Five New Cases of 46,XX Aromatase Deficiency: Clinical Follow-Up From Birth to Puberty, a Novel Mutation, and a Founder Effect

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Context: Aromatase is the key enzyme for estrogen biosynthesis and is encoded by the *CYP19A1* gene. Since 1991, several molecular *CYP19A1* gene alterations associated with aromatase deficiency have been described in both sexes.

Objective: The objective of the study was to detect *CYP19A1* mutations in five aromatase-deficient 46,XX patients and to describe the clinical follow-up from birth to puberty and to perform haplotype analysis associated with the high-frequency c.628G>A splice mutation in Argentinean patients.

Design: The design of the study was the sequencing of the coding and flanking intronic regions of the *CYP19A1* gene in all patients and parents. Haplotype analysis of patients carrying the c.628G>A mutation was also performed.

Patients: Clinical and biochemical findings in five new cases and one previously reported female aromatase-deficient patient (46,XX) are described. All patients presented with ambiguous genitalia at birth. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency as well as other steroidogenic defects were ruled out.

Results: Phenotypic variability among the affected patients was found during follow-up. Direct sequencing of the *CYP19A1* gene from genomic DNA revealed one novel mutation (c.574C>T) in two patients. In silico analysis predicted the c.574C>T mutation to be probably damaging. Four of six nonrelated patients presented with the c.628G>A splice mutation. Haplotype analysis showed that the c.628G>A splice mutation is associated with the same haplotype in our population.

Conclusions: Increased knowledge on phenotypical variability found in female aromatase-deficient patients is useful to improve the detection rate in this disorder. In our population, a genetic founder defect has probably contributed to an increase in the incidence of the c.628G>A splice mutation. **(*J Clin Endocrinol Metab* 100: 0000–0000, 2014)**

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Abbreviations: E, exon; P450arom, P450 aromatase; PolyPhen-2, Polymorphism Phenotyping-2; SIFT, sorting intolerant from tolerant.

Biosynthesis of estrogens from androgens is catalyzed by cytochrome P450 aromatase (P450arom). The human P450arom enzyme is located in the membrane of the endoplasmic reticulum of several tissues, such as gonad, brain, placental syncytiotrophoblast, breast, and adipose tissue (1).

Human P450arom is the product of a single gene, *CYP19A1*, located on chromosome 15q21.1. The protein-coding sequence is contained within nine exons (E2–E10), spanning approximately 35 kb (2, 3). There are multiple first exons that are involved in tissue-specific expression; however, because these exons are not translated, the protein sequence is conserved in every tissue (4, 5).

Aromatase deficiency is an autosomal recessive disorder first described by Shozu et al in 1991 (6). Since then, approximately 28 cases have been reported (18 females and 10 males) (7–13). The active human placental aromatization of androgens protects the fetus against the virilizing actions of fetal androgens. In congenital aromatase deficiency, the overload of androgens may cause signs of maternal virilization (acne, deep voice, clitoris enlargement) during pregnancy. After delivery these symptoms usually disappear gradually. In most female cases exposed in utero to excessive androgen levels, ambiguous genitalia with various degrees of masculinization of the external genitalia has been reported. Delayed skeletal maturation has also been described, and most affected girls have multiple bilateral ovarian cysts. Phenotype is dependent on sex and age and may be variable according to the level of enzyme activity (7, 14).

We previously reported an aromatase-deficient girl from Argentina who is a compound heterozygote for two point mutations: c.1235delA and c.628 G>A (15, 16).

The c.628G>A point mutation is a substitution of guanine for adenine at the consensus 5′ splice site of exon/intron 5, resulting in the disruption of this donor splice site. It has been demonstrated that this mutation is associated with the expression of an mRNA lacking exon 5. Alternative splicing of exon 5 of the *CYP19A1* gene occurs in normal human steroidogenic tissues as well as in the c.628G>A mutant, and it has been speculated that for the mutant a relative prevalence of the shorter over the full-length protein might explain the phenotype of partial aromatase deficiency (17).

Here we report five new cases of aromatase deficiency from Argentina. We describe one novel mutation: c.574C>T. A high frequency of the c.628G>A splice mutation was found in the Argentinean patients: in four of six in our study and in one homozygous aromatase-deficient man described by Maffei et al (18).

