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CONGENITAL ADRENAL HYPERPLASIA

CLINICAL CHARACTERISTICS AND GENOTYPE IN NEWBORN, CHILDHOOD AND ADOLESCENCE

TITANIA PASQUALINI[1], GUILLERMO ALONSO[1], ROSANGELA TOMASINI[1], ANA MARIA GALICH[2], NOEMI BUZZALINO[3], CECILIA FERNANDEZ[3, 4], CAROLINA MINUTOLO[3, 5], LILIANA ALBA[3], LILIANA DAIN[3, 4]

[1]*Sección de Endocrinología, Crecimiento y Desarrollo, Departamento de Pediatría y* [2]*Servicio de Endocrinología y Medicina Nuclear, Hospital Italiano;* [3]*Centro Nacional de Genética Médica, A.N.L.I.S. Dr. Carlos G. Malbrán;* [4]*Instituto de Biología y Medicina Experimental;* [5]*Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires*

Abstract Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is a disorder which can adopt three clinical expressions: two classical forms –salt-wasting (SW), with residual enzymatic activity (EA) ≤ 1% and simple virilizing (SV), with EA 1-2%– and a mild late onset or nonclassical (NC) form, with EA 10-60%. Our objective is to describe clinical characteristics, growth, and bone mass in a group of patients affected by 21-hydroxylase deficiency. Besides, molecular genetics studies were performed in patients, and also when available in their parents and siblings. Nine patients with neonatal diagnosis and 8 with pre or postpubertal diagnosis were studied. Analyses of 10-point mutations in the CYP21A2 gene were performed. We found that all the patients with the classical expression, except one with a *de novo* mutation R356W in one allele, were fully genotyped with predictive < 2% EA mutations. Signs of hyperandrogenism were present in 5/6 NC patients; one was diagnosed by searching for mutations in asymptomatic siblings. All the NC patients were compound heterozygotes carrying V281L mutation in one allele and a predictive low EA in the other, except for one not yet determined. In patients with neonatal diagnosis, mean height was low at one year of age, though it showed a significant increase before the onset of puberty. We conclude that neonatal diagnosis of classical CAH allows an adequate follow up enhancing growth. Molecular analyses of all members of an affected family may disclose asymptomatic patients. The presence of *de novo* mutations, as well as, the presence of mutations with low predicted EA in NC patients reinforces the importance of genotyping for appropriate genetic counseling. In fully genotyped NC patients, the lowest value of ACTH-stimulated 17OHP was 14 ng/ml. Lower cut-off values might overestimate the diagnosis of the NC form.

Key words: congenital adrenal hyperplasia; congenital adrenal hyperplasia molecular genetics; congenital adrenal hyperplasia clinical characteristics

Resumen ***Hiperplasia suprarrenal congénita. Características clínicas, seguimiento y genotipo en la etapa perinatal, la niñez y la adolescencia.*** La hiperplasia suprarrenal congénita por déficit de 21-hidroxilasa presenta tres formas clínicas: dos clásicas, perdedora de sal, con actividad enzimática (AE) ≤ 1% y virilizante simple, con AE 1-2% y una no clásica, con AE 10-60%. Nuestro objetivo es describir las características clínicas y el genotipo de un grupo de pacientes con hiperplasia suprarrenal congénita; este último también se determinó en todos los miembros de la familia. Se estudiaron 9 pacientes diagnosticados en la etapa perinatal y 8 durante la etapa pre y postpuberal. Se analizaron diez mutaciones en el gen CYP21A2 y se evaluó crecimiento y densidad mineral ósea. Once pacientes presentaron la forma clásica: 9 con diagnóstico perinatal y 2 diagnosticados más tardíamente, uno de ellos con agrandamiento testicular por restos adrenales. Todos los pacientes, salvo 1 con una mutación *de novo* R356W en un alelo, presentaron ambos alelos mutados con un genotipo que predice AE < 2%. Seis pacientes presentaron la forma no clásica, todos con signos clínicos de hiperandrogenismo salvo un familiar asintomático que se diagnosticó por el estudio molecular. Todos, a excepción de uno con un alelo aún no determinado, presentaron la mutación V281L acompañada de otra que predice AE < 2%. Durante la evolución de los pacientes de diagnóstico perinatal se observó talla baja al año con recuperación de la misma en la etapa prepuberal. La densidad mineral ósea fue normal. Podemos concluir que el diagnóstico en la etapa perinatal en pacientes con la forma clásica posibilita un mejor seguimiento y crecimiento. La genotipificación de todos los miembros de una familia permite el diagnóstico de formas asintomáticas. La presencia de mutaciones *de novo* y de un alelo con una mutación que predice baja AE en los pacientes con forma no clásica, refuerza la importancia de la genotipificación para un adecuado asesoramiento genético.

