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# 17α-Hydroxylase Deficiency

## Biochemical and Molecular Findings in Two Sisters and their Family

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## Abstract

**Objective:** To search for molecular changes in two Argentinian sisters with a clinical and biochemical diagnosis of 17α-hydroxylase deficiency.

**Subjects:** Both patients had 46 XX karyotype, with sexual infantilism, primary amenorrhea, and hypertension. Other member of the first degree family did not have this deficiency.

**Hormonal results:** The patients showed high levels of gonadotrophins and progesterone along with very low cortisol and androgen levels. Basal levels of corticosterone were very high, but aldosterone was normal. Both steroids had a high response after adrenocorticotropic hormone (ACTH) stimulation, with no changes in 17-hydroxyl progesterone and cortisol levels. Progesterone, corticosterone, and aldosterone decreased with the dexamethasone test, without modifications in 17-hydroxyl progesterone and cortisol levels. A corticosterone/aldosterone ratio was calculated from the results of the stimulation test; the ratios were similar in both patients. On administration of the ACTH test, both parents and one sister (S2) showed a marked response in corticosterone levels, their corticosterone/aldosterone ratios were also similar to each other and similar to the patients.

**Molecular results:** Molecular studies in the cytochrome P450 17 (*CYP17*) gene showed that exon 8 had a 4 bp duplication at codon 480 (CATC) in the two patients and their mother and in exon 1, a C to T transition at codon 96 was identified, changing CGG into TGG in the two patients, S2, and their father.

**Conclusions:** Both patients were shown to be compound heterozygous, carrying different alleles in exon 1 and exon 8, inherited from their father and mother, respectively. The molecular results obtained on S2 confirmed the heterozygosity suggested by the stimulated hormonal test and corticosterone/aldosterone ratio.



Cytochrome P450c17 is a microsomal enzyme expressed in the fasciculata and reticularis adrenal zones and in the testicular Leydig cells and ovarian follicles.[1] The enzyme has both 17α-hydroxylase and 17,20-lyase activity. It catalyzes the 17α-hydroxylation of both pregnenolone and progesterone, driving the products into the cortisol pathway. 17,20-Lyase activity cleaves the C 17-20 bond of the 17α-hydroxylated steroids leading to the production of the sex steroids dehydroepiandrosterone and androstenedione.[2] This process takes place in the reticularis adrenal zone, with a fundamental role in adrenarche and the gonads. Due to its properties, cytochrome P450c17 has a key role in human steroidogenesis in both the glands in which it is expressed.

Cytochrome P450c17 is encoded by the cytochrome P450 17 (*CYP17*) gene, located on chromosome 10q; it consists of 8 exons spanning 8549 bp. This gene has been completely sequenced.[3]

17α-hydroxylase deficiency is an autosomal recessive disease in which adrenal and gonadal steroidogenesis are impaired. Since Biglieri et al.[4] first described this disorder in 1966, over 120 cases of 17α-hydroxylase deficiency have been reported. This adrenal disorder causes hypertension and hypokalemia as a result of the patient having high levels of plasmatic mineralocorticoid; the impaired synthesis of androgens in gonadal glands causes sexual infantilism in females and pseudohermaphroditism in males.

We studied two Argentinian sisters with a 17α-hydroxylase deficiency and their healthy family, this paper presents our clinical, biochemical, and molecular findings.

**Table I.** Blood pressure modifications during treatment with the corticoid methylprednisolone

| | P1 | P2 | Normal values |
|---|---|---|---|
| Basal | 146/94mm Hg | 150/91mm Hg | 130/80mm Hg[a] |
| 6 mg/day | 136/83mm Hg | 133/81mm Hg | |
| 8 mg/day | 126/75mm Hg | 128/78mm Hg | |

a Blood pressure values obtained through ambulatory blood pressure monitor over 24 hours.

## Case Reports

### Patient 1

Patient 1 (P1) was a 33-year-old female with a 46XX karyotype; she was 1.81m tall and had primary amenorrhea. She had previously been diagnosed with gonadal dysgenesia and was undergoing estrogen and progesterone substitution therapy. Physical examination revealed scarce pubic and axillary hair, hypoplastic breasts and infantile external genitalia. The presence of a uterus and annexes structures were revealed by an ultrasound scan. Her blood pressure was 146/94mm Hg with no previous history of hypertension.

