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Androgen Insensitivity Syndrome at Prepuberty: Marked Loss of Spermatogonial Cells at Early Childhood and Presence of Gonocytes up to Puberty

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Androgen insensitivity syndrome · Germ cells · Prepuberty · Testicular dysgenesis

Abstract
Androgen insensitivity syndrome (AIS) is a hereditary condition in patients with a 46,XY karyotype in which loss-of-function mutations of the androgen receptor (*AR*) gene are responsible for defects in virilization. The aim of this study was to investigate the consequences of the lack of AR activity on germ cell survival and the degree of testicular development reached by these patients by analyzing gonadal tissue from patients with AIS prior to Sertoli cell maturation at puberty. Twenty-three gonads from 13 patients with AIS were assessed and compared to 18 testes from 17 subjects without endocrine disorders. The study of the gonadal structure using conventional microscopy and the ultrastructural characteristics of remnant germ cells using electron microscopy, combined with the immunohistochemical analysis of specific germ cell markers (MAGE-A4 for premeiotic germ cells and of OCT3/4 for gonocytes), enabled us to carry out a thorough investigation of germ cell life in an androgen-insensitive microenvironment throughout prepuberty until young adulthood. Here, we show that germ cell degeneration starts very early, with a marked decrease in number after only 2 years of life, and we demonstrate the permanence of gonocytes in AIS testis samples until puberty, describing 2 different populations. Additionally, our results provide further evidence for the importance of AR signaling in peritubular myoid cells during prepuberty to maintain Sertoli and spermatogonial cell health and survival.

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Androgen insensitivity syndrome (AIS) is part of a larger group of conditions usually denominated disorders of sex development (DSD), in which 46,XY patients show partial (PAIS) or complete (CAIS) defects in virilization [Quigley et al., 1995].

Histopathological changes in the gonads of patients with AIS have been previously documented [Rutgers and Scully, 1991; Regadera et al., 1999; Hannema et al., 2006]. These changes develop as the age of patients increases and may be influenced by 2 fundamental characteristics of the syndrome: decreased androgen receptor (AR) activity and abnormal gonadal location.

On the other hand, an increased risk of germ cell tumor development has been described in AIS [Manuel et al., 1976; Cools et al., 2006a], although its occurrence is very rare in childhood [Hurt et al., 1989]. However, noninvasive precursor lesions, referred to as intratubular germ cell neoplasia or carcinoma in situ (CIS), have been reported in an 18-year-old CAIS patient [Cools et al., 2005] and in a 17- and a 53-year-old CAIS patient [Hannema et al., 2006].

The diagnosis of CIS in children with AIS is difficult, given that atypical germ cells are commonly seen in their gonads [Müller, 1984; Müller et al., 1985; Cassio et al., 1990; Cortes et al., 2003]. The octamer-binding transcription factor (OCT3/4) is involved in the regulation of pluripotency and is normally expressed in embryonic germ cells. This factor is a well-established immunohistochemical marker to detect CIS and some invasive germ cell tumors in adult [Looijenga et al., 2003] and pediatric patients [Chemes et al., 2015]. Nevertheless, OCT3/4 is normally expressed in testicular germ cells shortly after birth [Honecker et al., 2004], and, in cases of undervirilization syndromes, its expression in patients younger than 1 year agrees with the expected maturation delay observed in these patients [Cools et al., 2005]. These developmentally delayed germ cells are prone to malignant transformation if they survive in an inappropriate environment [Rajpert-De Meyts et al., 1998; Cools et al., 2005].

In spite of requiring androgens for their survival and maturation, germ cells do not express the AR [Ruizeveld de Winter et al., 1991; Berensztein et al., 2006]. It has been widely accepted that the requirement of testosterone for spermatogenesis is mediated by AR-expressing somatic Sertoli, peritubular myoid (PTM), Leydig, vascular endothelial, and vascular smooth muscle cells of the testis [Ruizeveld de Winter et al., 1991; Berensztein et al., 2006]. However, because of their proximity to developing germ cells, Sertoli and PTM cells appear to be the most important for transmission of androgen signaling. In humans, the AR is either weakly detected or absent in Sertoli cells up to 5 years of age [Berensztein et al., 2006; Chemes et al., 2008; Rey et al., 2009], while the mesenchymal PTM cells are the first cells in the testis to express the AR [Shapiro et al., 2005].

It is well known that a functional AR is required at puberty to achieve normal spermatogenesis. Nevertheless, the importance of continuous AR expression in testicular somatic cells throughout male prepubertal life has not been elucidated. The study of the gonadal structure in patients with AIS and the ultrastructural characteristics of the remnant germ cells of their gonads by electron microscopy combined with the immunohistochemical analysis of specific germ cell markers may contribute to elucidate the role of AR in testicular development from birth to puberty.

In this study, we show that in an androgen-insensitive microenvironment germ cell degeneration starts very early on, with a marked decrease in number after 2 years of life. Furthermore, we demonstrate the presence of gonocytes in AIS testis until puberty and describe 2 different populations. Additionally, our results provide further evidence for the importance of PTM-AR signaling in the prepubertal period to maintain Sertoli and spermatogonial cell health.

Materials and Methods

Subjects

The clinical diagnosis of AIS was based on the patients' phenotype, hormonal studies, and detection of *AR* gene mutations by direct sequencing. The degree of normal development of the external genitalia was used in prepubertal subjects as a marker of virilization to clinically differentiate between CAIS and PAIS categories of the syndrome.

Twenty-three testes from 13 AIS patients, 10 CAIS and 3 PAIS (median age 8.8 years; range 0.4–23 years) were studied (Table 1). The patients belonged to 11 different families. The 8 prepubertal patients (median age 2.3 years; range 0.4–10.3 years) presented with inguinal gonads, while in the 5 pubertal patients (median age 19 years; range 16.2–23 years) the gonads were located abdominally. PAIS patients presented with different external masculinization scores (EMS), even between sisters (Table 1). The measurement of serum testosterone levels after hCG stimulation in prepubertal patients was used to detect the presence of steroidogenic testicular tissue [Rivarola et al., 1970]. The normal range of basal serum testosterone in the adult male was used as a criterion to define puberty. Serum testosterone was determined by DPC Immulite Assay System (Diagnostic Products, Los Angeles, CA, USA), and serum LH and FSH were determined using IMX systems (Abbott Laboratories, Abbott Park, IL, USA) (Table 1).