Palabras clave: hiperplasia suprarrenal congénita, genética molecular

Congenital adrenal hyperplasia (CAH) describes a group of autosomal recessive disorders of cortisol biosynthesis, among which 21-hydroxylase deficiency ac-

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Postal address: Dra. Titania Pasqualini, Mendoza 3479, 1430 Buenos Aires, Argentina

e-mail: tpasqualin@intramed.net

ABBREVIATIONS:

CAH:	congenital adrenal hyperplasia	**PRA:**	plasma renin activity
SW:	salt-wasting	**PR:**	plasma renin
EA:	residual enzymatic activity	**A_4:**	androstenedione
SV:	simple virilizing	**T:**	testosterone
NC:	nonclassical	**DHEA-S:**	dehydroepiandrosterone-sulphate
P:	progesterone	**BMD:**	bone densitometry
17OHP:	17-hydroxyprogesterone	**L2-L4:**	lumbar spine
ACTH:	corticotrophin	**FSH:**	follicular stimulating hormone
		LH:	luteinizing hormone.

counts for 90-95% of cases[1-4]. The disease includes a severely affected or classical form and a mild late onset or nonclassical (NC) form. The classical form includes salt-wasting (SW), 67-75% of the cases, and simple virilizing (SV) variants, depending on the degree of aldosterone deficiency. Poor synthesis of cortisol, with or without aldosterone deficiency, results in chronic stimulation of the adrenal cortex by corticotrophin (ACTH). Consequently, over-production of some cortisol precursors is shunted into the androgen biosynthetic pathway causing the signs and symptoms of androgen excess seen in this disorder. Due to the exposure of high systemic adrenal androgen levels from the 7th week of gestation, girls with classic CAH are typically born with ambiguous genitalia. In contrast, affected boys have no overt signs of the disease except for variable and subtle hyperpygmentation with or without penile enlargement[1-4].

Patients with NC CAH, present manifestations of hyperandrogenism, such as early pubarche, hirsutism (60%), oligomenorrhea or amenorrhea (54%), polycystic ovaries, acne (33%), and/or infertility (13% in women). Furthermore, NC CAH affects 5-10% of children with precocious pubarche and 1-6% of women with hyperandrogenism[4, 5].

Neonatal screening programmes performed since 1977 have shown an overall incidence of 1:15 000 live births for the classical form[4, 6, 7]. On the other hand, NC CAH is estimated to be more common than classical CAH, with a prevalence of 1:1000 in the white population [4], more frequent in certain ethnic groups such as Jews of Eastern Europe, Hispanics and Yugoslavs (1-3.7%).

Gene CYP21A2 encoding active 21-hydroxilase enzyme, and a pseudogene, CYP21AP with 98% nucleotide sequence identity, are located on chromosome 6p21.3. Due to the high grade of homology between gene and pseudogene, most of the common mutations arise from 2 types of unequal misalignment events between them. The most frequent is a process known as gene conversion, whereby aberrant pseudogene sequence is transferred to CYP21A2. On the other hand, about 20% of mutational events occur as unequal crossing-over during meiosis deleting a 30-kilobase-gene segment, which produces a nonfunctional chimeric pseudogene [4, 8, 9]. In addition, more than 90 rare point mutations have been described up to date[4, 10].

CYP21A2 mutations can be grouped into 3 categories according to the level of enzymatic activity (EA) predicted from *in vitro* expression studies. The first group includes large deletions and nonsense mutations that totally ablate EA (EA <1%) which are most often associated with SW form. In the second group mutations reduce EA to 1-2% of normal, enough for aldosterone synthesis (SV form). Mutations that retain 10 to 60% of normal EA, with normal sodium balance and only signs of androgen excess (NC form), are most often present in the third group[8, 11, 12]. Usually, phenotypic severity correlates with the EA of the underlying genotypes. Most patients are compound heterozygotes carrying 2 different CYP21A2 mutations, being the phenotype of the affected individual dependent on the milder gene defect[4, 8, 9, 11, 12].

The objective of this study is to describe clinical characteristics, growth, and bone mass in a group of patients affected by 21-hydroxylase deficiency. Besides, molecular genetics studies were performed in patients, and also when available in their parents and siblings. The patients were followed at the "Sección de Endocrinología, Crecimiento y Desarrollo del Departamento de Pediatría, Hospital Italiano de Buenos Aires".

Materials and Methods

This is a descriptive study of a group of Argentine patients followed at the "Sección de Endocrinología, Crecimiento y Desarrollo del Departamento de Pediatría, Hospital Italiano de Buenos Aires" with classical and nonclassical forms of CAH.

