Gruez, A. et al., 2000. Four crystal structures of the 60 kDa flavoprotein monomer of the sulfite reductase indicate a disordered flavodoxin-like module. J Mol Biol, 299(1): 199-212.
Gutierrez, A. et al., 2003. Interflavin electron transfer in human cytochrome P450 reductase is enhanced by coenzyme binding. Relaxation kinetic studies with coenzyme analogues. Eur J Biochem, 270(12): 2612-21.
Gutierrez, A., Paine, M., Wolf, C.R., Scrutton, N.S. and Roberts, G.C., 2002. Relaxation kinetics of cytochrome P450 reductase: internal electron transfer is limited by conformational change and regulated by coenzyme binding. Biochemistry, 41(14): 4626-37.
Hall, P.D., Stupans, I., Burgess, W., Birkett, D.J. and Mcmanus, M.E., 1989. Immunohistochemical Localization of Nadph-Cytochrome-P450 Reductase in Human-Tissues. Carcinogenesis, 10(3): 521-530.
Hamdane, D. et al., 2009. Structure and function of an NADPH-cytochrome P450 oxidoreductase in an open conformation capable of reducing cytochrome P450. J Biol Chem, 284(17): 11374-84.
Hart, S.N., Li, Y., Nakamoto, K., Wesselman, C. and Zhong, X.B., 2007. Novel SNPs in cytochrome P450 oxidoreductase. Drug Metab Pharmacokinet, 22(4): 322-6.
Hart, S.N. et al., 2008. Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism. Pharmacogenet Genomics, 18(1): 11-24.
Hassell, S. and Butler, M.G., 1994. Antley-Bixler syndrome: report of a patient and review of literature. Clin Genet, 46(5): 372-6.
Henderson, C.J. et al., 2003. Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. J Biol Chem, 278(15): 13480-6.
Herman, G.E., 2003. Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. Hum Mol Genet, 12 Spec No 1: R75-88.
Hershkovitz, E. et al., 2008. Homozygous mutation G539R in the gene for P450 oxidoreductase in a family previously diagnosed as having 17,20-lyase deficiency. J Clin Endocrinol Metab, 93(9): 3584-8.
Homma, K. et al., 2006. Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: implication for the backdoor pathway to dihydrotestosterone. J Clin Endocrinol Metab, 91(7): 2643-9.
Horecker, B.L., 1950. Triphosphopyridine Nucleotide-Cytochrome-C Reductase in Liver. Journal of Biological Chemistry, 183(2): 593-605.
http://www.ncbi.nlm.nih.gov/gene/5447.

Huang, N., Agrawal, V., Giacomini, K.M. and Miller, W.L., 2008. Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. Proc Natl Acad Sci U S A, 105(5): 1733-8.

Huang, N. et al., 2005. Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. Am J Hum Genet, 76(5): 729-49.

Hubbard, P.A., Shen, A.L., Paschke, R., Kasper, C.B. and Kim, J.J., 2001. NADPH-cytochrome P450 oxidoreductase. Structural basis for hydride and electron transfer. J Biol Chem, 276(31): 29163-70.

Hughes, A.L. et al., 2007. Dap1/PGRMC1 binds and regulates cytochrome P450 enzymes. Cell Metab, 5(2): 143-9.

Chun, K., Siegel-Bartelt, J., Chitayat, D., Phillips, J. and Ray, P.N., 1998. FGFR2 mutation associated with clinical manifestations consistent with Antley-Bixler syndrome. Am J Med Genet, 77(3): 219-24.

Idkowiak, J. et al., 2010. Pubertal Presentation in Seven Patients with Congenital Adrenal Hyperplasia due to P450 Oxidoreductase Deficiency. J Clin Endocrinol Metab.

Iijima, S., Ohishi, A. and Ohzeki, T., 2009. Cytochrome P450 oxidoreductase deficiency with Antley-Bixler syndrome: steroidogenic capacities. J Pediatr Endocrinol Metab, 22(5): 469-75.

Ilan, Z., Ilan, R. and Cinti, D.L., 1981. Evidence for a new physiological role of hepatic NADPH:ferricytochrome (P-450) oxidoreductase. Direct electron input to the microsomal fatty acid chain elongation system. J Biol Chem, 256(19): 10066-72.

Indra, R. et al., 2014. Modulation of human cytochrome P450 1A1-mediated oxidation of benzo[a]pyrene by NADPH:cytochrome P450 oxidoreductase and cytochrome b5. Neuro Endocrinol Lett, 35(Suppl 2): 105-113.

Iyanagi, T. and Mason, H.S., 1973. Some Properties of Hepatic Reduced Nicotinamide Adenine-Dinucleotide Phosphate - Cytochrome-C Reductase. Biochemistry, 12(12): 2297-2308.