### Patient 2

Patient 2 (P2) was a 38-year-old female with 46 XX karyotype; she was 1.78m tall. She had primary amenorrhea and had also been diagnosed with gonadal dysgenesia. P2 had similar clinical features to P1 and was also on hormonal therapy. The presence of a uterus was revealed by laparoscopy at the age of 20. Her blood pressure was 150/91mm Hg, even after undergoing three years of treatment for hypertension with enalapril 10 mg/day. This patients hypertension was more severe than that of P1.

### Clinical Management

After making a final diagnosis both patients were given corticoid therapy with different doses of methylprednisone (Deltisona B®[1], Aventis Pharma); doses were titrated until the correct dose was identified as indicated by the normalization of the patients blood pressure (table I). P1 was normalized on **methylprednisone** 6 mg/day and P2 was normalized on 8 mg/day.

On biochemical follow-up both sisters were found to have progesterone levels of less than 1 ng/mL. Substitutive therapy with estrogens-progestogens was also prescribed in order to maintain their secondary sexual features and to protect bone mass. P1 was given estradiol valerate 2mg + medroxyprogesterone acetate 5mg (Farludiol®, Rontag SA) and P2 was given ethinylestradiol 0.035mg + norgestimate 0.250mg (Cilest®, Janssen-Cilag Ltd).

### Family

Written informed consent was obtained from all the first degree relatives of P1 and P2 (the parents and siblings of the patients), they were then also studied. The father (F), mother (M), and two female siblings (S1 and S2) were 73, 67, 40, and 35 years old, respectively. Both parents had hypertension with normal serum potassium levels and both were undergoing treatment for hypertension. The siblings were both healthy and the eldest (S1) had three children.

## Materials and Methods

### Hormones

Blood samples were obtained between 8:00 and 9:00 (under fasting conditions) from all family members. The serum and plasma were separated immediately and stored at −20°C. The following hormone levels were analyzed for each patient: follicle-stimulating hormone, luteinizing hormone, and adrenocorticotropic hormone (ACTH) using immunoradiometric assays (IRMA; DPC Laboratory, Los Angeles, CA, USA); progesterone, estradiol, 17-hydroxyl progesterone, cortisol, androstenedione, dehydroepiandrosterone sulfate (DHEAS), and aldosterone using radioimmunoassays (RIA; DPC Laboratory, Los Angeles, CA, USA); and plasmatic renin activity (PRA) using RIA (PRA RIA/MAIA, Biochom Immunisistems, Bologna, Italy). Corticosterone was measured using a commercial RIA kit (ICN, Costa Mesa, CA, USA) without chromatographic separation. All blood tests were performed after a 1-month washout period.

The functional evaluation was carried out using both stimulation and inhibition tests. The adrenal stimulation was performed with synthetic ACTH (Synacthen®, Novartis Argentina SA) 2mg (one dose). The inhibition was achieved with dexamethasone (Decadron®, Instituto Sidus ICSA) 2 mg/day for 2 days.

### Blood Pressure

Blood pressure was evaluated using an ambulatory blood pressure monitor (ABPM) over a 24-hour period, we used a SpaceLab

1 The use of trade names is for product identification purposes only and does not imply endorsement.

model 90207 monitor (SpaceLabs Inc., Issaquah, WA, USA). According to British Hypertension Protocols, average normal blood pressure in individuals without a history of hypertension is 117/72mm Hg, and in treated patients is 130/80mm Hg.[5]

### Molecular Studies

Genomic DNA was prepared from the peripheral blood of the patients and family members using the Proteinase K-SDS method and phenol/chloroform extraction. Exons 8 and 1 of the *CYP17* gene were individually amplified by PCR using the oligonucleotide primers as previously described by Monno et al.[6]

