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Pharmacogenomics of human liver cytochrome P450 oxidoreductase: multifactorial analysis and impact on microsomal drug oxidation

Aims: NADPH:CYP oxidoreductase (POR) is an essential component of several enzyme systems, including the microsomal CYP monooxygenases. We investigated genetic and nongenetic POR variability and its impact on drug-oxidation activities in human liver microsomes. **Material and methods:** POR mRNA, protein and activity, as well as ten major drug-oxidation activities, were measured in the microsomes of 150 Caucasian surgical liver samples. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometric assays were established to determine the frequency of 46 selected *POR* SNPs. Multivariate log-linear regression models, including main effects and two-way interaction terms, and analyses of variance were used to identify statistically significant relationships. **Results:** POR phenotypes were less variable within the study population as compared with CYP phenotypes. Intronic SNPs g.18557G>A (intron 2), g.25676C>T (intron 3) and g.30986 G>A (intron 10) were associated with various drug-oxidation activities. The common allele *POR**28 (A503V) was not associated with any activity or expression changes. Haplotype analysis identified two novel composite alleles *POR**36 (P228L plus A503V) and *POR**37 (A503V plus V631I). **Conclusion:** Models that integrate POR and microsomal CYP function are complex and depend on the CYP isozyme, the substrate and numerous genetic and nongenetic factors. Intronic *POR* variants may influence microsomal CYP activities. These data provide a basis for further studies towards inclusion of *POR* polymorphisms in pharmacogenomic strategies.

KEYWORDS: atorvastatin • cytochrome P450 • liver microsomes • MALDI-TOF mass spectrometry • multiplex-genotyping • P450-oxidoreductase • pharmacogenetics/genomics • SNP

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The microsomal flavoprotein, NADPH:CYP oxidoreductase (POR), is an essential component of several oxygenase enzyme complexes including, for example, heme oxygenase, squalene monooxygenase, 7-dehydrocholesterol reductase and in particular all microsomal CYP monooxygenases, to which it transfers electrons originating from NADPH ([1,2] reviewed in [3]). Most of the 50 human microsomal CYPs are either involved in physiological functions, such as biosynthesis or catabolism of steroid hormones, bile acids, fatty acids or eicosanoids, or they catalyze the oxidative metabolism of xenobiotics including most drugs in clinical use [4]. In addition, POR was also found to directly bioactivate a number of anticancer prodrugs [3]. In contrast to the multiplicity of *CYP*s, mammals have only a single *POR* gene [5]. In humans it is located on chromosome 7q11.2 and spans approximately 72 kb, coding for a protein with 680 residues, which includes FAD and FMN domains as well as an NADPH binding and a hinge domain [6]. Complete deletion of the *Por* gene in mouse is embryonically lethal most likely due to deficient adrenocortical steroidogenesis [7,8]. By contrast, liver-specific knockout

of *Por* yields phenotypically and reproductively normal mice that accumulate hepatic lipids and have a drastically diminished capacity for hepatic drug metabolism [9,10]. In recent years, several *POR* missense mutations in humans were discovered that cause disordered steroidogenesis, ambiguous genitalia and Antley-Bixler syndrome [11–13]. Moreover, numerous common polymorphisms in the human *POR* were discovered that are present in various frequencies in different populations [14].

In human liver, the POR protein is expressed at severalfold lower stoichiometric amounts than the microsomal CYPs, giving rise to speculation that it might be a limiting factor for monooxygenase functions under certain circumstances [15,16]. Polymorphisms in human *CYP* genes have been intensely investigated over more than 20 years and their impact on drug metabolism and drug efficacy is well established [17,18]. By contrast, the question of whether common polymorphisms may have a role in interindividual variability of POR and xenobiotic metabolism has only recently been addressed. Using recombinant enzyme-coexpression systems, the functional consequences of at least 35 known

POR missense mutations on steroidogenic and drug-metabolism activities have been investigated [19–21]. Taken together, these studies revealed that some *POR* mutants elicit different functional effects depending on the CYP enzyme and substrate assayed [22,23]. However, most of the variants studied are very rare and common polymorphisms including the A503V polymorphism, which is found at frequencies varying from approximately 20–40% in various populations, had only minor effects on drug-oxidation activities [19]. Although highly valuable to study principal effects of nonsynonymous mutations on *POR* function, results from recombinant studies are difficult to extrapolate to the *in vivo* situation. In order to estimate the potential of *POR* polymorphisms as a source of variation for drug-metabolism activity, studies in human liver microsomes have advantages because they include the proteins in their natural environment. Additional variables such as P450 isoenzyme levels, *CYP* polymorphisms and nongenetic factors that are also present in this system can be appropriately dealt with by multivariate analysis tools. The only study in this direction, to our knowledge, investigated the variable expression and function of *POR* in relation to *POR* polymorphisms and drug metabolizing CYP activities in 99 human liver samples [24]. It was shown that microsomal *POR* and CYP activities are correlated to some extent, but common *POR* polymorphisms were not found to be related to the variable CYP activities, with the exception of a rather rare variant L577P that decreased *POR* and several CYP activities. Nevertheless, the authors suggested that common *POR* polymorphisms could affect CYP-catalyzed drug-oxidation activities thus serving as an additional biomarker for pharmacogenetic testing [24].

In this research article we describe analyses that extend the studies of *POR* expression and function in a large and well-documented human liver bank. We investigated a large selection of rare and common *POR* sequence variations, several of which had not been studied before. Their impact on drug-oxidation phenotypes was investigated by measuring additional drug-oxidation marker activities and by analyzing the data by multivariate methods, considering various *CYP* genotypes as well as several nongenetic confounding factors. The results demonstrate again that human *POR* is a highly polymorphic gene and they suggest complex relationships between *POR* and the different monooxygenase functions, each of which is best described by a different set of genetic and nongenetic factors.

In particular, we identified three intronic *POR* variants that significantly affected atorvastatin hydroxylation, a marker of CYP3A4 activity, in concert with the donor's sex. Whether *POR* can prove to become a predictive pharmacogenetic factor will depend on *in vivo* validation studies.

Materials & methods

■ General reagents & methods

Triethylammonium acetate buffer (TEAA) and acetonitril were purchased from Transgenomic (NE, USA) and Carl Roth GmbH+Co KG (Karlsruhe, Germany), respectively. Cytochrome C from horse heart, β -nicotinamide adenine dinucleotide phosphate reduced form as tetrasodium salt (NADPH) and sodium dihydrogen phosphate dihydrate ($\text{H}_2\text{NaPO}_4 \cdot 2\text{H}_2\text{O}$) were purchased from Sigma-Aldrich (MO, USA). Potassium cyanide (KCN) and disodium hydrogen phosphate dihydrate ($\text{HNa}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) were purchased from Merck EMD (Darmstadt, Germany). A Bradford assay was used for total protein content determination using bovine serum albumin as standard protein. Metabolite standards and internal standards were purchased from Toronto Research Chemicals (Ontario, Canada) and phenacetin from Fluka (Buchs, Switzerland). Oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany), Metabion International AG (Martinsried, Germany) or Applied Biosystems (CA, USA). All other chemicals were obtained at highest available grade from Sigma Aldrich.

■ Patient DNA & liver samples

Liver tissue and corresponding blood samples were previously collected [25] from patients undergoing liver surgery at the Department of General, Visceral and Transplantation Surgery (Nuessler AK and Neuhaus P, Campus Virchow, University Medical Center Charité, Humboldt University in Berlin, Germany). All tissue samples had been examined by a pathologist and only histologically normal liver tissue was used. Clinical patient documentation for all samples included age, sex, medical diagnosis, presurgical medication, alcohol and smoking habits (SUPPLEMENTARY TABLE 1). Samples from patients with hepatitis, cirrhosis or chronic alcohol use were excluded. A total of 150 liver samples from which high quality RNA and complete documentation could be obtained were finally included. The preparation of human liver microsomes used to determine microsomal phenotypes had been described before [26]. All samples have been

genotyped for major polymorphisms of *CYP2B6*, *CYP2C9*, *CYP2C19* and *CYP2D6* [26,27]. *CYP* genotypes taken into account were *2B6**6 and *11, *2C9**2 and *3, *2C19**2 and *2D6**2, *3, *4, *5, *6, *9, *10, *41 and gene-copy number. Genotype was digitized according to functional gene copy (i.e., the nonfunctional or low-functional alleles were counted as 0 and each allele of normal function was counted as 1), resulting in genotypes 0, 1 and 2 for *CYPs 2B6*, *2C9* and *2C19*, and 0, 1, 2 and 3 for *CYP2D6*.

■ Determination of microsomal enzyme activities

Cytochrome C reductase activity was determined with 25 µg of microsomal protein incubated for 3 min at 25°C in 300 mM sodium phosphate buffer (pH 7.7), 1 mM KCN and 40 µM cytochrome C. The reaction was started by the addition of 100 µM NADPH. Reduced cytochrome C was determined at 550 nm using an Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, CA, USA) for 3 min, essentially as described by [28–30]. The extinction coefficient (ϵ) of 21 mM⁻¹cm⁻¹ was used for the calculation of specific enzyme activity which was expressed as µmol of cytochrome C reduced per mg of microsomal protein per min.

Marker activities selective for individual *CYP* isozymes were determined in individual assays carried out at least in duplicate by incubation of 20–100 µg microsomal protein in an NADPH-regenerating system and the respective substrate (TABLE 1). Conditions for enzymatic assays and mass spectrometric detection have been published previously [31] for coumarin *O*-deethylation, bupropion hydroxylation, verapamil *N*-demethylation, *S*-mephenytoin 4'-hydroxylation and propafenone 5-hydroxylation. *CYP2C9* activity was determined using the commercially available P450-Glo™ assay (Promega, WI, USA) with luciferin-H as substrate. A total of 20 µg of microsomal protein was incubated for 30 min at 37°C in 96 well plates in triplicate according to the instructions by the manufacturer. Calibration curves were measured on each plate with D-luciferin (Promega; 0.8–100 pmol) as standard. Luminescence was measured with a Victor 1420 multilabel counter (Perkin Elmer Wallac, MA, USA). Activity was calculated as pmol luciferin produced per mg microsomal protein per min.

All other *CYP* activity assays were based on the HP 1100 LC-ESI-MS system (Agilent Technologies) using appropriate isotope-labeled compounds as internal standards. Details will be

published elsewhere [Turpeinen *et al.*, Unpublished data]. In brief, microsomal incubations were carried out in 0.1 M potassium phosphate buffer (pH 7.4), NADPH-generating system (5 mM MgCl₂, 4 mM glucose 6-phosphate, 0.5 mM NADP⁺, and 4.0 U/ml glucose 6-phosphate dehydrogenase) and a specified concentration of the substrate for each assay (TABLE 1). Formation of all metabolites was verified to be within linear range under these assay conditions. Reaction mixtures were preincubated for 2 min at 37°C in a water bath before reaction was initiated by addition of the NADPH-generating system. Each reaction was terminated after specified incubation period by adding 100 µl of ice-cold acetonitrile containing the internal standard. Samples were subsequently cooled in an ice bath and centrifuged at 16,000 g for 10 min. Supernatants were collected and stored at -20°C until analyzed.