Previously reported mutations in the *AR* gene [Gottlieb et al., 2012] were detected in 7 AIS patients (P1, P2, siblings P6 and P7,

Table 1. Clinical data of the patients at gonadectomy

Patient	CA, years	Family	Phenotype	Tanner stages		Gonadal position left/right	EMS	Serum levels		
				breast	pubic hair			T, ng/mL	LH, IU/L	FSH, IU/L
P1	0.4	F1	CAIS	I	I	I/I	2	8.8 (hCG)	1.0	0.8
P2	1.2	F2	CAIS	I	I	I/I	2	4.6 (hCG)	0.1	0.6
P3	1.8	F3	CAIS	I	I	I/I	2	4.7 (hCG)	NA	NA
P4	2.3	F4	PAIS	I	I	LS/I	6	11.7 (hCG)	NA	NA
P5	4.4	F5	CAIS	I	I	I/I	2	1.9 (hCG)	0.7	6.6
P6	8.8	F6	PAIS	I	I	I/I	3	1.7 (hCG)	NA	NA
P7	8.8	F6	PAIS	I	I	I/I	5	1.7 (hCG)	NA	NA
P8	10.3	F7	CAIS	I	I	I/I	2	NA	0.1	1.3
P9	16.2	F8	CAIS	IV	II	A/A	1	3.1 (basal)	NA	NA
P10	17.8	F9	CAIS	V	II	A/A	1	7.2 (basal)	17.2	2.8
P11	19.0	F10	CAIS	V	II	A/A	1	8.1 (basal)	24.8	3.9
P12	21.5	F11	CAIS	IV	II	A/A	1	NA	NA	NA
P13	23.0	F11	CAIS	IV	III	A/A	1	NA	9.4	2.2

CA, chronological age at gonadectomy; LS, labioscrotal; I, inguinal; A, abdominal; EMS, external masculinization score [Ahmed et al., 2000]; NA, not available; T, testosterone levels, either post-hCG in prepubertal patients (hCG) or in basal conditions in pubertal subjects (basal).

Table 2. *AR* gene mutations found in the patients

Patient	Family	Phenotype	Nucleotide change	Amino acid change	Exon	Mutation type	Domain	Reported
P1	F1	CAIS	c.2668G>A	p.Val890Met	8	missense	LBD	CAIS/PAIS
P2	F2	CAIS	c.2494C>T	p.Arg832*	7	nonsense	LBD	CAIS
P3	F3	CAIS	c.2177T>G	p.Phe726Cys	5	missense	LBD	no
P4	F4	PAIS	c.2410G>T	p.Glu803*	6	nonsense	LBD	no
P5	F5	CAIS	c.1550–1569del	p.Trp399fs*95	1	frameshift	N terminus	no
P6	F6	PAIS	c.2071–2073delGAC	p.Asp691del	4	deletion	LBD	PAIS
P7	F6	PAIS	c.2071–2073delGAC	p.Asp691del	4	deletion	LBD	PAIS
P8	F7	CAIS	c.2248A>G	p.Met750Val	5	missense	LBD	CAIS/PAIS
P9	F8	CAIS	c.2556C>T	p.Arg856Cys	7	missense	LBD	CAIS
P10	F9	CAIS	delEx2–8	–	2–8	deletion	DBD-LBD	CAIS
P11	F10	CAIS	IVS2–2 A>G	–	–	splicing	–	no
P12	F11	CAIS	c.1972delC	p.Gln658fs*2	4	frameshift	LBD	no
P13	F11	CAIS	c.1972delC	p.Gln658fs*2	4	frameshift	LBD	no

P8, P9, and P10), while novel mutations were found in the remaining 6 (P3, P4, P5, P11, and siblings P12 and P13) (Table 2).

As control samples, 18 testes from 17 subjects without endocrine disorders were collected at the time of necropsy. Thirteen were prepubertal samples (median 1.08 years; range 1 day to 8.92 years), while 5 had evident histological signs of pubertal development (median 12.8 years; range 12–16 years). All of them had scrotal localization and typical testicular histology for age.

Gonadal Histology Assessment

Gonadal samples were fixed in 10% formalin and embedded in paraffin. Hematoxylin-Eosin (H&E), Periodic Acid Schiff (PAS), and toluidine Blue staining was performed on 5-μm-thick sections from each sample. Overall gonadal tissue organization, markers of puberty, and abnormal histological phenomena were assessed by 2 specialists in gonadal histology.

Immunohistochemistry

Further germ cell analysis was performed using immunohistochemistry (IHC) with an anti-MAGE-A4 monoclonal antibody, kindly provided by Dr. Giulio C. Spagnolli [Aubry et al., 2001], as a specific premeiotic germ cell marker, and an anti-OCT3/4 monoclonal antibody (sc-5279, Santa Cruz Biotechnology), a pluripotency marker expressed in fetal gonocytes.

IHC was performed as previously reported [Berensztein et al. 2006]. Briefly, after heat-induced antigen retrieval with a citrate

