Clinical, Genetic, and Enzymatic Characterization of P450 Oxidoreductase Deficiency in Four Patients

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Context: P450 oxidoreductase (POR) deficiency causes disordered steroidogenesis; severe mutations cause genital ambiguity in both sexes plus the Antley-Bixler skeletal malformation syndrome, whereas mild mutations can cause adult infertility.

Objective: We describe four patients with POR deficiency and identify and characterize the activities of their mutations. A 46,XY male with micropenis and two 46,XX female infants with genital ambiguity presented with skeletal malformations, and a 46,XX adolescent presented with primary amenorrhea, elevated 17α-hydroxyprogesterone, and low sex steroids.

Methods: The coding regions of the POR gene were sequenced, and the identified mutations were recreated in human POR cDNA expression vectors lacking 27 N-terminal residues. POR and human P450c17 were expressed in bacteria. POR activity was measured by four assays: reduction of cytochrome c, oxidation of reduced nicotinamide adenine dinucleotide phosphate, and support of the 17α-hydroxylase and 17,20 lyase activities of P450c17.

Results: All four patients were compound heterozygotes for POR mutations, including five novel mutations: L577R, N185K, delE217, and frameshift mutations 1363delC and 697–698insGAAC. N185K and delE217 lacked measurable activity in the assays based on P450c17 but retained partial activity in the assays based on cytochrome c. As assessed by V_{max}/Km, L577R supported 46% of 17α-hydroxylase activity but only 27% of 17,20 lyase activity. Computational modeling of these novel mutants revealed the structural basis for their reduced or absent activities.

Conclusion: These patients illustrate the broad clinical spectrum of POR deficiency, including amenorrhea and infertility as the sole manifestation. POR assays based on P450c17 correlate well with hormonal and clinical phenotypes. ***(J Clin Endocrinol Metab* 94: 4992–5000, 2009)**

P450 oxidoreductase (POR) transfers electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to all microsomal (type II) cytochrome P450 enzymes, including three steroidogenic enzymes: P450c17 (17α-hydroxylase/17,20 lyase), P450c21 (21-hydroxylase), and P450aro (aromatase) (1). POR also transfers electrons to some non-P450 enzymes: squalene epoxidase (2), fatty acid elongase (3), and heme oxygenase (4). POR is an 82-kDa membrane-bound flavoprotein consisting of 680 amino acids and two bound flavins: a flavin adenine dinucleotide (FAD), and a flavin mononucleotide (FMN). The x-ray crystal structure of rat POR (5) and modeling of

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Abbreviations: ABS, Antley-Bixler syndrome; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Km, Michaelis constant; NADPH, reduced nicotinamide adenine dinucleotide phosphate; nl, normal; 17OHP, 17α-hydroxyprogesterone; POR, P450 oxidoreductase; PRA, plasma renin activity; V_{max}, maximum velocity.

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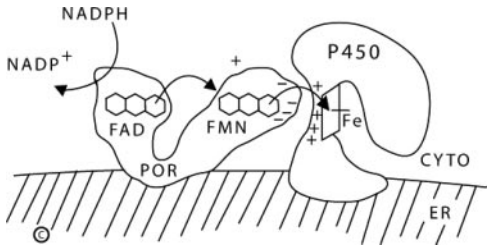

FIG. 1. The role of POR in electron transfer by microsomal (type II) P450 enzymes. Electrons from NADPH are first taken up by the FAD moiety of POR, eliciting a conformational change in the hinge domain bringing the FAD and FMN domains closer together. The electrons donated by NADPH travel from the FAD to the FMN moiety, which then docks with the redox partner binding site of the P450 by electrostatic interaction, and the electrons then travel to the heme iron of the P450, permitting enzymatic catalysis. For some P450 enzymes, such as P450c17, this interaction is facilitated by the allosteric action of cytochrome b5. Cyto, cytoplasm; ER, endoplasmic reticulum.

the 92% identical human POR (6) show that POR has two lobes comprising distinct domains: one lobe contains the NADPH-binding site and also binds the FAD moiety, whereas the other lobe binds the FMN moiety. POR functions by binding NADPH and accepting a pair of electrons via the FAD; this elicits a conformational change bringing the FAD and FMN close together so that the electrons pass to the FMN; this then restores the initial open conformation, permitting the FMN domain to interact with the P450, which receives the electrons and uses them to mediate catalysis on its heme group (Fig. 1).

Although disruption of the POR gene in mice causes gross disorders of embryogenesis and embryonic lethality (7, 8), in 2004 Flück *et al.* (9) reported POR mutations in three children with ambiguous genitalia and skeletal malformations [Antley-Bixler syndrome (ABS)] and in a phenotypically normal adult woman with primary amenorrhea and polycystic ovaries. The majority of patients with POR deficiency described to date have also had the ABS phenotype, characterized by craniosynostosis, radioulnar or radiohumeral synostosis, bowed femora, and other variable skeletal disorders (10). Our genetic studies demonstrated that indistinguishable ABS phenotypes result from two distinct genetic disorders: autosomal recessive POR deficiency, and dominant, gain-of-function mutations in fibroblast growth factor receptor 2 (6). These two genetic disorders are readily distinguished by the abnormal steroidogenesis and consequent genital anomalies seen in patients with POR mutations, but not in those with mutations in fibroblast growth factor receptor 2 (6). Some patients with milder POR mutations do not have ABS, and the steroidogenic defect may present as hypogonadism and/or infertility (6, 9, 11). The human POR gene consists of 16 exons, spanning approximately 70 kb on chromosome 7q11.2 (12). To date, more than 20 mutations in at least 70 patients have been described (13, 14), suggesting that this disorder may be relatively common. However, relatively few adults without ABS but having POR deficiency have been described. We now describe four patients with POR deficiency with varied presentations and identify and characterize the activities of their POR mutations.