■ Quantification of POR protein

POR protein was quantified in human liver microsomes by Western blotting. In brief, 25 µg of microsomal protein was separated on 10% sodium dodecyl sulfate/polyacrylamide gels and transferred to nitrocellulose sheets using standard methods. Detection of POR was performed by incubation with rabbit anti-POR polyclonal antibody raised against affinity-purified rat POR [Zanger UM & Meyer UA, University of Basel, Unpublished Data] and secondary IR Dye 680® goat anti-rabbit antibody using the infrared imaging system Odyssey (LI-COR Biosciences, NE, USA). Recombinant CYP3A4 lymphoblast microsomes (BD Biosciences, NJ, USA) were co-analyzed as standards on each blot (range of 2–0.125 pmol). The POR content in the lymphoblast microsomes had been determined using purified rat POR as the standard [32]. The amount of protein present in each sample was calculated by non-linear regression of the integrated intensities of the standards, using the Odyssey software version 2.1beta. All samples were measured at least in duplicate and mean values were calculated and expressed in pmol per mg of microsomal protein. Microsomal total P450 content was determined in all 150 samples by reduced carbon monoxide-difference spectroscopy [33].

■ Preparation of RNA, reverse transcription & quantitative PCR

Total RNA from liver tissue was prepared using Trizol (Invitrogen, CA, USA), extraction followed by RNeasy MiniKit (Qiagen, Venlo, The Netherlands) with on-column DNase I treatment according to the supplier's

Table 1. Marker activities for microsomal CYPs and POR determined in human livers.

Protein	Substrate (conc., μM)	Product	Median (pmol/mg*min) min–max, ratio max/min	Spearman coefficient (p-value)	Ref.
POR	Cytochrome C (40)	Reduced cytochrome C	138977 76136.0–257924, 3.39	0.47 (<0.001)	[28]
CYP1A2	Phenacetin (25)	Acetaminophen	1442 130.1–4536, 34.9	0.27 (<0.001)	[45]
CYP2A6	Coumarin (200)	7-OH-coumarin	1088 96.8–3624, 37.4	0.25 (0.002)	[31,46]
CYP2B6	Bupropion (50)	Hydroxybupropion	93.1 5.56–1332, 240	0.24 (0.004)	[31,47]
CYP2C8	Amodiaquine (2)	<i>N</i> -desethylamodiaquine	1213 204–3212, 15.7	0.41 (<0.001)	[48]
CYP2C9	Luciferin-H (100)	6'-deoxyluciferin	24.1 5.09–148, 29.1	0.39 (<0.001)	[49]
CYP2C19	S-mephenytoin (200)	4'-OH-mephenytoin	48.9 0.63–274, 435	0.15 (0.062)	[31]
CYP2D6	Propafenone (5)	5-OH-propafenone	87.0 0–433, >1000	0.26 (0.002)	[31,50]
CYP2E1	Chlorzoxazone (75)	6-OH-chlorzoxazone	163.4 21.5–860, 40	0.23 (<0.005)	[51]
CYP3A4	Verapamil (50)	Norverapamil (<i>N</i> -desmethylverapamil)	551.1 120–2144, 17.9	0.41 (<0.001)	[31,52]
CYP3A4	Atorvastatin (35)	Ortho-OH-atorvastatin	188.2 0.31–1397, 4506	0.47 (<0.001)	[53]

Spearman coefficients refer to the correlations between individual P450 marker activities and the cytochrome C reductase activity which was correlated to the total spectral P450 content of the microsomal samples. See Materials and methods section for further details.
Conc.: Concentration; POR: NADPH:CYP oxidoreductase.

instructions. RNA quality was controlled with the Agilent Bioanalyzer using the RNA6000 Nano LabChip® Kit (Agilent Technologies). Only RNA preparations with a RNA integrity number higher than seven were used for further analyses. Each sample was reverse transcribed using 1 μg of RNA, random hexamers and the TaqMan® Reverse Transcription Kit (Applied Biosystems) according to the supplier's instructions. Relative quantification of POR mRNA was performed using the standard protocol for the 7500 Real-Time PCR system (Applied Biosystems), primers (TQ-hCPR-628F: 5'-GACGACGATGGGAAGCTTGA-3'; TQ-hCPR-762R: 5'-GGTGTGGACCACAA-GCTCGTA-3') and probe (hCPR_MGB: 5'-VIC-AGGAGTCCAGCATTCGC-3'-MGB). POR mRNA levels were normalized to the 18S content determined using predeveloped TaqMan assay (Applied Biosystems). The full length POR-cDNA used for the standard curve was kindly provided by Jeroen Buters (Technical University of Munich, Germany).

■ DHPLC & sequencing

Fragments which included exons and at least 50 bases of adjacent intronic regions were amplified and subjected to denaturing high

performance liquid chromatography (DHPLC) analysis using a WAVE® System (Transgenomic). Primers (SUPPLEMENTARY TABLE 2) were designed using software from Transgenomic and OligoPerfect™ Designer (Invitrogen, CA, USA). Mobile phase gradient and denaturing temperatures were calculated by WaveMaker software v4.1. DHPLC results were confirmed and complemented by sequencing in a 4300 DNA Analyzer (LI-COR Inc., USA) using infrared-800 labelled nested primers and Thermo sequenase fluorescent labelled primer cyclor sequencing kit with 7-deaza-dGTP (GE Healthcare, Chalfont St. Giles, UK). Sequences were analyzed with e-Seq™ DNA Sequencing software (v3.0) and Vector NTI Advance 10.1.1 (Invitrogen Corporation). In some instances, sequencing was performed with amplification primers and BigDye® Terminator v1.1 (Applied Biosystems) in an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) and Vector NTI Advance 10.1.1 was used for sequence analysis.

■ Electronic database information & nomenclature

GenBank [101] accession number NT_007933 was used as the *POR* genomic reference sequence (referred to as *POR*1A*) and NM_000941.2 was

used as cDNA reference. The genomic sequence was used as reference in case of inconsistent nucleotides between both reference sequences. Numbering and nomenclature of *POR* alleles were consistent with the CYP allele nomenclature homepage [102] with the adenine of the first translated ATG being numbered +1 (the *POR* untranslated exon is not considered). cDNA positions (c.) refer to the same numbering system except that intronic sequences are disregarded. To select known SNPs of *POR*, the publicly available databases of the National Institute of Biological Information dbSNP [103] and of BioVentures Inc. (TN, USA [104]) were accessed through the internet.

■ MALDI-TOF MS genotyping

MassARRAY® Assay Design (version 3.0.0) was used in combination with manual adjustment for the design of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) primers. All MALDI-TOF MS amplification primers had a tag sequence (ACGTTGGATG) to permit a more efficient amplification as suggested by the used software. Three different assays were developed to include all 46 DNA variations analyzed (SUPPLEMENTARY TABLE 3). Each assay consisted of a pre-amplification by PCR, shrimp alkaline phosphatase (SAP) treatment, iPLEX™ primer extension and a clean resin step. Multiplex PCRs (performed in a 384 well microtiter plates, final volume 8 µl) contained 50 ng of DNA, 2.5 U/µl of HotstarTaq® Polymerase from HotstarTaq Master Mix (Qiagen), 0.125 µM of primers and 1.41 mM of MgCl₂ (Qiagen). PCR conditions were: denaturing step at 94°C for 15 min followed by 45 cycles at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. The excess of dNTPs was dephosphorylated in a final volume of 10 µl with 0.03 U/µl of SAP in 0.17 × SAP Buffer (Sequenom, CA, USA), at 37°C for 20 min followed by 10 min at 85°C and 20°C for 1 s. Following addition of 0.25 µl of iPLEX Buffer, 0.25 µl of iPLEX Terminator Mix, and 0.05 µl of iPLEX Enzyme (iPLEX Gold Reaction Kit, Sequenom), extension primers were added to a final concentration of 0.467 or 0.933 µM (final volume of 12 µl) depending on whether the primer mass was lower or higher than 6000 Da, respectively, in order to adjust for variable signal:noise ratios. Extension reaction conditions were: 94°C for 30 s; 40 cycles consisting of a 94°C step for 5 s and five subcycles of 52°C

for 5 s and 80°C for 5 s; and a final extension at 72°C for 3 min. A total of 6 mg of Clean Resin (Sequenom) and 16 µl of water were added to the reactions followed by incubation for 20 min to remove the salts and a 20 min centrifugation at 4000 rpm. For MALDI-TOF MS analysis, samples were dispensed onto a 384 SpectroCHIP® Array (Sequenom) using a nanodispenser, and introduced into a MassARRAY Compact mass spectrometer (Sequenom). Automated spectra acquisition was performed using Spectroacquire and data analysis was performed with MassArray Typer software version 3.4. Samples not automatically assigned were determined manually.

■ Molecular haplotype confirmation

Two methods were applied to investigate molecular haplotypes. The DNA sample was diluted and approximately 3 pg of genomic DNA were used as template for multiplex amplification of a single DNA copy (M1-PCR) followed by MALDI-TOF MS analysis as described [34]. The haplotype of allele *POR**36 was also confirmed by allele-specific amplification. Reverse transcription was performed as described above except the synthesis step, which was carried out at 25°C for 10 min, at 48°C for 90 min and at 95°C for 5 min. cDNA (template) and the full length *POR*-cDNA (positive control) were amplified and sequenced. To verify the sequence at position c.1508 (TABLE 2), either the reference primer 228C_Fw (cDNA forward) (SUPPLEMENTARY TABLE 2) or the variant primer 228T_Fw (cDNA forward) was used in combination with the reverse primer *POR*_18b (cDNA reverse) for amplification and the product was then sequenced. For position c.683 either the reference primer 503C_Rv (cDNA reverse) or the variant primer 503T_Rv (cDNA reverse) was used together with *POR*_5 (cDNA forward) accordingly.

■ Computational genetic analyses

Observed and expected allele and genotype frequencies within populations were tested for deviation from Hardy–Weinberg equilibrium using the online DeFinetti program [105]. Haplotype computations were performed with the assistance of the PHASE program version 2.1.1, obtained from homepage [106] and Haploview version 3.32 [107] was used to create linkage disequilibrium (LD) plots. Sequence variations were described according to the recommendations for the description of sequence variants in DNA [35,36].

Table 2. Characteristics and variant allele frequencies of *POR* SNPs investigated in this study.