Approval for this study was obtained from the Ethics Committee of the "Instituto de Biología y Medicina Experimental", the "Centro Nacional de Genética Médica" and the "Comité de Etica de Protocolos de Investigación del Hospital Italiano de Buenos Aires". The study group consisted of 11 patients with classic CAH, 8 SW and 3 SV (9 with neonatal diagnosis), and 6 NC patients diagnosed during prepubertal or pubertal development (Tables 1 and 2). SW CAH clinical characteristics included ambiguous genitalia at birth in females and/or the presence of a salt loss adrenal crisis. Diagnosis was confirmed by high concentration of basal 17OHP accompanied by high values of standing plasma renin activity (PRA) or direct plasma renin (PR) (Table 1). The SV form was diagnosed on the basis of genitalia ambiguity at birth in females or later signs of virilization in both sexes accompanied by elevated 17OHP level (Tables 1 and 2). Patients with NC form presented late-onset symptoms of androgen excess. Diagnosis involved an early-morning basal level of 17OHP > 2 ng/ml and a 60-min 17OHP post stimulation test with synthetic ACTH ≥ 10 ng/ml (Table 2).

Hormone assays: 17OHP, androstenedione (A_4), testosterone (T) and dehydroepiandrosterone-sulphate (DHEA-S) were assayed by radio-immunoassay (RIA) with the aid of a Diagnostic System Laboratory (DSL), Houston, TX, USA, commercial kit. PRA was measured using a Renin, BioChem Inmuno System, Rome, Italy, commercial kit and PR was measured by a radioimmunometric method[13]. Hormone control levels in prepubertal children were: 17OHP: < 2 ng/ml; A_4: <0.5 ng/ml; T: <0.5 ng/ml; DHEA-S: < 720 ng/ml; standing position PRA 1.5-5.6 ng Angiotensin I/ml/h; standing position RP: 0.38-7.2 pg/ml. Hormone control levels in adult females during early follicular phase were: 17OHP: < 2 ng/ml; A_4: 0.9-3.8 ng/ml; T: 0.3-0.9 ng/ml; DHEA-S: 350-4300 ng/ml.

Genotyping mutations in the steroid 21-OH gene: Analysis of 10-point mutations in the CYP21A2 gene was performed according to methods established in our laboratory, using DNA from peripheral blood leucocytes[13]. Fifteen parents and 8 asymptomatic siblings were also genotyped. Genotypes of parents, when available, allowed us to determine if the mutations found were in separate alleles. On the other hand, sibling genotyping was performed to achieve carrier status. Southern blot analysis using Taq I and BglII restriction enzymes was performed, to further exclude or confirm the pres-

TABLE 1.– *Patients with neonatal diagnosis*

Patient (sex)	Clinical manifestations	Age at diagnosis (days)	17OHP (ng/ml)	PRA (ng angiotensin I/ml/h)	Ionograme Na/K (meq/l)	Genotype	Parent and sibling genotypes
1)[a] (male)	Failure to thrive, vomits, weight loss	16	>20	NR	108 / 7.7	Cluster Ex 6 / A/C>G Intron 2	F: Cluster Ex 6 / N M: V281L / A/C>G Intron2
2)[a] (male)	Failure to thrive, vomits, weight loss	18	>20	>18	109 / 8.5	Q318X / V281L + InsT Ex 7	F: Q318X / N M: V281L + InsT Ex7 / N
3) (male)	Failure to thrive, vomits, weight loss, E. Cloacae sepsis	22	>20	60	114 / 9.1	Del 8pb Ex3 +V281L / Q318X	F: Del 8pb Ex3 + V281L / N M: Q318X / N
4)[a] (male)	Failure to thrive, vomits.	51	>25	>35	115 / 7.2	A/C>G Intron 2 / 5´ Macroconversion	Adopted
5) (female)	Ambiguous genitalia	3	24[b]	>20	136 / 4.8	Macroconversion / Del CYP21A2	F: Macroconversion / N M: Del CYP21A2 / Del CYP21A1
6) (female)	Ambiguous genitalia	36	>20	High	137 / 4.2	A/C>G Intron2 + Q318X / I172N	F: A/C>G Intron 2 + Q318X / N M: I172N / N
7)[a] (female)	Ambiguous genitalia; failure to thrive	37	>25	>26	-	R356W* / ND	F: ND / N M: ND / N Brother : N / N
8)[a] (female)	Ambiguous genitalia; diagnosis of undermasculinezed boy	49	33.6[b]	1	-	Q318X / R356W + P453S	F: Q318X / N M: R356W + P453S / N
9) (female)	Ambiguous genitalia; no treatment, salt-losing crisis at 10 days of age.	4	>20	PR:18 pg / ml	129	Q318X / A/C>G Intron 2	F: Q318X / N M: A/C>G Intron 2 / N Brother : N / N

