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Evaluation of Gonadal Function in 107 Intersex Patients by Means of Serum Antimüllerian Hormone Measurement*

RODOLFO A. REY, CORINNE BELVILLE, CLAIRE NIHOUL-FÉKÉTÉ, LAURENCE MICHEL-CALEMARD, MAGUELONE G. FOREST, NAJIBA LAHLOU, FRANCIS JAUBERT, IRÈNE MOWSZOWICZ, MICHEL DAVID, NURCIN SAKA, CLAIRE BOUVATTIER, ANNE-MARIE BERTRAND, CLAUDINE LECOINTRE, SYLVIE SOSKIN, SYLVIE CABROL, HÉLÈNE CROSNIER, JULIANE LÉGER, STEPHEN LORTAT-JACOB, MARC NICOLINO, WOLFGANG RABL, SERGIO P. A. TOLEDO, FIRDEVS BAŞ, ANNE GOMPEL, PAUL CZERNICHOW, PIERRE CHATELAIN, RAPHAËL RAPPAPORT, YVES MOREL, AND NATHALIE JOSSO

Unité de Recherches sur l'Endocrinologie du Développement (R.A.R., C.Be., N.J.), INSERM, Ecole Normale Supérieure, 92120 Montrouge, France; Services de Chirurgie Pédiatrique (C.N.-F., S.L.-J.), Endocrinologie Pédiatrique (R.R.), Anatomie Pathologique (F.J.) and Biochimie B (I.M.), Hôpital Necker-Enfants Malades, 75015 Paris, France; Service d'Endocrinologie Pédiatrique (C.Bo., N.J.) and Laboratoire de Biologie Hormonale (N.L.), Hôpital Saint Vincent de Paul, 75014 Paris, France; Service d'Endocrinologie et Diabétologie Pédiatrique (J.L., P.Cz.), Hôpital Robert Debré, 75019 Paris, France; Service d'Explorations Fonctionnelles Endocriniennes (S.C.), Hôpital Armand Trousseau, 75012 Paris, France; Service de Gynécologie-Obstétrique (A.G.), Hôpital Hôtel-Dieu, 75004 Paris, France; Unité de Recherches sur la Pathologie Hormonale Moléculaire, INSERM (L.M.-C., M.G.F., Y.M.), and Service d'Endocrinologie et Diabétologie Infantiles (M.N., P.Ch.), Hôpital Debrousse, 69005 Lyon, France; Service de Pédiatrie (M.D.), Centre Hospitalier Lyon Sud, 69495 Pierre-Bénite, France; Service de Pédiatrie 1 (A.-M.B.), Centre Hospitalier Universitaire, 25030 Besançon, France; Clinique de Pédiatrie (C.L.), Hôpital Charles Nicolle, 76000 Rouen, France; Service de Pédiatrie (S.S.), Hôpital de Hautepierre, 67098 Strasbourg, France; Service de Pédiatrie (H.C.), CHI Poissy - Saint Germain en Laye, 78104 Saint Germain en Laye, France; Institute of Child Health (N.S.) and Department of Pediatric Endocrinology (F.V.), University of Istanbul, 34390 Istanbul, Turkey; Kinderklinik und Poliklinik der Technischen Universität (W.R.), 80804 Munich, Germany; and Unidade de Endocrinologia Genética LIM-25 (S.P.A.T.), Faculdade de Medicina, Universidade de São Paulo, 01266-900 São Paulo, Brazil

ABSTRACT

Fetal male sexual differentiation is driven by two testicular hormones: testosterone (synthesized by interstitial Leydig cells) and antimüllerian hormone (AMH; produced by Sertoli cells present in the seminiferous tubules). Intersex states result either from gonadal dysgenesis, in which both Leydig and Sertoli cell populations are affected, or from impaired secretion or action of either testosterone or AMH. Until now, only Leydig cell function has been assessed in children with ambiguous genitalia, by means of testosterone assay.