SNP	Genomic (g.) position	cDNA (c.) position	Residue change	Region	SNP ID	VAF (this study)	VAF (published)	Ref.
1	15 A>G	15 A>G	5Gly	E#01	rs10262966	0.007	0.01–0.045	[103]
2	18557 G>A	237 +88G>A		I#02	rs17148944	0.087	0.08–0.10	[103]
3	18646 C>G	237 +177C>G		I#02	BV12003	0.000	0.03	[104]
4	25364 G>T	238 -95G>T		I#02	rs10225188	0.360	0.50	[103]
5	25565 C>T	344 C>T	Ala115Val	E#03		0.000	Rare	[8]
6	25600 C>T	366 +13C>T		I#03	BV12005	0.000	0.01	[104]
7	25676 C>T	366 +89C>T		I#03	rs10239977	0.337	0.36–0.47	[103]
8	25723 C>T	366 +136C>T		I#03	rs41299478	0.003	0.02	[103]
9	26367 A>G	387 A>G	129Pro	E#04	rs1135612	0.260	0.19–0.27	[103]
10	26404 A>G	424 A>G	Thr142Ala	E#04		0.000	Rare	[8]
11	26438 A>G	458 A>G	Gln153Arg	E#04		0.000	Rare	[8]
12	27080 T>G	541 T>G	Tyr181Asp	E#05		0.000	Rare	[33]
13	27210 C>T	641 +30C>T		I#05	BV12009	0.000	0.02	[104]
14	27498 G>A	642 -27G>A		I#05	rs2286820	0.017	0.010	[103]
15	27566 C>T	683 C>T	Pro228Leu	E#06	rs17853284	0.003	0.01–0.05	[103]
16	27615 G>A	731 +1G>A		I#06		0.000	Rare	[7]
17	27839 G>A	731 +225G>A		I#06	rs10954732	0.660	0.66–0.68	[103]
18	28045 C>T	732 -187C>T		I#06	rs2286821	0.667	0.70	[103]
19	28287 A>G	787 A>G	Met263Val	E#07		0.000	Rare	[8]
20	28446 C>T	830 +116C>T		I#07	rs3815455	0.323	0.23–0.28	[103]
21	29493 C>T	831 -35C>T		I#07	rs41301394	0.310	0.18–0.27	[103]
22	29556 G>C	859 G>C	Ala287Pro	E#08		0.000	Rare	[7]
23	29561 A>G	864 A>G	288Ala	E#08	rs2228103	0.000	0.02	[103]
24	29595 G>A	898 G>A	Glu300Lys	E#08	rs11540674	0.000	Rare	[103]
25	29782 C>T	984 C>T	328Ala	E#09		0.007	0.002	DHPLC
26	30954 C>T	1236 C>T	412Ser	E#10	rs41301424	0.000	0.00	[103]
27	30955 G>A	1237 G>A	Gly413Ser	E#10	BV12020	0.000	0.01	[104]
28	30978 C>T	1248 +12C>T		I#10	rs2286822	0.293	0.25–0.36	[103]
29	30986 G>A	1248 +20G>A		I#10	rs2286823	0.297	0.29–0.37	[103]
30	31192 T>C	1375 T>C	Tyr459His	E#11		0.000	Rare	[8]
31	31467 G>A	1399 -120G>A		I#11	rs2302429	0.207	0.20	[103]
32	31643 T>C	1455 T>C	485Ala	E#12	rs2228104	0.990	0.92–0.99	[103]
33	31663 T>A	1475 T>A	Val492Glu	E#12	rs28931606	0.000	Rare	[103]
34	31696 C>T	1508 C>T	Ala503Val	E#12	rs1057868	0.303	0.22–0.26	[103]
35	31698 G>A	1510 G>A	Gly504Arg	E#12	BV12031	0.000	0.01	[104]
36	31803 G>C	1615 G>C	Gly539Arg	E#12		0.000	Rare	[8]
37	31955 T>C	1694 T>C	Leu565Pro	E#13		0.000	Rare	[8]
38	31977 G>A	1716 G>A	572Ser	E#13	rs1057870	0.363	0.31–0.47	[103]
39	32173 G>T	1822 G>T	Val608Phe	E#14		0.000	Rare	[7]
40	32197 C>T	1846 C>T	Arg616Stop	E#14		0.000	Rare	[8]
41	32242 G>A	1891 G>A	Val631Ile	E#14		0.007	Rare	[8]
42	32383_32385 delTTC	1937_1939 delTTC	646Phe del	E#15		0.000	Rare	[8]
43	32696 C>G	2250 C>G		3'-UTR	rs41302348	0.000	0.01	[103]
44	32795 G>A	2349 G>A		3'-UTR	rs17685	0.300	0.20–0.24	[103]
45	32796 T>G	2350 T>G		3'-UTR	BV12040	0.000	0.03	[104]
46	32861 G>A	2415 G>A		3'-UTR	rs2286824	0.000	0.00	[103]

Frequency data were compiled from [11,12,14,24,40,103,104].

Genomic positions refer to *POR* reference sequence NT_007933 and cDNA positions refer to cDNA NM_000941.2 where (+) represents the number of nucleotides to the right of the last base translated in the genomic sequence and (-) represents the number of nucleotides to the left of the first base translated in the genomic sequence for intronic variants.

Del: Delete; DHPLC: Denaturing high performance liquid chromatography; E: Exon; I: Intron; VAF: Variant allele frequency.

■ Statistics

Statistical analyses were performed with the statistics software packages SPSS, version 16.0.2 (SPSS Inc., IL, USA) and R, version 2.6.2 [108]. Spearman rank correlation coefficients (r_s) were calculated to study the associations between *POR* and CYP enzyme activities. We performed Kruskal–Wallis rank sum tests to detect univariate associations between the nongenetic factors (age, sex, smoking history, drinking history and drug history) and the phenotypes for *POR* mRNA, *POR* protein and cytochrome C reductase as well as the ten measured microsomal CYP enzymes activities. The resulting p-values were adjusted for multiple testing with the method of Benjamini and Hochberg [37].

We applied multivariate log-linear regression models and analyses of variance in order to assess the combined effect of polymorphisms and nongenetic factors on the microsomal phenotypes. The initial models included all mentioned nongenetic factors, 16 of the 21 *POR* SNPs that occurred with at least five variant alleles in the population, and the genetic polymorphisms of CYPs 2B6, 2C9, 2C19 and 2D6 as main effects, as well as selected two-way interaction terms. These two-way interaction terms were chosen by comparing all possible full two-factor log-linear models. A stepwise model selection was then performed by the Akaike Information Criterion [38] to optimize the predictive power of each model, with the restriction that all the nongenetic confounding factors were always incorporated. All statistical tests were two-tailed and statistical significance was defined as $p < 0.05$.

Results

■ Microsomal *POR* & CYP phenotypes & impact of nongenetic factors

The average molar ratio between spectrally detected microsomal P450 and *POR* protein quantified as protein using a specific antibody in 150 human liver samples was 7.1 (range: 2:1–27:1) confirming that *POR* is expressed at substoichiometric levels thus potentially limiting P450-dependent monooxygenase reactions. Cytochrome C reductase activity as a nonphysiological surrogate marker of *POR* function and *POR* protein were significantly correlated ($r_s = 0.54$; $p < 0.0001$). Owing to the unknown and unpredictable nature of P450:*POR* interactions and because previous studies investigated only selected enzyme activities we measured marker activities for all major microsomal drug-metabolizing CYPs (i.e., 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4/5 [TABLE 1]).

Univariate analysis was performed to investigate whether sex, age, alcohol drinking and smoking habits or presurgical drug exposure were associated with CYP or *POR* phenotypes. Of note, we found that smoking donors had significantly increased CYP1A2 activity and that female donors had increased CYP3A4 activity, confirming previous findings (data not shown; [25,39]). Although women appeared to express slightly more *POR* protein (60.0 ± 29.6 pmol/mg, average $\pm$ standard deviation [SD]) than men (51.9 ± 29.9 pmol/mg) this was not statistically significant and no other significant associations were found between documented nongenetic factors and any of the *POR* phenotypes measured (see below for multivariate analyses).

■ *POR* polymorphisms & allele frequencies

We initially screened a subset of 53 samples selected for *POR* expression and/or activity in the higher or lower range systematically for sequence variations by DHPLC. All variants detected were described either in previous publications or in publicly available databases [12,14,24,40,102–104]. A total of 46 mutations and polymorphisms in exonic (seven silent, 19 nonsynonymous), intronic (16) and 3'-UTR (4) gene regions were then selected for complete analysis based on functional aspects and previously reported population frequencies. We developed a highly multiplexed MALDI-TOF mass spectrometry strategy that permits the detection of these 46 SNPs in three assays (SUPPLEMENTARY TABLE 3). A total of 21 SNPs were detected to be present at least once, including three nonsynonymous SNPs 15 (P228L), 34 (A503V), and 41 (V631I); five silent SNPs, 12 intronic SNPs and one SNP located in the 3'-UTR (TABLE 2). None of the known *POR* amino acid variants known to cause abnormal steroidogenesis [12] was detected confirming that these are very rare mutations not present in the normal population. The amino acid variant L577P, described in the Hart *et al.* study as decreasing *POR* and several CYP drug-metabolism activities [24], was screened by DHPLC in all 150 samples but was not detected.

Allele frequencies of the observed variants were generally similar to published data where available (TABLE 2). Of these, 14 had a variant allele frequency of greater than 10%. The common A503V variant was present at an allele frequency of 30.3%, similar to other studies. The P228L and V631I variants were present in one and two heterozygous individuals, respectively, and

shown to be part of novel composite alleles (see below). No deviations from Hardy–Weinberg equilibrium were observed.

■ Haplotypes & novel alleles

The genotyping results indicated that several SNPs analyzed in this study were tightly linked to each other. Linkage disequilibrium maps constructed by Haploview are presented in FIGURE 1. We used the PHASE program [41] and computationally a total of 24 haplotypes were deduced that we denominated according to the newly adopted CYP allele nomenclature homepage for *POR* [102]. The two most frequent haplotypes *POR*1B* (31.1%) and *POR*1C* (23.1%) were variants of the reference sequence not including any amino acid changes (TABLE 3). The reference sequence *POR*1A* itself was inferred to be present in only one individual with genotype *POR*1A/*28A*. However a total of 15 allelic variants of the reference sequence (i.e., not harboring any amino acid changes) were detected. The second most frequent allele *28 was defined by (A503V) and was found in a total of six variants of which only two were common (*POR*28A*, 17.7% and *POR*28B*, 8.4%).

Two novel combinatorial alleles were deduced by PHASE analysis, which we termed *POR*36* and *POR*37*. The haplotype *POR*37* (A503V + V631I) was directly deduced from one individual homozygous for SNP34 (T) and heterozygous

for SNP41 (G/A) and was confirmed by direct molecular haplotype analysis using M1-PCR in another individual heterozygous at both positions, yielding a frequency of approximately 0.7% in the entire study population. Haplotype *POR*36* could not be directly inferred because the only carrier was heterozygous for both amino acid changing polymorphisms SNP15 (C/T) and SNP34 (C/T). Molecular haplotype analysis by M1-PCR (FIGURE 2) was used to confirm *POR*36* as a novel allele with two amino acid changes (P228L + A503V).

