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ORIGINAL ARTICLE

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Is a CIS phenotype apparent in children with Disorders of Sex Development? Milder testicular dysgenesis is associated with a higher risk of malignancy

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SUMMARY

All malignant testicular germ cell tumors (TGCT) of adult men are preceded by an in situ stage (CIS) of protracted evolution. The adult CIS is well characterized, but there is debate on the phenotype of infantile CIS, its distinction from delayed maturation of germ cells and prognostic potential. A large series of 43 patients with Disorders of Sex Development (DSD) and dysgenetic testes (90% ranging from neonates to 12 years, mean age 4.7 years), was studied by quantifying dysgenetic features, degree of germ cell abnormalities/atypia (GCA), expression of OCT 3/4 (a pluripotency-undifferentiation marker), germ cell ploidy and evolution to CIS and invasive TGCT. Findings were compared with those of normal testes. The type of gonads present defined three groups of patients: bilateral testes (BT-DSD, $n = 21$), one testis and one streak gonad (CT-DSD, C for combined, $n = 13$), and ovarian-testicular combinations (OT-DSD, $n = 9$). There were 5 boys with infantile CIS, bilateral in 3 (total of 8 infantile CIS) and two patients with adult CIS, bilateral in one (total of 3 adult CIS). Two patients had bilateral seminomas one at 12–17 and the other at 23 years. Histological dysgenesis was significantly higher in CT-DSD ($p < 0.05$), that had only 1 CIS. The highest frequency of GCA was in BT-DSD ($p < 0.05$), which coincided with a total of 11 CIS. In all patients, aneuploidy was significantly higher (63%) than diploidy ($p < 0.02$), and GCA were more frequent in aneuploid than in diploid samples ($p < 0.02$). All CIS and TGCT were OCT 3/4 positive. Finally, there was a significant association between the triad Aneuploidy + GCA + OCT 3/4 positivity and the incidence of CIS (Fisher Exact test $p < 0.002$, relative risk 7.0). The degree of testicular dysgenesis (derived from abnormal organization of Sertoli cells in fetal testicular cords) is inversely related to the incidence of CIS. Our data demonstrate that the combined use of OCT 3/4 expression, quantification of germ cell abnormalities-atypia and ploidy in dysgenetic testes can satisfactorily identify infantile CIS with high risk of malignant evolution and set it aside from delayed germ cell maturation with lower or nil neoplastic potential.

INTRODUCTION

Testicular embryonic differentiation is a consequence of a cascade of early events involving various genes in the bipotential gonadal ridge (reviewed by Wilhelm *et al.*, 2007). This is followed by the sequential activation of the testis determining factor Sry and subsequent expression of SOX9 in somatic cells of the testicular primordium, a landmark of Sertoli cell differentiation (Kanai *et al.*, 2005). Sertoli cells cluster together with recently arrived primordial germ cells to form testis cords, the precursors of postnatal seminiferous tubules. Alterations in this process may lead to various types of testicular dysgenesis, the phenotypic expression of Disorders of Sex Development (DSD), congenital anomalies that involve impaired development of chromosomal, gonadal or anatomical sex (Hughes *et al.*, 2006). Testicular dysgenesis comprises a wide spectrum of developmental anomalies ranging from a well-differentiated testis with mild dysgenetic changes to a completely undifferentiated gonadal tissue (Robboy *et al.*, 1982; Chemes *et al.*, 2003). It is generally accepted that dysgenetic gonads carrying Y chromosome material are at increased risk of developing gonadal germ cell tumors (Bianchi *et al.*, 2006; Krausz & Looijenga, 2008).

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Testicular germ cell tumors (TGCT) are the most frequent malignant neoplasia in adult young males (Ulbright *et al.*, 1999). TGCT of the adult testis derive from pre-invasive testicular changes first described as Carcinoma in Situ of the testis (CIS, Skakkebæk, 1972, 1978) or Intratubular Germ Cell Neoplasia (ITGCN, Scully, 1993). It is accepted that CIS derives from malignant transformation of primordial fetal germ cells and gonocytes that have failed to differentiate to postnatal spermatogonia (Skakkebæk *et al.*, 1987; Rajpert-De Meyts *et al.*, 1998, 2004). The diagnosis of testicular CIS-ITGCN is based on well-established histologic and immunohistochemical criteria (Skakkebæk, 1972, 1978; Ulbright *et al.*, 1999; Rajpert-De Meyts *et al.*, 2003; Oosterhuis *et al.*, 2011). Müller *et al.* (1984, 1985) and Müller (1987) first reported on the existence of abnormal infantile germ cells and development of CIS in boys with DSD and cryptorchidism, but the validity of its presence in children has been questioned (Cools *et al.*, 2006; Fan & Ulbright, 2012). Isolated cases of CIS in the vicinity of prepubertal TGCT were described by Stamp *et al.* (1993) and Renedo & Trainer (1994), but these reports are not confirmed by the study of larger series of patients, which supports the notion of divergent pathogenetic mechanisms in prepubertal and adult TGCT (Guinand & Hedinger, 1981; Manivel *et al.*, 1988; Chemes *et al.*, 2003).

The main purpose of the present work was an objective, evidence-based characterization of testicular dysgenesis in children and adolescents with DSD and its relationship with neoplastic risk. Particular attention was paid to histopathologic and cytological findings, immunohistochemical markers of primordial germ cells, DNA quantification and their value to identify a recognizable CIS phenotype before puberty.

MATERIAL AND METHODS

Patients and tissue samples

Testicular biopsies or gonadectomy specimens obtained from 43 patients with various forms of DSD and displaying histopathologic features of testicular dysgenesis were selected from the files of the Pathology Laboratory of CEDIE. Thirty seven of them had been clinically evaluated at the Division of Endocrinology of the Hospital de Niños Ricardo Gutiérrez and the other six at the Endocrine Unit of the Hospital Nacional Alejandro Posadas. A report on the clinical and cytogenetic features of some of these patients has been previously published (Arcari *et al.*, 2007). Collected data included age, sex of rearing, karyotype and the degree of virilization. The latter was assessed using the Prader scale (Prader, 1954), a scoring system that evaluates the degree of masculinization of the phallus, scrotum/labia and location of the urethral opening. This scale ranges from 0 (female external genitalia) to 6 (male external genitalia).

A retrospective revision of all testicular samples was independently performed by two of the authors (HC and MV). All of them were identified as having varying degrees of testicular dysgenesis. According to the type of gonads present they were grouped into three categories:

- Bilateral testicular DSD (BT-DSD) (two dysgenetic testes present), previously referred to as Dysgenetic Male Pseudohermaphroditism (Bergadá *et al.*, 1994).
- Combined testicular DSD (CT-DSD) (the simultaneous presence of a dysgenetic testis and a streak gonad), previously known as Asymmetric Gonadal Differentiation (Bergadá *et al.*, 1962) or Mixed Gonadal Dysgenesis (Sohval, 1963), and
- Ovotesticular DSD (OT-DSD) (a variable combination of testicular and ovarian tissues), previously known as true hermaphroditism.