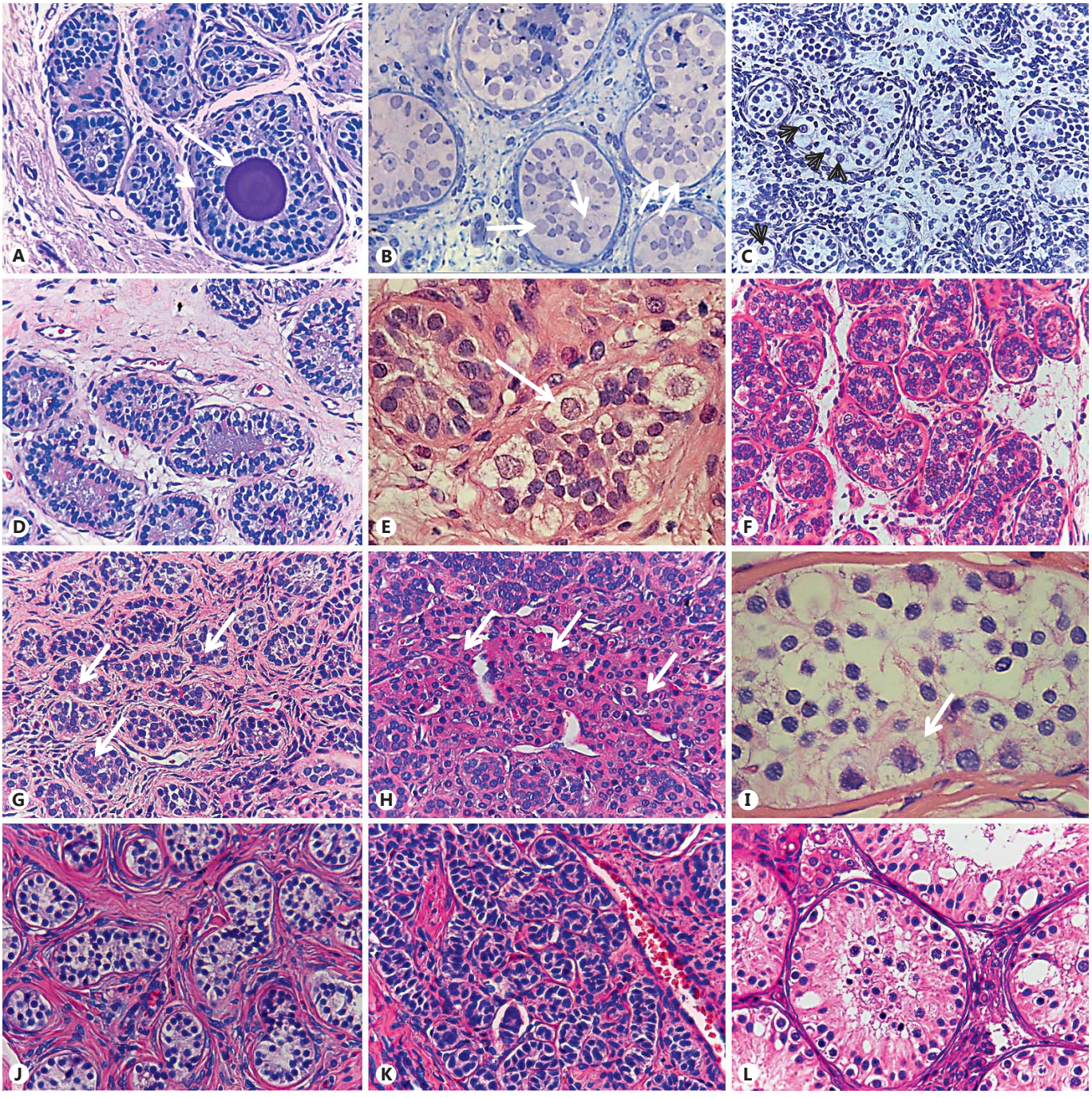


Fig. 1. Dysgenetic features in AIS testes. **A** P3 CAIS, 1.8 years old, ×40. Ring-like seminiferous cords, some with central PAS-positive substance (arrow), and germ cell apoptotic nucleus (arrowhead) (PAS technique). **B** P3 CAIS, 1.8 years old, ×63. Postnatal gonocytes (arrows) near the basement membrane and associated with immature Sertoli cells (Toluidine blue). **C** P5, CAIS, 4.4 years old, ×40. Clumps of highly condensed chromatin in germ cells (arrowheads) **D** P6, PAIS, 8.8 years old, ×40. Fibrous interstitium, scarce germ cells and intratubular substance. **E** P8, CAIS, 10.3 years old, ×100. Gonocytes nearby basement membrane (arrow). **F** Scrotal control testis, 1.0 year old, ×40. **G**, **H** P9, CAIS, 16.2 years old, ×40. Immature seminiferous cords without meiocytes, Sertoli cells with acidophilic cytoplasm (arrows in **G**), fibrous interstitium, and Leydig cell hyperplasia (arrows in **H**). **I** P11, CAIS, 19 years old ×100. Remnant postnatal gonocytes in a young adult testis (arrow). **J** P12, CAIS, 21.5 years old, ×40. Absence of meiosis, fibrous interstitium. **K** P13, CAIS, 23.0 years old, ×40. Sex cord tumor composed by solid cords of small immature Sertoli cells. **L** Scrotal control testis, 16 year old, ×40. Normal development of spermatogenesis.

Table 3. Histological determination of premeiotic germ cells and MAGE-A4 and OCT3/4 expression in patient samples

Patient	Family	CA, years	Phenotype	Germ cells	MAGE-A4	Gonocytes	OCT3/4
P1	F1	0.4	CAIS	yes	+	yes	+
P2	F2	1.2	CAIS	yes	+	yes	–
P3	F3	1.8	CAIS	yes	+	yes	+
P4	F4	2.3	PAIS	yes	+	yes	–
P5	F5	4.4	CAIS	yes	+	yes	+
P6	F6	8.8	PAIS	no	–	no	–
P7	F6	8.8	PAIS	no	–	no	–
P8	F7	10.3	CAIS	yes	+	yes	–
P9	F8	16.2	CAIS	no	–	no	–
P10	F9	17.8	CAIS	yes[a]	n.d.	n.d.	n.d.
P11	F10	19.0	CAIS	yes	+	yes	–
P12	F11	21.5	CAIS	no	–	no	–
P13	F11	23.0	CAIS	no	–	no	–

CA, chronological age at gonadectomy; n.d., not determined.
[a] All observed germ cells in P10 were degenerating or necrotic germ cells. Thus, P10 was excluded from further analysis.

buffer (pH 6), sections were incubated overnight with anti-OCT3/4 or anti-MAGE-A4 antibodies diluted 1:50. Staining was performed employing the streptavidin-biotin and peroxidase method according to the manufacturer's protocol (DAKO Catalyzed Signal Amplification System, K1500, HRP). After each incubation step, slides were washed in a 0.01% TBS-Tween 20 solution. As negative controls, normal serum was used instead of the primary antibody. No specific immunoreactivity was detected in these sections. Immunostudies were carried out twice, and no difference between duplicates was detected in the staining pattern.

The staining of MAGE-A4 and OCT3/4 was assessed in at least 100 cross sections of seminiferous tubules for each tissue sample. One tubule was considered positive for a marker if at least 1 germ cell in the tubule was clearly stained. The number of MAGE-A4-positive premeiotic germ cells was determined in every tubule cross section, and an average number was calculated per sample. All counts were performed by 2 observers (E.B. and P.A.) blinded to age and specimen origin (patient or control).

