

## CLINICAL CASE SEMINAR

# Hypothalamic-Pituitary-Ovarian Axis during Infancy, Early and Late Prepuberty in an Aromatase-Deficient Girl Who Is a Compound Heterozygote for Two New Point Mutations of the CYP19 Gene

ALICIA BELGOROSKY, CAROLINA PEPE, ROXANA MARINO, GABRIELA GUERCIO, NORA SARACO, ELISA VAIANI, AND MARCO A. RIVAROLA

*Laboratorio de Investigación, Hospital de Pediatría Garrahan, Buenos Aires, Argentina 1245*

A loss of function mutation of the CYP19 aromatase gene leads to excess circulating androgens in the fetus and in the mother, resulting in ambiguous genitalia in the female fetus. Later on, lack of aromatase is responsible for sexual infantilism, primary amenorrhea, tall stature, and multicystic ovaries, even in preadolescent girls. Up to now, 11 CYP19 aromatase point mutations and 10 well-documented cases have been reported. In the present case, we are reporting the clinical and hormonal follow-up, from birth to 7 yr of age, of an affected girl with ambiguous genitalia. Gene analysis showed that she was a compound heterozygote for two new CYP19 aromatase point mutations. In the father's allele, there was a consensus 5' splice donor sequence mutation, GAA-AAA at cDNA position bp 655 in exon 5, which probably results in a cryptic donor site. In the mother's allele, there was a base A deletion in exon 9 ( $\Delta$ A GLU 412X), causing a frame shift mutation, and a stop codon after 98 bp (33 codons) downstream, altering the critical heme-binding region. Basal serum LH and FSH levels were high at 8 d of age (42.9 and 51.3 U/liter), 26 d of age (76.2 and 119 U/liter), and 60 d of age (58.7 and 150 U/liter, respectively). Both gonadotropins dropped dramatically between the second and fifth months of age (to 1.79 and 14.9 U/liter) but remained higher than in normal control girls (0.64 and 8.5 U/liter, respectively). Serum testosterone (T) and androstenedione ( $\Delta_4$ A) levels were high during the first month, but  $\Delta_4$ A was normal at 2 months of age. However, at 5 months of

age, along with significant decrements of serum LH and FSH levels and increments in serum  $\Delta_4$ A and T levels, a large ovarian cyst was removed from each gonad. Relatively high levels of T [27.3 ng/ml (94.6 nmol/liter); control, 34.9 ng/ml (121 nmol/liter)], but not of estradiol [1.8 ng/ml (6.6 nmol/liter); control 62.9 ng/ml (231 nmol/liter)], and a high T/estradiol ratio [15.2; control < 1] were found in the follicular fluid.

Serum  $\Delta_4$ A and T levels remained normal from 1–5 yr of age, but they were high at the last visit (late prepuberty). A GnRH test was performed at 3.9, 6, and 7.1 yr of age. At 3.9 yr, a low prepubertal serum LH peak (2.12 U/liter) was found, but at the older ages, higher serum LH peaks (8.25 and 22.5 U/liter, respectively) were observed. Growth pattern and body mass index were normal, but after the age of 5.2 yr, delays in bone age greater than 2 yr were observed. We concluded that: 1) these two new CYP19 aromatase gene mutations are responsible for the phenotype of aromatase deficiency; 2) in girls, aromatase deficiency results in a decrease of the negative feedback of both serum LH and FSH, which can be detected as early as the second week after birth and persists up to the sixth month of life, and of FSH during the rest of prepuberty; and 3) because large ovarian cysts developed when serum LH and FSH dropped, aromatization of androgens might be required to prevent formation of cystic ovaries. (*J Clin Endocrinol Metab* 88: 5127–5131, 2003)

AROMATIZATION OF FETAL adrenal androgens is essential for the production of estrogens by the human placenta (1). In females, a disruptive mutation of P450 aromatase gene leads to excess circulating androgens in the fetus associated to virilization of the mother from the second trimester of pregnancy. Since 1992, 10 well-documented cases have been reported (nine families). There were four males, including three adults (2–4) and one neonate (5), and six females, including two adults (2, 6), one neonate (7), and three children (8–10).