■ Multivariate modeling of microsomal POR- & CYP-activity phenotypes

Multivariate log-linear regression models built in order to model the *POR* phenotypes mRNA, protein, cytochrome C reductase activity, and the ten measured drug-oxidation activities included *POR* SNPs, *CYP* genetic polymorphisms and the nongenetic factors age, sex, smoking history, drinking history and presurgical drug exposure as main factors, as well as possible two-way interaction terms. To reduce complexity of the results we only report results with a significance level of $p < 0.01$. As evident from TABLE 4, the resulting multivariate models were different with respect to the included factors for each phenotype. FIGURE 3 shows the corrected main effects for all significant *POR* SNP associations.

Figure 1. Pairwise linkage disequilibrium of analyzed *POR* SNPs. The graphic was prepared using the program Haploview version 3.32 [107]. Strong linkage disequilibrium is, for example, observed between SNPs 17, 18, 20 and 21 (see also TABLE 2).

Table 3. *POR* haplotypes determined in 150 human liver samples from Caucasian donors.

Haplotype	SNP #																					Predicted frequency
	1	2	4	7	8	9	14	15	17	18	20	21	25	28	29	31	32	34	38	41	44	
POR*1A	A	G	G	C	C	A	G	C	G	C	C	C	C	C	G	G	T	C	G	G	G	0.003
POR*1B	–	–	T	T	–	–	–	–	A	T	–	–	–	–	–	–	C	–	A	–	–	0.311
POR*1C	–	–	–	–	–	G	–	–	A	T	–	–	–	T	A	–	C	–	–	–	–	0.231
POR*28A	–	–	–	–	–	–	–	–	–	–	T	T	–	–	–	A	C	T	–	–	A	0.177
POR*28B	–	A	–	–	–	–	–	–	–	–	T	T	–	–	–	–	C	T	–	–	A	0.084
POR*1D	–	–	–	–	–	–	–	–	A	T	–	–	–	T	A	–	C	–	–	–	–	0.056
POR*1E	–	–	T	–	–	–	–	–	A	T	–	–	–	–	–	–	C	–	A	–	–	0.023
POR*1F	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	C	–	–	–	–	0.019
POR*28C	–	–	–	–	–	–	–	–	–	–	T	T	–	–	–	–	C	T	–	–	A	0.013
POR*28D	–	–	–	–	–	–	A	–	–	–	T	T	–	–	–	A	C	T	–	–	A	0.012
POR*1G	–	–	T	T	–	G	–	–	A	T	–	–	–	–	–	–	C	–	A	–	–	0.010
POR*1H	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	0.007
POR*1I	G	–	T	T	–	–	–	–	A	T	–	–	–	–	–	–	C	–	A	–	–	0.006
POR*37	–	–	–	–	–	–	–	–	–	–	T	T	–	–	–	A	C	T	–	A	A	0.006
POR*1J	–	–	T	T	–	–	–	–	A	T	–	T	–	–	–	–	C	–	A	–	–	0.006
POR*1K	–	–	–	–	–	G	–	–	A	T	–	–	–	–	–	–	C	–	–	–	–	0.005
POR*1L	–	–	–	–	–	–	–	–	–	–	T	T	–	–	–	–	C	–	A	–	–	0.003
POR*1M	–	–	–	–	–	G	–	–	–	T	–	–	–	–	–	A	C	–	–	–	–	0.003
POR*28E	–	–	–	–	–	G	–	–	–	T	–	–	–	–	–	A	C	T	–	–	–	0.003
POR*1N	–	–	–	–	–	G	–	–	A	T	–	–	T	T	A	–	C	–	–	–	–	0.003
POR*28F	–	–	–	–	–	–	A	–	–	T	T	T	–	–	–	A	C	T	–	–	A	0.003
POR*1O	–	–	–	–	–	G	–	–	A	T	–	–	T	–	–	–	C	–	–	–	–	0.002
POR*1P	–	–	–	–	–	G	–	–	A	T	–	–	–	T	–	–	C	–	–	–	–	0.002
POR*36	–	A	–	–	T	–	–	T	–	–	T	T	–	–	–	–	C	T	–	–	A	0.001

Haplotypes were deduced by PHASE (version 2.1.1.) based on genotype data from 150 Caucasian livers (see TABLE 2). Definitive haplotypes directly inferred from the data are shown in bold type. Haplotypes *POR**36 and *POR**37 are novel confirmed alleles (see text).

POR mRNA level was decreased by SNP2 as main effector and by three different two-way interaction terms including also sex and smoking habit (TABLE 4). *POR* protein expression was influenced by SNPs 4 and 44, as well as SNP7 and sex interactions. None of the factors included in the model for cytochrome C reductase activity reached a significance level $p < 0.01$, but several main effects and two-way interaction terms with p -values between 0.01 and 0.05 (not shown in TABLE 4) explained almost 50% of the observed variability. *POR* SNPs identified as main effectors of P450 drug-oxidation phenotypes were SNP2 (*CYP3A4* assayed by atorvastatin); SNP7 (*CYP1A2*, *CYP2C8*, *CYP2C19* and *CYP3A4* by atorvastatin); SNP20 (*CYP2D6*); SNP21 (*CYP2C9*); SNP29 (*CYP2C19* and *CYP3A4* by atorvastatin); and SNP44 (*CYP2E1*). These and additional SNPs also occurred in several two-way interaction terms (TABLE 4). The common SNP34 (A503V) was not a significant effector ($p < 0.01$) in any model and the two novel alleles were not included because they were too rare for meaningful statistical analysis. Calculation

of multiple R^2 (coefficient of determination), which describes the fraction of variance of each phenotype explained by the corresponding log-linear model, revealed that between 37.2% (amodiaquine *N*-desethylation) and 87.4% (propafenone 5-hydroxylation) of the variability in the investigated microsomal monooxygenase activities were explained by the factors included in each model.

Discussion

Since the *POR* enzyme has an essential role for all microsomal CYP-dependent monooxygenase enzyme activities, it is quite surprising that *POR* variability has only recently been the focus of systematic investigations, initially with respect to its role for pathological steroid hydroxylase deficiencies and more recently also with respect to oxidative drug metabolism [11,13,24]. A reason for this is certainly that *POR* was not generally considered as a highly variable gene. In our human liver bank the maximal variability expressed as ratio between the highest and lowest observed cytochrome C reductase activity

Figure 2. MALDI-TOF MS analysis of novel haplotypes *POR36 and *POR**37.** Mass spectra were recorded following M1-PCR. (A) and (B) correspond to the subject heterozygous for both SNP15 (green) and SNP34 (orange) and (C) and (D) correspond to one of the two subjects heterozygous for both SNP34 (orange) and SNP41 (red).

MALDI-TOF MS: Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry; M1-PCR: Multiplex amplification of a single DNA copy; UEP: Unextended primer.

was 3.39-fold (TABLE 1), which was much lower as compared with the mRNA and protein ranges (18.3 and 16.5, respectively), indicating that a part of the cytochrome C activity may not be due to POR. It should be noted that all microsomal POR activity measurements were carried out in the presence or absence of KCN to inhibit cytochrome C oxidase, a known mitochondrial contaminant (see Methods & materials section). Although no difference in activity was observed (data not shown) this does not exclude the possibility that other microsomal components are able to reduce the cytochrome C thus limiting the specificity of this nonphysiological assay, which also does not correlate well with clinical phenotypes of POR deficiency [12]. Comparing mRNA and protein levels also revealed much higher variability of CYPs (generally >100-fold; data not shown) than POR, confirming earlier findings that POR is much less variable in the general population as compared with the hepatic drug-oxidation CYP isoforms [15,42], which may be owing to its essential role for many physiological functions. POR variability was more similar to the overall spectrally determined heme-containing P450, which varied 8.8-fold among all livers (data not shown) as also reflected in a significant correlation between POR protein and P450 content ($r_s = 0.47$; $p < 0.001$; TABLE 1). This is most likely due to co-regulation of *POR* and *CYP* expression in liver, which may be physiologically reasonable in order to avoid limiting amounts of POR in situations where increased monooxygenase activity is required. In agreement with the study by Hart *et al.* we also observed significant correlations between microsomal monooxygenase activities and cytochrome C reductase activity although our results were different in some aspects [24]. In particular we used different marker substrates for CYPs 1A2, 2B6, 2C8, 2C9, 2D6 and 3A4/5 and we observed generally stronger correlations in particular for CYP1A2 (phenacetin vs 7-ethoxyresorufin) and 2D6 (propafenone vs dextromethorphan). Nevertheless, the results of both studies suggest that POR may be more limiting for some CYP isozymes than for others. Similar observations were also reported for steroidogenic CYP activities [22,23,43]. Interestingly, the correlation strength appears to depend on the relative expression of the individual CYP: whereas marker activities for the highly expressed isoforms 3A4 and 2C9 enzymes (which contribute approximately 30% to the total microsomal P450 pool in our liver bank) showed higher

correlation coefficients, those of the less expressed P450s were lower or even nonsignificant (2C19). Whether this observation reflects a causal relationship with mechanistic basis or coregulation between *POR* and major P450 forms (or heme biosynthesis) is an interesting area for future research.

We selected SNPs for genotyping by MALDI-TOF based on various aspects including frequency and potential functional effects from a variety of resources and publications. Many of the already known common *POR* gene polymorphisms were confirmed here and their frequencies were generally in agreement with previous studies (TABLE 2). Based on these data we performed haplotype calculations which revealed that the five most common haplotypes with frequencies greater than 5%, which do not include the reference haplotype *POR**1A, represent in approximately 85% of all haplotypes in Caucasians. The structures of these haplotypes are quite complex although they do not

yet contain promoter variants. As shown by our LD analysis, several of the common SNPs (see FIGURE 1 and TABLE 3) are strongly linked while others including the intronic SNPs 2 and 7 (TABLE 2) occur more independently. These data should be valuable in aiding the development of future *POR* genotyping strategies. Our haplotype analysis also revealed two novel composite alleles both of which include the common amino acid variant A503V plus one additional less common amino acid variant, which were termed *POR**36 and *POR**37. We used a MALDI-TOF based method (M1-PCR) to confirm the novel molecular haplotypes [34]. The P228L variant had been first described on the BioVentures Website and Huang *et al.*, who did not detect it in patients, found moderately reduced protein activity by recombinant expression analysis [12,104]. The V631I variant had been suggested by Huang *et al.* to be a polymorphism based on their detection of the variant in individuals with normal steroid profile and also moderately reduced

Table 4. Analyses of variance results showing the dependency of microsomal phenotypes on *POR* SNPs, *CYP* genotypes and nongenetic factors.