The high prevalence of the c.628G>A mutation may be due to a mutational hot spot at this position or to a founder effect. A hot spot, with multiple de novo mutational events would result in a wide geographic distribution of the mutation, and may thus be a more frequent event than previously reported. Using haplotype analysis, we show that this mutation is likely due to a founder effect. This information is of great importance for appropriate genetic counseling.

Subjects and Methods

Clinical and biochemical findings of six female patients with aromatase deficiency were analyzed. One patient was previously reported by us (15–17). Detailed information is presented in the Supplemental Methods. This study was approved by the Ethics Committee of the Garrahan Pediatric Hospital. Written informed consent for the study was obtained from the parents of all patients.

Sequence analysis

Genomic DNA was isolated from peripheral leukocytes of the affected subjects and their parents using standard techniques.

Each coding exon (E2–E10) and the flanking intronic regions of the *CYP19A1* gene were PCR amplified and automatedly sequenced.

The nucleotide sequences obtained were compared with the National Center for Biotechnology Information entry of the *CYP19A1* gene (NG_007982.1).

In silico tools

The sequence homology-based tool, sorting intolerant from tolerant (SIFT), version 2.0.6 (http://sift.jcvi.org/), and the structure-based tool, Polymorphism Phenotyping-2 (PolyPhen-2; http://www.genetics.bwh.harvard.edu/pph2) were used to predict the pathogenicity of the novel mutation identified using default settings.

Haplotype analysis

The haplotypes of the patients who were carriers of the same mutation were characterized with 21 polymorphic markers within the *CYP19A1* gene. We also characterized the haplotypes of an Argentinean control population (94 alleles) by genotyping three additional polymorphic markers within the *CYP19A1* gene for a total of 24 markers (see Table 2). The frequencies of the polymorphic markers were assessed according to the report by Ma et al (19) and the Ensembl database (http://www.ensembl.org).

Twenty-three single-nucleotide polymorphisms were genotyped by automated sequencing, whereas a (TTTA)n repeat at position 77 in intron 4 was analyzed by using GeneScan to detect the length of the polymorphism.

In all cases the haplotypes were determined genotyping the samples of both parents when necessary.

Statistical analysis

The haplotype frequency found in the same mutation carriers (21 polymorphic markers) was compared with the same haplotype frequency published from a control Caucasian American population (19) using a binomial test.

To confirm the association between the mutation and this haplotype, we characterized the haplotypes in an Argentinean control population (94 alleles) by genotyping three additional polymorphic markers within the *CYP19A1* gene (total 24 markers). Haplotype frequencies between patients and the Argentinean control population were compared using a binomial test.

Statistical analyses were performed using S-Plus version 6.0 software.

Results

The clinical description and biochemical findings of subjects carrying *CYP19A1* mutations are shown in Table 1. Detailed information is presented in the Supplemental Results.

All patients presented with ambiguous genitalia at birth. The karyotype was 46,XX in all of them. Congenital

Table 1. Clinical and Biochemical Findings of Subjects Carrying CYP19A1 Mutations