Electron Microscopy Imaging

To study the seminiferous cords in detail with the light microscope and the ultrastructure of the germ cells and surrounding somatic cells by electron microscopy, 2 small pieces of the gonadal tissue from one CAIS patient testis (1.8 years old) were processed for semi-thin in thick (0.5 μm) and thin (0.08 μm) sections, respectively. Electron micrographs were obtained with a Zeiss EM 109T equipped with a digital camera Gatan171 ES1000W, at the LANAIS laboratory (CONICET, Buenos Aires) [Sciurano and Solari, 2014].

Statistics

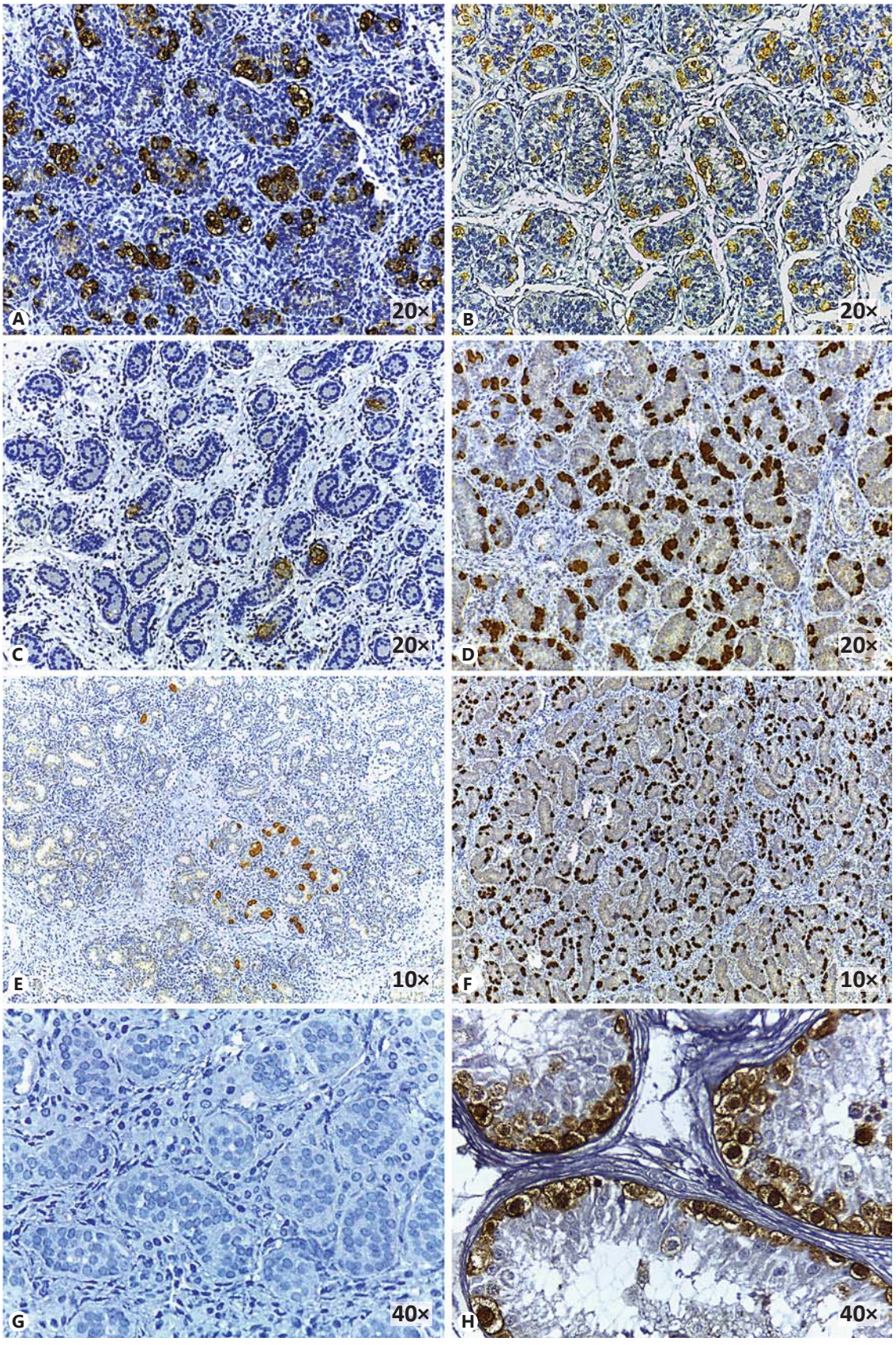
Spearman´s test was used to analyze the linear correlation between age and the number of germ cells per seminiferous cord, as well as between age and the percentage of seminiferous tubules with at least one germ cell in testes from prepubertal control subjects and patients with AIS.

Results

Germ Cell Characterization and Histological Description

On histological examination, different pathological features were found in the testes from patients with AIS. Examples of the most common characteristics are shown in Figure 1.

Germ cells were observed in the testes of all prepubertal patients, except the 2 sisters with PAIS (P6 and P7) (Table 3). Several of these germ cells were abnormally large with vacuolated cytoplasm. They were multinucleated, with clumps of highly condensed chromatin or with signs of apoptosis, and they were found both centrally and basally located within the seminiferous cords. On the other hand, nondifferentiated postnatal gonocytes were observed in all prepubertal AIS patient with germ cells (Fig. 1; Table 3). Those postnatal gonocytes were identified as very large, round cells with round nuclei, homogeneous chromatin, clearly stained cytoplasm with perinuclear mitochondria, and absence of a well-developed granular endoplasmic reticulum. Gonocytes can be differentiated from spermatogonia as the latter are smaller, their nucleus/cy-



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toplasm ratio is closer to 1, they are supported on the basement membrane, and they have typical corpuscles adhered to the karyotheca [Manku and Culty, 2015].

The most distinctive pathological features found in the prepubertal samples were Sertoli cells with an acidophilic cytoplasm (5 out of 8 samples), ring-like seminiferous cords with central hyaline, eosinophilic and PAS-positive substance, convoluted seminiferous cords, tubules with thickened basement membrane, and a fibrous interstitium (4/8) (Fig. 1). In the oldest prepubertal patient, mature Leydig cells were found. Normal prepubertal testicular histology was observed in the 1-year-old scrotal control testis shown in Figure 1F.

The pubertal patients had prepubertal-like seminiferous cords, although one presented with some signs of maturation, such as few seminiferous tubules with lumen and elongated Sertoli cells. Germ cells were not found in 3 out of the 5 pubertal patients, while in the other 2 they were very rare: in one patient all were in the process of degeneration or necrosis, and in the other postnatal gonocytes were found. Spermatogenesis was absent in both cases (Table 3).