*[a] data published partially previously [13]; [b] Values under treatment; N: Normal; ND: Not determined; Del: Deletion; ** de novo *mutation. M: mother; F: father.*

TABLE 2.– *Patients with diagnosis during prepubertal or pubertal development*

Patient (Sex)	Clinical manifestations	Chr Age / Bone age	17OHP (ng/ml)	Hormone values (ng/ml)	Genotype	Parent and sibling genotypes	Treatment
*10) (male)	Diagnosis by genotyping assymptomatic siblings W/H 35%, Height 0.44 SD	5.3/5	0.76	A_4: 0.44 T: 0.13	V281L / Q318X	F: V281L / N M: Q318X / N Sister: V281L / Q318X Brother: N / N	No therapy
11)[a] (male)	Precocious virilization, W/H 20%, Height 1.94 SD, Testis 3 cc, pubic hair Tanner II	9.2/13	>20	A_4: 2.6 DHEA-S: 543 T:0.7	V281L / Q318X	F: V281L / N M: Q318X / N	Hidrocortisone
12)[a] (male)	Precocious virilization, W/H 10%, Height 0.65 SD Testis 5cc, pubic hair Tanner II	9.4/13.5	53	A_4: 3.3 DHEA-S:2590 T: 1	A/C>G Intron 2 / V281L	F: A/C>G Intron2 / N M: V281L / N 2 sisters: N / N and A/C>G Intron2 / N	Hidrocortisone
13)[a] (male)	Precocious virilization, W/H -7%, Height 1.43 SD Testis 3cc, pubic hair Tanner III	10.5/ 12.7	18 Post ACTH: >40	A_4: 2.6 DHEA-S:2750 T:0.6	R356W / V281L	F: R356W / N M: V281L / N 2 sisters: V281L / N	Hidrocortisone
14)[a] (male)	Precocious puberty W/H -5%, Height -0.86 SD, Testis >25cc, pubic hair Tanner VI	13.8 / adult	48	A_4 >100 DHEA-S: 14000 T: 0.5	R356W / A/C>G Intron 2	F: R356W / N M: A/C>G Intron2 / N Brother: N / N	Dexa+fludrocortisone
15) (female)	Precocious pubarche Clitoromegaly	4.2 / 7	>20	A_4: 6.4 DHEA-S: 202 T:1.0	I172N / I172N	F: I172N / N M: I172N / N	Hidrocortisone
*16)[a] (female)	Precocious pubarche W/H 38%, Height 2.56 SD Tanner I, pubic hair Tanner II.	7.8/10	4.8 Post ACTH: 14	A_4 : 0.73 DHEA-S:650 T: 1.0	V281L / Q318X	F: V281L / N M: Q318X / N Brother: V281L / Q318X Brother: N / N	No therapy
17) (female)	Oligomenorrhea W/H: 32%, Height -0.27 SD Tanner V	16.2 / adult	2.4 Post ACTH: 12.3	A_4: 5.5 ng/ ml DHEA-S: 6990 T: 0.6	ND / V281L	F: ND / N M: V281L / N	Methylprednisone

*[a] data published partially previously [13]; * Siblings; Chr Age: chronological age; W/H: Weight/Height; N: normal; ND: not determined; M: mother; F: father.*

TABLE 3.– *Analyzed mutations in relation to their predicted residual enzyme activity (EA)*

– Macroconversions/deletions:	EA<1%
– Mutations:	
• 8bp Ex 3 deletion	EA <1%
• Cluster Ex 6	EA <1%
• Ins T Ex 7	EA <1%
• Q318X	EA < 1%
• R356W	EA <1%
• I172N	EA 1-2%
• A/C>G Intron 2	EA < 1% or 1- 2%
• P30L	EA 10-60%
• V281L	EA 20-50%
• P453S	EA 20-50%

ence of 5´macroconversions, macroconversions, deletions or homozygous genotypes. Paternity tests were performed whenever discrepancy appeared between the children's and parent's genotypes as described previously[13]. Two patients (# 3 and # 5), and a sibling of # 9, were genotyped in the "Laboratorio de Investigación, Hospital de Pediatría, Hospital Juan P. Garrahan"[14, 15]. Table 3 shows the analyzed mutations and their classification according to residual EA.