Jensen, K. and Moller, B.L., 2010. Plant NADPH-cytochrome P450 oxidoreductases. Phytochemistry, 71(2-3): 132-141.

Jick, S.S., 1999. Pregnancy outcomes after maternal exposure to fluconazole. Pharmacotherapy, 19(2): 221-222.

Klitz, W. et al., 2010. Genetic differentiation of Jewish populations. Tissue Antigens, 76(6): 442-58.

Kranendonk, M. et al., 2008. Impairment of human CYP1A2-mediated xenobiotic metabolism by Antley-Bixler syndrome variants of cytochrome P450 oxidoreductase. Arch Biochem Biophys, 475(2): 93-9.

Krone, N. et al., 2012. Genotype-phenotype analysis in congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. J Clin Endocrinol Metab, 97(2): E257-67.

Kurzban, G.P. and Strobel, H.W., 1986. Preparation and characterization of FAD-dependent NADPH-cytochrome P-450 reductase. J Biol Chem, 261(17): 7824-30.

Lamb, D.C. et al., 2006. A second FMN binding site in yeast NADPH-cytochrome P450 reductase suggests a mechanism of electron transfer by diflavin reductases. Structure, 14(1): 51-61.

Leclerc, D. et al., 1998. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A, 95(6): 3059-64.
LeHeup, B.P., Masutti, J.P., Droulle, P. and Tisserand, J., 1995. The Antley-Bixler syndrome: report of two familial cases with severe renal and anal anomalies. Eur J Pediatr, 154(2): 130-3.
Lu, A.Y. and Coon, M.J., 1968. Role of hemoprotein P-450 in fatty acid omega-hydroxylation in a soluble enzyme system from liver microsomes. J Biol Chem, 243(6): 1331-2.
Marohnic, C.C. et al., 2011. Mutations of human cytochrome P450 reductase differentially modulate heme oxygenase-1 activity and oligomerization. Arch Biochem Biophys, 513(1): 42-50.
Marohnic, C.C., Panda, S.P., Martasek, P. and Masters, B.S., 2006. Diminished FAD binding in the Y459H and V492E Antley-Bixler syndrome mutants of human cytochrome P450 reductase. J Biol Chem, 281(47): 35975-82.
Massey, V., 1959. The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim Biophys Acta, 34: 255-6.
McCall, A.A., Kirsch, C.F., Ishiyama, G. and Ishiyama, A., 2007. Otologic findings in Antley-Bixler syndrome: a clinical and radiologic case report. Int J Pediatr Otorhinolaryngol, 71(7): 1139-43.
McGlaughlin, K.L., Witherow, H., Dunaway, D.J., David, D.J. and Anderson, P.J., 2010. Spectrum of Antley-Bixler syndrome. J Craniofac Surg, 21(5): 1560-4.
Miller, W.L., 1986. Congenital adrenal hyperplasia. N Engl J Med, 314(20): 1321-2.
Miller, W.L., 2004. P450 oxidoreductase deficiency: a new disorder of steroidogenesis with multiple clinical manifestations. Trends Endocrinol Metab, 15(7): 311-5.
Miller, W.L., Huang, N., Fluck, C.E. and Pandey, A.V., 2004. P450 oxidoreductase deficiency. Lancet, 364(9446): 1663.
Munro, A.W., Noble, M.A., Robledo, L., Daff, S.N. and Chapman, S.K., 2001. Determination of the redox properties of human NADPH-cytochrome P450 reductase. Biochemistry, 40(7): 1956-63.
O'Leary, K.A., Beck, T.W. and Kasper, C.B., 1994. NADPH cytochrome P-450 oxidoreductase gene: identification and characterization of the promoter region. Arch Biochem Biophys, 310(2): 452-9.
Oda, S., Nakajima, M., Toyoda, Y., Fukami, T. and Yokoi, T., 2011. Progesterone receptor membrane component 1 modulates human cytochrome p450 activities in an isoform-dependent manner. Drug Metab Dispos, 39(11): 2057-65.
Oneda, B. et al., 2009. The P450 oxidoreductase genotype is associated with CYP3A activity in vivo as measured by the midazolam phenotyping test. Pharmacogenet Genomics, 19(11): 877-83.
Ono, T. and Bloch, K., 1975. Solubilization and partial characterization of rat liver squalene epoxidase. J Biol Chem, 250(4): 1571-9.
Otto, D.M. et al., 2003. Identification of novel roles of the cytochrome p450 system in early embryogenesis: effects on vasculogenesis and retinoic Acid homeostasis. Mol Cell Biol, 23(17): 6103-16.
Paine, M.J. et al., 2000. Cloning and characterization of a novel human dual flavin reductase. J Biol Chem, 275(2): 1471-8.
Panda, S.P. et al., 2013. Conditional deletion of cytochrome p450 reductase in osteoprogenitor cells affects long bone and skull development in mice