Phenotype	Nongenetic factor (main effect)	<i>POR</i> SNP (main effect)	<i>CYP</i> genotype (main effect)	Two-way interactions	Multiple R ²
POR mRNA	None	SNP2 (p = 0.008)	None	SNP14:alcohol (p = 0.001) SNP17:SNP21 (p = 0.006) Sex:smoking (p = 0.005)	0.460
POR protein	None	None	None	SNP4:SNP44 (p = 0.007) SNP7:sex (p = 0.002)	0.313
POR activity	None	None	None	None	0.488
Phenacetin O-deethylation	Smoking (p = 0.002)	SNP7 (p = 0.005)	None	SNP4:SNP31 (p = 0.001)	0.456
Coumarin O-deethylation	None	None	None	Age:drug exposure (p < 0.001)	0.468
Bupropion hydroxylation	None	None	2B6 (p < 0.001)	SNP20:alcohol (p < 0.001) Sex:drug exposure (p = 0.002)	0.516
Amodiaquine N-demethylation	None	SNP7 (p = 0.001)	None	Sex:age (p = 0.001)	0.372
Luciferin-H oxidation	None	SNP21 (p = 0.001)	2C9 (p = 0.005)	SNP9:2C9 (p = 0.003)	0.517
S-mephenytoin 4'-hydroxylation	Drug exposure (p < 0.001)	SNP7 (p = 0.001) SNP29 (p = 0.003)	2C19 (p = 0.008)	SNP2:SNP4 (p = 0.006) Age:drug exposure (p = 0.004)	0.562
Propafenone 5-hydroxylation	Age (p < 0.001) Smoking (p < 0.001)	SNP20 (p = 0.003)	2D6 (p < 0.001)	SNP7:age (p < 0.001) SNP18:2D6 (p < 0.001) Smoking:2D6 (p < 0.001)	0.874
Chlorzoxazone 6-hydroxylation	None	SNP44 (p = 0.001)	None	SNP28:sex (p = 0.002)	0.407
Verapamil N-demethylation	Sex (p < 0.001)	None	None	SNP20:SNP31 (p = 0.009) SNP21:SNP28 (p = 0.008)	0.493
Atorvastatin ortho-hydroxylation	Sex (p < 0.001)	SNP2 (p < 0.001) SNP7 (p = 0.003) SNP29 (p = 0.006)	None	None	0.607

Only factors (main effects and two-way interactions) with a statistical significance of $p < 0.01$ in the analyses of variance are shown. Multiple R² (coefficient of determination) describes the fraction of variance of each phenotype explained by the corresponding log-linear model. *POR* SNPs with a frequency of less than five observed variant alleles have not been incorporated in these models. Variant *CYP* alleles taken into account included CYP2B6*6 and *11; CYP2C9*2 and *3; CYP2C19*2; and CYP2D6*2, *3, *4, *5, *6, *9, *10, *41 and gene-copy number. For details on nongenetic factors including drug exposure see SUPPLEMENTARY TABLE 1.

Figure 3. Main effects of *POR* SNPs on various phenotypes. Estimates of main effects in different multivariate log-linear models ($\log_2$). Only *POR* SNPs with $p < 0.01$ in the analyses of variance are displayed. The estimates are displayed by diamonds, while the error bars represent the standard deviation. Heterozygotes (1) and variant homozygotes (2) are compared with the homozygous reference (0) (labeled as 1/0 and 2/0).

activity in the recombinant assay [12]. Although these variants were not included in our statistical analyses, inspection of the data revealed that all three samples carrying these haplotypes had comparatively low activities for all CYPs (data not shown). It is possible that the combination of A503V with the rare amino acid variants results in more deficient *POR* proteins as compared with the single variants. None of the other 17 non-synonymous variants tested were present in our population, indicating that *POR* mutations with strong functional impact (including the L577P variant [24]) are very rare and do not contribute significantly to drug-oxidation variability in the general population. The common A503V variant was previously investigated in recombinant systems and found to have moderately decreased function for cytochrome C reductase and steroid 17 α -hydroxylase activities [12] whereas no marked influence on steroid 21-hydroxylase activity [43] or CYP1A2 and CYP2C19 activities [19] was found. Because neither the liver microsomal

study by Hart *et al.* nor our study was able to identify any significant association of this polymorphism with a microsomal *POR*- or CYP-related phenotype, its role as a pharmacogenetic marker appears to be negligible [24].

Because the interaction between the single *POR* protein and the different microsomal CYP enzymes must be expected to depend on the CYP:*POR* ratio, conformational protein determinants and the particular substrate, it is expected that the factors contributing to the variability of a particular drug-oxidation phenotype are not the same in each case. Thus it is not surprising that the models that described each phenotype best were quite different from each other. For example, smoking was identified as a significant main factor for phenacetin *O*-deethylation, in agreement with CYP1A2 being the major enzyme for this conversion, an isozyme well known to be induced in smokers [39]. Smoking was also detected as significant factor for CYP2D6-dependent propafenone

hydroxylation, although the direction of influence was opposite to that of CYP1A2 and the effect was much smaller (detailed analysis of these data will be published separately). A further nongenetic factor identified is the donor's sex, which influenced both verapamil *O*-demethylation and atorvastatin hydroxylation as main factor, confirming our previous findings of increased expression of CYP3A4 in women as compared with men [25]. Smoking and sex also influenced the *POR* phenotypes mRNA and protein, as part of two-way interactions, although no effect on *POR* activity was seen. This may be due to the lower variability of cytochrome C reductase activity compared with *POR* protein and mRNA expression as discussed above.

The fact that several *POR* SNPs had a significant and strong effect on particular CYP-dependent functions but not on *POR* may thus be surprising at first sight but may be explained on this basis. It is conceivable that a *POR* SNP not identified as significant for *POR* itself may lead to a significantly changed *POR*:CYP interaction if actual combination of *POR*–CYP–substrate is particularly sensitive to changes in *POR* expression level. The most consistent SNP identified in this respect is SNP7 (g.25676 C>T) in intron 3. This particular SNP influenced four different monooxygenase activities in a similar fashion, in that it was associated with increased oxidative activity for phenacetin (CYP1A2), amodiaquine (CYP2C8), *S*-mephenytoin (CYP2C19) and atorvastatin (CYP3A4). In all four cases, only homozygous subjects ($n = 18$) were affected whereas no effect was seen on heterozygotes indicating a recessive mode (FIGURE 3). Also remarkable is the influence of SNP29 on *S*-mephenytoin (CYP2C19) and atorvastatin hydroxylation and of SNP2 on *POR* mRNA level and atorvastatin hydroxylation. The effects of these two intronic SNPs were rather strong at least in the homozygous condition (FIGURE 3). Investigations into possible mechanistic explanations for these relationships, which may include alternative splicing, as recently observed for common intronic and silent polymorphisms in the *CYP2D6* and *CYP2B6* genes [27,44], are currently in progress. Taken together, several *POR* SNPs were identified by multivariate analysis (i.e., correcting for CYP polymorphisms and for several nongenetic factors) to influence multiple CYP monooxygenase activities consistently. In particular atorvastatin hydroxylation, which

was the most variable of all CYP activities (>1000-fold), was found to be influenced by three different *POR* SNPs in addition to sex, explaining approximately 60% of its oxidative biotransformation variability in human liver.

Conclusion

The human *POR* gene is highly polymorphic but functionally it appears to be less variable between subjects as compared with CYP-dependent monooxygenases. Thus far, investigated common SNPs in the *POR* gene do not have a pronounced influence on *POR* expression or function. Effects on microsomal drug-oxidation activities are complex, depending on the individual CYP isozyme, the drug substrate and several genetic and nongenetic factors. Multivariate models including the patients' sex, age, drug exposure, alcohol or smoking habits, as well as CYP polymorphic status identified several *POR* intronic SNPs that influenced two or more drug oxidation activities. These may be good candidates for further research into their effects. In addition, the haplotype and LD studies provide a useful basis for developing future pharmacogenomic strategies.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Microsomal POR & CYP phenotypes & impact of nongenetic factors

- Hepatic NADPH:CYP oxidoreductase (POR) phenotypes (mRNA, protein, cytochrome C reductase activity) were less variable within the study population as compared with most CYP-dependent drug oxidation phenotypes but correlated to microsomal total P450 content.

POR polymorphisms & allele frequencies

- Using highly multiplexed matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometric assays the frequency of 46 selected *POR* SNPs was determined and used to construct haplotypes and linkage disequilibrium maps.

Haplotypes & novel alleles

- Two new composite alleles *POR**36 (P228L + A503V) and *POR**37 (A503V + V631I) were identified and confirmed by MALDI-TOF based molecular haplotyping.

Multivariate modeling of microsomal POR- & CYP-activity phenotypes

- Multivariate log-linear regression models investigated main effects and two-way interaction terms. The intronic SNPs g.25676C>T (SNP7, intron 3) and g.30986 G>A (SNP29, intron 10), were significantly associated ($p < 0.01$) with increased microsomal activities of CYPs 1A2, 2C8, 2C19 and 3A4 and of CYPs 2C19 and 3A4, respectively, whereas SNP g.18557G>A (SNP2, intron 2) decreased *POR* mRNA and atorvastatin hydroxylase activity.
- Additional statistically significant influences included genotype for CYPs 2B6, 2C9, 2C19 and 2D6, smoking (CYP1A2), sex (CYP3A4) and several two-way interactions. The common polymorphism A503V was not found to have an effect on any of the measured phenotypes.
- Atorvastatin hydroxylation as the most variable CYP activity was significantly influenced by three intronic *POR* SNPs as well as by sex, owing to the difference in hepatic CYP3A4 expression between males and females.

Conclusion

- The human *POR* gene is highly polymorphic but less variable between subjects as compared with CYP-dependent monooxygenases.
- Multivariate models which included the patients' sex, age, drug exposure, alcohol and smoking habits, as well as common *CYP* polymorphisms identified several *POR* intronic SNPs which influenced different drug oxidation activities.
- SNPs g.25676C>T (intron 3), g.30986 G>A (intron 10) and g.18557G>A (intron 2) may be good candidates for further research into their effects in drug-oxidation activities.

