Steroid Biomarkers and Genetic Studies Reveal Inactivating Mutations in Hexose-6-Phosphate Dehydrogenase in Patients with Cortisone Reductase Deficiency

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Context: Cortisone reductase deficiency (CRD) is characterized by a failure to regenerate cortisol from cortisone via 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), resulting in increased cortisol clearance, activation of the hypothalamic-pituitary-axis (HPA) and ACTH-mediated adrenal androgen excess. 11β-HSD1 oxoreductase activity requires the reduced nicotinamide adenine dinucleotide phosphate-generating enzyme hexose-6-phosphate dehydrogenase (H6PDH) within the endoplasmic reticulum. CRD manifests with hyperandrogenism resulting in hirsutism, oligo-amenorrhea, and infertility in females and premature pseudopuberty in males. Recent association studies have failed to corroborate findings that polymorphisms in the genes encoding H6PDH (R453Q) and 11β-HSD1 (Intron 3 inserted adenine) interact to cause CRD.

Objective: Our objective was to reevaluate the genetics and steroid biochemistry of patients with CRD.

Design: We analyzed 24-h urine collection for steroid biomarkers by gas chromatography/mass spectrometry and sequenced the *HSD11B1* and *H6PD* genes in our CRD cohort.

Patients: Patients included four cases presenting with hyperandrogenism and biochemical features clearly indicative of CRD.

Results: Gas chromatography/mass spectrometry identified steroid biomarkers that correlated with CRD in each case. Three cases were identified as homozygous (R109AfsX3, Y316X, and G359D) and one case identified as compound heterozygous (c.960G→A and D620fsX3) for mutations in *H6PD.* No mutations affecting enzyme activity were identified in the *HSD11B1* gene. Expression and activity assays demonstrate loss of function for all reported H6PDH mutations.

Conclusions: CRD is caused by inactivating mutations in the *H6PD* gene, rendering the 11β-HSD1 enzyme unable to operate as an oxoreductase, preventing local glucocorticoid regeneration. These data highlight the importance of the redox control of cortisol metabolism and the 11β-HSD1-H6PDH pathway in regulating hypothalamic-pituitary-adrenal axis activity. **(*J Clin Endocrinol Metab* 93: 3827–3832, 2008)**

Cortisone reductase deficiency (CRD) is a disorder in which there is a failure to regenerate the active glucocorticoid cortisol (F) from cortisone (E) via the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1) (1). A lack of cortisol regeneration stimulates ACTH-mediated adrenal hyperandrogenism, with males manifesting in early life with precocious pseudopu-

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Abbreviations: CRD, Cortisone reductase deficiency; GC/MS-SIM, gas chromatography/mass spectrometry selected-ion-monitoring method; H6PDH, hexose-6-phosphate dehydrogenase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PCOS, polycystic ovary syndrome; THE, tetrahydrocortisone; THF, tetrahydrocortisol; WT, wild type.

berty and females presenting in midlife with hirsutism, oligoamenorrhea, and infertility (2–7). Biochemically, CRD has been diagnosed through the assessment of urinary cortisol and cortisone metabolites and consists of measuring the tetrahydrocortisol (THF) plus 5α-THF/tetrahydrocortisone (THE) ratio (THF+5α-THF/THE ratio); in CRD patients, the ratio is typically lower than 0.1 (reference range 0.7–1.2) (1).

11β-HSD1 (encoded by the *HSD11B1* gene) is a bidirectional enzyme *in vitro* acting as a reductase (cortisone to cortisol) or as a dehydrogenase (cortisol to cortisone) but *in vivo* functions primarily as a reductase (1). It therefore appeared that *HSD11B1* was the obvious candidate gene mutated in CRD; however, no coding mutations directly affecting enzyme activity had been identified (3, 4, 6). The cortisol-regenerating reductase activity of 11β-HSD1 is critically dependent upon the provision of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the endoplasmic reticulum lumen and is provided by the enzyme hexose-6-phosphate dehydrogenase (H6PDH, encoded by the *H6PD* gene) (Fig. 1) (8). In humans, this implies that without NADPH, 11β-HSD1 may be inactive or indeed switched from reductase to dehydrogenase activity; the H6PDH enzyme therefore represents a candidate to explain CRD. In support of this, we have demonstrated that H6PDH-null mice lose 11β-HSD1 reductase activity but gain dehydrogenase activity through changes in NADPH cofactor provision (9). Others have shown that mice lacking *HSD11B1* lack both activities (10). Previously, we identified sequence variants in *H6PD* and *HSD11B1* in patients with CRD (3), but subsequent large-scale population-based studies from three centers including our own indicated that these were polymorphic variants rather than disease-causing mutations, with population frequencies that could not fully explain either the urinary cortisol biochemical abnormalities or clinical features seen in our CRD cases (11–13).

