Low Risk of Impaired Testicular Sertoli and Leydig Cell Functions in Boys with Isolated Hypospadias

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Context: Isolated hypospadias may result from impaired testicular function or androgen end-organ defects or, alternatively, from hormone-independent abnormalities of morphogenetic events responsible for urethral seam.

Objective: The objective was to evaluate the relative prevalence of hormone-dependent etiologies in boys with isolated hypospadias.

Design, Patients, and Main Outcome Measures: We studied endocrine testicular capacity in 61 patients with isolated hypospadias and 28 with hypospadias associated with micropenis, cryptorchidism, or ambiguous genitalia. Serum anti-Müllerian hormone and inhibin B were used as Sertoli cell markers. A human chorionic gonadotropin test was performed to evaluate Leydig cell function.

Results: Testicular dysfunction was observed in 57.1% and androgen end-organ defects in 7.2% of patients with hypospadias associated with cryptorchidism, micropenis, or ambiguous genitalia. In the remaining 35.7%, the disorder was idiopathic. The presence of ambiguous genitalia predicted the existence of testicular or end-organ dysfunction with 81.8% specificity. Isolated hypospadias was associated in 14.8% of patients with testicular dysfunction and in 6.5% of cases with end-organ defects; in 78.7% of cases, the condition was idiopathic. The occurrence of isolated hypospadias ruled out the existence of testicular or end-organ disorders with 80.0% sensitivity. Altogether our data indicate that the risk for the existence of an underlying testicular or end-organ dysfunction is low in patients with isolated hypospadias (odds ratio, 0.13; 95% confidence interval, 0.05–0.36; $P < 0.001$).

Conclusions: Boys with isolated hypospadias are more likely to have normal endocrine testicular and androgen end-organ functions, suggesting that transient disruption of morphogenetic events in early fetal life may be the predominant underlying cause.

HYPOSPADIAS, ONE OF the most prevalent congenital malformations affecting one in 125 to one in 300 male neonates (1–6), is the consequence of an incomplete fusion of the urethral folds present on each side of the urethral groove on the ventral surface of the fetal phallus. At 8 wk of fetal development, the external genitalia are undifferentiated. Afterward, in the male, the medial edges of the urethral folds progressively fuse in the midline on the ventrum of the phallus; the penile urethra is completely closed by wk 14. The glandular portion of the urethra forms during wk 16; the underlying mechanisms of this step remain controversial (7). Urethral organogenesis is mostly androgen dependent. Testosterone is produced by fetal Leydig cells in the interstitial compartment of the testis and subsequently converted in genital skin to dihydrotestosterone (DHT), which acts by binding to the androgen receptor. Hypospadias is an example of incomplete virilization where the urethral meatus is abnormally placed on the ventral part of the penis instead of the tip of the glans (7). Other androgen-dependent events in male sexual differentiation are the fusion of labioscrotal folds, the enlargement of the phallus, and testicular descent.

Therefore, irrespective of its etiology, abnormal androgen secretion or action may provoke an incomplete masculinization of the genitalia during fetal life, resulting in micropenis, hypospadias, incompletely fused scrotum, and/or cryptorchidism. Partial testicular dysgenesis, Leydig cell hypoplasia, steroidogenic enzyme defects, androgen insensitivity, and defective DHT formation in androgen target organs are the possible endocrine etiologies of incomplete genital virilization (8, 9). Although the diagnosis of steroidogenic defects relies on the assessment of the androgen synthesis pathway, it has been shown that other testicular and androgen end-organ dysfunctions can be more reliably detected in prepubertal patients by using other markers of testicular function, such as anti-Müllerian hormone (AMH) (10–12) or inhibin B (13).

How impaired testicular function or end-organ defects may result in dissociated undervirilization, *e.g.* hypospadias without other features of genital ambiguity, lacks a convincing explanation. Alternatively, hormone-independent abnormalities of morphogenetic events responsible for urethral

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Abbreviations: Δ4-A, Δ4-Androstenedione; AMH, anti-Müllerian hormone; CI, confidence interval; CV, coefficient of variation; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; hCG, human chorionic gonadotropin; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17OH-P4, 17OH-progesterone; 17OH-P5, 17OH-pregnenolone; OR, odds ratio; SDS, SD score.

seam could explain the vast majority of cases of isolated hypospadias. To evaluate the relative prevalence of hormone-dependent etiologies in boys with isolated hypospadias, we performed a comprehensive comparative screening of testicular function, including the steroidogenic pathway and the production of AMH and inhibins, in two cohorts of pediatric patients, one with isolated hypospadias and another where hypospadias was associated with other signs of defective virilization, such as micropenis, cryptorchidism, bifid scrotum, and/or penile chordee.