Growth: We recorded the following information: sex, age, height, weight and linear growth. In patients under 4 years of age, height was measured in supine position while standing position was used for older patients. Height Z score was calculated using the formula: height Z score = (observed value – mean value) / SD) (SD: the standard deviation for the normal population of the same chronological age and gender). Anthropometrical measurements were matched against the Argentine National Growth Charts[16]. Pubertal status was defined according to Marshall and Tanner[17, 18].

Bone densitometry (BMD): BMD was assessed using the technique of dual energy X-ray absorptiometry of the lumbar spine (L2-L4) and total body (g/cm^2) and expressed as Z-Score (Lunar DPXL/PED, Lunar Radiation, Madison, Wis., USA).

Statistical analysis. Paired Student's t-test was used for evaluating longitudinal gain of height.

Results

Clinical evaluation of patients

Patients with neonatal diagnosis

Table 1 shows clinical characteristics, laboratory data and molecular study of the nine classical CAH patients with neonatal diagnosis. Four boys presented salt loss crisis after 15 days of age. The five female infants had ambiguous genitalia at birth, four of them with enlarged clitoris, partly fused and rugose labia majora and a common urogenital sinus with one orifice. One patient presented enlarged clitoris but a separated urethra and vagina (# 6). One of the patients with ambiguous genitalia (# 8) came to our hospital at 49 days of age as an undermasculinized boy with diagnosis of 3-β-hydroxysteroid dehydrogenase deficiency. On clinical examination, no palpable gonads were found and an uterus was observed by ultrasonography. Chromosome analysis showed a 46, XX karyotype.

In three out of five girls with ambiguous genitalia vaginoplasty, clitoral and labia surgery was carried out between 0.4 and 1.8 years, while in another patient surgery was performed at 6.7 years of age. Finally, surgery was not necessary in the patient with separated vagina and urethra, as clitoral size decreased during glucocorticoid therapy (# 6).

During follow-up, testicular ultrasonography performed in two of our oldest classical CAH patients, at 15.6 (# 1) and 17 (# 2) years of age, was normal.

It is noteworthy that the mother of # 1 presented symptoms of precocious pubarche, enlarged clitoris, hirsutism and infertility. She was diagnosed as NC CAH based on elevated basal serum 17OHP (>20 ng/ml). She got pregnant after three months of low dose glucocorticoid replacement.

Patients with prepubertal or pubertal diagnosis

Table 2 shows clinical characteristics, laboratory data and molecular study of the eight patients with later diagnosis, six NC CAH and two classical SV CAH. Within the NC patients, three males presented early virilization, one female precocious pubarche, all with advanced bone age. One female with later diagnosis had oligomenorrhea and acne. An asymptomatic male sibling was disclosed after molecular study.

The SV patients, presented elevated concentration of 17OHP and signs of early virilization (Table 2). Premature pubarche, enlarged clitoris and growth acceleration with advanced bone age at 4.2 years was observed in patient # 15. In patient # 14 diagnosis was made at 13.8 years of age based on the presence of testicular adrenal rests. At physical examination, he had generalized hyperpygmentation, height of 150 cm, Tanner stage V, and testes > 25cc. Adult bone age had been reached 2.5 years before. Abdominal and testicular sonography showed bilateral adrenal hyperplasia and enlarged testes with multiple nodules, one dominant in right teste of 16 x 15 mm with calcifications. Hormonal measurements showed high adrenal androgens with prepubertal level of serum testosterone and undetectable levels of gonadotrophins (FSH <0.4, LH < 0.5 mUI/ml). Testicular biopsy confirmed the diagnosis of adrenal rests. Testicular size decreased with glucocorticoid and mineralocorticoid replacement.

Genotyping mutations in the steroid 21-OH gene

The molecular study of the nine classic CAH patients with neonatal diagnoses showed mutations in both alleles with

TABLE 4.– *Genotype-phenotype relationship according to the observed phenotype*

Patient	Genotype	Expectede phenotype	Observed phenotyp
1	Cluster Ex 6 / A/C>G Intron 2	SW / SV	SW
2	Q318X / V281L + Ins T Ex 7	SW	SW
3	Del 8bp Ex 3 + V281L / Q318X	SW	SW
4	A/C>G Intron 2 / 5´macroconversion	SW / SV	SW
5	Macroconversion / deletion	SW	SW
6	A/C>G Intron 2 + Q318X / I172N	SV	SV
7	R356W / ND	—	SW
8	Q318X / R356W + P453S	SW	SW
9	Q318X / A/C>G Intron 2	SW / SV	SW
10	V281L / Q318X	NC	Asymptomatic
11	V281L / Q318X	NC	NC
12	A/C>G Intron 2 / V281L	NC	NC
13	R356W / V281L	NC	NC
14	R356W / A/C>G Intron 2	SW / SV	SV
15	I172N / I172N	SV	SV
16	V281L / Q318X	NC	NC
17	ND / V281L	—	NC