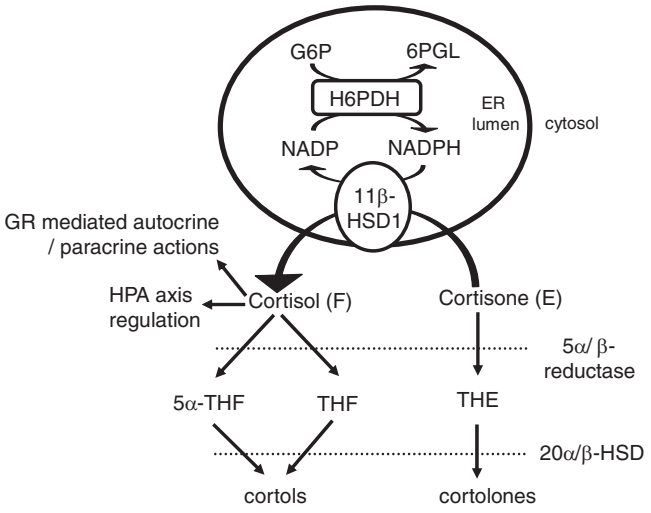

FIG. 1. Schematic representation of how H6PDH and 11β-HSD1 interact to regulate local cortisol metabolism and the HPA axis. G6P entering the endoplasmic reticulum (ER) lumen (via a specific G6P transporter not shown) is metabolized to 6-phosphogluconate (6PGL) and, in doing so, generates NADPH, allowing 11β-HSD1 reductase activity to convert inactive cortisone to active cortisol. Cortisol and cortisone metabolism via 5α- and 5β-reductase and 20α- and 20β-HSD generate metabolites detectable in the urine that serve as biomarkers of 11β-HSD1 activity. Any changes to cortisol clearance through impaired cortisone to cortisol conversion are offset by changes to cortisol secretion via the HPA axis to maintain normal circulating concentrations. GR, Glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; NADP, nicotinamide adenine dinucleotide phosphate.

We have now revisited three CRD cases previously studied, and a novel case, and extended the steroid, biochemical, and genetic findings. The data indicate that CRD can be caused by inactivating mutations in the *H6PD* gene that is fully consistent with the steroid biochemistry, functional evaluation, and the concept that 11β-HSD1 dehydrogenase activity predominates when H6PDH enzyme activity is absent.

Subjects and Methods

Cases

Approval for all studies was obtained from the local Hospital Ethics Committees, and each subject gave informed written consent; case B was a minor, and parental consent was obtained.

Case A presented at the age of 55 yr with androgenic alopecia, hirsutism, and a previous history of anovulatory infertility (2). Case B (individual 3 in Ref. 3) is a 7-yr-old boy who presented with precocious pseudopuberty (5). Case C (individual 2 in Ref. 3) presented with long-standing hirsutism at the age of 44 yr (3). Case D (individual 1 in Ref. 3) presented with oligoamenorrhea, hirsutism, and acne at the age of 36 yr (4). Cases A–D had urinary THF+5α-THF/THE ratios of 0.05 or lower (reference range, 0.7–1.2). None of the affected families were consanguineous.

Urinary steroid metabolite analysis

Urinary steroid metabolite excretion was analyzed using a previously described gas chromatography/mass spectrometry selected-ion-monitoring method (GC/MS-SIM) (7, 14). Steroids were enzymatically released from conjugation and, after extraction, chemically derivatized before GC/MS-SIM analysis. Metabolites quantified were cortisol (F), THF, 5α-THF, α-cortol, β-cortol, cortisone (E), THE, α-cortolone, and β-cortolone. Total metabolites of cortisol are represented as the sum of THF+5α-THF+F+cortols, and total metabolites of cortisone as the sum of THE+E+cortolones. Totaled cortisol and cortisone metabolites are an index of total daily cortisol secretion rate as previously validated (15, 16). The urinary THF +5α-THF/THE and cortols/cortolones were used as measures of 11β-HSD1 activity. Androgen excretion was quantified by the sum of androsterone plus etiocholanolone plus dehydroepiandrosterone excretion (14). Normal ranges for urinary steroid analysis are depicted as box and whisker plots and were determined using sex- and age-specific normal cohorts, which for the purposes of this report, are represented as adult females (n = 33), young males (n = 20 aged 8–16 yr), and adult males (n = 26).