PCR amplification of exon 8 was carried out in a 100µL reaction volume containing 100ng genomic DNA, 2.5 mmol/L $Cl_2Mg$, 10 pmol of each primer, 2.5 mmol/L of each dNTP, and 0.4UI of Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). An initial denaturation at 95°C for 5 minutes was followed by 30 cycles of denaturation (95°C for 1 minute), annealing (62°C for 30 seconds), and extension (72°C for 1 minute) with a final extension cycle (72°C for 5 minutes). The PCR products were electrophoresed on a 3% agarose gel, and purified straight from gel using the Concert™ gel extraction system kit (Gibco-BRL, Gaithersburg MD, USA). The PCR product was then cloned into the pGEM®-T vector (Promega, Madison WI, USA). Plasmid DNA was obtained using the Miniprep method (Concert™ Rapid Plasmid Miniprep System, Gibco-BRL, Gaithersburg MD, USA). The purified DNA was digested with EcoR1 restriction endonuclease and the fragments analyzed on a 1% agarose gel to check for the presence of the insert and then sequenced.

The PCR amplification of exon 1 was performed in a 100µL reaction volume containing 100ng genomic DNA, 2.5 mmol/L $Cl_2Mg$, 40 pmol of each primer, 2.5 mmol/L of each dNTP, 1% glycerol, and 0.4UI Taq polymerase. An initial denaturation at 96°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 15 seconds), annealling (59°C for 30 seconds), and extension (72°C for 1 minute 30 seconds), with a final extension (72°C for 6 minutes). The PCR product was analyzed after digestion with Pst I by single-strand conformational polymorphism (SSCP) gel electrophoresis and then sequenced.

### Single-Strand Conformational Polymorphisms Method

DNA resuspended in a formamide loading buffer (10mL formamide, 10 mmol/L EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol) was heated at 95°C for 3 minutes and then placed on ice before electrophoresis. SSCP was carried out on a 10% acrylamide **:** bisacrylamide (29 **:** 1) gel without glycerol. Gel was run at 4°C in buffer (Tris-HCl borate buffer and 0.2 mmol/L EDTA pH 8) over 22 hours at 140V followed by silver staining. Sequencing was carried out using the *f*mol® DNA Cycle Sequencing System (Promega, Madison, WI, USA) with a 2ng template and 10 pmoL reverse-specific primer ATP-labeled with 32P.

## Results

### Hormonal Studies

Table II shows the hormonal results from both patients. Plasmatic cortisol in basal conditions were remarkably low in both

**Table II.** Patients data

| Parameters | P1 | | | P2 | | | Normal values |
|---|---|---|---|---|---|---|---|
| | basal | synthetic ACTH | dexamethasone | basal | synthetic ACTH | dexamethasone | |
| Potassium (mEq/L) | 3.5 | | | 4.2 | | | 3.5–5.1 |
| Follicle-stimulating hormone (mUI/mL) | 70.5 | | | 87.0 | | | 4–13 |
| Luteinizing hormone (mUI/mL) | 34.7 | | | 42.3 | | | 1–18 |
| ACTH (pg/mL) | 19 | | | 25 | | | 9–52 |
| Progesterone (ng/mL) | 8.9 | 11.0 | 1.7 | 2.9 | 10.6 | 0.9 | 1.1 |
| Plasmatic renin activity (ng/mL/h) | 1.5 | 0.5 | | 1.3 | 1.2 | | 1.0–4.2 |
| Corticosterone (ng/mL) | 324.0 | 468 | 7.4 | 236 | 480 | 6.8 | 1–10 |
| Aldosterone (pg/mL) | 100 | 1376 | 60 | 350 | 1300 | 40 | 40–310 |
| Corticosterone/aldosterone ratio | | 0.34 | | | 0.37 | | |
| 17-hydroxy-progesterone (ng/mL) | 0.2 | 0.3 | <0.2 | 0.3 | 0.5 | <0.2 | 0.1–1.0 |
| Cortisol (µg/dL) | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | 1.0–25.0 |
| DHEAS (ng/mL) | 41 | | 66 | | | | 350–4000 |
| Androstenedione (ng/mL) | 0.7 | | 0.4 | | | | 0.4–4.5 |