Subject	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6[a]
Age at last evaluation, y	18	7	12	3	10	12
Genetic analysis						
Karyotype	46, XX	46, XX	46, XX	46, XX	46, XX	46, XX
CYP19A1	c.[628G>A]; [628G>A]	c.[628G>A]; [242A>G]	c.[628G>A]; [628G>A]	c.[574C>T]; [574C>T]	c.[574C>T]; [1369C>T]	c.[628G>A]; [1235delA]
Clinical features						
Maternal virilization	No	Acne, hirsutism	No	Acne, hirsutism	No	Yes
Fetal virilization	Prader II	Prader IV	Prader IV	Prader IV	Prader IV to III	Prader III
Prepubertal evaluation	No data[b]					
Bone age delay		2 y	2 y 6 months			2 y 6 months
Ovarian aspect (US)		Multiple cysts	Normal	Normal	Not visualized	Cysts
Pubertal evaluation						
Spontaneous breast development	Yes (11 y)[c]		Yes (9 y)		Yes (10 y)	Yes (7 y)
Androgenic signs in puberty	Yes		No		No	Yes
Ovarian aspect (US)	Cysts		Large cysts		Enlarged	Large cysts
Laboratory tests						
OGTT	Not done	Not done	Normal	Normal	Normal	Type 2 diabetes
Glucose, nmol/L, basal per 120 min			3.43/5.53	3.59/4.81	4.2/3.7	3.48/11
Insulin, μIU/mL, basal per 120 min			6.7/36.7	3.1/5.4	9/18.7	26.3/>300
Basal FSH, mIU/mL						
Neonatal (6.6 ± 5.2)						119
Infancy (5.4 ± 3.4)			27.5		19.5	>150
Childhood (2.8 ± 1.9)		14.5	14.1	25.7	10.2	22.8
Puberty (5.8 ± 2.9)	13.5		14.2		6.2	17
Basal LH, mIU/mL						
Neonatal (0.5 ± 0.4)						76
Infancy (0.2 ± 0.1)		1.9	3.1		0.6	58.7
Childhood (0.2 ± 0.9)			<0.1	2.5	0.3	0.58
Puberty (4 ± 3.2)	13.5		9.7		<0.1	22.3
Basal T, ng/mL						
Neonatal (<0.05)						0.7
Infancy (<0.05)			<0.05		0.7	0.5
Childhood (<0.2)		<0.2	<0.05	<0.05	<0.05	0.9
Puberty (0.05–0.7)	0.85		1.2		<0.05	1.68
Basal δ_4A, ng/mL						
Neonatal (<0.5)						0.9
Infancy (0.05–0.35)					0.8	0.3
Childhood (0.05–0.5)		<0.1	0.8	<0.1	0.3	0.8
Puberty (0.15–2.0)	4.2		1.5		0.3	2.1

Abbreviations: δ_4A, androstenedione; OGTT, oral glucose tolerance test; US, ultrasonography. Conversion factors to SI units: T (nanograms per milliliter) × 3.462 = T (nanomoles per liter); δ_4A (nanograms per milliliter) × 3.671 = δ_4A (nanomoles per liter). Reference values for LH, FSH, T, and δ_4A are shown in parentheses. Nomenclature of different mutations at protein and nucleotide level is as follows: c.242A>G: p.Tyr81Cys; c.574C>T: p.Arg192Cys, c.628G>A; c.1235delA: p.Glu412fs*445; c.1369C>T: p.Arg457* (NM_000103.3).

[a] References 15–17.

[b] First evaluation at 13 years.

[c] Referred by pediatrician.

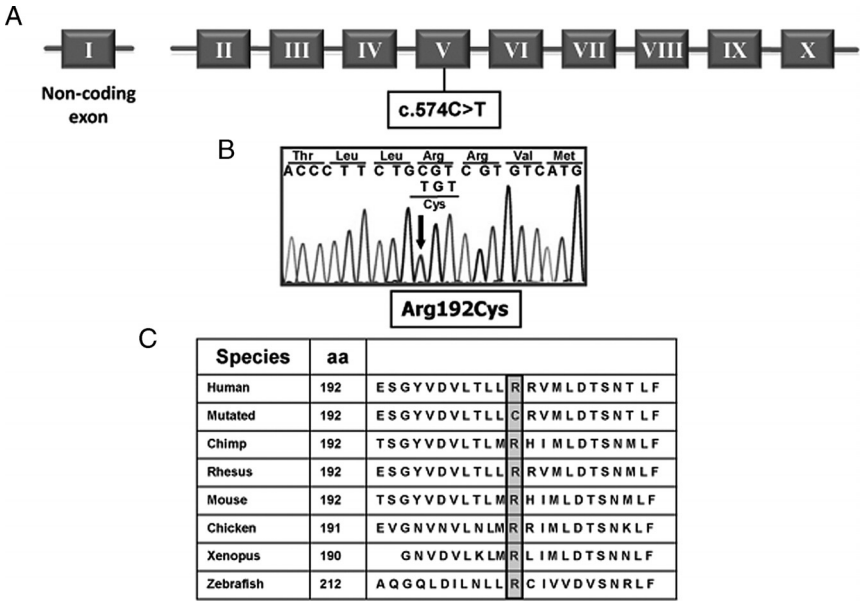

Figure 1. A, Schematic representation of the genomic structure of the *CYP19A1* gene showing the C-to-T transition in exon 5. The gene consists in nine coding exons (E2–E10), spanning approximately 35 kb. There are multiple first exons that are involved in tissue-specific expression. B, DNA sequence analysis of the affected patients 4 (homozygous) and patient 5 (compound heterozygous) showing C-to-T transition at cDNA position 574 bp (c.574C>T). This nucleotide change resulted in a novel substitution of arginine to cysteine at codon 192 of the mature protein: p.Arg192Cys. C, The mutated arginine at position 192 is highly conserved in homologues of P450arom from many species analyzed.