In all 5 patients, tubules with a thickened basement membrane, testicular parenchyma with fibrous interstitium, and Leydig cell hyperplasia were found. Additionally, Sertoli cells with acidophilic cytoplasm were observed in 2 patients, while 1 presented with ramified seminiferous cords and an eosinophilic, PAS-positive intratubular substance. Sex cord tumor, composed of small solid cords with cells resembling immature Sertoli cells inside a fibrous interstitium, was observed in 2 patients. One of them also had a Sertoli cell tumor with enlarged tubules consisting only of Sertoli cells with ample eosinophilic cytoplasm and a mild thickening basement membrane and a juxta-gonadal leiomyoma. Normal mature testicular histology, at both interstitial and tubular compartments, was observed in the 16-year-old scrotal control testis, including mature Leydig and Sertoli cells as well as developing spermatogenesis (Fig. 1L).

Fig. 2. MAGE-A4 immunostaining. **A** P1, CAIS, 0.4 years old, inguinal testis, high expression of MAGE-A4, ×20. **B** Scrotal control, 0.4 years old, ×20. **C** P4, PAIS, 2.3 years, labioscrotal testis, scarce positive cells, ×20. **D** Scrotal control 2 years old testis, ×20. **E** P5, CAIS, 4.4 years old inguinal testis, scarce positive cells, observed in clusters of few seminiferous tubules, ×10. **F** Scrotal control, 2 years old testis, ×10. **G** P9, CAIS, 16 years old abdominal testis, small immature tubules with Sertoli cells only, no MAGE-A4 positive cells, ×40. **H** Scrotal control testis, 13.8 years old, high expression of MAGE-A4 in many germ cells, ×40.

MAGE-A4 Immunostaining (Pre-Meiotic Germ Cells Study)

MAGE-A4 immunostaining analysis confirmed the complete absence of germ cells in 5 of the 13 samples of patients described above (Fig. 2; Table 3).

Due to the difficulty of carrying out a reliable germ cell count and identification because of the level of cell degeneration, pubertal patient P10 was excluded from further analysis.

The study of the number of germ cells per testicular seminiferous tubule as a function of age showed that during prepuberty, AIS patients suffer a drastic loss of germ cells as they grow older ($r = -0.8558$, $p = 0.0015$) (Fig. 2, 3A), with a marked decrease after 2 years of age. All patients older than 2 years had close to or 0 germ cells per seminiferous tubule, similar to the numbers observed in pubertal AIS subjects (Fig. 3). Given that only 3 patients had PAIS, it was not possible to analyze the effect of PAIS or CAIS on germ cell survival. In contrast, no linear correlation between age and the number of germ cells per tubule was observed in testes of control subjects ($r = 0.0372$, $p = 0.9039$). There was a significant increase in the number of premeiotic germ cells at puberty in control testes, completely opposite to the absence seen in AIS patients at this stage.

A negative correlation between age and the percentage of tubules with at least one germ cell was also observed at prepuberty in patients with AIS ($r = -0.8526$, $p = 0.0031$) (Fig. 2, 3B). In patients older than 2 years of age, germ cells, when present, showed a confined location only in clusters of few seminiferous tubules, whereas most tubules were composed of Sertoli cells only (Fig. 2). Until the onset of puberty, no significant change with age in the percentage of seminiferous tubules with germ cells was observed in samples from control subjects.

While all gonads from the prepubertal patients, except for a labioscrotal case, were located in the inguinal canal, in all pubertal patients they were found in the abdomen. The exception was the 2.3-year-old patient with PAIS, in whom the right gonad was located inguinally, while the left was found in the labioscrotum. The number of germ cells per tubule (0.3 and 0.2) and the percentage of seminiferous tubules with at least one germ cell (22.6 and 17%) were similar in both locations. Furthermore, compared to control scrotal testes from subjects of similar age (Fig. 2C, D), the presence of germ cells was greatly reduced in the patient with AIS regardless of the gonadal location: 2.0- and 2.8-year-old control subjects had 1.9 and 1.2 germ cells per tubule and 83.3 and 61.3% MAGE-A4 positive tubules, respectively.