Patients and Methods

Patients

Eighty-nine boys with moderate (penile) or severe (perineal-scrotal) hypospadias were included in this study (Table 1). Sixty-one patients had isolated hypospadias, whereas 28 had nonisolated hypospadias; in 17 cases, hypospadias was associated with micropenis, as defined by Lee *et al.* (14), or cryptorchidism, and in the remaining 11 cases there were other virilization defects, such as bifid scrotum and penile chordee, leading to the classification of genitalia as sexually ambiguous. A complete physical examination was performed in each patient. A karyotype was performed in all subjects with bilateral cryptorchidism or with ambiguous genitalia. Only 46,XY patients were included.

Reference serum AMH values were obtained by studying 107 boys with no endocrine disorders and not taking any medication (Table 2). We used an aliquot of blood samples obtained during routine preoperative assessments for nonacute orthopedic, dermatological, or ear-nose-throat diseases. All controls underwent a thorough clinical and genital examination, performed by one of our pediatric endocrinologists (S.G.) to exclude boys with history or signs of endocrine disorders. Blood samples were obtained in the morning, centrifuged, and stored at −20 C until assayed. Serum gonadotropins and testosterone were assayed to further exclude samples from boys with clinically unapparent gonadal disorders. Reference serum inhibin B values (Table 3) have been previously reported (15).

Only patients whose parents had given written informed consent were included. The study was approved by the local Institutional Review Boards of San Borja Arriarán and Ricardo Gutiérrez hospitals.

Study design

Both tubular and interstitial endocrine functions of the testis were assessed in all patients. Serum AMH and inhibin B were used as tubular markers for the evaluation of Sertoli cell function (11–13, 15–20). Because normal values of circulating AMH (Table 2) and inhibins (Table 3) vary with age in normal boys, results obtained in patients with hypospadias are reported as SD scores (SDS) for the age or pubertal development. Interstitial Leydig cell function was evaluated by measuring serum testosterone in basal conditions and 24 h after im administration of 100 IU/kg human chorionic gonadotropin (hCG) (Pregnyl, Organon, Roseland, NJ; or Profasi, Novartis, Basel, Switzerland) as previously described (21, 22). The post-hCG/basal testosterone levels ratio was used to evaluate testicular steroidogenic response.

To identify steroidogenic pathway defects, and because the first steroidogenic steps are the same in the testis and the adrenal, serum levels of 17OH-pregnenolone (17OH-P5), dehydroepiandrosterone (DHEA), 17OH-progesterone (17OH-P4), Δ4-androstenedione (Δ4-A), and cortisol were measured in basal conditions and after a standard ACTH stimulation test (0.25 mg iv) as previously described (23). The post-ACTH/basal cortisol levels ratio was used to evaluate overall adrenal steroidogenic response. The functional activities of P450c17 and 3β-hydroxysteroid dehydrogenase (3β-HSD) were assessed comparing the results of 17OH-P5, DHEA, 17OH-P4, and Δ4-A measurements in our patients with the normative values for glucocorticoids and sex steroids in healthy pediatric population published by Lashansky *et al.* (24). Furthermore, a molecular study of the 3β-HSD type 2 gene (HSD3B2) was performed, as already reported (23). The activity of testicular 17β-HSD was assessed with the testosterone/Δ4-A ratio after hCG stimulation. The association of a lack of testosterone increase after the hCG test and a testosterone/Δ4-A ratio less than 0.5 was considered as highly predictive of 17β-HSD deficiency (25). Finally, the activity of 5α-reductase was evaluated by using the testosterone/DHT ratio after hCG stimulation. A testosterone/DHT ratio higher than 35 was considered as highly predictive of 5α-reductase deficiency (26). A testosterone hCG/basal ratio of at least 2 associated with AMH SDS of at least 2 was considered as highly predictive of partial androgen insensitivity (17, 18, 20, 27, 28).

According to the results of the hormone assessments, the disorders were classified as shown in Table 4.