recapitulating antley-bixler syndrome: role of a redox enzyme in development. PLoS One, 8(9): e75638.
Pandey, A.V. and Fluck, C.E., 2013. NADPH P450 oxidoreductase: structure, function, and pathology of diseases. Pharmacol Ther, 138(2): 229-54.
Pandey, A.V., Kempna, P., Hofer, G., Mullis, P.E. and Fluck, C.E., 2007. Modulation of human CYP19A1 activity by mutant NADPH P450 oxidoreductase. Mol Endocrinol, 21(10): 2579-95.
Patki, K.C., Von Moltke, L.L. and Greenblatt, D.J., 2003. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. Drug Metab Dispos, 31(7): 938-44.
Pejznochova, M. et al., 2008. The developmental changes in mitochondrial DNA content per cell in human cord blood leukocytes during gestation. Physiol Res, 57(6): 947-55.
Peterson, R.E., Imperato-McGinley, J., Gautier, T. and Shackleton, C., 1985. Male pseudohermaphroditism due to multiple defects in steroid-biosynthetic microsomal mixed-function oxidases. A new variant of congenital adrenal hyperplasia. N Engl J Med, 313(19): 1182-91.
Phillips, A.H. and Langdon, R.G., 1962. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. J Biol Chem, 237: 2652-60.
Plewka, D. et al., 2000. Neoplastic lesions of the human liver in relation to the activity of the cytochrome P-450 dependent monooxygenase system. Med Sci Monit, 6(2): 244-8.
Polusani, S.R., Kar, R., Riquelme, M.A., Masters, B.S. and Panda, S.P., 2011. Regulation of gap junction function and Connexin 43 expression by cytochrome P450 oxidoreductase (CYPOR). Biochem Biophys Res Commun, 411(3): 490-5.
Porter, T.D., 1991. An unusual yet strongly conserved flavoprotein reductase in bacteria and mammals. Trends Biochem Sci, 16(4): 154-8.
Pursley, T.J., Blomquist, I.K., Abraham, J., Andersen, H.F. and Bartley, J.A., 1996. Fluconazole-induced congenital anomalies in three infants. Clin Infect Dis, 22(2): 336-40.
Reardon, W. et al., 2000. Evidence for digenic inheritance in some cases of Antley-Bixler syndrome? J Med Genet, 37(1): 26-32.
Reisch, N. et al., 2013. Prenatal diagnosis of congenital adrenal hyperplasia caused by P450 oxidoreductase deficiency. J Clin Endocrinol Metab, 98(3): E528-36.
Rendic, S.P. and Guengerich, F.P., 2014. Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Chemicals. Chem Res Toxicol.
Ribes, V. et al., 2007. Rescue of cytochrome P450 oxidoreductase (Por) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis. Dev Biol, 303(1): 66-81.
Sahakitrungruang, T. et al., 2009. Clinical, genetic, and enzymatic characterization of P450 oxidoreductase deficiency in four patients. J Clin Endocrinol Metab, 94(12): 4992-5000.
Saito, Y. et al., 2011. Genetic polymorphisms and haplotypes of por, encoding cytochrome p450 oxidoreductase, in a Japanese population. Drug Metab Pharmacokinet, 26(1): 107-16.