Patients and Methods

Case Reports

Patient 1

After an uncomplicated 39-wk pregnancy without maternal virilization, a 3.07 kg, 46,XY male was born to healthy, nonconsanguinous Caucasian parents. The baby had multiple anomalies, including prominent forehead, cloverleaf skull, brachycephaly, and midface hypoplasia. Radiographs showed right radiohumeral synostosis; the left proximal radius and proximal ulna appeared hypoplastic with abnormal humeral articulation. The phallus was 2.1 × 0.8 cm with chordee and a normal urethral meatus. The testes were 2.5 ml bilaterally. The newborn screening 17α-hydroxyprogesterone (17OHP) was 60 ng/ml [normal (nl), <50] at age 2 d and was 53 ng/ml (nl, <50) at 13 d. At 60 d, the baseline ACTH was slightly elevated (75 pg/ml), but plasma renin activity (PRA) was normal; an ACTH stimulation test (250 μg) showed basal cortisol of 9.3 μg/dl with subnormal response to 13 μg/dl and moderately elevated progesterone and 17OHP after 60 min (Tables 1 and 2). He was treated with hydrocortisone 10.4 mg/m^2/d and three-monthly im injections of 20 mg testosterone cypionate resulting in phallic growth to 3.1 cm. He underwent craniofacial reconstruction at 7 months and tonsillectomy and adenoidectomy for obstructive sleep apnea at 22 months. By age 3, he displayed poor speech articulation, but has a vocabulary of about 100 words. There was partial hearing loss on the right, but he was able to understand commands and conversations well; development was otherwise appropriate for age.

TABLE 1. Summary of mutation analysis and clinical findings

	Patient 1		Patient 2		Patient 3		Patient 4	
Mutation cDNA	delGGA/651–653	859G>C	555T>A	1730T>G	1363delC	1615G>A	697–698insGAAC	1615G>A
Mutation protein	delE217	A287P	N185K	L577R	Frameshift	G539R	Frameshift	G539R
Karyotype	46, XY		46, XX		46, XX		46, XX	
ABS phenotype	Yes		No		Yes		Yes	
Genitalia	Micropenis		Normal		Ambiguous		Ambiguous	

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TABLE 2. Summary of hormonal findings

	Patient 1		Patient 2		Patient 3		Patient 4	
	Basal	60 min after ACTH	Basal	60 min after ACTH	Basal	60 min after ACTH	Basal	60 min after ACTH
Cortisol (μg/dl)	9.3 (3–22)	13 (27–50)	9.7 (10–26)	8.5	12.3	14.2	2.3	15.6
ACTH (pg/ml)	75 (7–51)		54 (6–51)				146 (0–45)	
PRA (ng/ml · h)	0.5 (0.3–1.7)				2 (1.1–2.7)		33.9 (1.2–2.8)	
17OHP		1890 (85–250)	1187 (55–226)	2366			156	672
17OH pregnenolone							310	1505
Progesterone		5560 (74–200)	1000 (40–106)	3490				
11-Deoxycorticosterone		66 (40–158)	56 (8–29)	64			66	93
Corticosterone			2020 (240–3000)	3037				
Aldosterone					63 (3.6–16)		>119	
DHEA		170 (67–1453)						
DHEAS (μg/dl)					96 (33–426)		<29	
Androstenedione		44 (21–114)	74 (60–245)		130 (48–369)			
Testosterone	24 (60–400)	25[a]	22					
Estradiol			2.5		1.1			
LH (mIU/ml)			9		1.3			
FSH (mIU/ml)			3.9		8.8			

Hormonal values are expressed in nanograms per deciliter, unless otherwise indicated.

[a] Testosterone is not significantly changed after ACTH.

Patient 2

An 18 11/12-yr-old , 49 kg, 161 cm, 46,XX Caucasian with normal breast development, normal blood pressure, and no dysmorphic features was evaluated for primary amenorrhea. Basal gonadotropins (LH, 9 mIU/ml; FSH, 3.9 mIU/ml) and cortisol (9.7 μg/dl) were normal, but there was no cortisol response to ACTH stimulation (8.5 μg/dl at 60 min). Basal ACTH was at the upper limit of the normal range (54 pg/ml). Progesterone and 17OHP were grossly elevated and were hyperresponsive to ACTH, whereas androstenedione and testosterone were low (Table 2). Pelvic ultrasonography showed the presence of normal uterus and ovaries. She apparently had one hypoglycemic seizure in the past and is treated with hydrocortisone at times of stress. She is now 24 3/12 yr old, employed and married, but infertile.

Patient 3

This 46,XX Irish patient had ambiguous genitalia, craniosynostosis, and radiohumeral synostosis at birth, and was included in an early study of ABS, which indicated that she had a 17OHP of 13 nmol/liter (430 ng/dl), FSH of 18 U/liter (nl, <3), and LH of 9.8 U/liter (nl, <6) (15); these values were obtained at age 3 d. At 16 11/12 yr, she had severe scoliosis with height of 127.7 cm, bone age of 10 yr, and weight of 24 kg, and she had significant intellectual disability. She had no breast development or pubic hair, and her baseline LH (1.3 mIU/ml), FSH (8.8 mIU/ml), and estradiol (39 pmol/liter; 1.1 ng/dl on an extraction assay) were all prepubertal. Pelvic ultrasonography revealed a prepubertal uterus, but ovaries were not seen. She had a normal basal cortisol of 338 nmol/liter (12.3 μg/dl), which responded poorly to ACTH, rising to 391 nmol/liter (14.2 μg/dl) at 60 min. Dehydroepiandrosterone sulfate (DHEAS) and androstenedione were normal (Table 2). Electrolytes and PRA were also "normal," although aldosterone was 1750 pmol/liter (nl, 100–450 pmol/liter). The patient was given stress doses of hydrocortisone on sick days, but has never had adrenal crisis. She is presently 21 yr old and 129.7 cm tall, weighs 25 kg, and works in a sheltered environment.