Mutation analysis of *HSD11B1* and *H6PD* genes

PCR amplification of the coding region of *HSD11B1* and *H6PD* genes including exon/intron boundaries was performed using genomic DNA from cases A–D employing previously published primers (3). We compared *HSD11B1* sequences with GenBank entries for two overlapping clones covering chromosome 1q32.2-41, PAC 28010 and PAC 43014, and *H6PD* sequences to clone RP3-510D11 on chromosome 1p36.2-36.3.

Functional mutation analysis

The QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate wild-type (WT) *H6PD* and *HSD11B1* cDNA, contained in pcDNA3.1D/V5-His-TOPO (Invitrogen, Paisley, UK), to the respective alternative mutant sequences, with subsequent confirmation of the introduced mutations by direct sequencing. HEK 293 cells were transfected with WT and mutant *H6PD* and *HSD11B1* cDNAs

using a 293 cell-specific transfection reagent (Mirus, Madison, WI). Stably transfected cells were selected using G418 (Sigma, Poole, UK), and in each case, four cell lines were derived from four separate transfection experiments for WT and mutant enzyme studies, with cell lines mock transfected with empty vector as a further control.

Total RNA was extracted from transfected cell lines using a single-step extraction method (Tri-reagent; Sigma) and 1 μg total RNA reverse-transcribed to cDNA (Applied Biosystems, Warrington, UK). Subsequently, quantitative PCR was performed to determine HSD11B1 and H6PD mRNA expression levels using commercially available assays (Applied Biosystems).

H6PDH assays were performed on WT and mutant microsomes prepared from HEK 293 cells. Microsomal protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). H6PDH enzyme activity was measured by spectrofluorometric detection of NADPH generation using absorbance readings taken at 340 nm. Microsomes were permeabilized with 0.5% Triton X-100 and incubated in 50 mM glycine buffer (pH 9.0) at 37 C in the presence of 1 mM G6P and 0.4 mM NADP+. Absorbance readings were taken at 1-min intervals for 5 min using a luminescence spectrometer (excitation 340 nm, emission 456 nm).

All experiments were carried out in triplicate in at least three independent experiments, and data shown as mean ± SEM.

Results

Urinary steroid metabolite analysis

A low THF+5α-THF/THE ratio (see Fig. 1) was observed in all cases, with ratios of 0.05 or less in cases A–D [Fig. 2,A (adult female range) and C (young male range)]. In all cases, ratios were lower than age- and sex-specific reference cohorts. As an additional biochemical phenotype, we studied the ratio of urinary cortols to cortolones (Fig. 1), an adjunct ratio that reflects secondary metabolism of cortisol and cortisone, respectively. Again, this ratio was low when compared with age- and sex-specific reference cohorts, in keeping with a block in 11β-HSD1-mediated cortisone to cortisol conversion (Fig. 2, A and C). We also assessed the absolute levels of cortisol and cortisone metabolites (micrograms per 24 h). Although the metabolites of cortisol in cases A–D were low to normal, the metabolites of cortisone were extremely elevated in cases A–D (Fig. 2, B and D) compared with age- and sex-specific reference cohorts, again lending powerful evidence for a block in 11β-HSD1-mediated cortisone to cortisol conversion.

To examine any potential effect on the hypothalamic-pituitary-adrenal (HPA) axis, we totaled all cortisol and cortisone metabolites as an index of total cortisol secretion rate (micrograms per 24 h) (15). An increase in cortisol secretion rate was clearly seen in the adult female cases A, C, and D (A, 41,603; C, 33,213; and D, 36,564; reference range, 2,676–14,071 μg/24 h; median, 5118 μg/24 h). Cortisol secretion was moderately elevated toward the upper limit of the reference range in the young male case B (7411; reference range, 1,149–10, 212 μg/24 h; median, 3382 μg/24 h).

Molecular analysis of the *H6PD* gene

Sequencing revealed four novel and one previously reported sequence variant in the four unrelated cases A–D in either the homozygous or compound heterozygous state that were not detected in 120 control chromosomes. No mutations or sequence variants were identified in the *HSD11B1* gene in cases A–D.

Case A was homozygous for a c.325delC mutation in exon 2 that resulted in a frameshift with the generation of an in-frame stop codon, thereby truncating the protein by 781 amino acids (R109AfsX3) (Fig. 3A).