Aromatase deficiency results in virilization of the mother during pregnancy and in prenatal exposure of the female fetus to adrenal androgens with consequent ambiguous genitalia. Later on, aromatase deficiency is responsible for sexual infantilism, primary amenorrhea, tall stature, and multicys-

tic ovaries, developing not only in adolescent girls, but also during infancy and childhood (2, 7–9). It has been proposed that multicystic ovaries are secondary to high serum FSH concentrations and secondary to low serum estradiol ( $E_2$ ) levels, which are required to restrain FSH and LH secretion.

In the present case, we are describing the follow-up of the clinical features and hormonal studies from 8 d to 7 yr of age of a girl with ambiguous genitalia secondary to aromatase deficiency. She was a compound heterozygote for two new CYP19 aromatase point gene mutations. The follow-up of this patient with aromatase deficiency provided us with an experiment of nature to study the role of estrogens on gonadotropin regulation during infancy and childhood.

### Materials and Methods

Abbreviations:  $\Delta_4$ A, Androstenedione; aa, amino acid; BMD, bone mineral density;  $E_2$ , estradiol; T, testosterone.

Serum androstenedione ( $\Delta_4$ A) was determined by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX) (11). Assay sensitivity was 0.10

nmol/liter, and interassay coefficient of variation ranged from 7–9.8%. Serum  $E_2$  and serum testosterone (T) were determined by RIA (Biodata Ltd., Milan, Italy) (12). Serum LH and FSH were determined by the Imx systems (Abbott Laboratories, Abbott Park, IL). Assay sensitivities and coefficients of variation have been previously described (13).

### DNA isolation, amplification, and sequencing

Genomic DNA was isolated from peripheral leukocytes of the affected subjects and relatives as previously described (14). Each exon of the CYP19 gene, including the 5'-flanking region, was amplified using the primers already reported by Mullis *et al.* (7).

For this purpose, 500 ng of genomic DNA was added to a 50- $\mu$ l reaction mixture of 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.0 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide triphosphate, 0.3  $\mu$ M of each primer, and 2 U Taq DNA polymerase. The PCR mixture was denatured for 5 min at 95 C and cycled for 30 times (95 C, 1 min; 58 C, 1 min; and 72 C, 2 min) followed by a 10-min extension at 72 C. The resulting PCR products were purified with the commercial column QIA Quick PCR purification kit (QIAGEN, Valencia, CA). Each purified product was used as a template for direct sequencing of the whole fragment. Sequencing was performed by the dideoxy method using Thermosequence Kit (Amersham Pharmacia Biotech, Piscataway, NJ).

### Case report and clinical follow-up

The patient is the first child of nonconsanguineous parents. She was a normal weight product of a full-term pregnancy. No medication was taken during pregnancy. Her mother began with progressive virilization at about the second trimester of pregnancy. She had a 1.5-cm phallic-like structure, rugated labia majora, and posterior labial fusion, and no gonads were palpable. Chromosomal constitution showed a 46, XX karyotype. At 10 d of postnatal life, to rule out 46,XX true hermaphroditism, the infant was given 1500 U human chorionic gonadotropin im on two occasions, d 1 and d 4. No rise of serum T was observed on d 7, indicating the absence of functional Leydig cells. A blood test to rule out 21-hydroxylase and 11 $\beta$ -hydroxylase deficiencies indicated normal serum 17 $\alpha$ -hydroxyprogesterone and 11-desoxycortisol concentrations. Therefore, the diagnosis of a nonadrenal form of female pseudohermaphroditism was made. Aromatase deficiency was suspected.