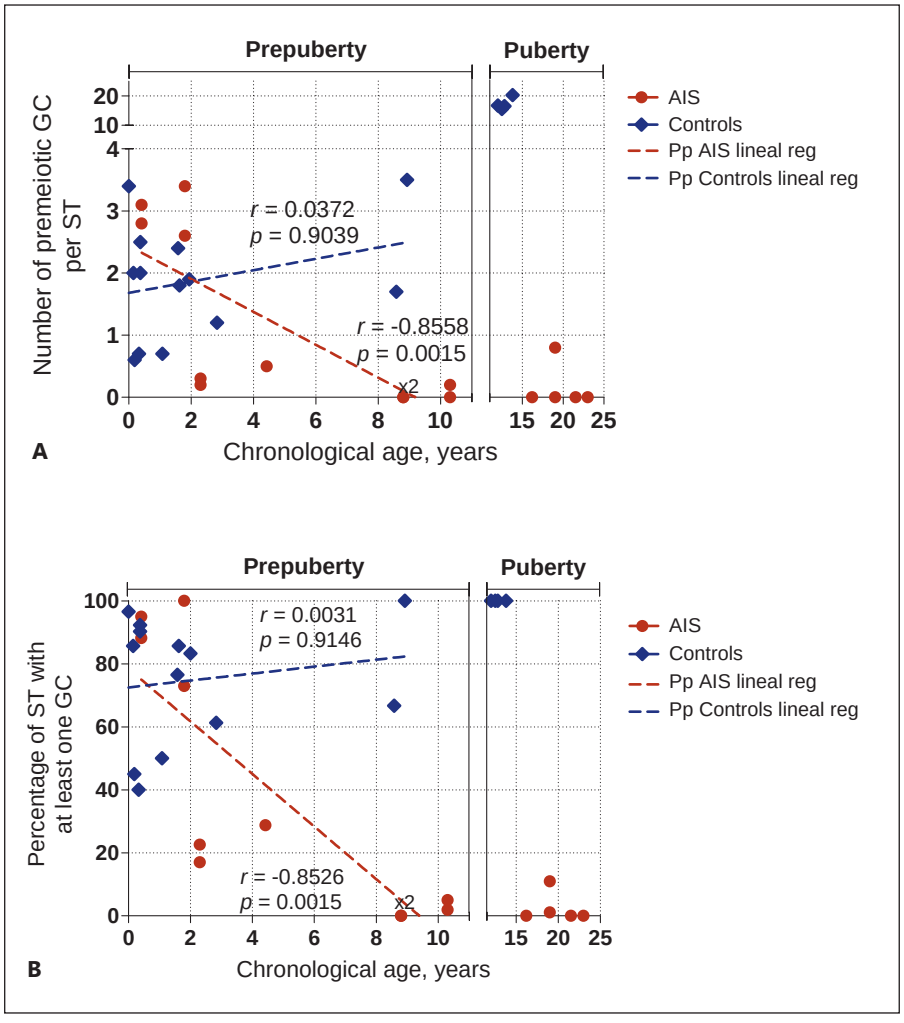

Fig. 3. A Number of premeiotic germ cells (GC) per seminiferous tubule (ST) in AIS and control subject testes. **B** Percentage of seminiferous tubules with at least one germ cell in AIS and control subject testes.

OCT3/4 Immunostaining

The immunoexpression of OCT3/4 was assessed in AIS and control testes. No expression of OCT3/4 was found in any scrotal control. Of the 7 patients with well-defined germ cells, 3 had positive OCT3/4 immunoexpression (Fig. 4; Table 3). All 3 were prepubertal CAIS patients, aged 0.4, 1.8, and 4.4 years. OCT3/4-positive germ cells were both located centrally and basally within the seminiferous tubule in all 3 patients (Fig. 4).

Electron Microscopy Imaging

The presence of gonocytes in the seminiferous tubules of a testis from a 1.8-year-old CAIS patient observed with conventional microscopy was confirmed by electron microscopy (Fig. 5). Gonocytes showed numerous typical mitochondria, scarcely elongated with symmetrically arranged crests. In occasional gonocytes, mitochondria were grouped in a cell pole close to the cell membrane. Most areas of the gonocyte cytoplasm were covered by non-dense aqueous vesicles. Most gonocyte nuclei were round and contained diffuse, relatively dense chromatin. Nucleoli with a prominent, amorphous (nongranular) portion were occasionally seen. In general, gonocytes were found lying on the tubular basement membrane, although this location varied with section incidence and with the functional status of these cells.

A large proportion of gonocytes had abnormal features, showing a dense cytoplasm, elongated protrusions towards the tubule lumen, and a nucleus with dense chromatin. These unhealthy germ cells were also identified in semi-thin sections with the light microscope because of their color intensity. Some of these germ cells have most probably underwent degenerative or necrotic changes.

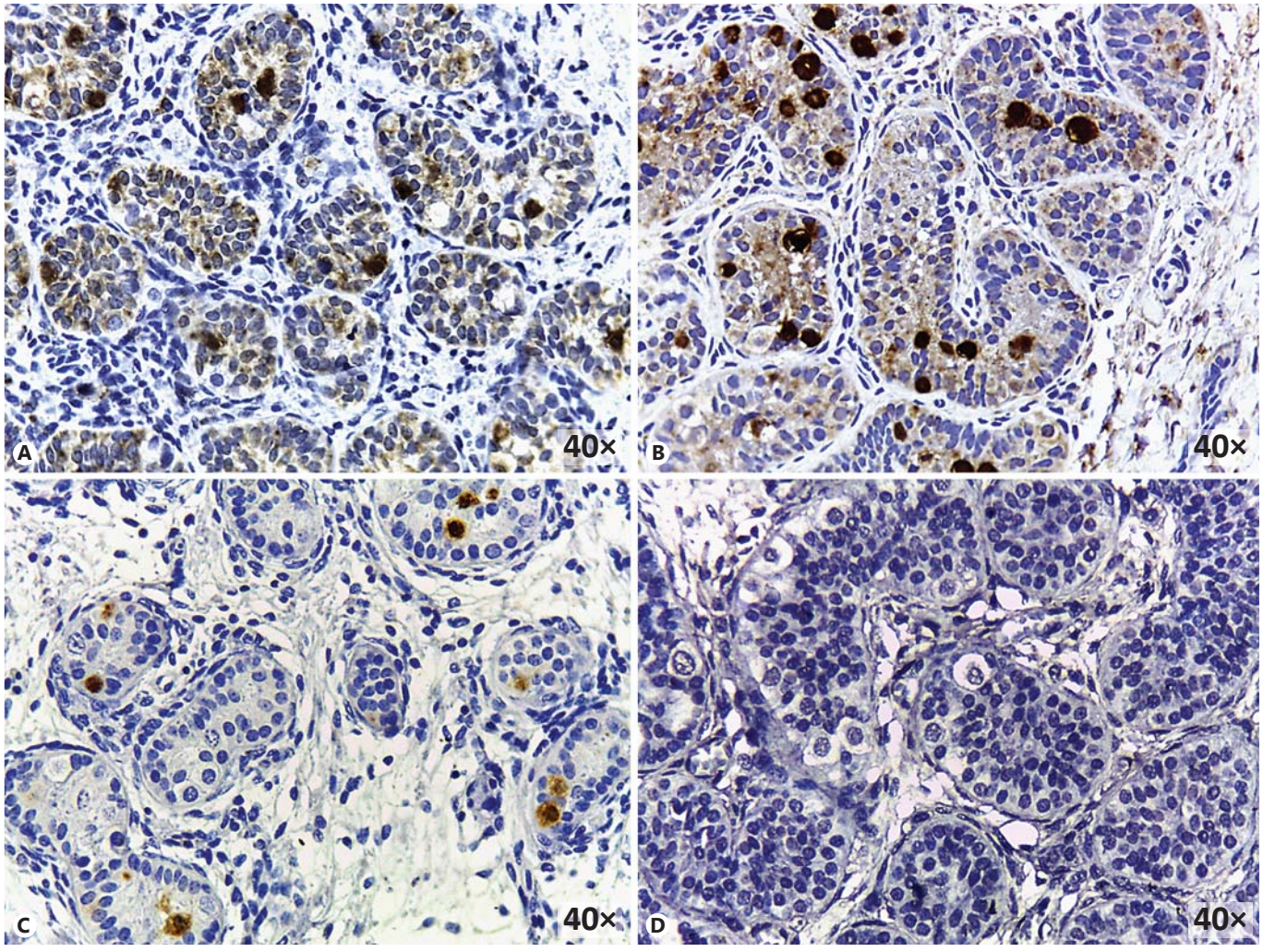

Fig. 4. OCT3/4 immunostaining. **A** P1, CAIS, 0.4 years old, positive OCT3/4 immunoexpression in abundant central and basal located germ cells, ×40. **B** P3, CAIS, 1.8 years old, positive OCT3/4 immunoexpression in abundant central and basal located germ cells, ×40. **C** P5, CAIS, 4.4 years old, positive OCT3/4 immunoexpression in abundant cells, mostly located basally, ×40. **D** P8, CAIS, 10.3 years old, negative OCT3/4 staining, ×40.