Hormone assays

Serum AMH was assayed using the AMH/MIS ELISA kit (Immunotech-Beckman, Marseilles, France) as previously described (17). The assay has a sensitivity limit of 0.7 pmol/liter. Serum inhibin B and Pro-αC were measured using specific two-site ELISAs (Oxford Bio-Innovation Ltd., Oxon, UK) as described previously (15). The assay sensitivity was 15 ng/liter for inhibin B and 2 ng/liter for Pro-αC. The interassay and intraassay coefficients of variation (CV) were less than 10% for all assays. 17OH-P5 was measured by RIA as previously described (23). In brief, the samples were extracted and purified in LC-18 columns. The antibody, tracer, and standards were from ICN Pharmaceuticals (Costa Mesa, CA). The interassay and intraassay CV were 10.1 and 8.3%, respectively. Testosterone, DHT, Δ4-A, 17OH-P4, DHEA, and cortisol were measured by competitive specific-binding RIAs (Diagnos-

TABLE 1. Clinical features of the external genitalia in 89 patients included in the study

Type of hypospadias	n	Associated genital malformation	
		Micropenis/ cryptorchidism	Ambiguous genitalia
Nonisolated	28	17	11
Endocrine testicular dysfunction	16	9	7
Testicular dysgenesis	12	6	6
Isolated steroidogenic deficiency	4	3	1
End-organ dysfunction	2		2
Idiopathic	10	8	2
Isolated	61		
Endocrine testicular dysfunction	9		
Testicular dysgenesis	3		
Isolated steroidogenic deficiency	6		
End-organ dysfunction	4		
Idiopathic	48		

TABLE 2. Serum AMH levels (mean ± SD) in normal boys

Age group	n	AMH (pmol/liter)[a]
<1 yr	11	699 ± 245
1–4.9 yr	15	793 ± 264
5–12 yr: Tanner I	29	516 ± 275
>10 yr		
Tanner II	12	249 ± 126
Tanner III	12	98 ± 65
Tanner IV–V	28	42 ± 38

[a] Divide by 7.14 to obtain ng/ml.

TABLE 3. Serum inhibin B and Pro-αC levels (mean ± SD) in normal boys (15)

Age group	n	Inhibin B (ng/liter)	Pro-αC (ng/liter)
0–6 months	7	477 ± 142	372 ± 152
7–24 months	9	322 ± 157	200 ± 178
2–3.9 yr	22	237 ± 76	139 ± 91
4–5.9 yr	15	153 ± 91	77 ± 43
6–9.9 yr	22	151 ± 76	98 ± 55
10–11.9 yr	7	179 ± 97	99 ± 66
12–19 yr	7	400 ± 186	357 ± 281

TABLE 4. Criteria for classification of patients with testicular or androgen end-organ dysfunction

	Partial testicular dysgenesis	Partial isolated steroidogenic deficiency			Partial end-organ dysfunction		Idiopathic
		3β-HSD	P450c17	17β-HSD	AIS	5α-reductase	
AMH SDS	<−1	−1 to +2	−1 to +2	−1 to +2	>2	−1 to +2	−1 to +2
T post-hCG/basal	<1.5	<1.5	<1.5	<1.5	>2	>2	>1.5
17OH-P4/17OH-P5		<0.5					>0.5
Δ4-A/DHEA		<0.5					>0.5
Δ4-A/17OH-P4			<0.5				>0.5
T/Δ4-A				<0.5			>0.5
T/DHT						>35	<35

AIS, Androgen insensitivity syndrome; T, testosterone.

tic System Laboratories, Webster, TX). The limit of detection for each assay was 0.04 nmol/liter for testosterone, 0.02 nmol/liter for DHT, 0.07 nmol/liter for Δ4-A, 0.03 nmol/liter for 17OH-P4, and 0.17 nmol/liter for DHEA. The interassay CV for T, DHT, Δ4-A, 17OH-P4, and DHEA were 6.4, 6.2, 6.1, 5.5, 7.7, and 5.2%, respectively, and intraassay CV were 5.1, 5.5, 3.2, 4.2, 5.3, and 3.1%, respectively.

Statistical analyses

χ^2 tests were performed to analyze contingency tables, and odds ratio (OR) with 95% confidence interval (CI), sensitivity, specificity, and predictive values were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

Results

In 16 of 28 patients (57.1%) with hypospadias associated with cryptorchidism, micropenis, or ambiguous genitalia, an endocrine testicular dysfunction, either testicular dysgenesis affecting tubular and interstitial compartments (12 cases) or isolated interstitial steroidogenic deficiency (four cases), was observed (Table 1). In two of 28 patients (7.2%), inadequate responsiveness of androgen target organs (*i.e.* partial androgen insensitivity or 5α-reductase deficiency) was found. In the remaining 10 patients (35.7%), no evidence of defects in testicular hormone secretion or end-organ responsiveness could be shown. The disorder was therefore classified as hormone independent, or idiopathic. As expected, in the subgroup of patients with ambiguous genitalia, there was a higher prevalence of testicular dysfunction (seven of 11 cases, 63.6%) and end-organ defects (two of 11 cases, 18.2%) than in those in whom the hypospadias was associated only with cryptorchidism or micropenis (testicular dysfunction in nine of 17 cases, 52.9%; end-organ defects in 0 of 17 cases).