Case B was homozygous for a c.948C→G mutation in exon 4, generating a premature stop codon replacing the encoded tyrosine and truncating the resulting protein by 575 amino acids (Y316X) (Fig. 3B).

Case C was homozygous for a c.1076G→A mutation in exon 5, generating a glycine to aspartate missense mutation (G359D). G359 is highly conserved within microsomal H6PDH and the cytosolic homolog G6PDH enzymes across species (Fig. 3C).

Case D was compound heterozygous for a paternally inherited c.960G→A mutation in exon 4 and a maternally inherited c.1860ins29bp insertion mutation in exon 5 (Fig. 3D). The c.960G→A mutation generates a strong donor splice site consensus sequence (AG.GTGCG to AG.GTACG) in exon 4. RT-PCR carried out on cDNA from case D indicated that the activated donor splice site is used and results in a 54-nucleotide truncated mRNA, with the loss of 18 amino acids in the resultant protein. An additional mRNA product retaining intron 4 was also observed, implying variable mutant splicing

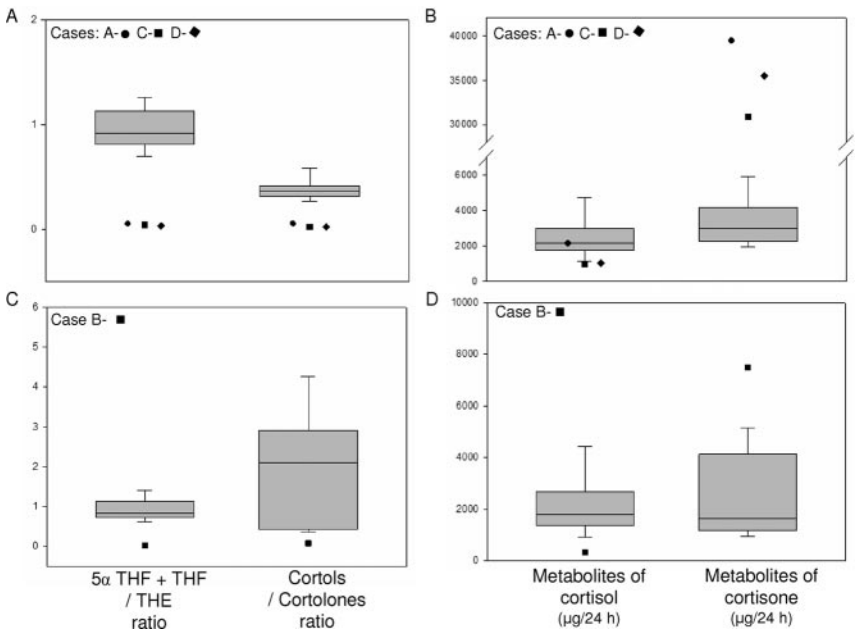

FIG. 2. Urinary cortisol and cortisone metabolites for cases A–D. For *panels* A and B, the *box plot* represents the normal range of the glucocorticoid metabolites or ratios measured for adult females (cases A, C, and D). For *panels* C and D, the *box plots* represent the normal range for young males (case B). Metabolites measured are listed in *Subjects and Methods*. In each case, the THF+5α-THF/THE and cortol/cortolone ratios are both exceptionally low. Metabolites of cortisone are elevated in all cases, most clearly in the adult females (note a break in the y-axis).