**ACTH** = adrenocorticotropic hormone; **DHEAS** = dehydroepiandrosterone sulfate.

**Table III.** Family data

| Parameters | Father | | Mother | | Sister 1 | | Sister 2 | | Normal values |
|---|---|---|---|---|---|---|---|---|---|
| | basal | synthetic ACTH | basal | synthetic ACTH | basal | synthetic ACTH | basal | synthetic ACTH | |
| Potassium (mEq/L) | 4.9 | | 3.9 | | 4.5 | | 4.4 | | 3.5–5.1 |
| Progesterone (ng/mL) | 0.3 | 2.9 | 0.3 | 15.3 | 6.3 | 11.5 | 0.6 | 5.4 | 1.1 |
| Corticosterone (ng/mL) | 24 | 168 | 8.0 | 200 | 20.0 | 64.0 | 16.0 | 128 | 1–15 |
| Aldosterone (pg/mL) | 95 | 620 | 68 | 650 | 530 | >1200 | 100 | 400 | 40–310 |
| Corticosterone/aldosterone ratio | | 0.27 | | 0.31 | | 0.05 | | 0.32 | |
| 17-hydroxy-progesterone (ng/mL) | 0.7 | 3.8 | 0.3 | 10.5 | 1.3 | 4.2 | 0.8 | 4.9 | 0.1–1.0 |
| Cortisol (μg/dL) | 15.8 | 56.0 | 16.5 | 70.0 | 25.0 | 90.0 | 18.4 | 64.6 | 0.5–25 |
| DHEAS (ng/mL) | 810 | 850 | 240 | 435 | 1525 | 2455 | 420 | 790 | 350–4000 |
| Androstenedione (ng/mL) | 0.8 | 2.0 | 0.3 | 2.4 | 2.2 | 5.0 | 1.3 | 2.6 | 0.4–4.5 |

**DHEAS** = dehydroepiandrosterone sulfate.

patients, while low levels of DHEA were detected in the serum of P1. The basal levels of gonadotrophins were typical for someone with primary gonadal failure. Serum levels of progesterone were very high in both patients, as high as levels observed in the luteal phase.

Basal levels of corticosterone were more than 30-fold higher than normal in both patients and showed a pronounced rise after ACTH stimulation. The basal aldosterone level was normal in P1 and slightly increased in P2, and showed the same elevated response after stimulation.

Basal levels of 17-hydroxy-progesterone, progesterone, cortisol, and PRA were unaffected after stimulation. All of these results were obtained using a high dose of ACTH in the stimulation test (2mg/24h); previously we had performed the test with a lower dose (0.25mg/60 min) but this did not produced any change in the parameters.

In both patients the plasma concentration of progesterone, corticosterone, and aldosterone decreased as a result of the administration of dexamethasone; 17-hydroxy-progesterone, progesterone and cortisol levels showed no changes after dexamethasone administration. The same situation was observed in P1 with DHEAS and androstenedione. DHEAS and androstenedione were not available in P2.

Table III shows the hormonal results for the family members. The basal level of progesterone in S1 is typical for a luteal phase. Both corticosterone and aldosterone were normal or slightly increased in all the healthy family members, with the exception of S1. Corticosterone levels in M, F, and S2 showed a marked response to ACTH while the rest of parameters were normal in the family members.

The ratio of corticosterone/aldosterone was calculated with the results of the stimulation test; P1 and P2 gave similar values (P1: 0.34, P2: 0.37). F, M, and S2 had similar corticosterone/aldosterone ratios (0.27, 0.31, and 0.32, respectively). There was no difference between these corticosterone/aldosterone values and those calculated for P1 and P2. S1 was the only family member with a very low corticosterone/aldosterone ratio (0.05).

## Molecular Studies

### *Exon 8*

The analysis of exon 8 with PCR products showed a different pattern for the patients and their family (figure 1). P1, P2, and M displayed 2 bands while F, S1, and S2 showed only 1 band. The two bands had a similar molecular weight, they were purified from the gel and cloned in pGEM®-T and then sequenced.