In contrast, Sertoli cells were arranged in cell palisades, showing elongated, ovoid nuclei with typically pale and homogenous chromatin and sometimes visible large nucleoli. Sertoli cell mitochondria were smaller and denser than spermatogonial mitochondria, and they were present in higher numbers. The cytoplasm reached the lumen of the seminiferous tubules with lipid droplets close to this apical region. Signs of some differentiation were indentations of the nuclear envelope and perinuclear filaments of vimentin (not shown). Finally, evidence of intercellular Sertoli-Sertoli joints was also observed (Fig. 5).

Discussion

As expected because of the importance of androgen signaling for the correct maturation of the testis, patients with AIS presented with different testicular histopathological features, both during childhood and in puberty and young adulthood, similar to previously reported cases [Rutgers and Scully, 1991; Hannema et al., 2006]. Most prepubertal patients had germ cells in their testes, albeit already presenting signs of degeneration. Although still present, their number per tubule was greatly reduced in testes of patients with AIS compared to normal scrotal samples, with a striking decline after 2 years of age. The

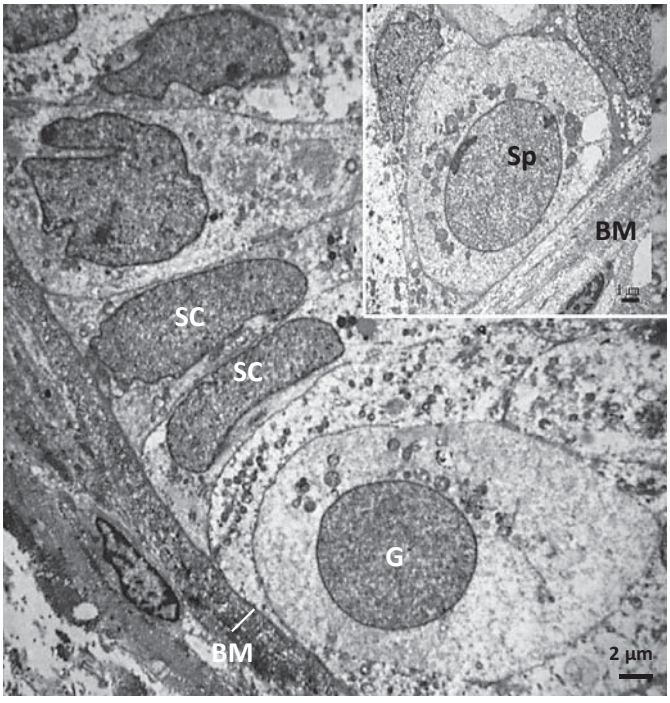

Fig. 5. Testicular electron microscopy imaging of P3, 1.8-year-old, CAIS. Gonocytes surrounded by immature Sertoli cells are still present near the basal membrane. The picture shows large round cells with a prominent spherical euchromatic nucleus associated with clusters of mitochondria and close to the basal membrane. Scale bar, 2 µm. **Inset** Spermatogonia found in the same patient. Scale bar, 1 µm. G, gonocyte; Sp, spermatogonia; SC, Sertoli cell; BM, basal membrane.

same was observed for the percentage of tubules with at least 1 germ cell. The early onset of germ cell loss observed in our patients is congruent with previous publications [Rutgers and Scully, 1991; Hannema et al., 2006; Kaprova-Pleskacova et al., 2014]. The number of germ cells per tubule and the percentage of MAGE-A4 positive tubules in testes of prepubertal and pubertal patients were very similar, further indicating that the damage in germ cells is established early in prepubertal life.

In samples of AIS patients older than 2 years, germ cells were present in confined locations. Whereas most tubules were composed of Sertoli cells only, germ cells were observed in clusters of few seminiferous tubules, suggesting that the microenvironment may be important for the maintenance of these cells. The factors of this microenvironment that are critical for their survival remain undetermined, but they may be androgen dependent.

The similarities in histopathological changes observed in testes from AIS patients and those reported in cryptorchid testes [Schindler et al., 1987; Huff et al., 2001] might suggest that an abnormal testicular position rather than androgen resistance is the underlying cause of abnormal testis development in both disorders. However, abnormal androgen signaling cannot be ruled out in patients with cryptorchidism, as reported by Regadera et al. [2001]. Furthermore, a study of 767 prepubertal boys with unilateral cryptorchidism that underwent bilateral testicular biopsies described similar changes in the undescended and contralateral descended testes [Huff et al., 2001]. This is consistent with our 2.3-year-old PAIS patient, in whom the right gonad was located inguinally while the left was in the labioscrotum. Even though the left gonad had descended further than the right, the number of germ cells per tubule and the percentage of seminiferous tubules with at least one germ cell were similar in both locations, and compared to control scrotal testes from subjects of similar age, the presence of germ cells was greatly reduced in the AIS patient regardless of the gonadal location. Indeed, the fact that all but one gonad from prepubertal patients with AIS were found in the inguinal canal whereas in all pubertal patients they were located in the abdomen, made it impossible to study the impact of the testis location without considering the age factor.

Germ cell maturation delay has been previously reported in AIS patients, and the presence of gonocytes in their testes up to the first year of life is considered as expected [Cools et al., 2005]. In our study, germ cells with histological characteristics of gonocytes were present in all 6 prepubertal AIS patients that had germ cells, 4 of whom older than 1 year of age. In contrast to the histological findings, OCT3/4 immunoexpression, a pluripotency marker normally expressed in gonocytes, was only detected in 3 patients. Historically, the term gonocyte has been used for the male germ cell from the point it becomes resident in the gonadal primordium to the time it reaches the basement membrane of the seminiferous cord and differentiates into a spermatogonial cell. According to many authors [McCarrey, 1993; Gaskell et al., 2004; Sasaki and Matsui, 2008; Culty, 2013], this term encompasses too many subsets of germ cell types that should be discriminated from each other. Gaskell et al. [2004] proposed a way to divide human fetal germ cells into different types according to their expression levels of a combination of 3 representative genes: gonocytes (OCT4pos/C-KITpos/MAGE-A4neg), intermediate germ cells (OCT4low/neg/C-KITneg/MAGE-A4neg), and prespermatogonia (OCT4neg/C-KITneg/MAGE-A4pos). The difference between the histological and immunohistochemical findings would indicate that at least

2 subclasses of gonocytes are present in the testis in an AIS prepubertal environment, one more differentiated than the other.