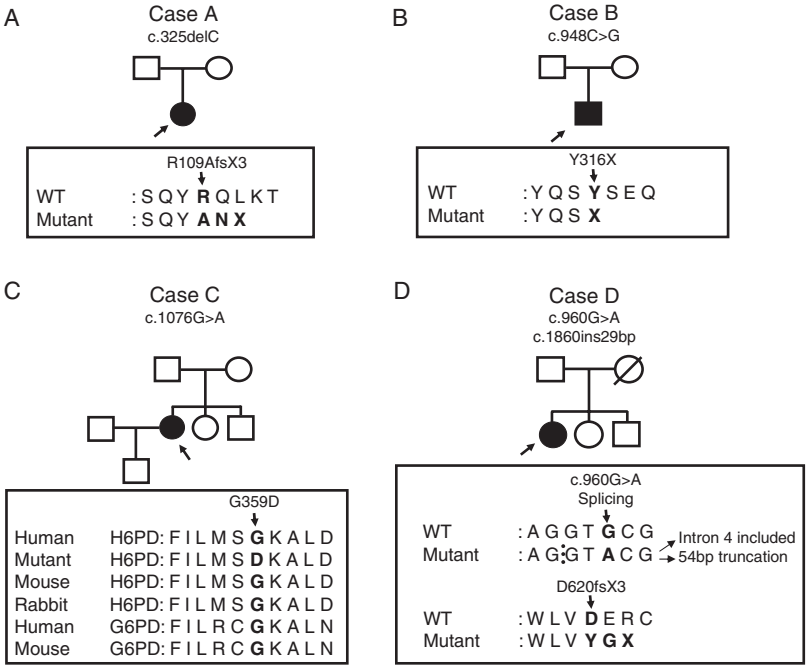

FIG. 3. Analysis of the *H6PD* gene mutations in CRD cases A–D. For each case, the family pedigree and position and alteration at the nucleotide and protein levels are given and shown. A, R109AfsX3 frameshift mutation results in a severe truncation; B, Y316X nonsense mutation results in a severe truncation; C, evidence for recessive inheritance of the G359D missense mutation and indication of sequence conservation across species; D, the affected female is compound heterozygous for a c.960G→A generating a cryptic donor splice site within exon 4 and a D620fsX3 frameshift mutation generating a truncated protein. RT-PCR on RNA isolated from sc adipose tissue from case D showed two alternate splice products as a result of the c.960G→A cryptic splice site activation (boundary defined by *three dots*). As a result of the mutation, a 54-nucleotide truncated and intron 4-included products were revealed.

(Fig. 3D). The c.1860ins29bp mutation would generate a frame shift and an in-frame stop codon. The resulting protein would be truncated by 268 amino acids (D620fsX3).

Functional analysis of *H6PD* mutations

Little is known of the three-dimensional structure of the H6PDH enzyme. However, most of the mutations detected would be expected to lead to complete loss of enzyme function. To further address function, the four coding sequence variants, R109AfsX3, Y316X, G359D, and D620fsX3, were stably expressed *in vitro* in HEK 293 cells and H6PDH enzyme activity measured by the ability to generate NADPH. HEK 293 cells mock transfected with empty vector had minimal H6PDH activity (Fig. 4). Upon transfection with WT H6PDH, there was a greater than 10-fold increase in H6PDH activity; however, when cells were transfected to similar levels of expression with the four mutations, we were unable to detect activity above cellular background levels (Fig. 4). We previously reported that the R453Q polymorphism reduces H6PDH enzyme activity; however, subsequent population-based studies have detected this mutant at high frequency and shown it not to correlate with parameters of cortisol metabolism. Indeed, in this assay, we were unable to detect any functional defect (Fig. 4). The discrepancy may lie in the assay conditions, because it is noted that this study reports assays from microsomal preparations from stably transfected cells, whereas our previous assays were carried out on cell lysates from stably transfected cells.

Discussion

This study shows that CRD can be explained solely by inactivation of the *H6PD* gene with recessive inheritance leading to pronounced manifestation of CRD due to a lack of cortisol generation causing activation of the HPA axis and subsequent androgen excess.

In 2003, we published a paper that first linked H6PDH to the 11-oxoreductase activity of 11β-HSD1 (3), a conclusion that has subsequently been confirmed through many studies (9, 17–19). In doing so we included *H6PD* sequence data on three cases reported in this manuscript (cases B, C, and D), data that focused on a R453Q mutation, which in functional assays, was associated with loss of H6PDH activity (3). This was a common finding in all cases of CRD. However, subsequent studies (12, 13), including our own (11), have characterized this R453Q mutation as a polymorphic variant that in retrospect could not explain either the biochemical or clinical features or CRD. In addition, a novel case with CRD (case A), revealed a novel homozygous mutation in *H6PD* that prompted a reinvestigation of cases B, C, and D. It is clear that mutations in *H6PD* were either missed (Y316X and G359D) or presumed to be silent (c.960G→A) and of no relevance. In light of this, we have readdressed the H6PDH enzyme activity data for the R453Q variant and show that this does not affect enzyme activity. The reason for this discrepancy remains to be fully elucidated; the assays employed initially were more protracted using cell lysates and the surrogate substrate galactose-6-phosphate (3) rather than purified microsomes incubated with the physiological substrate G6P.