To determine whether serum AMH would help in the diagnosis of intersex conditions, we assayed serum AMH levels in 107 patients with ambiguous genitalia of various etiologies. In XY patients, AMH was low when the intersex condition was caused by abnormal testicular determination (including pure and partial gonadal dysgenesis) but was normal or elevated in patients with impaired testosterone secretion, whereas serum testosterone was low in both groups. AMH was also elevated during the first year of life and at puberty in intersex states caused by androgen insensitivity. In 46,XX patients with a normal male phenotype or ambiguous genitalia, in whom the diagnosis of female pseudohermaphroditism had been excluded, serum AMH levels higher than 75 pmol/L were indicative of the presence of testicular tissue and correlated with the mass of functional testicular parenchyma.

In conclusion, serum AMH determination is a powerful tool to assess Sertoli cell function in children with intersex states, and it helps to distinguish between defects of male sexual differentiation caused by abnormal testicular determination and those resulting from isolated impairment of testosterone secretion or action. (*J Clin Endocrinol Metab* **84:** 627–631, 1999)

INTERSEX states are caused by defects in the process of fetal sexual differentiation. Although fetuses, irrespective of their genotype, develop female internal and external genitalia in the absence of testicular hormones, differentiation along the male pathway requires the development of normal testes producing adequate amounts of testosterone and antimüllerian hormone (AMH), also known as müllerian-inhibiting substance (MIS), and normal sensitivity of target tissues to these hormones. Before the 7th week of gestation, male and female fetuses have indifferent gonads, bipotential external genitalia, and two pairs of unipotential internal ducts (the müllerian ducts, which are the anlagen of the fallopian tubes, uterus and upper portion of the vagina, and the wolffian ducts, the primordia of the internal male genital ducts). In the XY fetus, the

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Address all correspondence and requests for reprints to: Dr. Rodolfo Rey, Present address for reprint requests: Centro de Investigaciones Endocrinológicas, Hospital de Niños, Gallo 1330, (1425) Buenos Aires, Argentina. E-mail: rodolforey@infovia.com.ar.

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expression of the SRY gene (1, 2) triggers testicular differentiation. In view of the determining role of the testis in the sexual differentiation of the internal and external genitalia, the development of the indifferent gonad along the testicular pathway has been called testicular determination (for review, see Ref. 3). The testis is formed by two morphologically and functionally distinct compartments: the seminiferous tubules, containing germ and Sertoli cells; and the interstitial tissue, containing Leydig cells. Sertoli cells produce AMH, which upon binding to a membrane receptor, induces the regression of the müllerian ducts (4). Leydig cells secrete androgens: human CG (hCG) and LH bind to the LH/hCG receptor present on Leydig cell membrane (5) and activate a chain of enzymatic reactions resulting in testosterone synthesis (6). In the fetal wolffian ducts, testosterone itself binds to the androgen receptor, whereas in the external genitalia, it is converted to dihydrotestosterone, which binds the androgen receptor with higher affinity.

Intersex states may result from abnormal testicular determination or from defects restricted to genital differentiation. In patients with abnormal testicular determination, including pure and partial gonadal dysgenesis, both Leydig cell and seminiferous tubule functions are affected, and variable degrees of external virilization impairment and persistence of müllerian structures may be observed, in correlation with the deficiency of androgen and AMH secretion. In abnormal genital differentiation, the gonads develop in accordance with the genetic sex, but tubular and interstitial testicular functions are dissociated, *i.e.* only one of the hormone-dependent developmental pathways is affected (for reviews, see Refs. 7–9). The most frequent forms of male pseudohermaphroditism result from impaired androgen secretion or action and are characterized by the presence of ambiguous or female external genitalia without müllerian duct remnants, because the AMH-dependent pathway is not affected. A less common form of male pseudohermaphroditism is the persistent müllerian duct syndrome, a disorder characterized by the persistence of the uterus and fallopian tubes in otherwise normally virilized boys, caused by abnormal AMH secretion or action (4).

Until now, assessment of gonadal function in children with ambiguous genitalia has relied on the measurement of serum testosterone in basal condition, and after hCG stimulation. This test provides information about Leydig (but not Sertoli) cell function. AMH is a glycoprotein specifically secreted by testicular Sertoli cells in the male, from the time of fetal testicular differentiation until puberty, when it is down-regulated by testosterone and meiotic entry (10–12). Because AMH is a reliable serum marker of Sertoli cell function in prepubertal boys (13–16), we undertook this study to determine whether serum AMH would assist in the diagnosis of intersex state conditions.