At 5 months of age, a laparoscopy and plastic surgery of external genitalia were performed. Two ovaries were identified; each one had a large follicular cyst (4  $\times$  2 cm). The two cysts were removed. T and  $E_2$  concentrations were measured in the follicular fluid. The fallopian tubes and the uterus were normal in appearance. Biopsies of the two ovaries demonstrated normal-appearing stromal elements and numerous follicular cysts; one of the enlarged cysts had evidence of luteinization. Later on, in monthly pelvic ultrasound examinations, carried out between 6 months and 1 yr of age, only small ovarian cysts, no larger than 2.4  $\times$  2.8 cm, were observed. No ovarian cysts were found during subsequent follow-up, up to 7 yr of age.

The patient grew and developed normally (Fig. 1). When she was 6.1 yr old, bone age showed a delay of 2.6 yr. At 6 yr of age, normal body and lumbar spine bone mineral density (BMD) was observed.

### Follow-up of serum gonadotropins and sex steroid levels

These determinations were followed from birth to late prepuberty. Gonadotropins and sex steroid concentrations were evaluated jointly at different maturational periods (infancy and childhood), including the time when she developed large ovarian follicular cysts.

The present study was approved by the Institutional Review Board of Garrahan Pediatric Hospital, and informed consent was obtained from the parents.

## Results

### Genetic analysis

The individual exons and flanking regions of the CYP-19 gene were amplified and sequenced, and a comparison with the published sequence of the human CYP-19 gene was performed (15–18). Sequence analysis revealed a compound het-

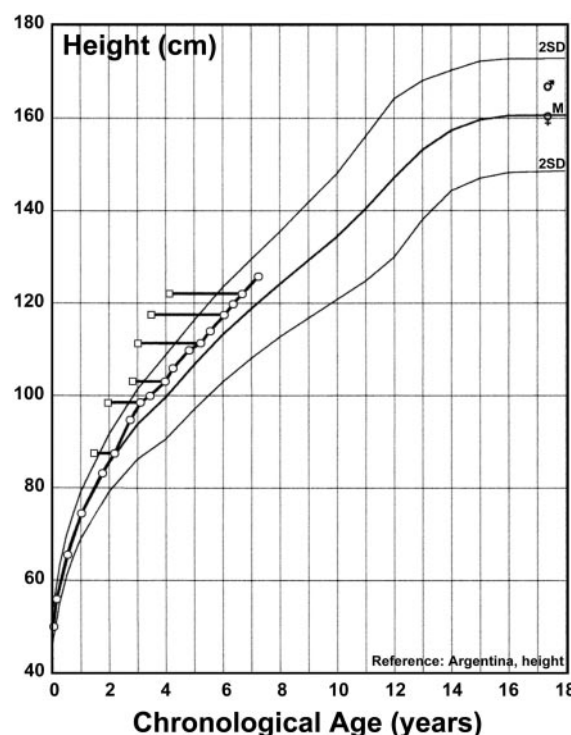


FIG. 1. Growth chart of the patient with aromatase deficiency. Bone ages are depicted by *small squares*, with *horizontal lines* indicating the corresponding chronological ages. After the chronological age of 2 yr, height was always above the mean for age, whereas a progressive delay of bone age was observed.

erozygosity for a point mutation from G to A at the consensus 5' splice donor sequence mutation (GAA to AAA), at cDNA position 655 bp in exon 5, resulting probably in a cryptic donor site (father's allele). In the allele inherited from the mother, there was a bp deletion (A) in Glu 412 (GAA, exon 9), causing a frame shift mutation generating a stop codon 98 bp downstream [33 amino acids (aa)], which results in a prematurely terminated protein, altering the critical heme binding region (15–18). We have also found the following polymorphism nucleotide sequences described by Kurosaki *et al.* (19): V30V (GTG-GTA Hm) at cDNA position 267 bp in exon 3, P146P (CCC-GCC Ht) at position 463 bp in exon 4, and  $\Delta$ TCT (del TCT Ht) in intron 4; as well as another described by Watanabe *et al.* (20): R264C (CGC-TGC Ht) at position 817 bp in exon 7.