Conversely, idiopathic disorders were more prevalent in the latter group (eight of 17 cases, 47.1%) than in patients with ambiguous genitalia (two of 11 cases, 18.2%). In patients with hypospadias, the occurrence of ambiguous genitalia was more strongly associated with the existence of an underlying testicular or end-organ dysfunction than the occurrence of solely cryptorchidism or micropenis (OR, 5.06; 95% CI, 0.83–30.76; $P < 0.05$). The presence of ambiguous genitalia predicted the existence of testicular or end-organ dysfunction with 52.9% sensitivity (95% CI, 48.2–97.7) and 81.8% specificity (95% CI, 48.2–97.7). The positive predictive value was 0.53 (95% CI, 0.28–0.77).

In the group of patients with isolated hypospadias (Table 1), only nine of 61 patients (14.8%) had hormone levels suggestive of testicular dysfunction. In three of them, testicular dysfunction affected both Sertoli and Leydig cell populations, as shown by low AMH (−1.9, −2.1 and −2.3 SDS for age) associated with poor testicular, but not adrenal, steroidogenic capacity, suggesting mild testicular dysgenesis (Table 5). In these patients, inhibin B and Pro-αC were also low. In the other six cases, the disorder was limited to the interstitial compartment; AMH levels were normal (−0.6 to +1.8 SDS), but an inadequate testicular response to hCG, with normal adrenal response to ACTH, was observed, suggesting a partially impaired testicular steroidogenic capacity. In two of these patients, low testosterone/androstenedione ratios (0.2 and 0.4) were suggestive of partial 17β-HSD deficiency, and in one patient, although no clear-cut pattern of 3β-HSD deficiency (*i.e.* altered Δ5/Δ4 steroid ratio) could be demonstrated, a deleterious heterozygous mutation of the HSD3B2 gene had been reported (23). In the remaining three patients with low testosterone levels after hCG stimulation, a specific pattern of steroidogenic blockade could not be identified, probably owing to the short hCG test used. Four patients (6.5%) were found to have partially inadequate responsiveness of androgen target organs. In two of them, the testosterone/DHT ratio was high (37.1 and 38.7) and AMH was normally down-regulated (−0.8 and 0 SDS for age, respectively), which is compatible with 5α-reductase defects (23), whereas in the remaining two patients, the testosterone/DHT ratio was normal (2.9 and 19.1) and AMH was abnormally elevated (+2.9 and +3.0 SDS for age, respectively), which is compatible with partial androgen insensitivity (11, 15, 22, 23). Both Sertoli and Leydig cell functions of the testis were normal, as revealed by circulating AMH, inhibin B, Pro-αC, and steroid levels, either in basal conditions or after adequate stimulation tests, in 48 of 61 patients (78.7%) (Table 5).

Altogether our data indicate that the risk for the existence of an underlying testicular or end-organ dysfunction was significantly decreased in patients with isolated hypospadias when compared with those with hypospadias associated with cryptorchidism, micropenis, or ambiguous genitalia (OR, 0.13; 95% CI, 0.05–0.36; $P < 0.001$). The occurrence of an isolated hypospadias ruled out the existence of endocrine testicular or end-organ disorders with 80.0% sensitivity (95% CI, 67.7–89.2) and 55.2% specificity (95% CI, 35.7–73.5). The predictive value of isolated hypospadias for normal endocrine testicular and end-organ functions was 0.79 (95% CI, 0.66–0.88).