Sequence analysis of the exon 8 of P1, P2, and M showed a 4 bp duplication (CATC) at codon 480. This mutation changes the reading frame encoding the Ct26 amino acids of cytochrome P450c17 and produces a premature stop codon at position 507 (figure 2).[7]

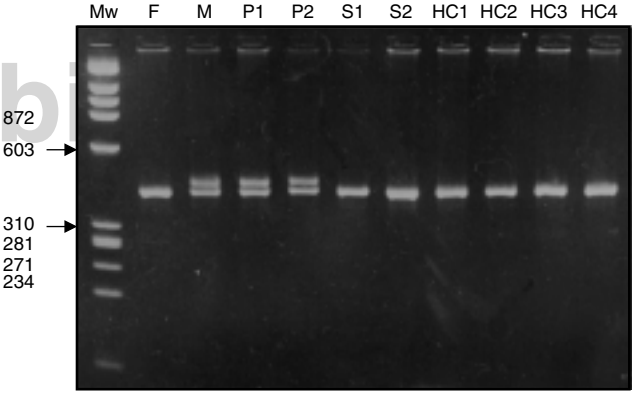


**Fig. 1.** Exon 8 PCR products electrophoresed in 3% agarose gel. Family members (father [F], mother [M], and sisters [S1 and S2]), patients (P1 and P2), and healthy control 1 to 4 (HC1, HC2, HC3, HC4). **MW** = molecular weight.

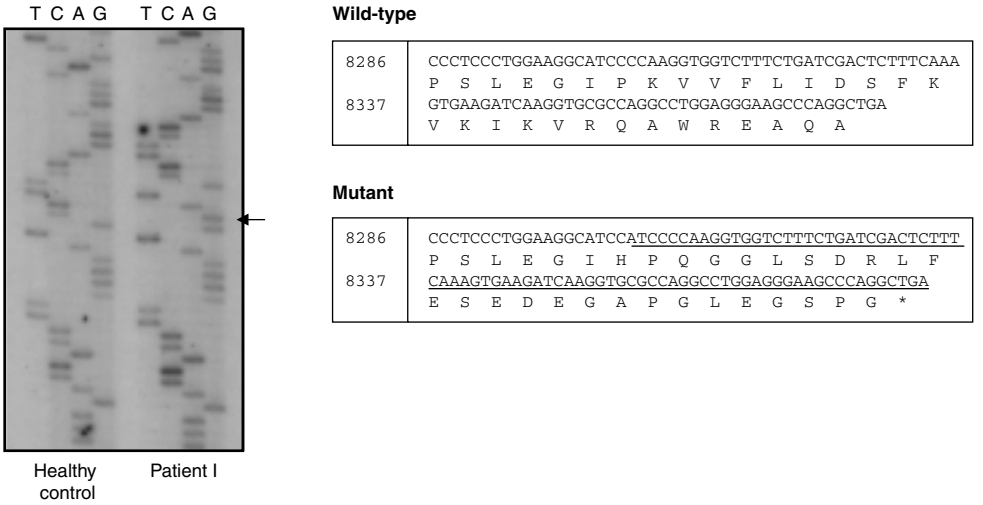


**Fig. 2.** Exon 8 nucleotide sequences of the normal and mutant *CYP17* gene, showing 4 bp duplication (CATC). The arrow indicates the mutation in patient 1 (P1). Patient 2 (P2) had an identical sequence to that of P1.

On a 3% agarose gel it was possible to distinguish the two bands, even with the small difference of 4 bp between them. This phenomenon could be explained by a heteroduplex formation, with the 4 bp (CATC) insertion generating a single-strand loop giving it a different migration pattern to the other chain.

F, S1, and S2 had a normal sequence for exon 8.

### *Exon 1*

After digestion with Pst I the 419 bp exon 1, the PCR products were analyzed using SSCP. P1, P2, S2, and F shared the same pattern while S1 and M were identical.

Sequence analysis revealed a heterozygous pattern at codon 96. This mutation was caused by a C to T transition, converting codon 96 CGG encoding arginine into the TGG encoding tryptophan (figure 3). This point mutation was described in 1996 by Laflamme et al.[8] in two French Canadian siblings. S1 and M had a normal exon 1 sequence.

## Discussion

Cytochrome P450c17 deficiency was suspected due to the clinical findings in both patients. The clinical diagnosis was later confirmed by biochemical studies because of the increased basal levels of progesterone and corticosterone, and response to ACTH. Molecular studies were used to identify the cause of the deficiency.