### Basal serum hormone studies: follow-up of serum LH, FSH, $E_2$ , $\Delta_4$ A, and T concentrations

Figure 2 shows basal serum LH, FSH (*upper panels*),  $\Delta_4$ A, and T (*lower panels*) concentrations during the first year of postnatal life.

An elevated serum LH level of 42.9 U/liter was found at 8 d of age. It increased to 76.2 and 58.7 U/liter at 26 and 60 d of age, respectively. Later on, serum LH levels dropped markedly between the second and the fifth month of age but remained higher (1.79 U/liter) than in normal control girls (0.64 U/liter) at 6.5 months of age (13). At the end of the first year of life, serum LH was in the normal range. A similar pattern was found for serum FSH during the first 6 months

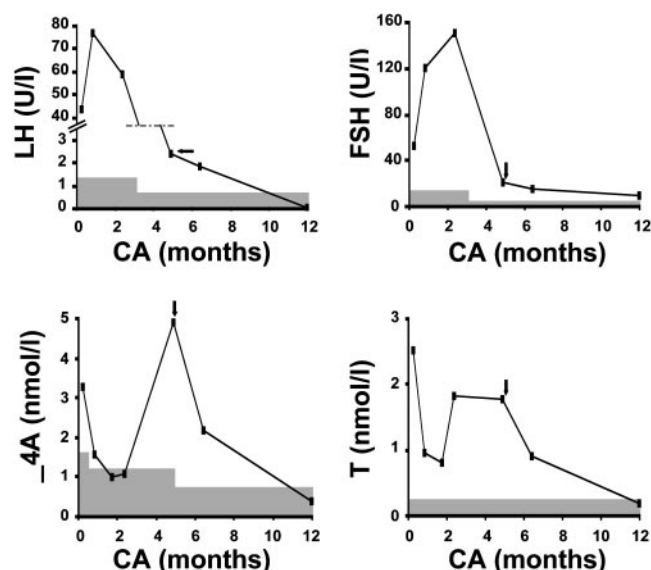


FIG. 2. Basal serum LH and FSH (*upper panels*), as well as serum  $\Delta_4$ A and T (*lower panels*) during the first year of life in the girl with aromatase deficiency. CA, Chronological age in months. Shaded areas, Normal control values. Arrows indicate age at follicular cyst removal. Conversion factors:  $\times 1/3.467$  from nanomoles per liter to nanograms per milliliter for T;  $\times 1/3.49$  from nanomoles per liter to nanograms per milliliter for  $\Delta_4$ A.

of life: an elevated serum level of 51.3 U/liter at 8 d of age, which increased to 119 U/liter at 26 d and to 150 U/liter at 2 months of age. Later on, the serum FSH level also dropped markedly between the second and the fifth months of age, but it was still higher than in normal control girls (13) at 6.5 months of age (14.9 and 8.5 U/liter, respectively). At the end of the first year of life, serum FSH remained elevated.