When germ cell maturation occurs normally, centrally located gonocytes differentiate into spermatogonial cells as they travel to the basement membrane of the seminiferous tubule. In our patients, gonocytes were found located both centrally and basally in the tubule, with no clear change in the location pattern as they became older, which was confirmed by electron microscope imaging. According to the literature, if the developmentally delayed germ cells can survive in their inappropriate environment, they are prone to malignant transformation [Cools et al., 2005]. The development of germ cell cancer is very rare in children with AIS, but noninvasive precursor lesions such as CIS have been repeatedly described in this age group [Cools et al., 2005; Hannema et al., 2006]. In agreement with this finding, of the 5 prepubertal patients with gonocytes, histological characteristics compatible with CIS were observed in the 2 oldest ones. Whether these lesions are predetermined to evolve into invasive cancers is still a matter of debate in the literature.

Surprisingly, OCT3/4-negative histologically identified gonocytes were present in the only pubertal sample that had analyzable germ cells. Even though gonocytes with abnormal features survived in an androgen-insensitive environment for 19 years, no germ cell precursor lesion or tumor was observed in the studied testicular tissue. This fact supports the hypothesis that the population of gonocytes present in the pubertal sample corresponds to Gaskell's definition of prespermatogonia [Gaskell et al., 2004], differentiated further than fetal gonocytes and thus less prone to malignant transformation.

On the other hand, somatic cell tumors, such as Sertoli cell tumor and a juxtagonadal leiomyoma, were found in 2 pubertal patients. Thus, in our study the incidence of testicular somatic cell tumors in patients with AIS was higher than that of germ cell neoplasia.

The risk for germ cell cancer development in AIS is very difficult to predict, because the approaches to manage patients have continually changed over time. Reports in the literature regarding the estimated risk of gonadal malignancy have varied from 0.8 to 22% from ages 14–51 years [Cools et al., 2006b].This has led to a controversy on the timing of gonadectomy. Some physicians advocate for orchidectomy before puberty to decrease the risk of malignancy, while most physicians nowadays are in favor of delaying gonadectomy to allow for spontaneous puberty [Deans et al., 2012]. While the shift toward delaying orchidectomy would allow autonomous decisions and avoid estrogen replacement therapy, earlier preservation of gonadal tissue may provide greater fertility potential.

In our study, we showed that germ cell loss starts very early in testes of patients with PAIS and CAIS, as young as 2 years of age. After puberty, most testes have none or very few degenerated germ cells. Even though spermatogenesis is not possible in an androgen-insensitive environment, as science and technology advance, the possibility of obtaining functional gametes from cultured spermatogonial stem cells acquired from cryopreserved immature testicular tissue is slowly becoming a reality [Hermann et al., 2012; Baert et al., 2015; Guo et al., 2015; Huleihel et al., 2015]. Furthermore, CRISPR/Cas9 mediated genome editing technology shows promising applications in gene therapy, possibly one day preventing or treating genetic diseases as AIS directly via genetic intervention [Huang et al., 2017]. Taken this into account, a testicular biopsy at a young age to harvest gonadal tissue for cryopreservation should be considered.

Although germ cells require androgens for their survival and maturation, they do not express the AR [Ruizeveld de Winter et al., 1991; Berensztein et al., 2006]. Because of their proximity to developing germ cells, Sertoli and PTM cells appear to be the most important for transmission of androgen signaling. Therefore, it would be logical to assume that the pathological changes observed in germ cells are a product of abnormal AR function in these somatic cells; however, in humans, Sertoli cell AR is either weakly detected or not present up to 5 years of age [Berensztein et al., 2006; Chemes et al., 2008; Rey et al., 2009]. Given that all prepubertal AIS patients had germ cell abnormalities in their testes and that more than half of them were younger than 5 years of age, a functional PTM-AR seems to be necessary for the adequate germ cell niche development early in life. Moreover, experiments in mice have shown that the specific genetic ablation of *AR* in Sertoli cells results in testes with a block in germ cell maturation at meiosis but a normal number of spermatogonia [De Gendt et al., 2004], whereas PTM-AR knockout mice have a reduction of germ cell number at all stages, including spermatogonia [Welsh et al., 2009], further supporting the hypothesis that the loss of PTM-AR function is mainly responsible for spermatogonial loss in prepubertal patients with AIS.

Sertoli cells presenting an acidophilic cytoplasm compatible with oncocytic changes or seminiferous tubules with an eosinophilic PAS positive substance within were commonly observed in our patients. Given that more than half of the samples with these features were from patients younger than 5 years of age, a functional PTM-AR also seems to be essential for Sertoli cell integrity.

In summary, here we describe the histological consequences of a lack of androgen action in the testis from birth to young adulthood. We show that in an androgen-insensitive environment, germ cell degeneration starts at a very early age, with a marked decrease in their number after only 2 years of life. We describe the presence of at least 2 populations of gonocytes in testis samples from AIS patients and demonstrate the presence of OCT3/4-positive gonocytes in prepuberty up to 4.4 years of age and of more differentiated OCT3/4-negative gonocytes until puberty. Our results provide further evidence for the importance of PTM-AR signaling in the prepubertal period to maintain Sertoli and spermatogonial cell health and survival.

We suggest that future management of patients with PAIS or CAIS should include a biopsy at a young age, if possible within the first 2 years of life, to evaluate the risk of neoplasia with concurrent harvest of gonadal tissue for cryopreservation.

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Statement of Ethics

The experimental design, data analysis, and consent form were approved by the Ethical Committee of the Hospital de Pediatría 'Prof. Dr. Juan P. Garrahan', Buenos Aires, Argentina. In all cases, sample collection and use for scientific purposes were carried out after a written consent was signed by the patients or the legal guardians before surgery or necropsy.

Disclosure Statement

The authors have no conflicts of interest to declare.

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