The diagnosis of this unusual type of congenital adrenal hyperplasia (CAH) is often made when a patient experiences puberal delay and/or arterial hypertension; nevertheless, arterial hypertension may sometimes have a late onset. The late manifestation of arterial hypertension may lead to a misdiagnosis of the disease in women, where it may be confused with gonadal dysgenesia (characterized by sexual infantilism, mammary hypoplasia, and primary amenorrhea). This was the case for P1 and P2.

Clinical suspicion of CAH is stronger in patients bearing a 46XX karyotype, together with hypokalemia and arterial hypertension.

Table II shows the high basal levels of corticosterone and progesterone in P1 and P2 that account for the accumulation of

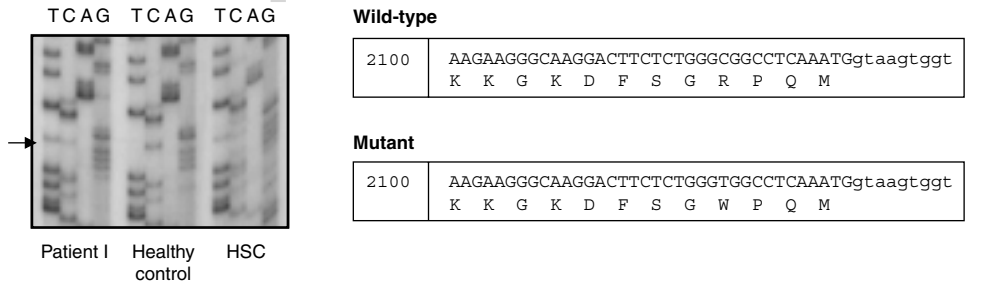


**Fig. 3.** Exon 1 nucleotide sequences of normal and mutant of *CYP17*, showing C to T transition. The arrow indicates the mutation in patient 1 (P1). Patient 2 (P2) had an identical sequence to that of P1. **HSC** = healthy sister carrier (S2).

metabolic intermediates that are present before enzymatic failure in the adrenal fasciculated zone. The lack of 17α-hydroxylated steroids in the cortisol pathway causes the low levels of end product observed in the two patients; however, ACTH levels are at the low end of normal since its regulation is strongly influenced by corticosterone production.[9]

The enzymatic adrenal and gonadal defect is also reflected by lower than normal in the levels of androgens, DHEAS, and androstenedione, a phenomenon observed in P1. These profiles are very similar to those originally described by Biglieri and Kater,[10,11] except for the levels of aldosterone. According to these authors, this enzymatic deficiency is accompanied by hypokalemia and hypoaldosteronism. However, several trials demonstrated that in some individuals affected by this disease, aldosterone levels may be normal or slightly elevated, with a suppressed PRA.[12-14] The later features are in accordance with our findings.

While several mechanisms have been proposed to explain these findings, none of the hypothesis has been confirmed. Yamakita et al.[13] states that the more severe the 17α-hydroxylase deficiency, the more active corticosterone-methyl-oxidase type II (which is present in the fasciculated zone) becomes, so favoring the synthesis of 18-hydroxy-corticosterone and aldosterone from corticosterone. This system would, thus, behave as a glucocorticoid-suppressible aldosteronism.[15]

The manipulation of the degree of ACTH stimulation of the elevated hormones allows for the evaluation of enzymatic failure in a more objective way. This was achieved by using a high dose of the ACTH stimulant (Synacthen® 2mg in 24 hours, with sampling 12 hours afterwards), as a low dose (Synacthen® 0.25mg with sampling 60 minutes afterwards) did not induce any effect; these results are in accordance with those described by D'Armiento et al.[16]

The partial lack of adrenal responsive to stimulation could be interpreted as a consequence of an augmented adrenal retroregulation produced for the high concentration of corticosterone,[9] since cortisol levels remained at undetectable levels. The high dose of ACTH stimulant produced a significant degree of response in corticosterone and aldosterone levels, a slight variation in the progesterone level, and no effect on the levels of 17-hydroxyl progesterone and cortisol.