Elevated levels of serum  $\Delta_4$ A [0.93 ng/ml (3.24 nmol/liter)] and T [0.72 ng/ml (2.51 nmol/liter)] were found at 8 d of age. At 2 months of age, serum  $\Delta_4$ A was within the normal reference range of 0.05–0.35 ng/ml (0.17–1.22 nmol/liter) (11), but serum T levels remained higher [0.52 ng/ml (1.80 nmol/liter)] than the normal reference range for girls [ $<0.05$  ng/ml ( $<0.17$  nmol/liter)] (12). However, at 5 months of age, along with a significant decrement of serum LH and FSH, serum  $\Delta_4$ A increased to 1.41 ng/ml (4.92 nmol/liter), whereas no change in the high serum T levels was found. Along with these serum hormone patterns, two enlarged follicular cysts, one per gonad, were found at laparoscopy, suggesting that follicular androgens might play a role in follicular cyst enlargement. The concentration of T,  $E_2$ , and T/ $E_2$  ratio in the follicular liquid was also measured. The concentration of T in the antral fluid of the follicular cysts was 27.3 ng/ml (94.6 nmol/liter), that of  $E_2$  was 1.8 ng/ml (6.6 nmol/liter), and the T/ $E_2$  ratio was 15.2. According to McNatty *et al.* (21), the concentration of T and  $E_2$  in the antral fluid of large human follicular cysts was  $34.9 \pm 6.0$  ng/ml ( $121 \pm 20.8$  nmol/liter) and  $62.9 \pm 19.0$  ng/ml ( $231 \pm 69.7$  nmol/liter, mean  $\pm$  SEM), respectively, with a T/ $E_2$  ratio below 1. After the removal of the two follicular cysts, during the second half of the first year of life, there was a gradual decrease of serum  $\Delta_4$ A and T toward normal levels.

Basal serum gonadotropins and sex hormone concentra-

tions between the first and seventh year of postnatal life are shown in Fig. 3. Between 1 yr of age and the last visit at 7 yr of age, serum LH levels remained within the normal girl range (13) (Fig. 3, *top left panel*), but from 5.7 yr of age a tendency to an increment was observed in three consecutive samples. Serum FSH varied between 23.0 and 9.23 U/liter; all values were above the normal girl range (Fig. 3, *top right panel*).

Serum  $\Delta_4$ A and T levels between 1 and 7 yr of age are also shown in Fig. 3 (*bottom left and bottom right panels*, respectively). Serum  $\Delta_4$ A remained within the normal girl range (11) up to 5 yr of age. Thereafter, serum  $\Delta_4$ A levels increased above the normal girl range to 0.6 and 0.75 ng/ml (2.1 and 2.62 nmol/liter) at the last visits. Starting after 5 yr of age, serum T levels also increased above the normal girl range (12), from 0.11–0.61 ng/ml (0.38–2.11 nmol/liter) at 7 yr of age.

Serum  $E_2$  level was always below the detection limit of the assay during the entire follow-up study.

#### GnRH tests

Three GnRH tests are shown in Table 1. They were performed at the following ages: 3.9, 6.0, and 7.2 yr. Peak LH values were 2.12, 8.25, and 22.5 U/liter, and peak FSH values were 38, 47, and 40 U/liter, respectively.

#### Discussion

In this patient, most of the phenotypic characteristics of aromatase deficiency were present. A consensus 5' splice donor sequence mutation from GAA to AAA at cDNA position 655 bp in exon 5 was found in the allele inherited from the father. According to the reported structural analysis of this gene (15–18), this point mutation is located within the nucleotide sequences of the exon/intron boundary. This base

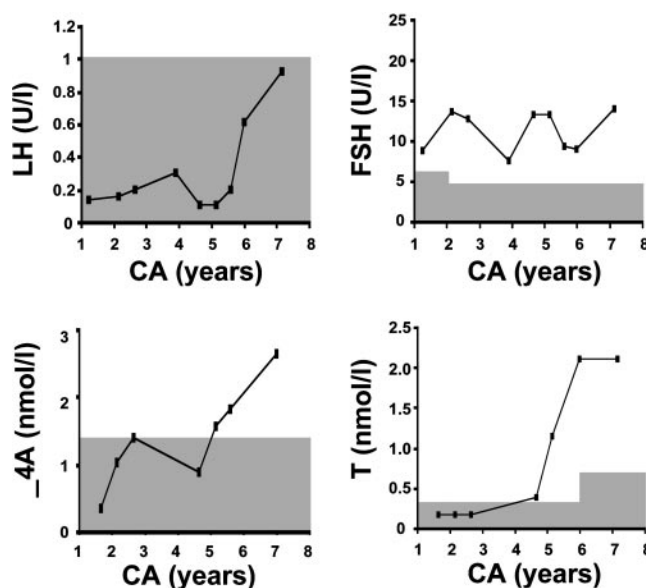


FIG. 3. Basal serum LH and FSH (*upper panels*), as well as serum  $\Delta_4$ A and T (*lower panels*) from the first year to the seventh year of life in the girl with aromatase deficiency. CA, Chronological age in years. Shaded areas, Normal control values.