Patient 4

A dysmorphic, 46,XX, 2780 g, 37-wk gestation infant was born to an Australian Aboriginal mother and a Caucasian father; a male sibling was normal. The phallus was 1.5 cm long and 0.7 cm wide with perineal hypospadias, fused labioscrotal folds, and no palpable gonads. Head circumference was 31.5 cm, with coronal craniosynostosis, facial asymmetry, choanal stenosis, prominent eyes, shallow orbits, divergent strabismus, prominent forehead, ridging of the right coronal suture, low-set ears with overfolded helices, and a flat nasal bridge with broad flat nasal tip, but no syndactyly. There was no radiographically detectable radioulnar or radiohumeral synostosis. On the first day of life, a random cortisol was 162 nmol/liter (5.9 μg/dl), 17OHP (after extraction) 119.5 nmol/liter (nl, <24) (3955 ng/dl), cholesterol 4.4 mmol/liter (nl, 1.3–3.6), and testosterone 6.2 nmol/liter (nl, <3.2) (179 ng/dl). At 5 d of age, the baseline ACTH and PRA were greatly elevated; the results of an ACTH stimulation test with 45 μg cosyntropin are shown in Table 2. Five days after starting hydrocortisone replacement therapy, the serum sodium was 128 mmol/liter, and fludrocortisone was added. At 16 d of age the ACTH was 18 pmol/liter (81 pg/ml), PRA was 0.1 ng/ml · h, and 17OHP was 1.6 nmol/liter (53 ng/dl). At 4 months, a large ovarian cyst was drained, which has not recurred; feminizing genitoplasty was done at 5 months and frontoorbital remodelling was done at 7 months. Despite steroid replacement therapy, by age 3 6/12 yr, she experienced nine hospitalizations for adrenal insufficiency associated with other illnesses; speech development has been delayed.

DNA sequencing

With informed consent, the 15 protein-coding exons and at least 100 bp of flanking intronic DNA of the POR gene were amplified by PCR from leukocyte genomic DNA using described primers and conditions (6). PCR products were sequenced, and the data were analyzed using Sequencher version 4.2 (Gene Codes, Ann Arbor, MI).

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Construction of POR expression vectors

Human POR cDNA lacking codons for 27 N-terminal residues was subcloned into pET22b for bacterial expression (6, 9). Mutant cDNA expression vectors were generated by PCR-based, site-directed mutagenesis (primers shown in Supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The methylated parental wild-type cDNA was digested with 10 U *Dpn* I at 37 C for 180 min, and the remaining unmethylated, mutagenized cDNA plasmid was used to transform *Escherichia coli* DH5α. The sequences of the mutagenized cDNAs were verified by sequencing.

Bacterial expression of POR and P450c17 and membrane preparation

E. coli BL21DE3(pLysS) transformed with pET22b were grown at 30 C to OD 0.4, and POR expression was induced with 0.4 mM isopropyl-1-thio-β-d-galactopyranoside at 28 C for 60 h. The bacterial membranes were prepared as described (6), and mutant POR was quantitated by Western blotting compared with a standard curve of wild-type POR (6, 16).

The pCWori-mod(His)4 expression plasmid containing human P450c17 cDNA (17) with amino-terminal modifications that facilitate bacterial expression (18) was transformed into *E. coli* JM109. P450c17 expression was induced, membranes were prepared, and their P450c17 content was determined by CO-difference spectra, all as described (6).

POR assays based on cytochrome c

The reduction of cytochrome c and oxidation of NADPH by bacterially expressed POR were assayed by quantitating changes in A550 when oxidized cytochrome c is reduced (extinction coefficient, 21.1 mM^{-1}). The 1-ml assay volume contained 0.1 M Tris-HCl (pH 7.8), 2 mM glucose-6-phosphate (Sigma, St. Louis, MO), and 3 U glucose-6-phosphate dehydrogenase (Sigma) to regenerate NADPH (19). The cytochrome c concentration was 40 μM when NADPH was the variable substrate, and the NADPH concentration was 5 μM when cytochrome c was the variable substrate (9). Assays were performed with bacterial membranes containing 2 pmol of POR. A550 was monitored against time; velocities were expressed as (nanomoles reduced cytochrome c)/(picomoles POR)/min. Maximum velocity (V_{max}) and apparent Michaelis constant (Km) were determined by nonlinear Michaelis-Menten plots using Graph Pad Prism 3 (GraphPad Software, Inc., San Diego, CA). Results are mean ± SD of at least three independent experiments.

POR assays based on P450c17

To assay 17α-hydroxylase and 17,20-lyase activities, membrane fractions containing 30 pmol of bacterially expressed human P450c17 (20, 21) were combined with membranes containing 60 pmol of bacterially expressed POR, 20 μg of 1,2-didodecanoyl-sn-glycero-3-phosphocholine (Sigma) in 100 mM K phosphate, and 20 μg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma) and sonicated in an ice bath for 10 min, followed by adding 6 mM K acetate, 10 mM $MgCl_2$, 1 mM reduced glutathione, 20% glycerol, and radiolabeled substrate in a total volume of 200 μl. Substrates were [^{14}C]progesterone for 17α-hydroxylase activity and [^{3}H]17α-hydroxypregnenolone for 17,20 lyase activity, with 20 pmol of cytochrome b5 added as an allosteric facilitator (22). Reactions were started by adding 4 μl of 100 mM NADPH, incubated at 37 C for 1 h, and stopped by adding 400 μl of ethyl acetate:iso-octane (1:1) to extract the steroids. Steroids were analyzed by thin-layer chromatography and quantitated by phosphorimaging (6, 20, 21). Data were analyzed as Lineweaver-Burk plots using Graph Pad Prism 3 (GraphPad Software) and expressed as mean ± SD of at least three independent experiments.