Discussion

The present results show that in boys with hypospadias, abnormal testicular function is most frequently associated

TABLE 5. Analysis of hormone determinations in 89 patients included in the study

Type of hypospadias	n	Age (yr)	AMH (SDS for age)	Inhibin B (SDS for age)	Pro-αC (SDS for age)	T (post-hCG/basal)	T/DHT	T/Δ4-A	Δ4-A/ 17OH-P4	Δ4-A/ DHEA	17OH-P4/ 17OH-P5
Nonisolated	28										
Testicular dysgenesis	12	3.2 ± 1.1	−1.99 ± 0.17	−1.11 ± 0.31	−0.48 ± 0.38	2.9 ± 0.8	3.30 ± 1.00	3.30 ± 1.00	1.86 ± 1.22	2.21 ± 0.59	1.12 ± 0.35
Isolated steroidogenic deficiency	4	7.3 ± 0.8	−0.02 ± 0.12	−0.58 ± 0.31	−0.45 ± 0.26	1.3 ± 0.3	0.62 ± 0.14	0.62 ± 0.14	1.37 ± 0.79	2.11 ± 0.97	1.11 ± 0.32
End-organ dysfunction	2	8.9 ± 3.1	3.69 ± 0.26	−0.42 ± 0.05	0.26 ± 0.89	5.0 ± 1.3	2.33 ± 0.51	1.46 ± 0.37	2.82 ± 2.21	2.48 ± 1.28	2.44 ± 1.28
Idiopathic	10	7.5 ± 1.5	−0.22 ± 0.18	0.03 ± 0.30	−0.49 ± 0.18	8.9 ± 2.7	6.03 ± 1.40	5.54 ± 1.59	1.60 ± 0.73	1.40 ± 0.25	1.02 ± 0.21
Isolated	61										
Testicular dysgenesis	3	4.4 ± 1.4	−2.11 ± 0.17	−0.61 ± 0.50	0.26 ± 0.35	5.4 ± 1.8	2.84 ± 0.60	2.84 ± 0.60	0.94 ± 0.29	1.67 ± 0.35	0.73 ± 0.18
Isolated steroidogenic deficiency	6	7.0 ± 0.7	0.92 ± 0.37	1.03 ± 0.50	−0.76 ± 0.22	1.3 ± 0.2	2.11 ± 0.97	2.11 ± 0.97	0.95 ± 0.21	1.69 ± 0.42	0.98 ± 0.19
End-organ dysfunction	4	10.5 ± 3.4	1.27 ± 0.98	−0.89 ± 0.29	0.46 ± 0.18	3.0 ± 0.8	24.45 ± 8.43	6.80 ± 1.82	1.94 ± 0.57	1.97 ± 0.40	0.89 ± 0.23
Idiopathic	48	6.5 ± 0.7	−0.05 ± 0.14	0.18 ± 0.17	−0.13 ± 0.11	4.4 ± 0.4	7.66 ± 0.85	2.99 ± 0.35	1.65 ± 0.33	1.98 ± 0.28	0.90 ± 0.08

Results are expressed as mean ± SD. T, Testosterone.

with a phenotype that includes several features of impaired virilization during fetal life. Comparatively, boys with isolated hypospadias have a low risk of abnormal hormone secretion by the gonads or androgen end-organ defects.

Defective fetal virilization is more frequently the consequence of testicular dysgenesis, affecting both the tubular and the interstitial compartments, than of isolated steroidogenic defects (8). Hypospadias might be a clinical manifestation of a testicular dysgenesis syndrome (29). The diagnosis of testicular dysgenesis in children can be reliably made by the existence of low serum AMH, reflecting impaired Sertoli cell function (11, 16, 17, 20). Conversely, the interpretation of the steroidogenic response to hCG to evaluate Leydig cell function is less clear-cut. Various hCG stimulation protocols have been used by different groups, and no consensus exists on unequivocal cutoff levels of response to predict defective androgen production (22). The difficulties in identifying partial testicular dysfunction through the evaluation of the steroidogenic response to hCG may have curtailed the detection of cases of testicular dysgenesis in previously reported cohorts of boys with isolated hypospadias. To overcome this difficulty, we have systematically analyzed both tubular (Sertoli cell) and interstitial (Leydig cell) markers of testicular function. We identified a significant proportion of patients with testicular dysgenesis among those with severe defects of virilization. Apart from decreased AMH, these patients also had low circulating levels of inhibin B and Pro-αC, compared with reference values previously reported by our group (15) and by Andersson and colleagues (30). As expected, in this group, the post-hCG/basal ratio of serum testosterone was decreased, but the other steroid ratios were normal in response to hCG or ACTH, indicating that no specific steroidogenic enzyme was affected. Contrasting with the relatively high prevalence of testicular dysfunction in our patients with ambiguous genitalia, testicular dysgenesis syndrome was infrequent in our large series of boys with isolated hypospadias.

