Supplementary on line material

Materials and Methods

Endocrine Studies

Serum was analyzed for cortisol, 17OHP, testosterone, DHEA sulfate (DHEAS), LH and FSH by highly sensitive and specific competitive immunoassays.
Cortisol was determined by a competitive immunoassay (ADVIA – Bayer diagnostic, Germany); the assay coefficient of variance was between 4.2-6.5%. 17OHP was determined by a competitive immunoassay ((MP – Biomedicals, NY); the assay coefficient of variance was between 10-13%. Testosterone was measured by a competitive immunoassay (ADVIA – Bayer diagnostic, Germany); the assay coefficient of variance was between 2.7- 7.6 %. DHEAS was determined by a competitive immunoassay (Immulite 2000-Siemense, Germany); the assay coefficient of variance was between 7.9-13%. LH was determined by highly sensitive competitive immunoassay (Immulite 2000-Siemense, Germany); the assay coefficient of variance was between 7-9.4%. FSH was determined by a competitive immunoassay (Immulite 2000-Siemense, Germany); the assay coefficient of variance was between 4-8%.

Gas chromatography/mass spectrometry (GC-MS)

Urinary steroids were profiled using GC-MS and selected ion monitoring analysis as previously described by Wudy et al*. The hydrolyzed steroids were recovered by Sep-Pak extraction. Known amounts of three internal standards (5α-androstane-3α,17α-diol, stigmasterol, and cholesteryl butyrate) were added to a portion of each extract before formation of methyloxime-trimethylsilyl ethers.

Genetic studies in Israel

Genomic DNA was extracted from peripheral leukocytes of all patients. Primers for PCR amplification of the 15 coding exons of the *POR* gene and their borders were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR amplification of *POR* exon 12, which carries the G539R mutation, was done with forward primer 5' CTGCAGAACGGGACTTGG 3', and reverse primer 5' CCCGGTACAGGTAGTCCTCA 3'; cycling consisted of 30 cycles at 94^{o} for 1 min, 61^{o} for 1 min, and 72^{o} for 1 min. PCR amplification of *CYP17A1* exon 1 to detect the frameshift mutation described by Biason-Lauber et al. (12) was done with forward primer 5' AGTTGAGCCAGCCCTTGAG 3', and reverse primer 5' GGCCGACAATCACTGTAGTCT 3'; cycling consisted of 30 cycles at 94^{o} for 1 min, 59^{o} for 1 min, and 72^{o} for 1 min. Following the PCR reaction, the remaining free primers were digested by a combination of 0.6 U shrimp alkaline phosphatase and 6 U Exonuclease I (both from Fermentas, Lithuania). Amplification *CYP17A1* exon 8 to examine the F417C mutation was done with forward primer 5' CATCCTCAGATCAGGGTTCC 3', and reverse primer 5' CAGGCCATGATGAGGAAGAG 3' cycling consisted of 30 cycles at 94^{o} for 1 min, 57^{o} for 1 min, and 72^{o} for 1 min. PCR products were directly sequenced on an AB373 apparatus.

Genetic studies in San Francisco

DNA prepared in Israel was sent to San Francisco for independent analysis. Primers for PCR amplification of *POR* exon 12 and *CYP17A1* exons 1 and 8 were designed using the University of California, Santa Cruz genome browser (http://genome.ucsc.edu) and Primer 3 server (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR amplification of *POR*

exon 12 was done with forward primer 5`-GAGGGGGCCTCTGAGGTTTG-3`, and reverse primer 5`- ACAGGTGCTCTCGGTCTTGCTT -3`. PCR was performed under touchdown cycling conditions: 95C for 4 min, then 15 touchdown cycles of 95C for 30 sec, 67C for 30 sec (decreasing by 0.5C with each cycle), and 72C for 45 sec, followed by 35 cycles of 95C for 30sec, 60C for 30 sec, and 72C for 45 sec. The final extension was held at 72C for 7 min. PCR of *CYP17A1* exon 1, to detect the frameshift mutation, was done with forward primer 5`-GGGCTCCAGGAGAATCTTTC-3`, and reverse primer 5`-TCCCTTCACATCATCCCACT-3`. Amplification was performed under touchdown condition as described above, except that annealing started at 62C and finished at 55C after the touchdown cycles, and then continued at 55C for the remaining 35 cycles. Amplification of *CYP17A1* exon 8, to detect the F417C mutation, was done with forward primer 5`-CTACCTGAAAGCAGGGCTGT-3`, and reverse primer 5`-CATTGCCACAAGCTGAAAAA-3`. PCR was performed as above, except that annealing started at 58C for 15 touchdown cycles and continued at 51C for the remaining 35 cycles. Automated direct sequencing of PCR products employed ABI BigDye terminator, version 3.1 (Applied Biosystems). Data were displayed with ABI 3730#1 DNA Analyzer and analyzed using Sequencher 4.5 (Gene Code Corporation, Ann Harbor, MI).

Restriction endonuclease digestion

According to NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php), no commercially available restriction endonuclease will recognize the putative frameshift mutation site in *CYP17A1* exon 1. However the site of the putative F417C mutation in exon 8 can be cut by Hpy188III, which recognizes TCNNGA. The 591 bp PCR products of exon 8 from the four patients and from control DNA were digested with 2 units of Hpy188III in a 50 μL reaction, the products were separated on a 2% agarose gel and visualized by staining with ethidium bromide.

* **Wudy SA, Hartmann MF** 2004 Gas chromatography-mass spectrometry profiling of steroids in times of molecular biology. Hormone Metabolic Res 36:415-422