Testicular tissue from four prepubertal gonadectomies was included as control. These samples were obtained from the normal testicular parenchyma peripheral to three testicular teratomas and 1 yolk sac tumor.

Histology and immunohistochemistry

Histopathologic reevaluation of all testicular biopsies and gonadectomies was blindly performed to confirm the diagnosis and review the following histologic parameters: thickness of the tunica albuginea, cortico-medullar differentiation, branching of seminiferous cords in the cortical region of the testis, penetration of the tunica albuginea by seminiferous cords, presence of cortical ovarian-like stroma, increase of the interstitial space, presence of annular tubules and microscopic calcifications. The presence of these dysgenetic traits was individually recorded in every specimen and their incidence was expressed as the respective percentage in each of the three groups of patients. In gonadectomy specimens, a longitudinal section of the testes was evaluated and the mean thickness of the tunica albuginea was obtained from three different measurements avoiding the rete testis area.

In all specimens careful evaluation of germ cell nuclear features was undertaken including hyperchromasia (increased nuclear staining with hematoxylin), anisokaryosis (nuclear size heterogeneity), and irregularly shaped nuclei. Their presence will be referred to as nuclear atypia, a characteristic of numerous malignant cells but also present in some highly proliferative states or as a cell response to injuries in viral infections and inflammation.

Immunohistochemical localization of OCT-3/4 (POU5F1) was assessed in all testicular specimens. OCT-3/4 is a transcription factor expressed by primordial germ cell nuclei and gradually downregulated through the differentiation process, without expression in normal testicular germ cells after the first postnatal months (Looijenga *et al.*, 2003; Honecker *et al.*, 2004; Rajpert-De Meyts *et al.*, 2004). Paraffin sections (4–5 μm) were mounted on positively charged slides, dewaxed, rehydrated and boiled in citric acid buffer (pH 6) for antigen retrieval. Nonspecific binding sites were blocked with Tris-buffered saline containing 1% bovine serum albumin (TBS–1% BSA). The sections were incubated overnight in wet chambers at 4 °C with the corresponding primary antibodies. OCT- 3/4 primary antibody(C-20: sc-8629 Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used at 1 : 500 dilution in TBS. Ready-to-use streptavidin peroxidase LSAB + System-HRP (DAKO, Carpinteria, CA, USA) or alkaline phosphatase Multilink AP/Fast Red (Biogenex, San Ramon, CA, USA) detection systems were used. When processing with the streptavidin peroxidase kit, slides were pretreated with 3% H_2O_2 in water to block endogenous peroxidase activity. Antibody localization was accomplished by application of diaminobenzidine/H_2O_2 for peroxidase kits (liquid DAB+, DAKO) or with Fast

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Table 1 Features of testicular dysgenesis and incidence of neoplasia (CIS + TGCT) in the three groups

Patients	Age mean (years)	Albuginea (μm) mean ± SEM	Cortico-medullar differentiation (%)	Dichotomic bifurcations (%)	Penetration of albuginea (%)	Germ cell abnormalities (%)	CIS + TGCT (*n*)
Controls *n* = 4	1.75	385 ± 34	–	–	–	–	–
BT-DSD *n* = 21	5	202 ± 24	10	33	52	85*	11
CT-DSD *n* = 13	3.3	142 ± 33	69*	85*	100*	42	1
OT-DSD *n* = 9	6.2	186 ± 48	44	78	66	67	3

Within each column, figures bearing asterisks are significantly higher (chi-squared test, $p < 0.05$).

Red diluted in naphthol phosphate for alkaline phosphatase detection (Biogenex). Some slides were slightly counterstained with hematoxylin. Negative controls were incubated in the absence of primary antibody or with nonimmune serum at similar protein concentrations.

Static quantitative analysis of DNA

A quantitative image analyses were performed to estimate nuclear DNA in testicular germ cells. Histologic sections of biopsies and gonadectomies were cut at 10 μm-thicknesses and the Feulgen reaction was performed to stain nuclear DNA in stoichiometric conditions. Germ cell nuclei were selected that were entirely included in the width of the section, avoiding the evaluation of partially cut nuclei. DNA quantification was accomplished through densitometric analysis using the imaging software NIS-Elements Version 3.0 BR and *SumDensity* (Nikon Instruments Inc. Melville, NY, USA). At least 100 germ cell nuclei were analyzed in each specimen. In very small biopsies all available nuclei were evaluated and, when possible, more than 2 non-consecutive sections were assessed for quantification (Müller, 1987). In each case, Sertoli cell nuclei of the same specimen were used as normal diploid nuclear controls. The ratio of the modal values of Go–G1 peak between germ and Sertoli cells was expressed as the DNA index (DI). An index between 0.9 and 1.1 was considered diploid and aneuploid for values equal or higher than 1.2. The aneuploid range included triploidy (DI around 1.5) and tetraploidy (DI around 2.0) which are of particular interest in the study of germ cell neoplasia.

Statistical analysis

Quantitative data were processed to evaluate correlations or statistical significance of differences with the MedCalc 11.6.1 software (Belgium). Different data were processed by analysis of variance, chi-squared test and Fisher's Exact test and the results were considered statistically significant when the *p* value was equal or lower than 0.05.

RESULTS

Clinical findings

From the 43 patients, 22 were reared as males and 21 as females. All of them consulted for ambiguous genitalia. Their degree of masculinization was between III and IV of the Prader scale (phallus between a hypertrophic clitoris and a small penis, complete, or almost, labial fusion and perineo-scrotal urethral opening). Patient ages ranged between 0.08 and 23 years (mean 4.7 ± 5.7, median 2.0). Fifteen patients (35%) were up to1 year of age, 19 (44%) were more than 1 and up to 8 years old, five (12%) were pubertal (11–14 years old) and four (9%) between 15 and 23 years old. All patients were grouped according to the type of gonads present: BT-DSD (n = 21), CT-DSD (n = 13) and OT-DSD (n = 9).

Testicular dysgenesis and cytogenetics

Bilateral testicular DSD

Twenty one patients presented BT-DSD (Table 1). Mean age was 5 years (range 1 month–23 years). They had bilateral testes with dysgenetic changes of moderate intensity (Table 1). The width of the tunica albuginea (202 ± 24 μm) was significantly reduced to 52% of the normal value (385 ± 34 μm). Main histologic changes included cortico-medullar differentiation, dichotomic bifurcation of testicular cords and penetration of thin albugineas by cords (Fig. 1). Other less frequently found features included increased interstitium, microscopic calcifications, annular tubules, and fusiform stroma. The prevalent karyotype was 46,XY (72%), there were four mosaic patients (45,X/46,XY) and two trisomies of the sex pair (47,XXY and 47,XYY).