Subjects and Methods

Study subjects

We studied 107 patients evaluated for intersex disorders: 186 serum samples were collected for AMH assay as part of the evaluation procedure. Criteria for inclusion of patients in this study were the following: patients with a 46,XY genotype, or a mosaicism including at least one XY lineage, presenting with female or ambiguous external genitalia [types I-IV of Prader's classification (17)], and patients with a 46,XX genotype presenting with ambiguous external genitalia or normal male phenotype, in whom a diagnosis of congenital adrenal hyperplasia had been excluded. Patients with simple hypospadias or micropenis, or with intersex states associated with polymalformative syndromes were not included. Patients with abnormal testicular determination were classified into 5 groups: pure gonadal dysgenesis, partial testicular dysgenesis (also known as dysgenetic male pseudohermaphroditism), asymmetric gonadal differentiation (also known as mixed gonadal dysgenesis), and true hermaphroditism. Patients with normal testicular determination, but abnormal genital differentiation, were classified into 2 groups: those with impaired testosterone biosynthesis, including Leydig cell aplasia or hypoplasia caused by identified LH receptor mutations (18), impaired Steroidogenic acute regulatory (StAR) protein function [also known as lipoid congenital adrenal hyperplasia (19)] and impaired function of steroidogenic enzymes (8); and those with an androgen insensitivity syndrome (AIS), including the complete (CAIS) and partial (PAIS) forms (20, 21). Diagnoses were confirmed by the following criteria:

Disorders of gonadal determination. Disorders of gonadal determination include: 1) pure gonadal dysgenesis: 46,XY karyotype and histologically verified bilateral streak gonads; 2) partial testicular dysgenesis: 46,XY karyotype and histologically verified bilateral dysgenetic testes; 3) asymmetric gonadal differentiation: one testis and one streak gonad, histologically verified; 4) true hermaphroditism: histologically verified coexistence of testicular and ovarian tissue (presence of ovarian follicles, not only of ovarian-like stroma, was required); and 5) XX male: 46,XX karyotype, male external genitalia, and two scrotal testes.

Disorders of genital differentiation

Leydig cell aplasia or hypoplasia. Leydig cell aplasia or hypoplasia are characterized by: 46,XY karyotype, extremely low serum testosterone in basal (postpubertal patients) or hCG-stimulated (prepubertal patients) conditions, as compared with normal age-matched males, and either LH receptor mutation or absence or extremely low number of Leydig cells in a biopsy performed in basal (postpubertal patients) or hCG-stimulated (prepubertal patients) conditions.

Defects in gonadal steroidogenesis. Defects in gonadal steroidogenesis include: 1) steroidogenic enzyme defects: 46,XY karyotype, low serum testosterone after hCG stimulation as compared with normal age-matched boys, and either an abnormal ratio between serum concentrations of testosterone precursors after hCG stimulation or a mutation in a gene encoding one of the enzymes involved in testicular steroidogenesis; 2) StAR deficiency: 46,XY karyotype, low serum testosterone after hCG stimulation (as compared with normal age-matched boys), lipoid adrenal hyperplasia, and *StAR* gene mutation.

Androgen insensitivity. Androgen insensitivity includes: 1) CAIS: 46,XY karyotype, complete female external phenotype, normal or high serum testosterone in basal (postpubertal patients) or hCG-stimulated (prepubertal patients) conditions, as compared with normal age-matched males, and either abnormal DHT binding in genital skin fibroblasts or an androgen receptor mutation or a familial history of established CAIS; 2) PAIS: 46,XY karyotype, incompletely virilized external genitalia, normal or high serum testosterone in basal (postpubertal patients) or hCG-stimulated (prepubertal patients) conditions, as compared with normal age-matched males, and either abnormal DHT binding in genital skin fibroblasts or an androgen receptor mutation or a familial history of established PAIS.

Control AMH levels were determined in serum samples of 75 prepubertal, 63 pubertal, and 21 adult male subjects. In all cases, serum samples had been taken for a reason independent of the present study: adults were apparently healthy blood donors, and children were patients free of disorders of the hypothalamic-pituitary-gonadal axis and of chronic illnesses.