**TABLE 1.** GnRH tests carried out at three different ages during prepuberty

CA (yr)	LH (U/liter)			FSH (U/liter)			T (nmol/liter)	$\Delta_4$ A (nmol/liter)	E <sub>2</sub> (pmol/liter)
	0	30 (min)	60	0	30 (min)	60			
3.88	0.3	2.03	2.12	17.5	20.4	38	0.17	0.69	<31
6.0	0.61	8.25	3.58	9	23.8	47	2.11	2.08	<31
7.16	0.92	8.99	22.5	14.3	25.2	40.2		2.98	<31
Range of normal prepuberty values	Normal basal values 0–14 (13)			Normal basal values 0.2–5.0 (13)			0–0.20 (12)	0.05–0.45 (11)	

Corresponding basal values of T,  $\Delta_4$ A, and E<sub>2</sub> are shown. Conversion factors:  $\times 1/3.462$  from nanomoles/liter to nanograms/milliliter for T;  $\times 1/3.49$  for  $\Delta_4$ A;  $\times 1/3.671$  for E<sub>2</sub>. CA, Chronological age.

change probably results in a cryptic donor site. Another explanation that could be proposed is the generation of a GLU210LYS aa change in the protein, but the GLU210 is located at the amphipathic F-helix, which has not been described among the most conserved regions of aromatase (22, 23). Therefore, it is not probable that an aa change could explain the alteration of the biological activity of aromatase resulting in the phenotype described. In the allele inherited from the mother, an A base deletion in exon 9 of the CYP19 aromatase gene ( $\Delta$ A GLU412X) was found, generating a frame shift mutation. This frame shift produced a stop codon 98 bp downstream (33 aa). The resulting peptide is probably completely inactive because there is an alteration of the substrate binding pocket at the electron-accepting heme-binding site (15–18). In view of obvious virilization of the mother during pregnancy and the degree of masculinization of the external genitalia of the newborn, we suggest that these two new point mutations generate a nascent peptide with very poor biological activity.

In the present case, we have analyzed the pattern of serum basal LH, FSH, T, and  $\Delta_4$ A concentrations during the neonatal period, as well as during infancy and childhood. For ethical reasons, the study is based on single serum gonadotropin determinations, drawn along the follow-up period of several years. During the first month of postnatal life, serum basal LH, FSH, and androgens were high. This abnormal hormonal pattern might reflect a central change in the activity of GnRH pulse generator and/or an effect at the pituitary level, presumably induced by the increment of androgens and the aromatase deficiency during fetal and neonatal life. As had been proposed by Mullis *et al.* (7), a low level of E<sub>2</sub> is an essential component of the restraint of FSH and LH secretion during infancy. However, from 2–6 months of age, serum basal FSH and LH levels dropped dramatically, but whereas serum basal FSH levels remained clearly high, serum basal LH levels decreased gradually to reach normal levels at the end of the first year of life (13). On the other hand, in the same age period, whereas serum basal FSH and LH levels decreased, basal levels of serum T and  $\Delta_4$ A were high, suggesting that androgens could play a negative feedback role on gonadotropin secretion at this age. This decrement in serum gonadotropins and the lower values measured during the second semester of the first year of life are in contrast with the increase in serum LH and FSH reported by Conte *et al.* (24) in Turner syndrome during the first year of life. It has to be pointed out that, in contrast to expectations in severe gonadal dysgenesis, serum inhibins are normal in aromatase deficiency (7). However, inhibin A is undetectable and in-

hibin B is very low in normal prepubertal girls after the sixth month of age (25). It has been reported that androgens, acting directly through the androgen receptor-mediated pathway, repress GnRH gene expression in hypothalamic GnRH-secreting neurons (26). At the pituitary level, a suppression of LH  $\beta$  gene transcription through a direct interaction of the androgen receptor, reducing the Sp1 binding site in the distal GnRH-responsive promoter region (27), has been described.