Combined testicular DSD

Thirteen patients presented CT-DSD. Mean age was 3.3 years (range 2 months–14 years).They had one dysgenetic testis on one side and a rudimentary gonad (streak gonad) on the other side. These two types occasionally combined in a 'streak-testis' configuration (Scully *et al.*, 1998; Chemes *et al.*, 2003). The tunica albuginea (142 ± 33 μm) was further reduced to 37% of the normal value. The degree of testicular dysgenetic change was very pronounced and significantly increased in relation to that of BT-DSD (Table 1). Tubular penetration of the tunica albuginea was observed in all cases (Fig. 1), followed by dichotomic bifurcations of seminiferous tubules (85%), and cortico-medullar differentiation (69%). The more frequent karyotype was the mosaic 45,X/46,XY (72%), and there were three 46,XY patients.

Ovotesticular DSD

Nine patients presented OT-DSD. Mean age was 6.2 years (range 2 months–16 years). The combination of gonads was variable. There were five bilateral ovotestes, three left ovotestes with right ovary or testis and one right ovotestis with left ovary. The albuginea was 186 ± 48 μm thick (48% of the normal value).The degree of testicular dysgenesis was intermediate between that

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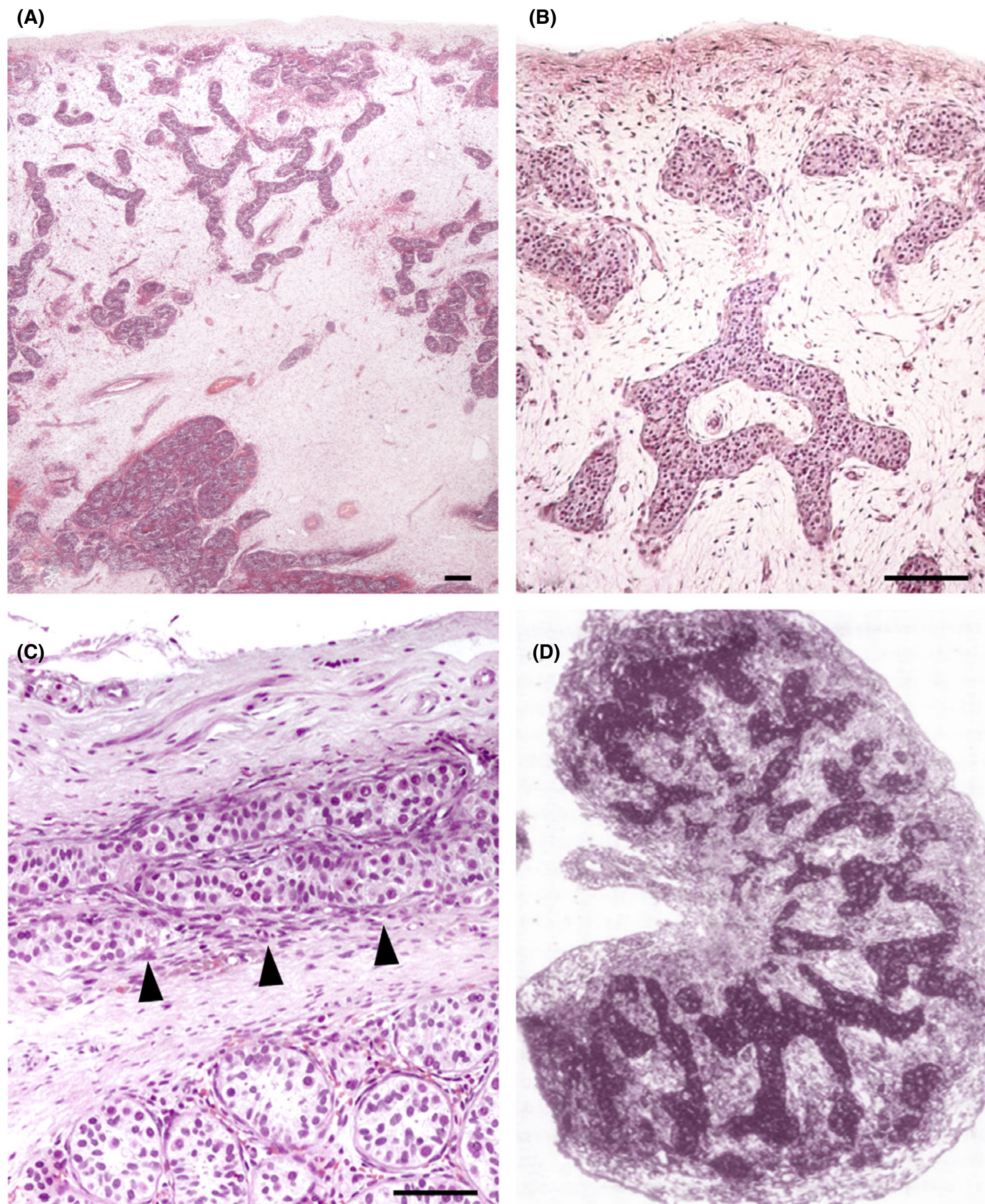
Figure 1 Main features of testicular dysgenesis. (A) CT-DSD, 2 months old. Thin albuginea, cortico-medullar separation, branching of cortical seminiferous cords, increased interstitial tissue. Dysgenetic traits were located in the cortical area. Relatively normal medullar region (bottom). (B) CT-DSD, 2 months old. Thin albuginea, plexiform seminiferous cords, increased interstitium. (C) BT-DSD, 1.5 years old. Penetration of the tunica albuginea by seminiferous cords (arrowheads). (D) Normal fetal testis (8th week of gestation). Notice similarity with dysgenetic features. Bars indicate 150 μm (A), 50 μm (B), 60 μm (C).



found in BT-DSD and CT-DSD (Table 1). More common dysgenetic features included dichotomic bifurcations of seminiferous tubules, tubular penetration in the albuginea and sharp differentiation between cortex and medulla (Fig. 1). Also noticed were increased interstitium, fusiform stroma, annular tubules, and microscopic calcifications. Karyotypes included three patients with 46,XX, and two different mosaics (46,XX/46, XY and 46,XX/47,XXY) present in three other patients each.

Percentages of the main dysgenetic features were significantly higher in CT-DSD patients when compared with those of children with BT or OT-DSD (Table 1).

This report deals exclusively with histopathologic features and neoplastic transformation of testicular dysgenetic testes in the three groups of patients here presented. Microscopic features of rudimentary gonads of CT-DSD patients and those of the ovarian parts of OT-DSD gonads will be dealt with in future reports.