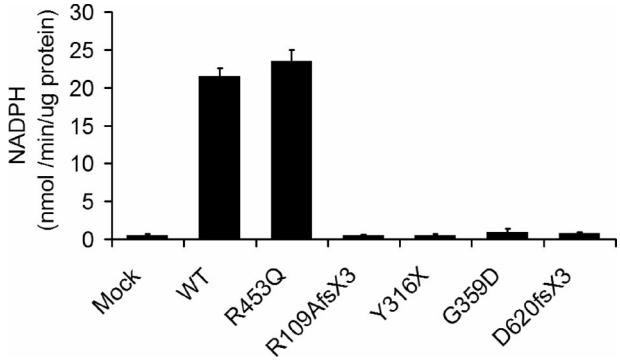

FIG. 4. *H6PD* gene functional activity in mutants. H6PDH activity (NADPH generation) from microsomes isolated from HEK 293 cells were mock or stably transfected with the WT and mutant cDNA constructs. Data are presented as nanomoles NADPH generated per minute per microgram of protein (n = 3 in triplicate, mean ± SEM). Compared with mock transfected cells (0.5 ± 0.2), activity in cells stably transfected with H6PDH constructs was 21.5 ± 1.1 (WT), 23.45 ± 1.6 (R453Q), 0.9 ± 0.5 (G359D), 0.4 ± 0.2 (R109AfsX), 0.4 ± 0.3 (Y316X), and 0.7 ± 1.2 (D620fsX3).

We postulated that H6PDH deficiency would result in a florid biochemical phenotype. 11β-HSD1 is a bidirectional enzyme with dehydrogenase activity, inactivating cortisol, clearly present in some tissues (1, 20). H6PDH null mice lose 11β-HSD1 reductase activity but gain dehydrogenase activity, whereas mice lacking *HSD11B1* lack both activities (9, 10). This led us to reason that the exceptionally low THF+5α-THF/THE ratios (<0.1) seen in subjects with *H6PD* mutations is a combination of both a gain in 11β-HSD1 dehydrogenase activity and loss of oxoreductase activity, which acts to clear cortisol at a greater rate than deficient 11β-HSD1 oxoreductase alone. It is of interest to note that heterozygosity for *H6PD* inactivation leads to no biochemical or clinical manifestation as determined in heterozygote carriers for G359D and D620fsX3 (data not shown), suggesting that NADPH provision from one H6PDH allele is adequate to maintain normal 11β-HSD1 reductase activity.

We have recently identified a novel skeletal myopathy in our H6PD knockout mice that results in reduced muscle mass, vacuolated fibers, and activation of the unfolded protein response pathway (21). The underlying mechanism for the myopathy is unknown. To date, we have no report or clinical findings to suggest myopathy in our CRD cohort. It is our intention to assess these observations further during our prospective studies aiming to identify novel cases of CRD and understand the metabolic consequences for these patients.

The genetic mechanism underlying hyperandrogenism and premature adrenarche still remain largely unknown. In females, androgen excess is a robust biochemical marker of polycystic ovary syndrome (PCOS), with 70% of all cases presenting with elevated circulating testosterone and/or androstenedione levels (22–24). PCOS is the most common endocrine disorder affecting 10–15% of all premenopausal women and characterized by oligoamenorrhea, hirsutism, anovulatory infertility, obesity, and insulin resistance, but the underlying cause in the majority of cases has remained obscure (23, 24). PCOS is regarded as a polygenic disorder with influences from the environment and lifestyle modulating the hyperandrogenic trait. We speculate that variants in *H6PD* might impact upon the variable phenotype of PCOS and in particular upon the associated metabolic abnormalities in patients with PCOS, who are frequently insulin resistant, obese, and prone to type 2 diabetes mellitus. Paradoxically, inhibition of 11β-HSD1 is being pursued as a novel therapy to treat patients with obesity and type 2 diabetes, with the resultant lack of regeneration of cortisol via 11β-HSD1 within liver and adipose tissue reducing hepatic glucose output and adipogenesis, respectively (25–27).

In conclusion, we present a novel monogenic cause of CRD leading to hyperandrogenism masquerading as a PCOS-like phenotype in females and precocious pseudopuberty in males. In general, we would assume that individuals of both sexes affected by CRD may present with premature pubarche or even precocious pseudopuberty; phenotypic presentation in adult females would typically be equivalent to PCOS. Although urinary GC/MS analysis of cortisol metabolites offers a sensitive and specific biochemical screening tool, we do not recommend screening for CRD in every case of PCOS at present. Before recommendations for general screening, we need to obtain more clinical data on the phenotypic presentation, in particular to delineate the patient characteristics that should prompt further work-up by GC/MS analysis. Further research in this area will inform about the feasibility of screening strategies.

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