At 5.5 months of age, two enlarged ovarian cysts (one in each ovary) were diagnosed by ultrasound examination. They were removed to prevent acute ovarian torsion. Ovarian cysts respond to low estrogen therapy in aromatase deficiency (7), but this information was not available at that time. Large hemorrhagic cystic follicles have been described in ArKO mice (28) and in human aromatase deficiency (2, 7–9). It had been proposed that chronic exposure to abnormally high levels of LH determines ovarian cyst development (29, 30). However, female mice that are homozygous for a targeted disruption of the FSH  $\beta$ -subunit gene exhibit an approximate 5-fold increase in serum LH but do not show indications of enlarged cystic follicles in the ovary (31), indicating a role for FSH in this process as well. In the rat, granulosa cells of primary, secondary, and mature follicles show intense androgen receptor expression (32). Weil *et al.* (33) showed that T increased follicular FSH receptors, and it had been suggested that androgens, such as those present in polycystic ovary syndrome, promote follicular growth by amplifying the FSH effect. Therefore, we can speculate that in aromatase-deficient patients, an amplification of FSH signaling might have occurred in the presence of high intraovarian androgens and that this mechanism could be involved in the development of ovarian follicular cysts.

Basal serum  $\Delta_4$ A and T levels were normal during the early prepubertal period. However, starting at 5.5 yr of age, a gradual increase in serum basal androgen levels was found. This is in agreement with the report of Oerter Klein *et al.* (34) indicating that the ovary is not hormonally quiescent during prepuberty.

Indeed, GnRH testing was performed at three different ages, once in early prepuberty and twice at ages older than 5.5 yr, *i.e.* after the onset of the gradual increase in serum androgens during late prepuberty. It was interesting to find that in early prepuberty, along with normal serum androgen levels, peak serum LH response was 10 times lower than in late prepuberty. It has been known for many years that sex hormones of extragonadal origin (adrenal), or administered exogenously, produce not only the development of secondary sexual characteristics, but also an advance in the induc-

tion of the onset of GnRH-dependent puberty, in boys and in girls. However, this could be a direct effect of androgens or an indirect effect mediated by local conversion into estrogens.

Finally, although this girl grew normally, a delay in bone age maturation was noted, as has been already reported (2, 3, 7–10). It is accepted that estrogens are important in preserving adequate BMD (35). However, information about the role of estrogens on bone mineralization during childhood is scarce. In the Mullis *et al.* (7) case, low BMD was reported. However, in our case, delay in bone age maturation and normal BMD was found during the last visit.

In conclusion, 1) these two new CYP19 aromatase gene mutations are responsible for the phenotype of aromatase deficiency; 2) in girls, aromatase deficiency results in a decrease of the negative feedback of both serum LH and FSH, which can be detected as early as the second week after birth and persist up to the sixth month of life, and of FSH during the rest of prepuberty; and 3) because large ovarian cysts developed when serum LH and FSH dropped markedly, aromatization of androgens might be required to prevent formation of cystic ovaries.

## Acknowledgments

Received March 13, 2003. Accepted July 19, 2003.

Address all correspondence and requests for reprints to: Alicia Belgorosky, Laboratorio de Investigación, Hospital de Pediatría Garrahan, C. de los Pozos 1881, Buenos Aires, Argentina 1245. E-mail: abelgo@elsitio.net.

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas and Fondo para la Investigación Científica y Tecnológica of Argentina, as well as by Pharmacia (Pfizer) Endocrine Care International Fund for Research and Education.

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