Histopathology, immunohistochemistry, and ploidy

Control group

Seminiferous cords of the infantile testis are composed of abundant immature Sertoli cells with scarce cytoplasm and

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ovoid or elongate nuclei with finely dispersed chromatin. In the neonatal testis Sertoli cells are intermingled with low numbers of gonocyte-like cells and spermatogonia. The former, are large cells generally located in the center of the cords with abundant clear cytoplasm and large, vesicular, euchromatic nuclei with few strands of heterochromatin and big nucleoli. During postnatal life gonocytes and large fetal spermatogonia are gradually replaced by postnatal spermatogonia, smaller round to ovoid cells that rest on the base of the seminiferous cords and depict round nuclei with small nucleoli and finely dispersed chromatin of variable density. In normal prepubertal testes, the percentage of seminiferous cords containing germ cells in histological sections increases from 50–60% in younger boys to 80–90% near puberty and the number of spermatogonia per tubular cross section ranges between 0 and 5.

Dysgenetic testes

Dysgenetic testes of the three groups were carefully studied with special attention paid to cytological characteristics and magnitude of germ cell proliferation, incidence of histochemical markers of germ cell differentiation/pluripotency, and densitometric quantification of DNA.

In prepubertal dysgenetic testes germ cell cytologic features ranged from normal configurations, as those described for control testes, to various degrees of hyperchromasia, prominent nucleoli, increased cell size and cytoplasmic vacuolization that were either diffusely distributed or in small isolated groups of seminiferous cords. The prevalence of these germ cell abnormalities was significantly higher in dysgenetic testes of BT-DSD patients with lowest values in CT-DSD (Table 1). In a sub group of dysgenetic testes nuclear features of frank atypia and extensive germ cell proliferation were present and will be described in the next section.

OCT 3/4 expression was observed in small numbers of germ cell nuclei of central location randomly distributed throughout the testis (Fig. 2). OCT 3/4 positive cells were present in 10/21 patients with BT-DSD, 4/13 with CT-DSD and 6/9 with OT-DSD. Their numbers were higher during the first postnatal year and progressively decreased to disappear in older patients. Germ cell aneuploidy, in the hypertriploid to hypotetraploid range, was detected in 75% BT-DSD biopsies (DNA index 1.7 $\pm$ 0.3), 42% CT-DSD biopsies (DNA index 1.6 $\pm$ 0.1) and 67% OT-DSD biopsies (DNA index 1.9 $\pm$ 0.4).

Incidence of CIS and TGCT in dysgenetic testes

Extensive atypical germ cell proliferations of intratubular or invasive nature were observed in the testes of seven patients (4 BT-DSD, 1 CT-DSD, and 2 OT-DSD).

Intratubular proliferations were of two varieties (Figs 2 & 3). The first of them occurred exclusively in biopsies of prepubertal or early pubertal patients. Many infantile seminiferous cords depicted abundant gonocyte-like germ cells with atypical features randomly intermingled with small immature Sertoli cell nuclei. Atypical germ cells were basal or central and their numbers per seminiferous cord cross section far exceeded that of prepubertal germ cells in the control testes. They were much bigger than normal spermatogonia, had large vacuolated cytoplasm and hyperchromatic nuclei with prominent nucleoli and big irregular clumps of heterochromatin. OCT 3/4 was intensely positive in the nuclei of most proliferated germ cells of basal or central location. Mitotic activity was low or undetectable. Seminiferous cords containing atypical cells gathered in focal or lobulillar areas separated from nonproliferating regions by thin connective tissue septa (Fig. 3). These changes were present in five patients (mean age 7 $\pm$ 5.6 years) and will be referred to as infantile CIS (infantile Carcinoma in Situ), as validated in the discussion section. This phenotype was bilateral in three patients, which amounted to a total of eight infantile CIS. Most germ cells in these atypical areas were aneuploid (Table 2).

The second type of intratubular proliferations was present in one 15-year-old late pubertal boy and one 23-year-old adult man, both of them of the BT-DSD group. It was composed of large basally located gonocyte-like germ cells with very large, glycogen rich, vacuolated cytoplasm, and prominent heterochromatic nuclei with big nucleoli and large chromatin clumps. Variable numbers of mitosis were present. Sertoli cell nuclei with prominent nucleoli were displaced to a second row by atypical germ cells. The distribution of these proliferated areas was also lobulillar and they corresponded to the phenotype of adult CIS (adult Carcinoma in Situ, Fig. 3) as first described by Skakkebæk (1972).

The infantile CIS phenotype is clearly predominant in younger prepubertal patients and, as they grow older, CIS cells localize more and more over the basal lamina where they predominate at and after puberty (Fig. 2B).

Malignant invasive germ cell tumors were present in the testes of two patients of the BT-DSD group. One of them was first seen when he was 4 years old because of ambiguous external genitalia and cryptorchidism. A bilateral testicular biopsy was taken and diagnosed as bilateral dysgenesis with areas of proliferated hyperchromatic infantile germ cells. These were noted but their meaning was not fully understood. The boy subsequently consulted at 12 years of age because of unilateral left typical seminoma with foci of immature teratoma. Reevaluation of the original biopsies did not result in agreement on its neoplastic nature because the histological phenotype did not conform to the standard adult CIS characteristics accepted at the time. At 17 years of age a typical seminoma with peripheral areas of adult CIS was diagnosed in the right testis. The delayed development of bilateral invasive seminomas led to the conclusion that the atypical germ cell proliferations present in the original prepubertal biopsies corresponded to an infantile variety of intratubular germ cell malignancy (infantile CIS). The other patient that presented invasive germ cell malignancies consulted at the age of 23 years because of ambiguous genitalia. Bilateral testicular biopsies lead to the diagnosis of adult CIS with intratubular seminoma and focal invasion of the testicular interstitium. This was confirmed in the gonadectomy specimens. In the first of these two patients there was a long interval of 8–13 years separating bilateral infantile CIS and seminomas, while the diagnosis of adult CIS and seminoma was synchronous in the 23-year-old patient.

The total incidence of CIS (infantile + adult) and TGCT (mostly seminomas) amounted to 15 samples (Table 1). Highest frequency was observed in BT-DSD (11 samples), which also had peak incidence of germ cell abnormalities ($p < 0.05$) and lowest values of dysgenetic features. Conversely, the presence of only one infantile CIS in the CT-DSD group coincided with lowest germ cell abnormalities and significantly higher numbers of dysgenetic traits.