Sandee, D. et al., 2010. Effects of genetic variants of human P450 oxidoreductase on catalysis by CYP2D6 in vitro. Pharmacogenet Genomics, 20(11): 677-86.
Scott, R.R., Gomes, L.G., Huang, N., Van Vliet, G. and Miller, W.L., 2007. Apparent manifesting heterozygosity in P450 oxidoreductase deficiency and its effect on coexisting 21-hydroxylase deficiency. J Clin Endocrinol Metab, 92(6): 2318-22.
Scott, R.R. and Miller, W.L., 2008. Genetic and clinical features of p450 oxidoreductase deficiency. Horm Res, 69(5): 266-75.
Sevrioukova, I.F., Li, H.Y., Zhang, H., Peterson, J.A. and Poulos, T.L., 1999. Structure of a cytochrome P450-redox partner electron-transfer complex. Proceedings of the National Academy of Sciences of the United States of America, 96(5): 1863-1868.
Shackleton, C., Marcos, J., Arlt, W. and Hauffa, B.P., 2004. Prenatal diagnosis of P450 oxidoreductase deficiency (ORD): a disorder causing low pregnancy estriol, maternal and fetal virilization, and the Antley-Bixler syndrome phenotype. Am J Med Genet A, 129A(2): 105-12.
Shen, A.L., O'Leary, K.A. and Kasper, C.B., 2002. Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. J Biol Chem, 277(8): 6536-41.
Shephard, E.A. et al., 1989. Isolation of a human cytochrome P-450 reductase cDNA clone and localization of the corresponding gene to chromosome 7q11.2. Ann Hum Genet, 53(Pt 4): 291-301.
Schacter, B.A., Nelson, E.B., Marver, H.S. and Masters, B.S., 1972. Immunochemical evidence for an association of heme oxygenase with the microsomal electron transport system. J Biol Chem, 247(11): 3601-7.
Schinzel, A., Savoldelli, G., Briner, J., Sigg, P. and Massini, C., 1983. Antley-Bixler syndrome in sisters: a term newborn and a prenatally diagnosed fetus. Am J Med Genet, 14(1): 139-47.
Smith, G.C., Tew, D.G. and Wolf, C.R., 1994. Dissection of NADPH-cytochrome P450 oxidoreductase into distinct functional domains. Proc Natl Acad Sci U S A, 91(18): 8710-4.
Soneda, S. et al., 2011. Proximal promoter of the cytochrome P450 oxidoreductase gene: identification of microdeletions involving the untranslated exon 1 and critical function of the SP1 binding sites. J Clin Endocrinol Metab, 96(11): E1881-7.
Strittmatter, P. and Velick, S.F., 1956. The isolation and properties of microsomal cytochrome. J Biol Chem, 221(1): 253-64.
Subramanian, M. et al., 2012. Effect of P450 oxidoreductase variants on the metabolism of model substrates mediated by CYP2C9.1, CYP2C9.2, and CYP2C9.3. Pharmacogenet Genomics, 22(8): 590-7.
Suzuki, K. et al., 1987. Antley-Bixler syndrome in a sister and brother. Jinrui Idengaku Zasshi, 32(3): 247-52.
Szczesna-Skorupa, E. and Kemper, B., 2011. Progesterone receptor membrane component 1 inhibits the activity of drug-metabolizing cytochromes P450 and binds to cytochrome P450 reductase. Mol Pharmacol, 79(3): 340-50.
Tee, M.K., Huang, N., Damm, I. and Miller, W.L., 2011. Transcriptional regulation of the human P450 oxidoreductase gene: hormonal regulation and influence of promoter polymorphisms. Mol Endocrinol, 25(5): 715-31.
Tomalik-Scharte, D. et al., 2010. Impaired hepatic drug and steroid metabolism in congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. Eur J Endocrinol, 163(6): 919-24.

Tomkova, M. et al., 2012. Identification of six novel P450 oxidoreductase missense variants in Ashkenazi and Moroccan Jewish populations. Pharmacogenomics, 13(5): 543-54.

Vincent, B. et al., 2012. The closed and compact domain organization of the 70-kDa human cytochrome P450 reductase in its oxidized state as revealed by NMR. J Mol Biol, 420(4-5): 296-309.

Wang, M. et al., 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc Natl Acad Sci U S A, 94(5): 8411-6.

Wang, S.L., Han, J.F., He, X.Y., Wang, X.R. and Hong, J.Y., 2007. Genetic variation of human cytochrome P450 reductase as a potential biomarker for mitomycin C-induced cytotoxicity. Drug Metabolism and Disposition, 35(1): 176-179.

Williams, C.H., Jr. and Kamin, H., 1962. Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. J Biol Chem, 237: 587-95.

Woillard, J.B. et al., 2013. Effect of CYP3A4*22, POR*28, and PPARA rs4253728 on Sirolimus In Vitro Metabolism and Trough Concentrations in Kidney Transplant Recipients. Clin Chem.

Woodcock, J. and Lesko, L.J., 2009. Pharmacogenetics--tailoring treatment for the outliers. N Engl J Med, 360(8): 811-3.

Xia, C. et al., 2011. Structural basis for human NADPH-cytochrome P450 oxidoreductase deficiency. Proc Natl Acad Sci U S A, 108(33): 13486-91.

Yasui, Y. et al., 1983. The first case of the Antley-Bixler syndrome with a consanguinity in Japan. Jinrui Idengaku Zasshi, 28(3): 215-20.

Yasukochi, Y. and Masters, B.S.S., 1976. Some Properties of a Detergent-Solubilized Nadph-Cytochrome C (Cytochrome-P-450) Reductase Purified by Biospecific Affinity Chromatographrome P-450) Reductase Purified by Biospecific Affinity Chromatography. Journal of Biological Chemistry, 251(17): 5337-5344.

Yu, L.J. et al., 2001. P450 enzyme expression patterns in the NCI human tumor cell line panel. Drug Metab Dispos, 29(3): 304-12.

Zhang, X., Li, L., Ding, X. and Kaminsky, L.S., 2011. Identification of cytochrome P450 oxidoreductase gene variants that are significantly associated with the interindividual variations in warfarin maintenance dose. Drug Metab Dispos, 39(8): 1433-9.

Zhao, Q. et al., 1999. Crystal structure of the FMN-binding domain of human cytochrome P450 reductase at 1.93 angstrom resolution. Protein Science, 8(2): 298-306.