Results

POR mutations

Patient 1 was a compound heterozygote for delGGA/651–653, encoding delE217, and 859G>C, encoding A287P (NCBI cDNA reference sequence NM_000941.2 and protein reference sequence NP_000932.2). The A287P mutation comprises approximately 40% of mutant alleles in individuals of European ancestry (6), has not been found in non-European individuals, and has been well-characterized in multiple assays (6, 16, 23, 24). Patient 2 was compound heterozygous for 555T>A, encoding N185K, and 1730T>G, encoding L577R. Patient 3 carried the mutations 1615G>A, encoding G539R and a frameshift mutation (1363delC). The G539R mutation has been described in a homozygote initially mistaken for having isolated 17,20 lyase deficiency (11), and as a heterozygote with a frameshift mutation different from that found in patient 3 (6). Patient 4 was a compound heterozygote for G539R and a frameshift mutation (697–698insGAAC). The mutations delE217, N185K, L577R, and the two frameshift mutations, 1363delC and 697–698insGAAC, are novel.

Activities of the novel POR mutations

To assess the activities of the novel POR mutations, we built vectors for each in the N-terminally truncated form of POR (N-27 POR) used in other studies (6, 9, 16, 25), expressed these in bacteria, and prepared bacterial membranes containing the wild-type or mutant human POR. The classical assay of POR activity is the spectrophotometric assessment of the reduction of cytochrome c (19, 26). This assay is nonphysiological because cytochrome c, located in mitochondria, is not a normal substrate for POR in the endoplasmic reticulum, but it is simple and also permits direct assessment of the oxidation of NADPH by POR. The Km, V_{max}, and V_{max}/Km for cytochrome c reduction and NADPH oxidation show that delE217 has 19–39% of wild-type activity in these assays, whereas N185K and L577R have only 1–6% of wild-type activity (Table 3 and Fig. 2, A and B). We previously found that assays of the capacity of POR to support the catalytic activities of human P450c17 provide a reliable correlation with the patients' clinical phenotypes (6, 9). Neither the N185K nor delE217 mutants could support detectable 17α-hydroxylase or 17,20-lyase activity, whereas L577R,

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TABLE 3. Catalytic activities supported by wild-type (WT) and mutant POR: cytochrome c assays

	Cytochrome c reduction				NADPH oxidation			
	Km[a]	V_{max}[b]	V_{max}/Km	%WT	Km[c]	V_{max}[b]	V_{max}/Km	%WT
POR WT	1.92 ± 0.26	1.402 ± 0.059	0.730	100	0.166 ± 0.036	0.486 ± 0.027	2.927	100
N185K	2.01 ± 0.83	0.083 ± 0.011	0.041	6	0.434 ± 0.191	0.011 ± 0.002	0.024	1
delE217	1.20 ± 0.23	0.167 ± 0.009	0.139	19	0.096 ± 0.027	0.109 ± 0.006	1.138	39
L577R	2.44 ± 0.54	0.093 ± 0.007	0.038	5	0.06 ± 0.021	0.007 ± 0.0004	0.113	4

Data are the mean ± SD of at least three independent experiments. POR content of each membrane preparation was measured by Western blot analysis, and all samples were normalized against wild-type POR.

[a] Micromoles of cytochrome c.

[b] Nanomoles of cytochrome c per picomole POR per minute.

[c] Micromoles of NADPH.

found in patient 2, supported 46% of 17α-hydroxylase activity but only 27% of 17,20 lyase activity (Table 4 and Fig. 2, C and D). Even when the incubation time was extended to 150 min, far beyond the linear range of wild-type, there was no conversion of progesterone to 17OHP or 17OH-pregnenolone to dehydroepiandrosterone (DHEA) for delE217 and N185K (Fig. 2, E and F). Thus the activities of these mutants, especially delE217 and L577R, differed substantially between the cytochrome c assays and P450c17 assays, and the POR assays based on P450c17 correlated well with the hormonal and clinical phenotype.

Structure/function correlations of the POR mutations

To understand how the mutants decreased activity, we recreated each in our model of human POR (6), which is based on the crystallographic structure of the 92% identical rat POR (5) (Fig. 3). Residue N185 directly participates in binding the FMN moiety; the N185K mutation changes the residue's electrostatic charge and ablated nearly all activity, similar to the previously described nearby Y181D mutant (6, 27). The delE217 mutation deletes an acidic residue from the highly acidic P450 interaction domain (residues 194–231) (5, 28), which interacts with basic residues in the redox partner binding site of microsomal P450 enzymes by electrostatic interactions (29–31). Deletion of E217 had a modest effect on the nonphysiological assays based on cytochrome c but ablated all detectable P450c17 activity.

The NADPH binding domain is a parallel five-stranded β-sheet sandwiched by α-helices (5). L577 lies in β-strand P and participates in maintaining the domain structure through hydrophobic interactions. The L577R mutation, which substitutes a basic for an aliphatic residue and may affect NADPH binding, lies near the highly conserved C terminus of the protein and supported 46% of 17α-hydroxylase activity and 27% of 17,20- lyase activity. A somewhat similar mutation, C569Y, found in a woman with amenorrhea and polycystic ovaries and who had a very mild POR mutation on her other allele, retained 28% of 17α-hydroxylase activity and 13% of 17,20-lyase activity (6, 9). G539 is also in the NADPH-binding pocket; G539R, changing a small aliphatic residue to a large charged one, affects NADPH-binding, impairing function. Thus, mutations in NADPH binding domain may result in relatively milder impairment of POR activity. By contrast, mutations near the FMN domain were more deleterious.