Bibliography

Papers of special note have been highlighted as:

▪ of interest

▪▪ of considerable interest

- Yasukochi Y, Masters BS: Some properties of a detergent-solubilized NADPH-cytochrome C (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* 251(17), 5337–5344 (1976).
- Backes WL: NADPH-cytochrome P450 reductase: Function. In: *Handbook of Experimental Pharmacology (Volume 105)*. Schenkman JB, Greim H (Eds). Springer-Verlag, Germany (1993).
- Hart SN, Zhong XB: P450 oxidoreductase: genetic polymorphisms and implications for drug metabolism and toxicity. *Expert Opin. Drug Metab. Toxicol.* 4(4), 439–452 (2008).
- Lewis DF: 57 varieties: the human cytochromes P450. *Pharmacogenomics* 5(3), 305–318 (2004).
- Yamano S, Aoyama T, McBride OW, Hardwick JP, Gelboin HV, Gonzalez FJ: Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus-mediated expression and localization of the *CYPOR* gene to chromosome 7. *Mol. Pharmacol.* 36(1), 83–88 (1989).
- Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ: Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl. Acad. Sci. USA* 94(16), 8411–8416 (1997).
- Shen AL, O'Leary KA, Kasper CB: Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J. Biol. Chem.* 277(8), 6536–6541 (2002).
- Otto DM, Henderson CJ, Carrie D *et al.*: Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis. *Mol. Cell Biol.* 23(17), 6103–6116 (2003).
- Gu J, Weng Y, Zhang QY *et al.*: Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J. Biol. Chem.* 278(28), 25895–25901 (2003).
- Finn RD, McLaren AW, Carrie D, Henderson CJ, Wolf CR: Conditional deletion of cytochrome P450 oxidoreductase in the liver and gastrointestinal tract: a new model for studying the functions of the P450 system. *J. Pharmacol. Exp. Ther.* 322(1), 40–47 (2007).
- Fluck CE, Tajima T, Pandey AV *et al.*: Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat. Genet.* 36(3), 228–230 (2004).
- Authors reported for the first time mutations in the NADPH:CYP oxidoreductase (*POR*) gene and its influence in activity using recombinant systems.
- Huang N, Pandey AV, Agrawal V *et al.*: Diversity and function of mutations in P450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. *Am. J. Hum. Genet.* 76(5), 729–749 (2005).
- Fluck CE, Pandey AV, Huang N, Agrawal V, Miller WL: P450 oxidoreductase deficiency – a new form of congenital adrenal hyperplasia. *Endocr. Dev.* 13, 67–81 (2008).
- Huang N, Agrawal V, Giacomini KM, Miller WL: Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. *Proc. Natl Acad. Sci. USA* 105(5), 1733–1738 (2008).
- Authors reported *POR* variability in four ethnicities and the influence in activity of some nonsynonymous variants using recombinant systems.
- Schmucker DL, Woodhouse KW, Wang RK *et al.*: Effects of age and gender on *in vitro* properties of human liver microsomal monooxygenases. *Clin. Pharmacol. Ther.* 48(4), 365–374 (1990).
- Guengerich FP: Cytochrome P450: what have we learned and what are the future issues? *Drug Metab. Rev.* 36(2), 159–197 (2004).
- Interesting overview on open problems in the field of cytochromes P450.

- 17 Meyer UA: Pharmacogenetics – five decades of therapeutic lessons from genetic diversity. *Nat. Rev. Genet.* 5(9), 669–676 (2004).
- **Overview on the field of pharmacogenetics/genomics.**
- 18 Zanger UM, Turpeinen M, Klein K, Schwab M: Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Annal. Bioanal. Chem.* 392(6), 1093–1108 (2008).
- 19 Agrawal V, Huang N, Miller WL: Pharmacogenetics of P450 oxidoreductase: effect of sequence variants on activities of CYP1A2 and CYP2C19. *Pharmacogenet. Genomics* 18(7), 569–576 (2008).
- 20 Kranendonk M, Marohnic CC, Panda SP *et al.*: Impairment of human CYP1A2-mediated xenobiotic metabolism by Antley–Bixler syndrome variants of cytochrome P450 oxidoreductase. *Arch. Biochem. Biophys.* 475(2), 93–99 (2008).
- 21 Miller WL, Huang N, Agrawal V, Giacomini KM: Genetic variation in human P450 oxidoreductase. *Mol. Cell Endocrinol.* 300(1–2), 180–184 (2008).
- 22 Dhir V, Ivison HE, Krone N *et al.*: Differential inhibition of CYP17A1 and CYP21A2 activities by the P450 oxidoreductase mutant A287P. *Mol. Endocrinol.* 21(8), 1958–1968 (2007).
- 23 Pandey AV, Kempna P, Hofer G, Mullis PE, Fluck CE: Modulation of human CYP19A1 activity by mutant NADPH P450 oxidoreductase. *Mol. Endocrinol.* 21(10), 2579–2595 (2007).
- 24 Hart SN, Wang S, Nakamoto K, Wesselman C, Li Y, Zhong XB: Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism. *Pharmacogenet. Genomics* 18(1), 11–24 (2008).
- **First report demonstrating correlations between POR and microsomal CYP activities and first *POR* polymorphism analysis in human liver.**
- 25 Wolbold R, Klein K, Burk O *et al.*: Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* 38(4), 978–988 (2003).
- **Best documented example of the influence of sex on microsomal P450.**
- 26 Lang T, Klein K, Fischer J *et al.*: Extensive genetic polymorphism in the human *CYP2B6* gene with impact on expression and function in human liver. *Pharmacogenetics* 11(5), 399–415 (2001).
- 27 Toscano C, Klein K, Blievernicht J *et al.*: Impaired expression of CYP2D6 in intermediate metabolizers carrying the *41 allele caused by the intronic SNP 2988G>A: evidence for modulation of splicing events. *Pharmacogenet. Genomics* 16(10), 755–766 (2006).
- 28 Vermilion JL, Coon MJ: Purified liver microsomal NADPH-cytochrome P-450 reductase. Spectral characterization of oxidation-reduction states. *J. Biol. Chem.* 253(8), 2694–2704 (1978).
- 29 Tamura S, Korzekwa KR, Kimura S, Gelboin HV, Gonzalez FJ: Baculovirus-mediated expression and functional characterization of human NADPH-P450 oxidoreductase. *Arch. Biochem. Biophys.* 293(2), 219–223 (1992).
- 30 Dudka J, Jodynis-Liebert J, Korobowicz E *et al.*: Activity of NADPH-cytochrome P-450 reductase of the human heart, liver and lungs in the presence of (–)-epigallocatechin gallate, quercetin and resveratrol: an *in vitro* study. *Basic Clin. Pharmacol. Toxicol.* 97(2), 74–79 (2005).
- 31 Richter T, Murdter TE, Heinkele G *et al.*: Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J. Pharmacol. Exp. Ther.* 308(1), 189–197 (2004).
- 32 Vermilion JL, Coon MJ: Highly purified detergent-solubilized NADPH-cytochrome P-450 reductase from phenobarbital-induced rat liver microsomes. *Biochem. Biophys. Res. Commun.* 60(4), 1315–1322 (1974).
- 33 Omura T, Sato R: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 239, 2370–2378 (1964).
- 34 Ding C, Cantor CR: Direct molecular haplotyping of long-range genomic DNA with M1-PCR. *Proc. Natl Acad. Sci. USA* 100(13), 7449–7453 (2003).
- 35 Antonarakis SE: Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum. Mutat.* 11(1), 1–3 (1998).
- 36 den Dunnen JT, Antonarakis SE: Nomenclature for the description of human sequence variations. *Hum. Genet.* 109(1), 121–124 (2001).
- 37 Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300 (1995).
- 38 Venables WN, Ripley BD: *Modern Applied Statistics with S*. Springer, Germany (2002).
- 39 Landi MT, Sinha R, Lang NP, Kadlubar FF: Human cytochrome P4501A2. *IARC Sci. Publ.* (148), 173–195 (1999).
- 40 Arlt W, Walker EA, Draper N *et al.*: Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. *Lancet* 363(9427), 2128–2135 (2004).
- 41 Stephens M, Donnelly P: A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am. J. Hum. Genet.* 73(5), 1162–1169 (2003).
- 42 Shephard EA, Palmer CN, Segall HJ, Phillips IR: Quantification of cytochrome P450 reductase gene expression in human tissues. *Arch. Biochem. Biophys.* 294(1), 168–172 (1992).
- 43 Gomes LG, Huang N, Agrawal V, Mendonca BB, Bachega TA, Miller WL: The common P450 oxidoreductase variant A503V is not a modifier gene for 21-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* 93(7), 2913–2916 (2008).
- 44 Hofmann MH, Blievernicht JK, Klein K *et al.*: Aberrant splicing caused by single nucleotide polymorphism c.516G>T [Q172H], a marker of *CYP2B6**6, is responsible for decreased expression and activity of CYP2B6 in liver. *J. Pharmacol. Exp. Ther.* 325(1), 284–292 (2008).
- 45 Tassaneeyakul W, Birkett DJ, Veronese ME *et al.*: Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *J. Pharmacol. Exp. Ther.* 265(1), 401–407 (1993).
- 46 Yamazaki H, Tanaka M, Shimada T: Highly sensitive high-performance liquid chromatographic assay for coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by human liver cytochrome P450 enzymes. *J. Chromatogr. B Biomed. Sci. Appl.* 721(1), 13–19 (1999).
- 47 Faucette SR, Hawke RL, LeCluyse EL *et al.*: Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab. Dispos.* 28(10), 1222–1230 (2000).
- 48 Li XQ, Bjorkman A, Andersson TB, Ridderstrom M, Masimirembwa CM: Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. *J. Pharmacol. Exp. Ther.* 300(2), 399–407 (2002).
- 49 Cali JJ, Ma D, Sobol M *et al.*: Luminogenic cytochrome P450 assays. *Expert Opin. Drug Metab. Toxicol.* 2(4), 629–645 (2006).
- 50 Hofmann U, Pecia M, Heinkele G, Dilger K, Kroemer HK, Eichelbaum M: Determination of propafenone and its phase I and phase II metabolites in plasma and urine by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* 748(1), 113–123 (2000).

51 Peter R, Bocker R, Beaune PH, Iwasaki M, Guengerich FP, Yang CS: Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.* 3(6), 566–573 (1990).

52 Busse D, Cosme J, Beaune P, Kroemer HK, Eichelbaum M: Cytochromes of the P450 2C subfamily are the major enzymes involved in the *O*-demethylation of verapamil in humans. *Naunyn Schmiedebergs Arch. Pharmacol.* 353(1), 116–121 (1995).

53 Jacobsen W, Kuhn B, Soldner A *et al.*: Lactonization is the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin. *Drug Metab. Dispos.* 28(11), 1369–1378 (2000).

■ **Websites**

101 Genbank/NCBI homepage
www.ncbi.nlm.nih.gov

102 The CYP allele nomenclature homepage
www.cypalleles.ki.se/por.htm

103 NCBI dbSNP homepage (Build 129)
www.ncbi.nlm.nih.gov/projects/SNP/

104 BioVentures Inc.
www.bioventures.com/

105 Strom TM and Wienker TF, DiFinetti Program homepage for Hardy–Weinberg equilibrium test
<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>

106 The Matthew Stephens homepage
<http://stephenslab.uchicago.edu/home.html>

107 The Broad Institute haploview homepage
www.broad.mit.edu/mpg/haploview/

108 The R-project for statistical computing
www.r-project.org

Supplementary material

Supplementary Table 1. Demographic information on the liver samples.