adrenal hyperplasia due to 21-hydroxylase deficiency as well as other steroidogenic defects were ruled out. The presence of testicular tissue was ruled out based on the lack of serum T response to human chorionic gonadotropin stimulation. Maternal virilization was reported in three of six cases.

At prepubertal evaluation, bone age was found to be delayed in three patients, and ovarian cysts were found in two. Basal serum FSH levels were elevated in all patients studied.

All four patients who had reached puberty presented with spontaneous breast development, associated with clinical signs of virilization in two of these. Basal serum LH and FSH levels were elevated. Serum androgen (T and androstenedione) levels were high compared with normal values for age and stage of sexual development in almost all patients. Ovarian enlargement or the presence of bilateral ovarian cysts wasd seen on pelvic ultrasound in all patients.

An oral glucose tolerance test was requested in four patients; all but one had normal glucose tolerance.

All siblings were included in pedigrees in Supplemental Figure 1.

Mutation analysis

Table 1 also summarizes molecular studies of the five aromatase-deficient patients reported here as well as the previously reported case. Direct sequencing of the *CYP19A1* gene from genomic DNA revealed a novel mutation in two patients (numbers 4 and 5). Patient 4 was found to be homozygous for a C-to-T transition at cDNA position 574 bp in exon 5 (c.574C>T, the A of the ATG of the initiator Met codon is denoted as nucleotide +1). This nucleotide change resulted in a arginine-to-cysteine substitution at codon 192 of the mature protein: p.Arg192Cys (Figure 1). Both parents were found to be heterozygous for this alteration, whereas a 7-year-old brother was homozygous for this mutation. This variant was not found in the 1000 Genomes or Exome Sequencing Projects (http://www.ensembl.org). To determine whether this alteration is present in the general population, 60 control subjects (120 alleles) were screened for this mutation using DNA sequencing. No allele carrying this mutation was detected, suggesting that it would not be a common polymorphism. Patient 5 revealed to be a compound heterozygote for the same mutation c.574C>T and the previously reported c.1369C>T nonsense mutation. The father and mother were heterozygous carriers of the c.574C>T and the c.1369C>T mutations, respectively.

Four of six nonrelated patients presented with the previously described c.628G>A splice mutation; two were homozygotes (patients 1 and 3) and two (patients 2 and 6) were found to be compound heterozygotes for the c.628G>A and either the earlier reported c.242A>G or c.1235delA (15, 20) mutations, respectively. Both parents of patient 3 and the mother of patient 1 were found to be heterozygous for c.628G>A. The father of patient 1 was not available for blood sampling.

In silico analysis

The evolutionary conservation of the amino acid affected by the c.574C>T mutation was examined by sequence alignment of P450arom proteins from different species. The amino acid substitution affects a highly conserved amino acid of the aromatase protein from other species (Figure 1).

We also applied an evolutionary perspective to screen the c.574C>T substitution using the sequence homology-based SIFT tool, which predicted this variant to affect

protein function with a highly deleterious tolerance index score of 0.00.

In addition, the effect of the c.574C>T mutation was predicted to be probably damaging with a score of 1.000 (sensitivity 0.00; specificity 1.00) using the structure-based approach PolyPhen-2.

Haplotype analysis

To investigate a possible common ancestry linked to the highly frequent c.628G>A mutation in our population, the haplotypes of the four c.628G>A patients (six alleles) were characterized using 21 polymorphic markers within the *CYP19A1* gene (Table 2). To discriminate between alleles, both parents were analyzed for segregation analysis. Interestingly, all six alleles shared a common haplotype (HH1) in the c.628G>A allele. The c.628G>A-linked haplotype frequency in our sample population was calculated (100%, n = 4 alleles) and compared with the same haplotype frequency from the control Caucasian American population (27%, n = 120 alleles). For these calculations two c.628G>A alleles were excluded because consanguinity could not be ruled out. This mutation was found to be linked to the same haplotype significantly more frequently than expected ($P = .019$).