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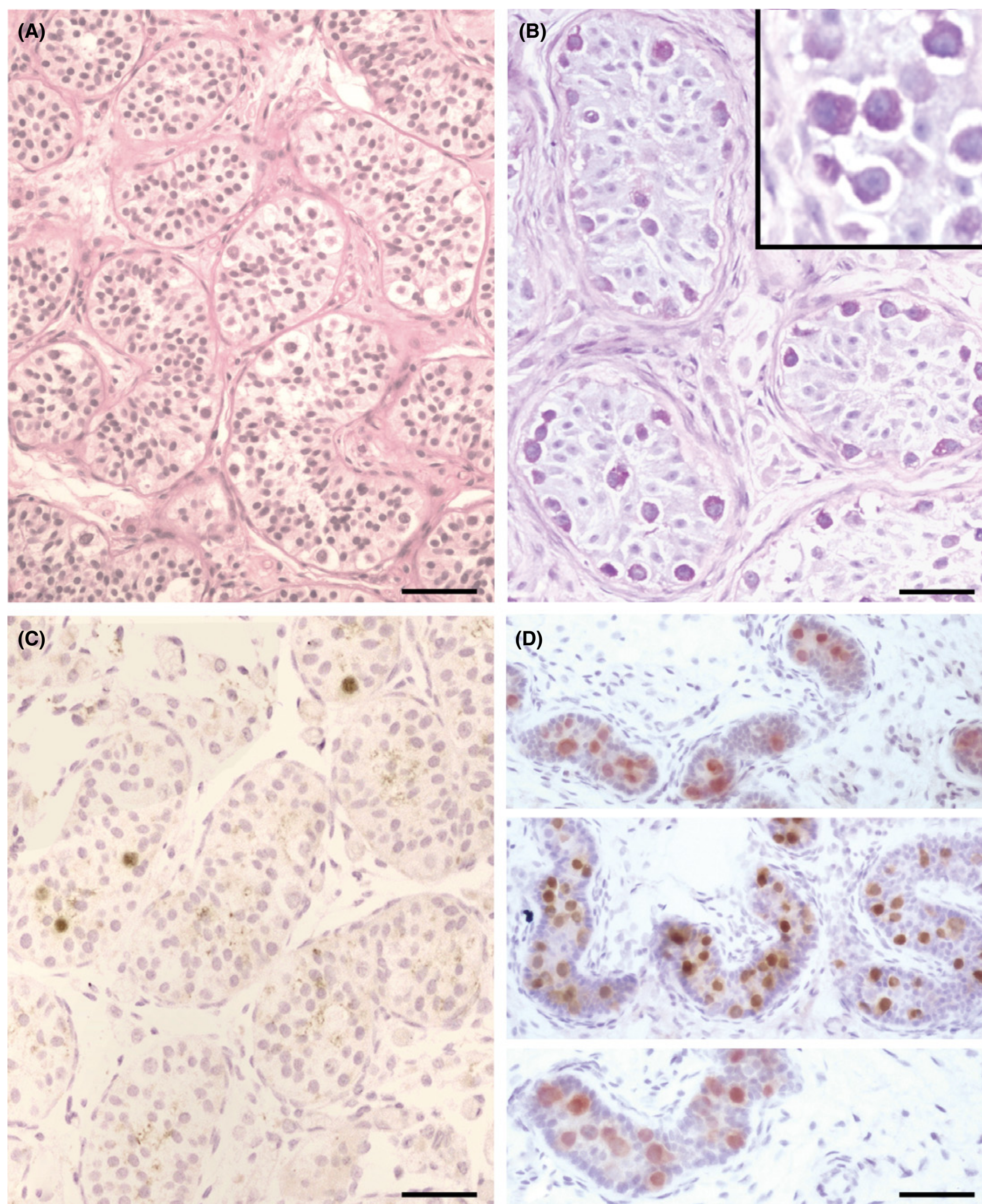
Figure 2 Dysgenetic testes with various degrees of germ cell proliferation. A and C correspond to nonmalignant changes, B and D to atypical CIS proliferations. (A) BT-DSD, 6.5 years old. Focal, relatively scarce, big vacuolated germ cells. (B) OT-DSD, 13.5 years old. Very abundant glycogen-rich, predominantly basal atypical CIS cells. Inset: detail of basal and central CIS cells. Changes in A and B are paralleled by OCT 3/4 nuclear reactivity present in few disperse cells in C (nonmalignant BT-DSD 5 years old), and in numerous, intensely reactive cells in all seminiferous cords in D (CIS in BT-DSD, 3 years old). Bars indicate 30 μm (A and C) and 50 μm (B and D).



Of particular interest was the finding of two OT-DSD patients with CIS in the dysgenetic testicular component of their ovotestes. The CIS was bilateral in one of them and histologically identical to that seen in BT-DSD patients. All ovotestes showed a clear cut separation between their testicular and ovarian portions and CIS changes localized exclusively in the testicular areas. The ovarian parts were essentially normal depicting numerous primary follicles embedded in fusocellular cortical stroma. Some of these follicles were cystic and or hemorrhagic.

All 13 CT-DSD patients had severely dysgenetic testes on one side, one of them harboring infantile CIS. The contra lateral gonad was a thin band of ovarian-like fusocellular stroma with varying amounts of anastomosing cords formed by a mixture of sustentacular somatic cells and primitive germ cells similar to those previously described by Robboy *et al.* (1982) and Chemes *et al.* (2003). This combination is typical of the syndrome of Asymmetric Gonadal Differentiation (Bergadá *et al.*, 1962) or Mixed Gonadal Dysgenesis (Sohval, 1963). Given that the present report is focused on the neoplastic risk in testicular dysgenesis,

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Figure 3 The three left panels correspond to infantile CIS and the three right ones to adult CIS. (A) BT-DSD, 4 years old. Infantile CIS with abundant big gonocyte-like germ cells of basal and central location (arrowheads). Notice large hyperchromatic nuclei with big chromatin clumps and mitosis (M). (B) BT-DSD, 12 years old. Adult CIS. Atypical germ cells line the basement membrane (arrowheads) and displace Sertoli cell nuclei to a second row. (C) BT-DSD, 4 years old. Infantile CIS. Intense and widespread OCT 3/4 expression in basal and central germ cell nuclei. (D) BT-DSD, 15 years old. Adult CIS. Intense OCT 3/4 reactivity in atypical cells of basal location. E (infantile CIS, OT-DSD, 12 years old) and F (adult CIS, BT-DSD, 15 years old): OCT 3/4 positive seminiferous cords have a lobulillar distribution clearly demarcated from OCT 3/4 negative areas. Bars indicate 16 μm (A), 20 μm (B), 30 μm (C), 35 μm (D), and 250 μm (E and F).