Discussion

POR deficiency is the most complex form of congenital adrenal hyperplasia because POR supports the activities of

TABLE 4. Catalytic activities supported by wild type (WT) and mutant POR: P450c17 assays

	17α-hydroxylase				17,20 lyase			
	Km[a]	V_{max}[b]	V_{max}/Km	%WT	Km[c]	V_{max}[b]	V_{max}/Km	%WT
POR WT	1.62 ± 0.23	0.057 ± 0.003	0.035	100	1.40 ± 0.42	0.062 ± 0.015	0.044	100
N185K	NC	NC	NC	NC	NC	NC	NC	NC
delE217	NC	NC	NC	NC	NC	NC	NC	NC
L577R	2.60 ± 1.94	0.041 ± 0.014	0.016	46	3.79 ± 1.21	0.045 ± 0.012	0.012	27

Data are the mean ± SD of at least three independent experiments. POR content of each membrane preparation was measured by Western blot analysis, and all samples were normalized against wild-type POR. NC, Could not be calculated.

[a] Micromoles of progesterone.

[b] Picomoles per picomole P450c17 per minute.

[c] Micromoles of 17α-hydroxypregnenolone.

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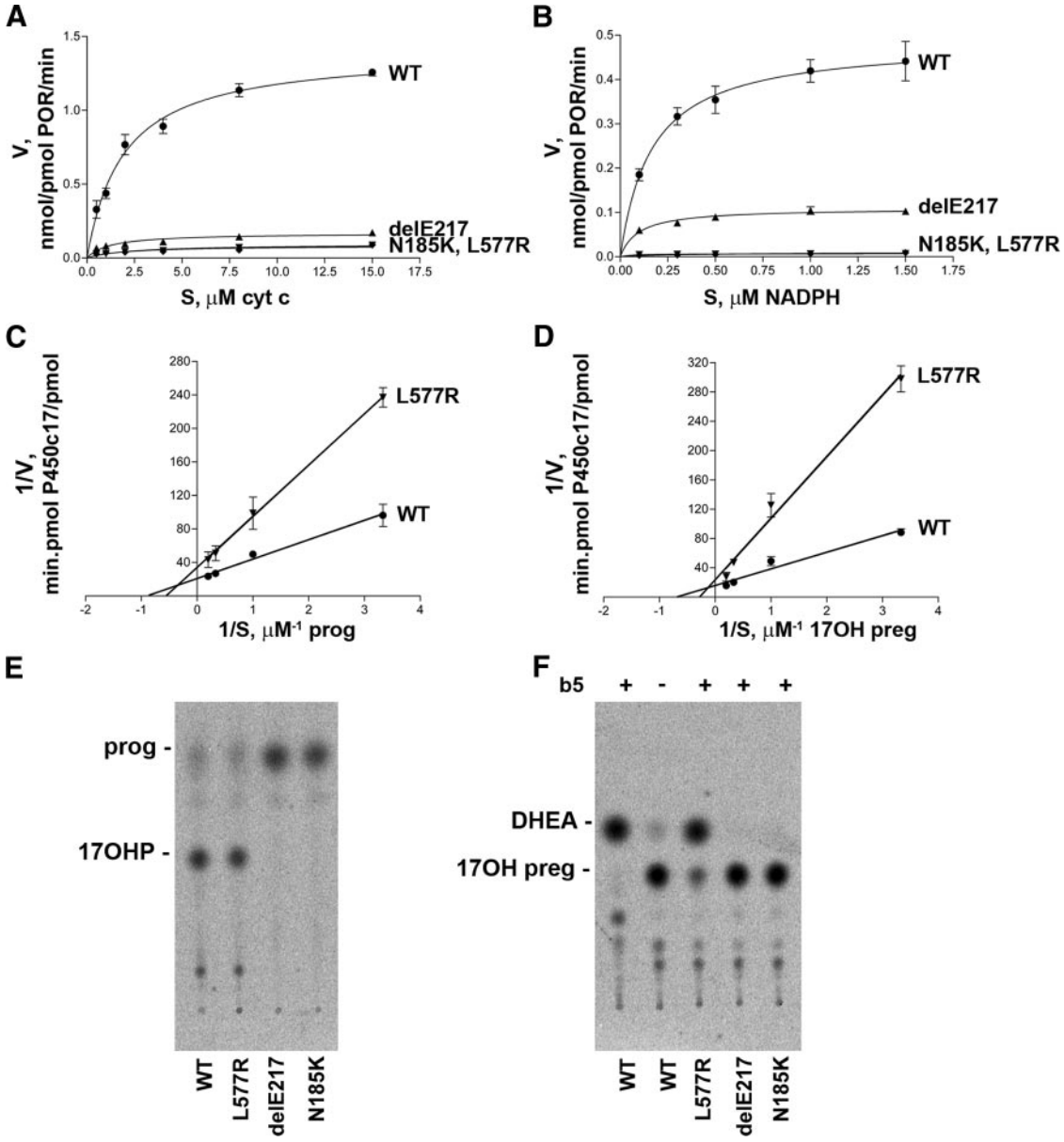

FIG. 2. Activities of wild-type and mutant POR expressed in bacteria. Error bars represent the mean ± SD of at least three independent experiments. The apparent Km and V_{max} values obtained from these data are shown in Tables 3 and 4. A, Assays of cytochrome c reduction, using cytochrome c (cyt c) as the variable substrate (S). B, Assays of NADPH oxidation, using NADPH as the variable substrate. C, Lineweaver-Burk analyses of 17α-hydroxylase activity, using progesterone (prog) as the variable substrate. D, Lineweaver-Burk analyses of 17,20-lyase activity, using 17OH pregnenolone (17OH preg) as the variable substrate. E and F, Autoradiograms of thin layer chromatograms after 150-min incubations. Wild-type (WT) POR converts progesterone (prog) to 17-hydroxyprogesterone (17OHP) (E) and 17OH pregnenolone (17OH preg) to DHEA (F). L577R showed robust conversion from prog to 17OHP and some conversion from 17OH preg to DHEA, whereas the mutations delE217 and N185K have no detectable 17α-hydroxylase or 17,20-lyase activity. In panel F (17,20-lyase activity), the membranes were incubated with (+) and without (−) cytochrome b5 (b5), as indicated above the figure.