SW: salt-wasting; SV: simple virilizing; NC: nonclassical; ND: Not determined, not currently predicted

a genotype that predicts < 1% EA, except in two patients, # 6 and # 7 (Table 1). A genotype that predicts an EA of 1-2%, associated with the SV form of the disease was disclosed in patient # 6 (born with separated vagina and urethra). On the other hand, a R356W mutation found in one allele of patient # 7, was absent in both parents. Paternity tests confirmed a *de novo* mutation. In addition, two mutations in the same allele were found in 5/18 analyzed alleles. The most frequent mutations for the classical form of the disease were A/C to G in intron 2 and Q318X mutation.

Within the eight patients with later diagnosis (Table 2), six with the NC form presented V281L mutation in one allele. All but one- with a non-characterized allele (# 17) were compound heterocygotes with a mutated allele that predicts low EA (<1%).

Two patients with classical SV form of CAH diagnosed during childhood and puberty (# 15 and # 14) presented a genotype that predicts low EA (<2%).

A total of fifteen parents were also genotyped. In 26 out of 30 chromosomes analyzed, one mutated allele was characterized. The mother of # 1 with NC CAH presented mutations in both alleles (V281L/ A/C>G Intron 2). No mutations were identified in 3 parents (both parents of # 7; one parent of # 17). When searching for mutations in asymptomatic siblings, a 5-year-old brother (# 10) was genotyped as V281L/Q318X, as was the index case (# 16). No mutations were found in 5, while one mutated allele was identified in 3 (Tables 1 and 2).

Table 4 shows the observed and expected phenotypes, according to the predicted EA and the genotype found. There was a high correlation between expected and observed phenotypes.

Growth during follow-up

In seven patients with neonatal diagnosis, median height Z score was –1.9 (range -2.6 to 0.03) at one year of age, –1.15 (-1.7 to 0.55) at three years of age, –1.1 (-1.3 to 0.25) at six years of age, –0.17 (-0.61 to 0.14) at the beginning of pubertal development, and -0.53 (-0.85 to -0.26) between 13.8 and 17 years of age (Fig. 1). There was a significant mean increment in height Z score between 1 and 3 years of age (mean 0.83 ± 0.21; p=0.0077), and between 1 year of age and the beginning of pubertal development (1.5 ± 0.34; p=0.0217). In four patients (2 males), pubertal development began between 7.9 and 12.6 years of age. One patient (# 7) with low compliance, presented early puberty, with telarche at 7.9 years of age accompanied by an advanced bone age of 8.7 years. Two male patients (# 1 and # 2) reached a near final height Z score of –0.5 and –0.53, 1.05 and 1.74 SD below the target height, respectively.

Bone densitometry (BMD)

BMD was evaluated in seven treated patients, five with the classical form and two with NC CAH. Lumbar spine (Fig. 2) and whole body (data not shown) BMD was within the normal range in six patients treated with 7-20.5 mg/m^2 hydrocortisone. The only patient treated with a low dose of dexametasone (# 14) (equivalent hydrocortisone dose 9.05 mg/m^2) presented low BMD for chronological age, though it was normal when corrected for height age (Fig. 2).

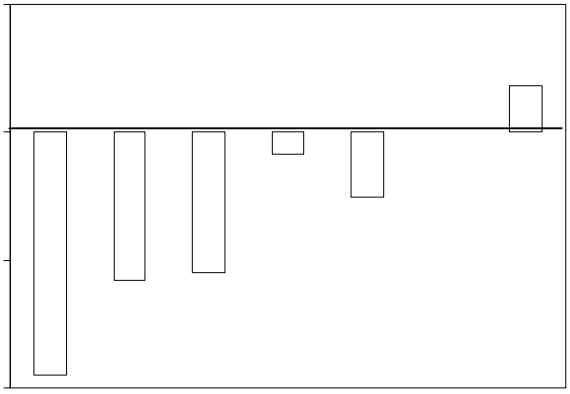

Fig 1.– *Height Z score during longitudinal follow-up in patients with neonatal diagnosis:* The graph shows longitudinal evolution of median height Z score and target height of patients with neonatal diagnosis of classical CAH. There was a significant mean increment in height Z score between 1 and 3 years of age (mean 0.83 ± 0.21; p = 0.0077), and between 1 year of age and the beginning of pubertal development (1.5 ± 0.34; p = 0.0217).