The search for an endocrine etiology of hypospadias has led several authors to study testosterone biosynthetic defects as a possible cause. Although early studies were controversial about the relative frequency of impaired steroidogenesis in boys with isolated hypospadias (31–37), more recent genetic surveys have not detected an increased prevalence of steroidogenic enzyme defects (23, 38, 39). Our present results in a large cohort of boys support the hypothesis that steroidogenic enzyme defects are not a common etiology of isolated hypospadias. Abnormalities in androgen target organs also seem to be an infrequent finding in patients with isolated hypospadias. In concordance with results reported by other groups showing a low prevalence of androgen receptor (38, 40–42) or 5α-reductase (42, 43) gene mutations in such patients, only four of 61 boys with isolated hypospadias in our series had a hormonal tableau compatible with androgen insensitivity or defective DHT production. Differential diagnosis between partial androgen insensitivity and 5α-reductase type 2 deficiency is not always possible based solely on androgen levels, owing to the activity of 5α-reductase type 1, which can mask defective DHT production in external genitalia or, on the contrary, to secondary 5α-reductase deficiency described in patients with androgen insensitivity

(reviewed in Ref. 44). However, the combined analysis of Sertoli cell markers and androgens in basal and hCG-stimulated conditions enhances the ability to diagnose the underlying endocrine defect (28). AMH production is inhibited by high intratesticular testosterone concentration independently of DHT levels, but provided that the androgen receptor is adequately expressed in Sertoli cells (for review, see Ref. 45). Therefore, serum AMH is elevated in patients with androgen insensitivity (27) but normally down-regulated in patients with 5α-reductase defects (28). Our four patients suspected to have an end-organ defect had normal to high testosterone levels in response to hCG. Two of them had high AMH and normal testosterone/DHT ratio and DHT response to hCG, which strongly suggests a partial androgen insensitivity as probable etiology. Gene mutation screening could definitively confirm the diagnosis, although this has proved unsuccessful in a high proportion of patients with clinical and hormonal features of partial androgen insensitivity (8, 38). In the other two patients, DHT response to hCG was poor, testosterone/DHT ratio was clearly elevated, and AMH levels were normally down-regulated, which was highly suggestive of a partial deficiency of 5α-reductase with normal androgen receptor activity.

Normal development of the urethra during fetal male differentiation involves the functional integration of the androgen signaling pathway, *i.e.* testosterone being transformed to DHT, which binds to the androgen receptor present in the mesenchyme lateral to the urethral epithelium, and local morphogenetic factors. The molecular mechanisms underlying early urethragenesis have remained poorly understood until recently, when a number of elegant studies conducted in rodents have identified Hoxa13, Shh, Bmp2, Bmp4, Bmp7, Fgf8, Fgf10, and Wnt5a as local factors critically involved in male urethral organogenesis (46–49). Most of these factors have widespread effects on multiple organ systems, which makes it unlikely that boys with isolated hypospadias carry mutations in their encoding genes. However, specific signaling pathways may exist in the urethra, and their malfunction could explain the existence of hypospadias in familial cases. For example, Fgf10 signals in the genital tubercle specifically through Fgfr2-IIIb, whose targeted deletion results in hypospadias in the mouse (49).

Another mechanism possibly responsible for isolated hypospadias is transient exposure to environmental factors acting as morphogenetic disruptors in a critical stage of development (49). Several reports have shown an association between increased risk of hypospadias and exposure to hazardous waste (50, 51), maternal high intake of phytoestrogens (52), or exposure to other endocrine disruptors (53, 54). Maternal exposure to harmful environmental factors in early pregnancy might represent the likeliest reason for the increasing trends in the epidemiology of hypospadias. These factors may have deleterious actions in a critical period of development resulting, *e.g.* in hypospadias or cryptorchidism without further affecting future endocrine testicular function. However, the decreasing quality of semen quality detected in European countries and an increasing incidence of testicular neoplasia (reviewed in Ref. 29) may indicate that limited chronological exposure to environmental factors during embryogenesis could have long-life effects in the germ cell population of the gonads.

In summary, we have conducted a comprehensive study of the endocrine functions of the testis in a large series of boys with either isolated hypospadias or hypospadias associated with other clinical manifestations of undervirilization. We have found that boys with isolated hypospadias are more likely to have normal endocrine testicular and androgen end-organ functions. Other possible determinants of hypospadias, including mutations of morphogenetic factors involved in the formation of the urethra in early fetal life and *in utero* exposure to environmental disruptors, will need to be explored to clarify the multifactorial etiology of this relatively frequent malformation.

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