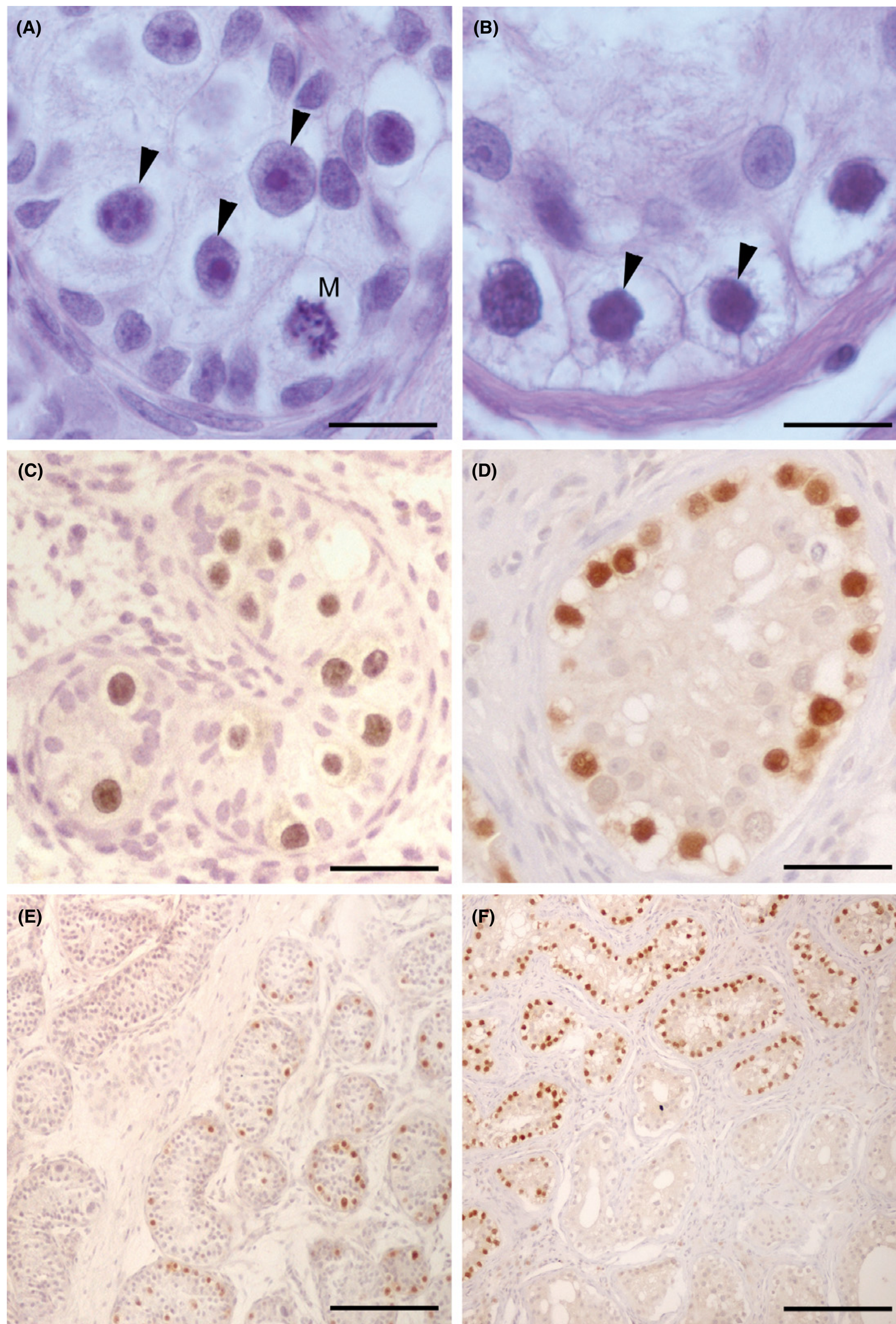

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Table 2 Germ cell ploidy and embryonic stem cell markers in the diagnosis of CIS

	Diploid		Aneuploid	
	OCT 3/4−	OCT 3/4+	OCT 3/4−	OCT 3/4+
Number of patients (*n*)[a]	11	4	10	16
DNA Index	1.0 ± 0.01	1.0 ± 0.01	1.7 ± 0.01	1.8 ± 0.01
Germ cell abnormalities (GCA, *n*)	2	3	8	15
CIS (*n*)	0	2	0	9

There is a significant predominance of Aneuploid over Diploid samples ($p < 0.02$). The incidence of Germ Cell Abnormalities is significantly higher in Aneuploid over Diploid samples ($p < 0.02$) and in OCT 3/4+ over OCT 3/4− samples ($p < 0.02$). The number of cases with the triad Aneuploidy/OCT 3/4+/GCA+ (shaded column) is significantly related to the number of CIS (Fisher's exact test, $p < 0.002$). When this triad is present in dysgenetic testes there is a 7.0 higher relative risk of CIS development. [a]There was insufficient data in two samples; total number in this table is 41 patients.

incidence of gonadoblastoma in CT-DSD streak gonads is not reported here.

Table 2 illustrates the relationship between ploidy, OCT 3/4 positivity, germ cell abnormalities, and incidence of CIS in the entire population. There was numerical predominance of aneuploid over diploid samples ($p < 0.02$). The incidence of germ cell abnormalities was significantly higher in aneuploid over diploid cases and in OCT 3/4 positive over OCT 3/4 negative ones ($p < 0.02$).The number of specimens with the triad Aneuploidy/OCT 3/4+/Germ Cell Abnormalities (shaded column) was significantly related to the number of CIS (Fisher's exact test, $p < 0.002$). Dysgenetic testes bearing this triad had a 7.0 higher relative risk of CIS development.

DISCUSSION

This is a report of the largest series of DSD patients presented to date including an evidence-based description of testicular dysgenesis, quantification of cytological germ cell abnormalities, incidence of immunohistochemical markers of undifferentiation-multipotency and densitometric quantification of germ cell DNA (ploidy). We have studied dysgenetic testes from 43 DSD patients with ambiguous external genitalia. Bilateral dysgenetic testes were present in 21 patients (BT-DSD), in 13 other patients one dysgenetic testis was combined with one streak gonad (CT-DSD) and nine had variable arrays of dysgenetic testes and ovaries (OT-DSD). Our main purpose was to analyze the relationship between the degree of objectively characterized testicular dysgenesis with various forms of prepubertal germ cell proliferations and germ cell tumors to define the phenotype of an infantile form of CIS and differentiate it from nonspecific germ cell proliferations and delayed maturation of testicular germ cells.

We have previously demonstrated that testicular dysgenesis mimics the changes during the first stages of testicular embryogenesis when the albuginea is not well formed and branching sex cords reach the testis surface. Testicular dysgenesis represents a form of immaturity, as if the gonad had failed to complete full embryonic maturation (Chemes *et al.*, 2003). The changes reported in Table 1 and Fig. 1, such as high incidence of cortico-medullar differentiation, dichotomic bifurcation of seminiferous cords and their penetration of a thin albuginea are probably the result of arrested testicular development, an important component of the of testicular dysgenesis syndrome (Skakkebæk *et al.*, 2001).