Discussion

In this study we evaluated patients with 21-hydroxylase CAH attended at Hospital Italiano from Buenos Aires. Eleven, 8 SW and 3 SV, presented the classical form, nine of whom were diagnosed at birth. In 4 SW male infants diagnosis was based on hypovolemia and electrolyte disturbances. On the other hand, it is well documented that CAH due to 21-hydroxilase deficiency is the most common cause of ambiguous genitalia in 46, XX infants[9]. Indeed, the five female infants with classical CAH diagnosed at birth, presented ambiguous genitalia. Among the three SV patients, diagnosis at birth was possible in only one female due to clitoromegaly (# 6). Signs of virilization developed later on in one female (# 15), while in patient # 14 diagnosis was performed by the presence of adrenal testicular rests. Testicular adrenal rests may develop during periods of absence or insufficient glucocorticoid therapy with sustained elevation of plasma ACTH, and may regress when glucocorticoid therapy is instituted

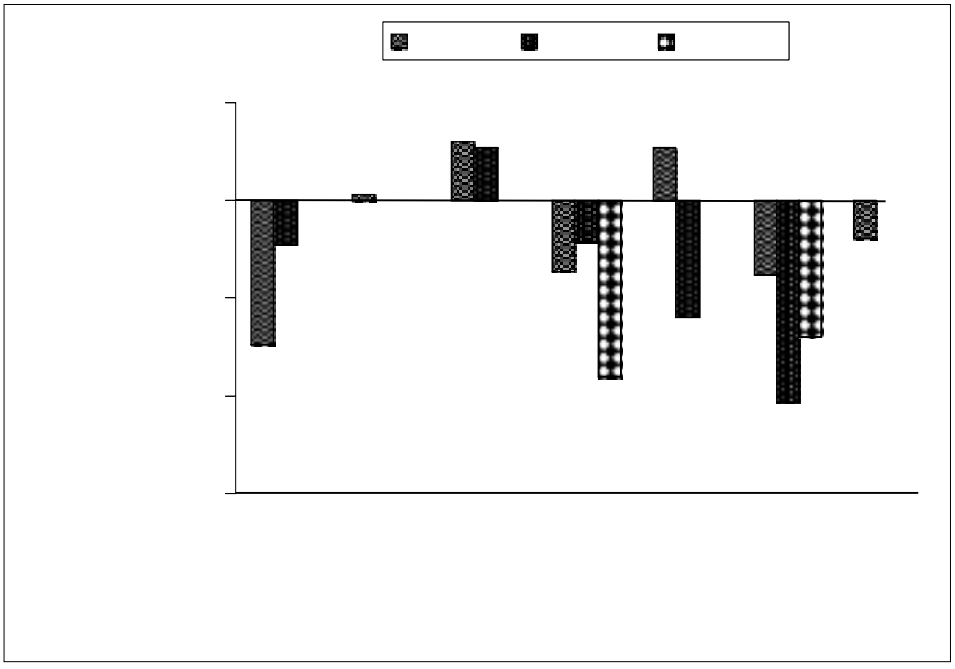

Fig 2.– *BMD: Lumbar (L II-L IV) spine Z score in 7 patients with CAH:* The graphic shows lumbar spine Z score BMD according to chronological age and Tanner stage at the time of the study.

or intensified[19, 20]. In fact, testicular size decreased in this patient under glucocorticoid replacement. Another two of our oldest treated CAH patients with the classical form of the disease, presented normal testicular sonography. Nevertheless, as patients with the classical form of CAH are at high risk of testicular adrenal rest tumors, ultrasonography should be proposed during follow up for early nodules detection and glucocorticoid dose adjustment[19-21].

Nonclassical CAH manifests with varying symptoms of hyperandrogenism outside the neonatal period. Males in our cohort presented precocious virilization, while females showed premature pubarche or oligomenorrhea. One of our NC patients was detected by searching for mutations in asymptomatic siblings. Even though, corticoid replacement is recommended for those with clinical manifestations[22, 23], asymptomatic NC patients do not need treatment. Nevertheless, long-term follow-up is recommended, since it was suggested that these patients –as well as carriers– might be at risk of incidentally discovered adrenal masses (incidentalomas)[24, 25].