such a wide variety of enzymes. Consequently, POR deficiency has a broad range of clinical phenotypes and is unique because it also affects nonendocrine systems, such as skeletal development and drug metabolism. POR deficiency can cause ambiguous genitalia in both sexes. 46,XY males are typically undervirilized because decreased 17,20-lyase activity reduces androgen synthesis. 46,XX females are frequently virilized at birth, but unlike girls with untreated 21-hydroxylase deficiency, this virilization is not progressive postnatally. There are two mechanisms for this virilization, whose relative contributions vary with the individual POR mutations. Patients carrying the common Japanese mutation R457H, either as homozygotes or heterozygotes with a more severely affected allele, typically have the most severe skeletal findings and are often born to mothers who virilized during pregnancy (6, 9, 13, 32), suggesting fetoplacental aromatase deficiency. By contrast, patients carrying the common European muta-

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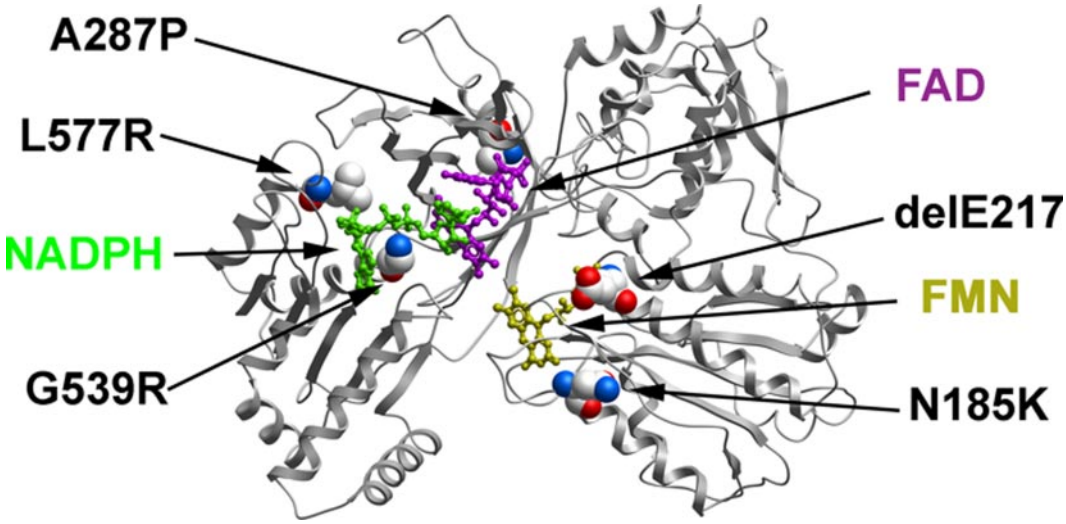

FIG. 3. Model of human POR, showing the locations of the POR mutations identified. The model is based on the x-ray crystal structure of rat POR (pdb id: 1AMO) lacking 65 N-terminal residues. The α-carbon backbone is depicted as a *narrow ribbon*, and the FAD (*magenta*) and FMN (*yellow*) moieties and NADPH (*green*) are depicted as *ball and stick models*. Residues N185, E217, A287, G539, and L577 are depicted as packed sphere images: *white*, carbon; *red*, carboxyl groups; *blue*, amine groups. The figure was created with Molsoft ICM (Molsoft L.L.C., La Jolla, California).

tion A287P are less severely affected, and their mothers do not virilize during pregnancy (6, 9, 27). Consistent with this, the A287P POR mutant supports 40% of 17α-hydroxylase and 21% of 17,20 lyase activities (6), and 100% of aromatase activity (24), whereas R457H supports only 3% of 17α-hydroxylase activity, no detectable 17,20 lyase activity (6, 9), and only 1% of aromatase activity (24). Thus, the effects of different POR mutations will vary with the P450 to which it donates electrons: the R457H mutation severely affects aromatase activity, whereas A287P does not. The virilization of 46,XX fetuses carrying A287P appears to involve the "backdoor pathway" to fetal androgen production (27, 33), in which 21-carbon steroid precursors are 5α-reduced and ultimately converted to dihydrotestosterone, bypassing the conventional precursors androstenedione and testosterone (34, 35).

Most POR-deficient patients described to date have had skeletal malformations (14), but some have been phenotypically normal adults with infertility (6, 9, 11); this distribution may reflect ascertainment bias because mildly affected patients are only detected by assaying multiple plasma steroids on an ACTH test (6,9) or performing mass spectrometric analysis of urinary steroids (11). For example, our patient 2 was a normal-appearing young female with primary amenorrhea and normal basal gonadotropins; her mild, atypical congenital adrenal hyperplasia was only detected by measuring ACTH-stimulated cortisol, 17OHP, progesterone, and androstenedione, similar to case 4 in our initial report (9). Thus, it is possible that many patients with mild forms of POR deficiency may remain undiagnosed.

The long-term outcome of severe POR deficiency is incompletely understood. Patient 3 with severe ABS is now a young adult, but she lacked spontaneous development of breasts and pubic hair. Hypergonadotropic hypogonadism in affected females will frequently lead to polycystic ovaries in infancy or childhood (13) and may be a presenting complaint in adulthood (9). However, in contrast to women with the polycystic ovary syndrome, the ovarian cysts in POR deficiency are associated with hypoandrogenemia. Patient 3 was very short, primarily because of severe scoliosis. A survey of Japanese patients found that many affected children and adults were tall, although some had scoliosis that compromised height (13). Puberty was typically absent, but two R457H homozygous male patients had normal spontaneous pubertal development (13), although R457H supported no 17,20 lyase activity *in vitro* (6, 9). Thus, physicians must remain cautious about predicting clinical outcomes based on mutation analysis.