Subgroup	Number
All	150
Males	71
Females	79
Age	
Age <20 years	2
Age ≥20 and ≤70 years	122
Age >70 years	26
Smoking*	
Nonsmoker	117
3–20 cigarettes/day	18
>20 cigarettes/day	11
Alcohol*	
Nondrinker	96
1- to 2-times/week	38
Daily	10
Drug exposure*	
No drug intake	40
P450 Inducers	40
Other drugs	70

*Numbers do not add up to 150 because patients with occasional nicotine or alcohol consumption are not listed.

*Regular drug intake preceding liver surgery; known P450 inducers include atorvastatin, budesonid, metamizol, nifedipin, omeprazole, pantoprazol, prednisolon, simvastatin and tamoxifen.

Supplementary Table 2. *POR* amplification and sequencing primers.

Name	Genomic position	cDNA position	Primer sequence (5'>3')	Amplification product (bp)
POR1 (for)	-90– -71		GCCCAGCATTAGGTGGGCA	340
POR1 (rev)	250–230		GAACTGCTTGAGTGGTGAC	
POR2 (for)	18360–18380		CGTGACACCTGTGTCCCATGA	190
POR2 (rev)	18549–18529		CCAGCCGTCTGCTAGACTTGA	
POR3 (for)	25401–25418		CACCGGAGCCGTGGCTGA	247
POR3 (rev)	25647–25628		GACCTGCTCCCTGTCTTACC	
POR4 (for)	26583–26562		CGGGTTGAACCTGAACAGGCT	299
POR4 (rev)	26583–26561		GGGCTGCCTTTCAGATACATGG	
POR5 (for)	26951–26969		CCAGCAGCTCAGCCAGTGC	295
POR5 (rev)	27245–27228		CCTCACGTTCACGGCCC	
POR6 (for)	27461–27481		CACCAGGTACCGTTGCCACAT	213
POR6 (rev)	27673–27656		GCTGCCCTCGGCACAGGA	
POR8 (for)	29463–29483		CCTTGAGACGGAGACTCAGA	274
POR8 (rev)	29736–29718		GATGTGAGGCTCGGGAGGG	
POR9 (for)	29682–29699		TGGGCCTCCTGACCTGGG	244
POR9 (rev)	29925–29907		CCTACAGGCCCAATCCCGC	
POR10 (for)	30690–30710		CAGAGCTGGCCCAAGGTGTCA	342
POR10 (rev)	31031–31013		GGCCACGGAGCTCGGAAA	
POR11 (for)	30952–30969		TCCGGCGAGGGCAAGGTG	323
POR11 (rev)	31274–31256		CTGCTGTGAGCAGGGCTGG	
POR12 (for)	31509–31528		CCTGCAGAACGGGACTTGGG	431
POR12 (rev)	31939–31922		CCTCCTTGCTGCGTGGC	
POR13 (for)	31848–31866		CGACAGCAGGGTGAGTGGG	289
POR13 (rev)	32136–32118		CACTGTGGCTGTGGCCCTG	
POR15B (for)	32545–32564		CTCCCTCCCGTAGTCTCCTG	402
POR15B (rev)	32946–32927		CCACCACATGGGTTCTCTGGG	
228C_Fw (cDNA for)		665–683	GGCGAGAGCAGTTCTGGCC	1473
POR_18b (cDNA rev)		2127–2108	acgttgatgAAACACACCCAGGAGACTAC	
228T_Fw (cDNA for)		665–683	GGCGAGAGCAGTTCTGGCT	1473
POR_18b (cDNA rev)		2127–2108	acgttgatgAAACACACCCAGGAGACTAC	
POR_5 (cDNA for)		516–534	acgttgatgaGGTGTTGGTCTTGGGAAC	1020
503C_Rv (cDNA rev)		1526–1508	CGGCCGCCGTCTCCCCG	
POR_5 (cDNA for)		516–534	acgttgatgaGGTGTTGGTCTTGGGAAC	1020
503T_Rv (cDNA rev)		1526–1508	CGGCCGCCGTCTCCCCGA	
PORS4F (seq)	26294–26315		CCTTGAACAGGCTCAGTCATGG	
PORS4R (seq)	26578–26557		GCCTTTCAGATACATGGATCTG	
PORS6F (seq)	27465–27483		AGGTACCGTTGCCACATGG	
PORS6R (seq)	27670–27653		GCCCTCGGCACAGGACCC	
PORS8F (seq)	29472–29492		CGGAGACTCAGATCAAAGCCC	
PORS8R (seq)	29727–29709		CTCGGGAGGGGCGGAAGGC	
PORS9F (seq)	29686–29702		CCTCCTGACCTGGGGCA	

Primers used for exon amplification, allele-specific amplification (cDNA for, cDNA rev) or for sequencing are given with their genomic position referring to the POR reference sequence NT_007933 and, where applicable, with their cDNA position referring to the POR reference sequence NM_000941.2. Nucleotides not capitalized denote a noncomplementary tag sequence that permits more efficient amplification. Allele-specific nucleotides are highlighted in bold.

F: Forward orientation; for: Forward primer; R: Reverse; orientation; rev: Reverse primer; seq: Sequencing.

Supplementary Table 2. <i>POR</i> amplification and sequencing primers (cont.).				
Name	Genomic position	cDNA position	Primer sequence (5'>3')	Amplification product (bp)
PORS9R (seq)	29921–29903		CAGGCCCAATCCCGCGGCT	
PORS10F (seq)	30694–30714		GCTGGCCCAAGGTGTCACCCC	
PORS10R (seq)	31025–31007		CGGAGCTCGGAAAACGGCG	
PORS10BF (seq)	30924–30941		GGAGCTGCTGCGCAAGAT	
PORS12F (seq)	31513–31530		CAGAACGGGACTTGGGGC	
PORS12R (seq)	31935–31918		CTTGCCTGCGTGGCAGGG	
PORS12bR (seq)	31623–31604		CAACCACCGCACAGATGTGC	
PORS13F (seq)	31853–31871		GCAGGGTGAGTGGGGTCCC	
PORS13R (seq)	32118–32101		GCCAGCCTCATGCCCACC	
PORS15BF (seq)	32549–32568		CTCCCGTAGTCTCCTGGGTG	
PORS15R (seq)	32943–32924		CCACATGGGTCCTGGGGTC	
<i>Primers used for exon amplification, allele-specific amplification (cDNA for, cDNA rev) or for sequencing are given with their genomic position referring to the POR reference sequence NT_007933 and, where applicable, with their cDNA position referring to the POR reference sequence NM_000941.2. Nucleotides not capitalized denote a noncomplementary tag sequence that permits more efficient amplification. Allele-specific nucleotides are highlighted in bold.</i>				
<i>F: Forward orientation; for: Forward primer; R: Reverse; orientation; rev: Reverse primer; seq: Sequencing.</i>				

Supplementary Table 3. Amplification and extension primers used for MALDI-TOF mass spectrometry analysis.

Name	Assay	Genomic position	Primer sequence (5'>3')	Amplification product (bp)	Genotyped SNPs
POR 1 (MS for)	1	-94– -75	ACGTTGGATGCAAGGCCAGCATTAGGTG	318	SNP1
POR 1 (MS rev)		204–185	ACGTTGGATGGAGAGGCGGCACTTACAATG		
POR 2 (MS for)	2	25245–25264	ACGTTGGATGTGTGTGAGATTGCCTTGGTG	231	SNP4
POR 2 (MS rev)		25455–25436	ACGTTGGATGAGGAAGGCAGACACAGGGTT		
POR 3 (MS for)	1, 2	25466–25485	ACGTTGGATGACATCATCGTGTCTACGGC	343	SNP6, SNP7, SNP8
POR 3 (MS rev)		25788–25769	ACGTTGGATGTGGCTGCAACTGCAGGAATG		
POR 4 (MS for)	1	26290–26309	ACGTTGGATGTGAACCTTGAACAGGCTCAG	249	SNP9, SNP10, SNP11
POR 4 (MS rev)		26518–26499	ACGTTGGATGTAGCAGTCTCTGGGTGACTC		
POR 5 (MS for)	1, 2	27054–27073	ACGTTGGATGAGGTGTTTGGTCTTGGGAAC	272	SNP12, SNP13
POR 5 (MS rev)		27305–27286	ACGTTGGATGTTCCCTCTGTGTTGGAGGTG		
POR 6 (MS for)	1, 2	27379–27398	ACGTTGGATGATGCTCTGGGTTTATGTCGC	361	SNP14, SNP15, SNP16
POR 6 (MS rev)		27719–27700	ACGTTGGATGTCTTCTAACCTTGCTGCGAC		
POR 7+8 (MS for)	2	28013–28030	ACGTTGGATGTTCTGGGCGTCTGGCCCC	484	SNP20
POR 7+8 (MS rev)		28476–28457	ACGTTGGATGACAGGTCTGGACACTCACA		
POR 9 (MS for)	1, 2	29383–29402	ACGTTGGATGGGGAGAGCCCTTGATGTAAC	326	SNP21, SNP23, SNP24
POR 9 (MS rev)		29688–29669	ACGTTGGATGAGGCCAGAGTGAGGGTTCA		
POR 12 (MS for)	1	30876–30894	ACGTTGGATGCAACGTGCTGTACGAGCTG	223	SNP26, SNP27, SNP28, SNP29
POR 12 (MS rev)		31078–31059	ACGTTGGATGTCAGGTACAGCTCCTAAGAG		
POR 14 (MS for)	2	31380–31398	ACGTTGGATGTGGCTTGTGAGATTCTCAG	158	SNP31
POR 14 (MS rev)		31517–31498	ACGTTGGATGTTCTGCAGGCCAGCCCCACT		
POR 15 (MS for)	1	31524–31543	ACGTTGGATGTTGGGGCCGGGGCTGGGCAA	244	SNP34
POR 15 (MS rev)		31747–31728	ACGTTGGATGTGGGACTTGCGCACGAACAT		
POR 16 (MS for)	1	31773–31791	ACGTTGGATGACGCCGTGTCATCATGGTGG	251	SNP36, SNP37
POR 16 (MS rev)		32003–31984	ACGTTGGATGTCTCCCGGTACAGGTAGTC		
POR 17 (MS for)	1, 2	32039–32057	ACGTTGGATGAGCTCAACGTGGCCTTCTC	291	SNP39, SNP40, SNP41
POR 17 (MS rev)		32309–32290	ACGTTGGATGACTGTGGCAGGCAGGGCCTT		
POR 19 (MS for)	1, 2	32601–32620	ACGTTGGATGCAGTGACAAAGACTCCTCTG	350	SNP43, SNP44, SNP45, SNP46
POR 19 (MS rev)		32930–32911	ACGTTGGATGTGGGCTCTGAGTTAGTTAAG		
POR 20 (MS for)	1, 2	18490–18509	ACGTTGGATGTACTCTTCTCAGGAAGCCTC	314	SNP2, SNP3
POR 20 (MS rev)		18783–18764	ACGTTGGATGGTGAGATTACCTCTGCTCC		
POR 21 (MS for)	2	27748–27767	ACGTTGGATGTTGGTCCCAGCCAAGGACTC	189	SNP17
POR 21 (MS rev)		27916–27897	ACGTTGGATGTTTTGGCCAAGTCTGTGGAG		
POR 3b (MS for)	3	25500–25519	ACGTTGGATGTGCAGAGGAGTTTGCCAACC	309	SNP5
POR 3b (MS rev)		25788–25769	ACGTTGGATGTGGCTGCAACTGCAGGAATG		
POR 7+8b (MS for)	3	28011–28028	ACGTTGGATGGCTTCTGGGCGTCTGGCC	334	SNP18, SNP19
POR 7+8b (MS rev)		28324–28305	ACGTTGGATGTGGTTCTCGTAGCTCTTCAG		

Genomic (g.) positions refer to the POR reference sequence NT_007933; MALDI-TOF MS analysis was performed with four different assays for genotype (assays one to three) and for haplotype (assay four) determination. All amplification primers have a tag sequence ACGTTGGATG that permits more efficient amplification. Nucleotides not capitalized are mismatches introduced by the software MassArray Assay Design to enhance detection by MS. F: forward; MS for: MALDI-TOF MS amplification forward primer; MS rev: MALDI-TOF MS amplification reverse primer; MS ext: MALDI-TOF MS extension primer; POR: NADPH:CYP oxidoreductase; R: reverse orientation.