To confirm this association, we characterized the haplotypes in the Argentinean population by genotyping three additional polymorphic markers within the *CYP19A1* gene. The c.628G>A-linked haplotype (HH2) frequency found (100%, n = 4 alleles) was compared with the same haplotype frequency found in the Argentinean population (27%, n = 94 alleles). This association was observed to be statistically significant ($P = .019$). Moreover, when the c.628G>A-linked HH2 frequency was compared with the reported frequency for the Caucasian American population (<1%, n = 120 alleles), the significance was even higher ($P < 10^{-6}$).

Taken together, these results show that the c.628G>A mutation is associated with the same haplotype in our population, suggesting a common ancestral origin rather than a recurrent event.

Discussion

In this paper, we report five new cases of aromatase deficiency and one novel mutation (c.574C>T) in the *CYP19A1* gene.

The follow-up of these five 46,XX patients plus a previously reported girl (15) from birth to late prepuberty or puberty provided an opportunity to evaluate phenotypes of subjects with varying degrees of partial deficiency of the aromatase enzyme (Table 1). The first striking clinical consequence was the masculinization of the female external genitalia of the female fetus, which, to a varying de-

Table 2. Human *CYP19A1* Genetic Polymorphisms Genotyped

SNP Identification	Location	Nucleotide	Nucleotide Change	Haplotype 1	Haplotype 2
rs61203654	Exon 2	42	C>G	C	C
rs58282176	Exon 2	109	T>C	T	T
rs2236722	Exon 2	115	T>C	T	T
Rs3759811	**Intron 2**	**−59**	**A>G**	N/A	**G**
rs28377729	Intron 2	−27	T>C	T	T
rs60308277	Exon 3	186	C>T	C	C
rs700518	Exon 3	240	A>G	A	A
rs61292383	Intron 3	48	G>A	G	G
	Exon 4	436	C>G	C	C
rs28892004	Intron 4	8	G>A	G	G
rs11575899	Intron 4	27	TCT(I>D)	D	D
rs60271534	Intron 4	77	(TTTA)n	7	7
rs28757184	Exon 5	602	C>T	C	C
rs4324076	Intron 5	−16	T>G	T	T
rs61317221	Exon 6	633	T>C	T	T
rs1143704	Intron 6	36	A>T	A	A
rs59196885	Intron 6	44	G>C	G	G
rs2304463	**Intron 6**	**−106**	**T>G**	N/A	**T**
rs700519	Exon 7	790	C>T	C	C
rs17601241	Intron 7	26	C>T	C	C
rs2289105	**Intron 7**	**−79**	**A>G**	N/A	**A**
rs59359360	Exon 8	963	C>G	C	C
rs28757194	Intron 8	29	C>T	C	C
rs56658716	Exon 9	1091	T>C	T	T

Abbreviations: N/A, not analyzed; SNP, single-nucleotide polymorphism.

The haplotypes of the four c.628G>A patients were characterized with 21 polymorphic markers (HH1). Three additional polymorphic markers (in bold) were genotyped in the four c.628G>A patients or Argentinean control population (94 alleles) (HH2).

gree, was seen in all cases. This stresses the important physiological role of the aromatase enzyme in inactivating biologically active androgens generated in the fetoplacental unit in females. Ambiguous genitalia are a good marker of fetal androgen action but other, less visible consequences may certainly be taking place during development, including effects on ovarian function (21), brain programming of future behavior (22), and metabolic programming of adult fat and glucose metabolism (23), as well as cardiovascular physiology, among others.

The second striking clinical consequence was a resetting of central gonadotropin feedback, resulting in moderate (patients 1, 2, 3, and 4) or high (patients 5 and 6) increases in serum FSH, and occasionally, mild increases in serum LH. This dysfunction is probably responsible for the frequent detection of ovarian cysts, which may require surgery because of the risk of acute cyst rupture, even before puberty (16). Serum T and δ4-androstenedione might have been temporarily elevated during the postnatal activation period (patient 1) but remained low during prepuberty and tended to increase at puberty in some patients. Moreover, enlarged ovarian cysts were detected by ultrasonography in the four girls who started puberty during follow-up.