The pathogenesis of the skeletal malformations in POR deficiency is unclear. Four lines of evidence support a role for cholesterol biosynthesis in bone formation (36). First, cholesterol biosynthesis requires squalene epoxidase and 14α-demethylase (CYP51), which both require POR; 14α-demethylase activity was reduced in fibroblasts from a patient with ABS caused by POR deficiency. Second, ABS has been reported after maternal ingestion of fluconazole, which acts by inhibiting fungal CYP51 activity. Third, skeletal malformations are found in other disorders of cholesterol biosynthesis such as Smith-Lemli-Opitz syndrome (OMIM 270400). Finally, cholesterol derivatization of hedgehog proteins is required for signaling in bone formation. Two recent studies support this hypothesis. First, tissue-specific POR knockout in the limb bud mesenchyme of mice induces the expression of genes through-

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out the cholesterol biosynthetic pathway, suggesting that cholesterol deficiency could explain the skeletal phenotypes (37). Second, POR knockdown by RNA interference in rat chondrocytes decreased cell proliferation and differentiation, induced apoptosis, and reduced expression of *Indian hedgehog*, but these effects were reversed by providing cholesterol (38). Thus, cholesterol synthesis is probably involved in the skeletal phenotype of POR deficiency, but other mechanisms may also be operative.

The diagnosis of POR deficiency requires an ACTH test with measurement of baseline and stimulated cortisol, 17OHP, progesterone, DHEA, and androstenedione. The basal ACTH is typically minimally elevated; basal cortisol is often normal, but poorly responsive to ACTH; 17OHP values are mildly elevated and modestly hyperresponsive to ACTH; normal ratios of $\Delta 5$ to $\Delta 4$ steroids exclude 3β-hydroxysteroid dehydrogenase deficiency. Some patients have had adrenal crisis (9, 13, 39), and our patient 4 required ongoing replacement therapy. The decision whether or not to treat with glucocorticoid replacement therapy will vary with the individual patient and the specific POR mutation(s) involved. As in other causes of adrenal insufficiency, this clinical judgment is best made on the basis of the cortisol response to ACTH stimulation; we typically recommend glucocorticoid supplementation during surgery or severe stress. Mineralocorticoid deficiency has not been described in POR deficiency, and PRA is generally normal, although patient 4 had hyponatremia and hyperreninemia with abundant aldosterone in infancy. Low maternal serum estriol and a characteristic pattern of urinary steroid precursors may suggest the diagnosis in an affected fetus (24, 32, 40), which could then be confirmed by sequencing the POR gene in fetal DNA obtained by chorionic villus biopsy. Preliminary preimplantation genetic diagnosis has been reported recently (41). Because most drugs are metabolized by POR-dependent P450 enzymes and POR mutants affect drug metabolism *in vitro* (16), abnormal drug metabolism is likely in these patients; thus caution should be exercised in treating POR-deficient patients with drugs metabolized by P450 enzymes.

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References

1. **Miller WL** 2005 Minireview: regulation of steroidogenesis by electron transfer. Endocrinology 146:2544–2550
2. **Ono T, Bloch K** 1975 Solubilization and partial characterization of rat liver squalene epoxidase. J Biol Chem 250:1571–1579
3. **Ilan Z, Ilan R, Cinti DL** 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 256:10066–10072
4. **Wilks A, Black SM, Miller WL, Ortiz de Montellano PR** 1995 Expression and characterization of truncated human heme oxygenase (hHO-1) and a fusion protein of hHO-1 with human cytochrome P450 reductase. Biochemistry 34:4421–4427
5. **Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ** 1997 Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc Natl Acad Sci USA 94:8411–8416
6. **Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, Jabs EW, Van Vliet G, Sack J, Flück CE, Miller WL** 2005 Diversity and function of mutations in P450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. Am J Hum Genet 76:729–749
7. **Shen AL, O'Leary KA, Kasper CB** 2002 Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. J Biol Chem 277:6536–6541
8. **Otto DM, Henderson CJ, Carrie D, Davey M, Gundersen TE, Blomhoff R, Adams RH, Tickle C, Wolf CR** 2003 Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis. Mol Cell Biol 23:6103–6116
9. **Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, Jabs EW, Mendonça BB, Fujieda K, Miller WL** 2004 Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. Nat Genet 36:228–230
10. **Antley R, Bixler D** 1975 Trapezoidocephaly, midfacial hypoplasia and cartilage abnormalities with multiple synostoses and skeletal fractures. Birth Defects Orig Artic Ser 11:397–401
11. **Hershkovitz E, Parvari R, Wudy SA, Hartmann MF, Gomes LG, Loewental N, Miller WL** 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 93:3584–3588
12. **Scott RR, Gomes LG, Huang N, Van Vliet G, Miller WL** 2007 Apparent manifesting heterozygosity in P450 oxidoreductase deficiency and its effect on coexisting 21-hydroxylase deficiency. J Clin Endocrinol Metab 92:2318–2322
13. **Fukami M, Nishimura G, Homma K, Nagai T, Hanaki K, Uematsu A, Ishii T, Numakura C, Sawada H, Nakacho M, Kowase T, Motomura K, Haruna H, Nakamura M, Ohishi A, Adachi M, Tajima T, Hasegawa Y, Hasegawa T, Horikawa R, Fujieda K, Ogata T** 2009 Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype-phenotype correlations in 35 Japanese patients. J Clin Endocrinol Metab 94:1723–1731
14. **Scott RR, Miller WL** 2008 Genetic and clinical features of P450 oxidoreductase deficiency. Horm Res 69:266–275
15. **Reardon W, Smith A, Honour JW, Hindmarsh P, Das D, Rumsby G, Nelson I, Malcolm S, Adès L, Sillence D, Kumar D, DeLozier-Blanchet C, McKee S, Kelly T, McKeehan WL, Baraitser M, Winter RM** 2000 Evidence for digenic inheritance in some cases of Antley-Bixler syndrome? J Med Genet 37:26–32
16. **Agrawal V, Huang N, Miller WL** 2008 Pharmacogenetics of P450