The dexamethasone test employing low doses (2mg for 2 days) inhibited the secretion of corticosterone, aldosterone, and progesterone, with all returning to normal levels.

The basal biochemical profiles of family members (table III) were within normal ranges except for corticosterone which was slightly increased. Blood sampling was carried out on the healthy sister (S1) during the luteal phase, as confirmed by the progesterone value. S1 does not carry any mutations. Her progesterone levels were connected to the high basal aldosterone levels, since the renin-angiotensin system shows variation over the menstrual cycle.[17] Corticosterone displayed a wide response to the ACTH test in F, M, and S2, with levels being lower in S1. With regards to aldosterone levels, the responses achieved by F, M, and S2 were also significant when compared with the basal levels, whereas in S1 there was a dramatic rise, which could be related to the modifications in the renin-angiotensin system during the menstrual cycle.

The corticosterone/aldosterone ratio was calculated using the values obtained in the ACTH test. This ratio accounted for the differences in the production/synthesis between the fasciculated (corticosterone) and glomerulus (aldosterone) zones. The corticosterone/aldosterone ratio in both patients was similar, for P1 it was 0.34 and for P2 0.37. This ratio could be a useful tool for the identification of heterozygosity in the putatively affected sibling of patients with 17α-hydroxylase deficiency.[16-18]

The high dose synthetic ACTH test was also performed on the patients' family in order to compare the degree of response across the family (between the affected and unaffected members). After calculating the corticosterone/aldosterone ratio for F, M, and S2, it was found that the values were similar to those of the patients (F: 0.27, M: 0.31, S2: 0.32) whereas for S1 the index was quite different (0.05). The fact that the index was similar for S2, P1, P2, M, and F led us to suspect the possibility of heterozygosity in the healthy sister (S2) and this was confirmed by the molecular studies.

The biochemical profile for these patients did not allow us to determine whether the enzymatic deficiency in cytochrome P450c17 was a combined deficiency of 17α-hydroxylase/17,20-lyase or a single deficiency in 17α-hydroxylase. To determine this we needed to carry out further molecular studies.

The mutations identified in the *CYP17* gene of patients with 17α-hydroxylase deficiency include deletions, insertions, and single base changes; all of these are spread throughout the gene. From our results, it can be concluded that P1 and P2 are compound heterozygous, carrying two different inherited mutant alleles in the *CYP17* gene; one in exon 8, which is inherited from M and the other in exon 1 inherited from F. Both F and S2 were heterozygous for the mutation in exon 1, and M was heterozygous for the mutation in exon 8.

Alteration in the sequence at codon 480 had led to the loss of both the 17α-hydroxylation and 17,20-lyase activities of the enzyme.[19] The role of Arg 96 is not clear, but it could be involved in the formation of the steroid binding pocket.[8] Furthermore, this amino acid is conserved in several cytochrome P450c17 enzymes that have been characterized in humans, rats,[20,21] pigs,[22] porcine,[23] chickens,[24] and rainbow trout.[25]

Substitutive therapy with glucocorticoids, to normalize blood pressure, relies on the hypertension being a consequence of a mineralocorticoid increase, therefore glucocorticoids would be the only way to induce a drop in blood pressure. Antihypertensive therapy failed in P2. Hypertension must be treated shortly after diagnosis in order to avoid the development of malignant hypertension over time.

Substitutive treatment with estrogens and progestogens should also be considered in order to develop and maintain the well-being, secondary sexual characteristics, and bone mass of the patient.[18]

This unusual enzymatic deficiency must be suspected in tall females presenting sexual infantilism and primary amenorrhea, which may or may not be accompanied by hypertension. At a biochemical level, high concentrations of gonadotrophins and progesterone at luteal phase concentrations in addition to suppressed levels of cortisol, can confirm the diagnosis. As a second step in the diagnosis it is important to check for any signs of the deficiency in siblings to be able to give genetic advice.

In conclusion, a calculation of the corticosterone/aldosterone ratio after ACTH testing, as used in this case study, could be useful in the screening of the patients when 17α-hydroxylase deficiency is suspected. Molecular studies can be used to confirm the disorder in patients and the condition of healthy carriers (heterozygous) in first grade members of the family.



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