Supplementary Table 3. Amplification and extension primers used for MALDI-TOF mass spectrometry analysis (cont.).

Name	Assay	Genomic position	Primer sequence (5'→3')	Amplification product (bp)	Genotyped SNPs
POR 9b (MS for)	3	29421–29440	ACGTTGGATGGAGATCTCTGAGATTCCCTG	288	SNP22
POR 9b (MS rev)		29688–29669	ACGTTGGATGAGGCCAGAGTGAGGGTTCA		
POR 10b (MS for)	3	29731–29750	ACGTTGGATGCACATCTCCCTCCAGGTATG	167	SNP25
POR 10b (MS rev)		29877–29858	ACGTTGGATGATGTGGCACTCACCATCCAG		
POR 13b (MS for)	3	31098–31116	ACGTTGGATGGAGGCACATCTGGCCATC	244	SNP30
POR 13b (MS rev)		31321–31303	ACGTTGGATGAGCTCAGGGCTCAGATCTC		
POR 15b (MS for)	3	31591–31610	ACGTTGGATGACCCCAACTCTGTGCACATC	177	SNP32, SNP33, SNP35
POR 15b (MS rev)		31747–31728	ACGTTGGATGTGGGACTTGCGCACGAACAT		
POR 16b (MS for)	3	31773–31791	ACGTTGGATGACGCCTGTCATCATGGTGG	251	SNP38
POR 16b (MS rev)		32003–31984	ACGTTGGATGTCTCCCGGTACAGGTAGTC		
POR 18b (MS for)	3	32343–32361	ACGTTGGATGAGGGATGCACGGAACATGG	232	SNP42
POR 18b (MS rev)		32573–32554	ACGTTGGATGAAACACACCCAGGAGACTAC		
POR 1 F (MS ext)	1	–4–14	cccatTTTCATGATCAACATGGG	–	SNP1
POR 2 R (MS ext)	2	25379–25365	gcttGCAGGCGGGGGCCCT	–	SNP4
POR 3#2 R (MS ext)	1	25617–25601	gaATCTGGGGCCAGCACGC	–	SNP6
POR 3#3 R (MS ext)	2	25691–25677	ACCCCACTCCCTGCC	–	SNP7
POR 3#4 R (MS ext)	1	25740–25724	cttATGGCAATGACCCTCAC	–	SNP8
POR 3b F (MS ext)	3	25549–25564	aATGCGAGGCATGTCAG	–	SNP5
POR 4#1 R (MS ext)	1	26382–26368	GGCGTTGTCGATCTC	–	SNP9
POR 4#2 R (MS ext)	1	26423–26405	ccccGGGTCTCCCTCACCGTAGG	–	SNP10
POR 4#3 R (MS ext)	1	26454–26439	CCAGTCGTAGAAGTCC	–	SNP11
POR 5#1 R (MS ext)	2	27096–27081	GCATTGAAGTGCTCGT	–	SNP12
POR 5#2 F (MS ext)	1	27192–27209	tgccccCCCTGCCACCATGATCAG	–	SNP13
POR 6#2 F (MS ext)	2	27483–27497	ttGGCCTCCCTGAGCC	–	SNP14
POR 6#3 F (MS ext)	2	27549–27565	GCGAGAGCAGTTCTGGC	–	SNP15
POR 6#4 R (MS ext)	1	27632–27616	ggagaaaCTGCGGGCACTTGCTCA	–	SNP16
POR 7+8b#1 F (MS ext)	3	28027–28044	tCCCCGGCAGCTCCACGC	–	SNP18
POR 7+8b#2 F (MS ext)	3	28272–28286	cagGCGGCCAAGGTGTAC	–	SNP19
POR 7+8#5 F (MS ext)	2	28429–28445	TCTCACCAGACCCCGTG	–	SNP20
POR 9#3 F (MS ext)	2	29477–29492	ctatACTCAGATCAAAGCCC	–	SNP21
POR 9#5 R (MS ext)	1	29576–29562	ggCCGTTGGTGGTGAC	–	SNP23
POR 9#6 R (MS ext)	2	29610–29596	gaggcGCATGAGGTGGCGCT	–	SNP24
POR 9b#1 F (MS ext)	3	29539–29555	CCAAGAATCCGTTCTCTG	–	SNP22
POR 10b R (MS ext)	3	29798–29783	gtgcCGAGAGCAGAGTCGTT	–	SNP25
POR 12#1 F (MS ext)	1	30936–30953	ccctCAAGATGGCTCTCTCTC	–	SNP26
POR 12#2 R (MS ext)	1	30971–30956	CGCACCTTGCCCTCGC	–	SNP27
POR 12#3 F (MS ext)	1	30961–30977	tggtGGCAAGGTGCGCCCCCT	–	SNP28
POR 12#4 R (MS ext)	1	31001–30987	ACGGGGCGGAGGTTG	–	SNP29
POR 13b#2 R (MS ext)	3	31207–31193	ATGAGGCGATGGAGT	–	SNP30

Genomic (g.) positions refer to the POR reference sequence NT_007933; MALDI-TOF MS analysis was performed with four different assays for genotype (assays one to three) and for haplotype (assay four) determination. All amplification primers have a tag sequence ACGTTGGATG that permits more efficient amplification.

Nucleotides not capitalized are mismatches introduced by the software MassArray Assay Design to enhance detection by MS.

F: forward; MS for: MALDI-TOF MS amplification forward primer; MS rev: MALDI-TOF MS amplification reverse primer; MS ext: MALDI-TOF MS extension primer;

POR: NADPH:CYP oxidoreductase; R: reverse orientation.

Supplementary Table 3. Amplification and extension primers used for MALDI-TOF mass spectrometry analysis (cont.).

Name	Assay	Genomic position	Primer sequence (5'>3')	Amplification product (bp)	Genotyped SNPs
POR 14#2 F (MS ext)	2	31452–31466	gggtGCCTCTGAGGTTTGG	–	SNP31
POR 15#5 F (MS ext)	1	31679–31695	GCGGGCCAAGGAGCCTG	–	SNP34
POR 15b#1 F (MS ext)	3	31628–31642	GTACGAGACCAAGGC	–	SNP32
POR 15b#2 F (MS ext)	3	31647–31662	CGCATCAACAAGGGCG	–	SNP33
POR 15b#3 R (MS ext)	3	31713–31699	GGCCGCCGTTCTCCC	–	SNP35
POR 16#1 R (MS ext)	1	31821–31804	gaagaCTATGAAGGGTGCCACCC	–	SNP36
POR 16#4 F (MS ext)	1	31940–31954	gagtTGGGGGAGACGCTGC	–	SNP37
POR 16b#2 R (MS ext)	3	31997–31978	CGGTACAGGTAGTCTCATC	–	SNP38
POR 17#3 F (MS ext)	2	32155–32172	ccaaCCTTGCCCCAGGTCTAC	–	SNP39
POR 17#4 F (MS ext)	1	32180–32196	gggACCTGCTAAAGCAAGAC	–	SNP40
POR 17#5 F (MS ext)	2	32227–32241	GGTGCCACATCTAC	–	SNP41
POR 18b R (MS ext)	3	32398–32384	cctGCCACGATGTCGTAG	–	SNP42
POR 19#1 R (MS ext)	2	32714–32697	GGAGCCCGTGCCCCCAT	–	SNP43
POR 19#3 F (MS ext)	2	32780–32794	tGCCCAGCCCCTCCAC	–	SNP44
POR 19#4 R (MS ext)	1	32817–32797	TATTACACTCACTGGAAATC	–	SNP45
POR 19#5 F (MS ext)	2	32837–32860	CCTTGGAATAAAGTTCTGTTTCT	–	SNP46
POR 20#1 R (MS ext)	1	18576–18558	ggaCACCGGGACATCCTCTATG	–	SNP2
POR 20#2 F (MS ext)	2	18628–18645	ggaaCAAAGCCCTGAAGCAGCT	–	SNP3
POR 21 F (MS ext)	2	27822–27838	agcgCGACAAGGACACATCGC	–	SNP17
228 (MS for)	4	27526–27545	ACGTTGGATGTTGGAGGAGGACTTCATCAC	91	SNP15
228 (MS rev)		27596–27577	ACGTTGGATGGCTTCCACCCCAAAGTGTTTC		
503 (MS for)	4	31621–31640	ACGTTGGATGTTGTGGAGTACGAGACCAAG	143	SNP34
503 (MS rev)		31743–31725	ACGTTGGATGACTTGCGCACGAACATGGG		
631 (MS for)	4	32207–32226	ACGTTGGATGTGTGGAAGTTGATCGAAGGC	121	SNP41
631 (MS rev)		32307–32288	ACGTTGGATGTGTGGCAGGCAGGGCCTTGT		
228 F (MS ext)	4	27549–27565	GCGAGAGCAGTTCTGGC	–	SNP15
503 R (MS ext)	4	31715–31697	ACGGCCGCCGTTCTCCCCG	–	SNP34
631 F (MS ext)	4	32225–32241	GCGGTGCCACATCTAC	–	SNP41

Genomic (g.) positions refer to the *POR* reference sequence NT_007933; MALDI-TOF MS analysis was performed with four different assays for genotype (assays one to three) and for haplotype (assay four) determination. All amplification primers have a tag sequence ACGTTGGATG that permits more efficient amplification. Nucleotides not capitalized are mismatches introduced by the software MassArray Assay Design to enhance detection by MS.

F: forward; MS for: MALDI-TOF MS amplification forward primer; MS rev: MALDI-TOF MS amplification reverse primer; MS ext: MALDI-TOF MS extension primer; *POR*: NADPH:CYP oxidoreductase; R: reverse orientation.