The prevalence of germ cell cytological abnormalities varied widely among dysgenetic testes. In samples without malignant changes they occurred in small numbers of random distribution, but could gather focally in few neighbor seminiferous cords as collections of cells depicting large vacuolated cytoplasm. These areas may be wrongly interpreted as malignant germ cell proliferations because of their superficial resemblance to adult forms of intratubular malignancies from which they can be differentiated by their restricted extension, lack of nuclear atypia, mostly diploid DNA and negative or weak OCT 3/4 reactivity. Their presence in the vicinity of infantile teratomas was taken as proof of the existence of peritumoral CIS during infancy (Stamp *et al.*, 1993; Renedo & Trainer, 1994). However, the study of the testicular parenchyma around larger series of infantile germ cell tumors does not validate this concept and suggests that they correspond to reactive events without neoplastic potential (Guinand & Hedinger, 1981; Manivel *et al.*, 1988; Hawkins *et al.*, 1997; Chemes *et al.*, 2003).These pseudo-CIS areas in prepubertal testes should be distinguished from genuine CIS and from delayed germ cell maturation characterized by the persistence of small numbers of OCT 3/4-positive gonocyte-like germ cells of diffuse distribution. The replacement of central gonocytes by basal spermatogonia and the downregulation of markers of pluripotency and undifferentiation that normally occurs in the first postnatal months may fail in testicular dysgenesis resulting in delayed germ cell maturation, a risk factor for germ cell malignancies at or after puberty (Rajpert-De Meyts *et al.*, 1998, 2004; Skakkebæk *et al.*, 2001; Looijenga *et al.*, 2003; Cools *et al.*, 2005). The distinction of CIS from delayed maturation may be quite difficult even to experienced pathologists because early neoplastic changes in the prepubertal testis do not conform to the classic adult CIS phenotype. A careful cytologic characterization of atypical features in proliferated germ cells, histopathologic assessment of their distribution, adequate immunohistochemical markers and ploidy studies may allow a proper distinction between these two conditions (Müller 1987, Chemes *et al.*, 2003; Cools *et al.*, 2005; Oosterhuis *et al.*, 2011).

Microscopic features suggestive of malignant potential had variable incidence in the three groups of patients and changed accordingly with the incidence of CIS + TGCT. Indeed, cytologic abnormalities, OCT 3/4 positivity and germ cell aneuploidy were higher in testes of BT-DSD patients (that had maximal numbers of CIS and TGCT), intermediate in OT-DSD ones and lowest in CT-DSD testes that had only 1 CIS.

The basic features of adult CIS and its evolution to invasive germ cell tumors were originally described by Skakkebæk (1972, 1978). They consist in small seminiferous tubules with severe germ cell depletion containing big basally located germ cells with atypical nuclei, increased mitotic rate and aneuploid DNA content. Sertoli cell nuclei are usually displaced to a second row. As previously reported in a number of studies, and more extensively in the present work, infantile CIS is characterized by solid seminiferous cords with increased numbers of atypical gonocyte-like germ cells of basal and central location, most of them strongly reactive for OCT 3/4, with infrequent mitotic figures and DNA content in the hypertriploid to hypotetraploid range

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(Müller *et al.*, 1984, 1985; Słowikowska-Hilczer, 2001; Chemes *et al.*, 2003). Seminiferous cords with these features gather in areas of lobulillar distribution separated from normal testicular parenchyma by thin connective tissue septa or, more rarely, have a cortical location immediately beneath the albuginea. The neoplastic potential of infantile CIS is confirmed by its evolution to invasive germ cell cancer after puberty, as documented in the present population of dysgenetic testes and by Müller *et al.* (1985) in cryptorchidism. During late infancy, there is a progressive evolution from the infantile to the adult CIS phenotype. In a preliminary report of boys with cryptorchidism, Oosterhuis *et al.* (2011) proposed that co expression of OCT 3/4 and TSPY in basal and central gonocytes with focal positivity of SCF may define the existence of a pre-CIS condition with risk of malignant transformation. However, in the absence of clinical data, it is difficult to predict malignant evolution because infantile and adult CIS are 'dormant' forms of intratubular malignancy with unpredictable and sometimes protracted progression. With methods currently available it is very difficult to anticipate the moment when CIS will develop to invasive germ cell cancer. The observation of very rapid advance from CIS to micro invasive seminoma in two infertile patients (H.E. Chemes, personal unpublished observations) only emphasizes our uncertainty about the timing of CIS evolution to invasive forms.

There was a wide range in the extent of dysgenetic changes in our population of DSD patients. Testicular dysgenesis was inversely related to the incidence of CIS as shown by the highest numbers of CIS + TGCT in BT-DSD patients that had milder signs of dysgenesis. Conversely, maximal dysgenesis in CT-DSD coincided with low values of germ cell cytological abnormalities and smallest incidence of CIS. The dissociation between dysgenesis and neoplastic risk is an intriguing finding that was previously noted in dysgenetic gonads and infertile patients (Słowikowska-Hilczer *et al.*, 2003; Guminska *et al.*, 2010). Histological signs of dysgenesis are a consequence of disordered sex cord formation and insufficient segregation between the albuginea and the testicular parenchyma. As such they point toward an abnormal condition of testicular somatic components (Sertoli, mesenchyme). On the other hand, development of CIS depends on the failure of gonocyte differentiation beyond its original immature multipotent character. The paradox is that somatic cells (Sertoli) remain immature in dysgenesis and so are germ cells in CIS, but without correlation between the magnitudes of these two conditions. This dissociation points to a disrupted interaction between germ and somatic cells in the fetal testis that may upset the fine tuning between gonocyte pluripotency, proliferation, and differentiation capacities. Skakkebæk *et al.* (2001) first advanced the concept of Testicular Dysgenesis Syndrome and suggested its origin in a disturbed somatic-germ cell interaction in the fetal gonad. The present results are an objective documentation of such disruption, but the fact that testes with most dysgenetic Sertoli cells are not the ones harboring the majority of CIS remains a puzzling observation. It could be hypothesized that disrupted Sertoli cell function in severe dysgenesis may not only fail to elicit gonocyte differentiation but also compromise their viability and proliferative capacity. In milder forms of dysgenesis the mitotic potential of arrested gonocytes may not be compromised which would facilitate their evolution to intratubular germ cell proliferations.