Breast development started spontaneously in all four subjects who reached 9 years of age, pointing to the partial nature of the enzyme deficiency. Patient 1 had spontaneous menarche followed by regular menses. Estrogen replacement therapy was indicated in patients 3 and 6 in an attempt to prevent consequences of estrogen insufficiency. Remarkably, even though systemic estrogen administration is followed by clinical signs of estrogen response, it is not able to normalize some signs of estrogen insufficiency, particularly elevation of serum FSH and improvement of images of polycystic ovaries on ultrasonography. This observation was previously reported by Guercio et al (16) and Janner et al (24) in aromatase-deficient girls under estrogen therapy. It is speculated that this atypical response may be secondary to poor local estrogen synthesis from androgen precursors in peripheral tissues, such as the hypothalamus and ovary.

As found for many genes, our study shows that loss-of-function mutations in the aromatase gene show variable expressivity. Part of the phenotypic variations observed in prebubertal and pubertal years in our six subjects might be related to the type of mutation (Table 1). As discussed below, an alternative splicing mutation was found, in either the homozygous or heterozygous state, in four of six patients showing abnormalities ranging from mild (patient 1) to severe (patient 6).

Here we report a novel mutation, c.574C>T (p.Arg192Cys), in two patients. The amino acid substitution takes place in a highly conserved site among species observed in in silico analysis.

Availability of the crystal structure of aromatase has enabled the investigation of protein dynamics (25). The Arg192 is important in the structure of the channel that is probably the major transport route to and from the active site for water, oxygen, and steroid molecules (26). The Arg192 side chain forms a salt bridge with Glu483 and is linked through two water molecules to Ser478 by a weak hydrogen bond. The salt-bridging Arg 192-Glu 483 pair as well as Asp309 and Ser478 line this channel. The p.Arg192Cys substitution may disrupt this structure, affecting the aromatase activity. Two different bioinformatics approaches (SIFT and Polyphen2) confirmed this prediction (Supplemental Figure 2).

Recently in vitro functional characterization of another residue alteration in the same position (p.Arg192Hys) was assessed (13), and it was found that catalytic efficiency of the mutant was 19% compared with the wild type. This is consistent with the importance of this residue for catalytic activity.

We previously described the c.628G>A mutation associated with an aberrant spliced mRNA lacking the coding sequence from exon 5 (17). This mutation has also been reported by Maffei et al (18) in a homozygous aromatase-deficient man from Argentina. In this study we report new cases of aromatase deficiency carrying the c.628G>A mutation. We describe four of six unrelated patients presenting with the c.628G>A splice mutation, two that were homozygous and two that were found to be compound heterozygotes for c.628G>A and the previously reported c.242A>G and c.1235delA mutations. Considering the low incidence of aromatase deficiency described (28 cases) and the high frequency of this mutation, it may be a hot spot or a founder mutation. Individuals who harbor hot-spot mutations are usually not related to each other, and thus, their DNA will vary as is typical of unrelated people. Haplotypes of the four c.628G>A patients (six alleles) were characterized using 21 polymorphic markers within the *CYP19A1* gene. All six alleles shared the same haplotype HH1. Moreover, when comparing with the HH1 frequency published from a control Caucasian American population, we found that this haplotype HH1 is linked to the mutation significantly more frequently than expected. We confirmed these results, genotyping three additional polymorphic markers within the *CYP19A1* gene in our Argentinean population. These findings do not support the hypothesis of a hot-spot mutation. A high frequency of markers associated with a mutation is usually attributed to a founder effect in a population and is usually evidenced by haplotype conservation. Here we observed a significantly higher frequency of hap-

lotype HH1 associated with the mutation, suggesting a founder effect. Therefore, the identification of this mutation and genetic counseling of heterozygous carriers from affected families of our population seems to be important. We believe that this mutation might be present in other populations, mainly because our Argentinean population has a greatly diverse origin. The search for this mutation in other populations will reveal whether it has a wider ethnic distribution.

In summary, the follow-up of these 46,XX patients with aromatase deficiency during their first years of life has increased our knowledge on the clinical course, emphasizing the need for early diagnosis and appropriate interventions in this unique chronic condition, which combines excessive androgens with deficient estrogens, starting early in fetal life. The results show that in our population a genetic founder defect has probably contributed to an increase in the incidence of this rare mutation.

Acknowledgments

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