One of the most troubling signs of classical 21-hydroxylase deficiency is genital ambiguity in affected females and adrenal crisis in the first weeks of life in both sexes. Besides, adrenal androgenization *in utero* may be at least partially prevented by administration of dexamethasone to the mother starting as early as possible in each pregnancy[4, 22, 23]. In view of these considerations, once an index case is detected, it is important to ascertain patient and parental genotypes for prenatal counseling[4, 22, 23]. We have analyzed presence of the 10 most frequent mutations in CYP21A2 gene in affected families. As reported for other populations[8, 12], we found that one of the most frequent mutations for the classical form of the disease was A/C to G in intron 2. A total of 95.4% of the alleles studied were identified, and all presented mutations that predict EA < 2%. It should also be stressed that due to the complexity of the locus, coexistence of two or more mutations on an allele is not uncommon in 21-hydroxylase deficiency. Therefore, molecular diagnosis must include the segregation of parental genotypes, for accurate prenatal diagnosis[26]**.** In fact, 23.8% of the alleles in our cohort of classical patients, presented more than one point mutation. Besides, gene conversions or deletions between CYP21A2 and CYP21A1P are detectable in $1{:}10^3$ to 10^5 sperm cells. These germ line mutations produce apparent *de novo* mutations in about 1-2% of affected alleles[27, 28]. Indeed, a *de novo* R356W mutation was found in patient # 7. Prenatal diagnostic studies in the same couple's second pregnancy would be useless, since only rare gametes might be expected to harbor CYP21A2 mutations[28].

In addition, a total of 90% of the alleles were genotyped in the cohort of NC patients. V281L was the most prevalent mutation, as reported in most of the populations analyzed [5, 12, 29]. It is noteworthy that all NC patients but one –with a non characterized allele– were compound heterozygote for mutations with ≤ 2% EA. These individuals are therefore at risk for conceiving and giving birth to an infant with classic CAH if their partner also carries a mutation with low EA. Indeed, a SW CAH classical patient (#1) was born from a mother with the NC form of the disease. Recently, it was described that the risk that a woman with NC CAH will give birth to a child with the classic variant of the disease is approximately 2.5%. This is higher than the calculated risk of 1:480 (*i.e.* $1/60 \times 1/2 \times 1/4$), assuming a prevalence of heterozygosis for severe CYP21 gene mutation in the general population of 1:60 and that 50% of mothers with NC CAH are compound heterozygotes carrying one mild and one severe mutation[29]. Therefore we suggest that genetic counseling may include genotyping the partner of a NC patient carrying one mutated allele with predicted EA ≤ 2%, to further exclude its carrier status.

Even though clinical diagnosis of NC 21-hydroxylase deficiency was established on the basis of late-onset symptoms of androgen excess and an abnormal 60-minute 17OHP response to synthetic ACTH stimulation > 10 ng/ml, it is actually considered that this cut-off value might overestimate the diagnosis of the NC form. As in a previous study[13], we have found that the lowest value of ACTH-stimulated 17OHP in the NC group with both alleles identified was ≥ 14 ng/ml, while lower levels were found in cases where only one allele or no allele had been identified. However, in non-identified alleles the presence of less frequent, already identified mutations or still unidentified ones cannot be excluded. Currently, single-stranded conformation polymorphism and sequencing experiments are being performed in our laboratory to allow the identification of the genetic defects in these alleles. Alternatively, the presence of only one mutated allele might contribute to the clinical expression of hyperandrogenism, in combination with other genetic or environmental risk factors[29, 30].

During the follow-up of our patients with neonatal diagnosis, height was low at 1 year of age, with a significant increment during pre-puberty, though this does not exclude a potential loss of height during pubertal development. A meta-analysis from 18 other centers data showed a mean final height of 1.37 SD (10 cm) below the control population in patients with the classical form[31]. Several reports have suggested that early diagnosis, the use of more physiological cortisol equivalent dosages during the first year of life, and the extension of mineralocorticoid therapy to all genetically classical patients, can improve the auxological outcome[3, 33]. Unfortunately, the number of patients we followed up to final height is too small to draw any reliable conclusion. However, two of our patients reached lower near final height than the tar-

get height. Besides, elevated androgen levels during insufficient adrenal suppression can lead to pseudo precocious puberty in boys and true precocious puberty in both sexes[4, 32, 33]. Indeed, one of our patients (#14) with late diagnosis and thus untreated reached adult bone age at 13.8 years of age, while another (# 7) with poor compliance presented early pubertal development.

BMD is a result of the balance between glucocorticoid doses and androgen excess and conflicting data in patients with CAH have been published. While some authors found a decreased BMD in adult classical patients[34], others reported normal BMD in children[35] and adults with both classical and NC CAH[36, 37]. In the present study, all of our patients presented a normal BMD.

In conclusion, accurate diagnosis of neonatal classical CAH allows adequate follow up and enhances growth as well. Molecular analyses of all members of an affected family may disclose asymptomatic patients. Presence of *de novo* mutations and mutations with low predicted EA in NC patients reinforces the importance of genotyping for appropriate genetic counseling. In fully genotyped NC patients, the lowest value of ACTH-stimulated 17OHP was 14 ng/ml. Lower cut-off values might overestimate the diagnosis of the NC form.

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Oscar Wilde (1854-1900)

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