Differences observed in the phenotypes of infantile and adult CIS probably reflect the dissimilarities introduced by development of the blood-testis barrier around puberty. Infantile CIS develops in seminiferous cords that are not compartmentalized and germ cell distribution is not restricted by a permeability barrier. This is the clue to understand why infantile CIS cells may have both basal and central location. In the adult testes spermatogonia reside in the basal compartment, under the blood-testis barrier. CIS cells, their malignant counterparts, have similar location and organize in single rows that rest on the basal lamina. Some CIS cells may traverse the barrier and be shed from the seminiferous epithelium as proven by their reported presence in semen (Almstrup *et al.*, 2011). Even if the barrier is partially disrupted (Tarulli *et al.*, 2013), some degree of compartmentalization may still exist in adult CIS tubules.

The incidence of CIS in our population of OT-DSD seems higher than what has been generally understood. Isolated cases of CIS in limited series of ovotestes have been previously reported (Ramani *et al.*, 1993; Słowikowska-Hilczer *et al.*, 2003; Wang *et al.*, 2005), and van Niekerk & Retief (1981) communicated a neoplastic incidence of 2.6% in a revision of 409 cases of OT-DSD. However, this last figure does not refer to CIS but to invasive GCT or gonadoblastoma, and it may be biased because until very recently most ovotestes were resected during infancy or puberty, before the possible appearance of TGCT (Beheshti *et al.*, 1987). This possible underestimation was also noted by Cools *et al.* (2006). Interestingly, there have been four recent reports of seminoma arising in ovotestes in coincidence with current trends to preserve the testicular component in cases of male sex assignment (Nichter, 1984; Malavaud *et al.*, 2000; Malik *et al.*, 2007; Hua *et al.*, 2012). The problem is further complicated because in largest series of OT-DSD the identification of testicular CIS has not even been attempted. In summary, the few properly studied cases reported to date (including those here presented) are not enough to draw reliable percentages until larger series can be compounded.

We believe that the data presented here clearly delineate the CIS phenotype in testicular dysgenesis and demonstrate that the combined use of markers of germ cell multipotency, quantification of germ cell abnormalities-atypia and ploidy can satisfactorily identify infantile CIS with high risk of malignant evolution and set it aside from delayed germ cell maturation with lower (or nil) neoplastic potential. However, it seems reasonable to remember that, from the standpoint of the pathologist, the diagnosis of infantile CIS should primarily rest in a careful histopathologic examination of cytological signs of malignancy and their topographic distribution, and be confirmed by specific immunohistochemical markers and (in most cases) aneuploidy.

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MEETING COMMENTS

KATE LOVELAND (CLAYTON, AUSTRALIA)

When you examined the infantile CIS testicular samples, did you use markers to determine the maturation status of the somatic/Sertoli cells?

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HECTOR CHEMES (BUENOS AIRES, ARGENTINA)

Our study did not include Sertoli cell maturation markers. In prepubertal patients with CIS, Sertoli cells depicted immature nuclei and distributed randomly in the center and basal areas of seminiferous cords. In pubertal and adult CIS big nucleoli appear in Sertoli cells that organize in rows toward the base of seminiferous tubules, as they do in normal adult testes. This indicates some degree of Sertoli cell maturation. There is a recent publication (Tarulli *et al.*, 2013) that reports on Sertoli cell undifferentiation (dedifferentiation?) and disruption of the blood-testis barrier in cases of CIS. We plan to study protein markers of the barrier in children and post-pubertal patients with CIS. The signs of testicular dysgenesis are indicative of alterations in somatic cell (Sertoli) differentiation. Testicular sex cords in fetal testes form by assembling of primitive Sertoli cells. In dysgenetic testes, disruption of the cords with bifurcations and penetration of the tunica albuginea, are a reflection of arrested differentiation of testicular somatic cells. However, as stated above, there are clear signs of Sertoli cell differentiation (partial?) in pubertal-adult CIS (Fig. 3).

MARTINE COOLS (GHENT, BELGIUM)

I was very surprised at your high incidence of CIS (4/9 cases) in cases of ovotesticular disorders of sexual development (OT-DSD). We found a very low incidence in our series and we attribute this to normal combined development of the ovarian and testicular tissue in these cases. We never find co-expression of FOXL2 and SOX9. Is there a greater degree of testicular dysgenesis in your cases of OT-DSD?

HECTOR CHEMES

Different parts of our study were performed independently and when completed all results were collated. The evidence of an inverse relationship between the degree of dysgenesis and the risk of neoplastic development was unexpected and surprising. Among OT-DSD patients, the intensity of testicular dysgenesis (indicative of altered testicular development) is intermediate between those with BT-DSD and CT-DSD (see manuscript). The ovarian and testicular parts in ovotestes are clearly separated, that is why you do not see co-expression of FOXL2 (granulosa cell marker) and SOX9 (Sertoli cell marker). In comparison to other series, our study contains a relatively high proportion of CIS (on this point see discussion of the paper). Please note that the incidence is three samples in two patients (in one of them CIS was bilateral).

LEENDERT LOOIJENGA (ROTTERDAM, NETHERLANDS)

The diagnosis of OT-DSD relies very much on definition and diagnostic criteria. Oocytes must be present in the ovarian region, and detection of CIS or pre-CIS must only be seen in the testicular region. We are very reluctant to make a diagnosis of CIS or pre-CIS, especially in young individuals, purely on OCT 3/4 staining. Additional staining for KITLG or TSPY is required for confirmation. Are you sure that your cases are true OT-DSD individuals, because such patients are considered to be a low-risk population?

HECTOR CHEMES

We are completely sure about the diagnosis of OT-DSD patients. In a previous workshop of this series we presented a detailed characterization of different forms of gonadal dysgenesis including ovotestes (Chemes *et al.*, 2003). Its diagnosis requires the coexistence of normal ovarian tissue with many primary follicles, clearly separated from a testicular section that is moderately dysgenetic. At the interphase between them a few seminiferous cords may contain isolated oocyte-like germ cells. We are aware that OT-DSD has been generally considered a low-risk condition, but larger series studied with the emphasis on intratubular germ cell proliferations are necessary (for a more complete discussion of this point please see manuscript). Abnormal proliferations of germ cells and CIS configurations were only found within testiculat tissue, completely separated from the ovarian portion. We are aware of other markers like TSPY, SCF, and others, but we have found OCT 3/4 to be very reliable diagnostically. In our hands, the diagnosis of CIS depended not only on the existence of OCT ¾ positive seminiferous cords well demarcated from OCT ¾ negative ones, but also on the number and cytology of abnormal cells and ploidy of germ cells. In relation with markers that may predict invasive potential we should bear in mind that infantile CIS cells (or pre-CIS according to others) are dormant in young individuals. In particular, two of our BT-DSD patients who developed seminoma did so at or after puberty. Adult CIS is also dormant because seminoma develops predominantly in the 3rd to 5th decades although these patients should have harbored CIS at least since puberty.

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