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oxidoreductase: effect of sequence variants on activities of CYP1A2 and CYP2C19. Pharmacogenet Genomics 18:569–576
17. **Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL** 1987 Cytochrome P450c17 (steroid 17 α-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. Proc Natl Acad Sci USA 84:407–411
18. **Brock BJ, Waterman MR** 1999 Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species. Biochemistry 38:1598–1606
19. **Lamb DC, Warrilow AG, Venkateswarlu K, Kelly DE, Kelly SL** 2001 Activities and kinetic mechanisms of native and soluble NADPH-cytochrome P450 reductase. Biochem Biophys Res Commun 286:48–54
20. **Pandey AV, Mellon SH, Miller WL** 2003 Protein phosphatase 2A and phosphoprotein SET regulate androgen production by P450c17. J Biol Chem 278:2837–2844
21. **Pandey AV, Miller WL** 2005 Regulation of 17,20 lyase activity by cytochrome b5 and by serine phosphorylation of P450c17. J Biol Chem 280:13265–13271
22. **Auchus RJ, Lee TC, Miller WL** 1998 Cytochrome b5 augments the 17,20-lyase activity of human P450c17 without direct electron transfer. J Biol Chem 273:3158–3165
23. **Dhir V, Ivison HE, Krone N, Shackleton CH, Doherty AJ, Stewart PM, Arlt W** 2007 Differential inhibition of CYP17A1 and CYP21A2 activities by the P450 oxidoreductase mutant A287P. Mol Endocrinol 21:1958–1968
24. **Pandey AV, Kempná P, Hofer G, Mullis PE, Flück CE** 2007 Modulation of human CYP19A1 activity by mutant NADPH P450 oxidoreductase. Mol Endocrinol 21:2579–2595
25. **Huang N, Agrawal V, Giacomini KM, Miller WL** 2008 Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. Proc Natl Acad Sci USA 105:1733–1738
26. **Horecker BL** 1950 Triphosphopyridine nucleotide-cytochrome *c* reductase in liver. J Biol Chem 183:593–605
27. **Arlt W, Walker EA, Draper N, Ivison HE, Ride JP, Hammer F, Chalder SM, Borucka-Mankiewicz M, Hauffa BP, Malunowicz EM, Stewart PM, Shackleton CH** 2004 Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. Lancet 363:2128–2135
28. **Shen AL, Kasper CB** 1995 Role of acidic residues in the interaction of NADPH-cytochrome P450 oxidoreductase with cytochrome P450 and cytochrome c. J Biol Chem 270:27475–27480
29. **Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, Deisenhofer J** 1995 Structure and function of cytochromes P450: a comparative analysis of three crystal structures. Structure 3:41–62
30. **Geller DH, Auchus RJ, Mendonça BB, Miller WL** 1997 The genetic and functional basis of isolated 17,20-lyase deficiency. Nat Genet 17:201–205
31. **Auchus RJ, Miller WL** 1999 Molecular modeling of human P450c17 (17α-hydroxylase/17,20-lyase): insights into reaction mechanisms and effects of mutations. Mol Endocrinol 13:1169–1182
32. **Fukami M, Horikawa R, Nagai T, Tanaka T, Naiki Y, Sato N, Okuyama T, Nakai H, Soneda S, Tachibana K, Matsuo N, Sato S, Homma K, Nishimura G, Hasegawa T, Ogata T** 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 90:414–426
33. **Homma K, Hasegawa T, Nagai T, Adachi M, Horikawa R, Fujiwara I, Tajima T, Takeda R, Fukami M, Ogata T** 2006 Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: implication for the backdoor pathway to dihydrotestosterone. J Clin Endocrinol Metab 91:2643–2649
34. **Wilson JD, Auchus RJ, Leihy MW, Guryev OL, Estabrook RW, Osborn SM, Shaw G, Renfree MB** 2003 5α-Androstane-3α,17β-diol is formed in tammar wallaby pouch young testes by a pathway involving 5α-pregnane-3α,17α-diol-20-one as a key intermediate. Endocrinology 144:575–580
35. **Auchus RJ** 2004 The backdoor pathway to dihydrotestosterone. Trends Endocrinol Metab 15:432–438
36. **Flück CE, Miller WL** 2006 P450 oxidoreductase deficiency: a new form of congenital adrenal hyperplasia. Curr Opin Pediatr 18: 435–441
37. **Schmidt K, Hughes C, Chudek JA, Goodyear SR, Aspden RM, Talbot R, Gundersen TE, Blomhoff R, Henderson C, Wolf CR, Tickle C** 2009 Cholesterol metabolism: the main pathway acting downstream of cytochrome P450 oxidoreductase in skeletal development of the limb. Mol Cell Biol 29:2716–2729
38. **Aguilar A, Wu S, De Luca F** 2009 P450 oxidoreductase expressed in rat chondrocytes modulates chondrogenesis via cholesterol- and Indian Hedgehog-dependent mechanisms. Endocrinology 150:2732–2739
39. **Adachi M, Tachibana K, Asakura Y, Yamamoto T, Hanaki K, Oka A** 2004 Compound heterozygous mutations of cytochrome P450 oxidoreductase gene (POR) in two patients with Antley-Bixler syndrome. Am J Med Genet A 128A:333–339
40. **Shackleton C, Marcos J, Arlt W, Hauffa BP** 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:105–112
41. **Alberola TM, Bautista-Llácer R, Fernández E, Vendrell X, Pérez-Alonso M** 2009 Preimplantation genetic diagnosis of P450 oxidoreductase deficiency and Huntington disease using three different molecular approaches simultaneously. J Assist Reprod Genet 26: 263–271

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