Serum AMH assay

Serum AMH was measured using the AMH/MIS enzyme-linked immunosorbent assay kit (Immunotech, Marseilles, France). Briefly, 25 μL of each serum sample was incubated in duplicate on a polystyrene plaque precoated with a monoclonal anti-AMH antibody. After 1 h incubation, a second monoclonal anti-AMH antibody, coupled to biotin, was added, together with a streptavidin-horseradish peroxydase complex. After addition of TMB substrate, the resulting color reaction was quantified using a MRX spectrophotometer (Dynatech Corp., Chantilly, Virginia) at 450 nm. A preparation of purified recombinant human AMH

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was used to construct a standard curve. The limit of sensitivity of the assay was 0.7 pmol/L (0.1 ng/mL); inter- and intraassay coefficients of variation were 8.7% and 5.3%, respectively, for a serum AMH concentration of 35 pmol/L and 7.8%, and 4.9% for a serum AMH concentration of 1100 pmol/L. No cross-reaction was observed with pure transforming growth factor β.

Statistical analysis

Comparisons among multiple groups were performed using Dunn's multiple comparison test after a Kruskal-Wallis nonparametric ANOVA. For statistical analyses in the case of patients from whom several serum samples had been taken for AMH assay, only the first sample obtained in each patient per age group was considered. In all cases, a difference was considered significant when the two-tailed P value was less than 0.05. The 99% confidence intervals of the means were calculated to determine normal serum AMH ranges for each age group. All statistical analyses were performed using InStat software, version 2.05 (GraphPad Software, Inc., San Diego, California).

Results

Serum AMH in control subjects

Serum AMH levels in control male subjects from birth to adulthood are shown in Table 1. As previously reported (13–16), serum AMH concentration is low in the first 1–2 weeks after birth; it subsequently increases and remains high during infancy and childhood, then decreases progressively during pubertal development, and falls to low or undetectable levels in the adult. Mean values obtained with the AMH/MIS enzyme immunoassay kit used in this work are in keeping with those previously reported by Lee *et al.* (16) in a large series of normal boys.

Serum AMH in patients with abnormal testicular determination

In XY patients with pure gonadal dysgenesis, serum AMH was always undetectable, indicating the absence of functional Sertoli cells (Fig. 1). In patients with partial testicular dysgenesis, asymmetrical gonadal differentiation, or true hermaphroditism, serum AMH levels were usually lower than in normal boys, although with individual variability: very low serum AMH was found in patients with small, severely dysgenetic testes, coexisting with müllerian remnants, whereas normal values were found in patients with mildly dysgenetic testes, in whom no persistence of müllerian ducts was observed. As predicted by its high Kd (22), AMH should be present in high local concentrations to induce müllerian duct regression, which explains the fact that a hemi-uterus was generally found on the side of the ovary in true hermaphrodites and on the side of a severely dysgenetic testis or a streak gonad in patients with partial testicular dysgenesis or asymmetric gonadal differentiation (results not shown).

TABLE 1. Serum AMH in normal males

Age group	n	Serum AMH (pmol/L)[a]	
		Mean ± SEM	Range[b]
<15 days	6	229 ± 59 (32.1 ± 8.3)	76–381 (10.6–53.4)
15 days–1 yr	22	465 ± 93 (65.1 ± 13.0)	251–679 (35.2–95.1)
1.01–4 yr	17	499 ± 66 (69.9 ± 9.2)	360–638 (50.4–89.4)
4.01–7 yr	16	438 ± 61 (61.3 ± 8.4)	309–566 (43.3–79.3)
7.01–9 yr	14	336 ± 47 (47.0 ± 6.6)	234–438 (32.8–61.3)
>9 yr[c]			
I	22	249 ± 26 (34.9 ± 3.7)	194–304 (27.2–42.6)
II	25	159 ± 25 (22.2 ± 3.5)	107–211 (15.0–29.6)
III	8	79 ± 28 (11.0 ± 3.9)	12–145 (1.7–20.3)
IV–V	8	48 ± 14 (6.7 ± 1.9)	14–81 (2.0–11.3)
Adult	21	30 ± 4 (4.2 ± 0.6)	22–38 (3.1–5.3)

[a] Between parentheses, values are also given in ng/mL to allow comparisons with previously reported data. Only pmol/L units will be used throughout this work.
[b] 95% confidence interval.
[c] Pubertal stages according to Marshall and Tanner (27).

Serum AMH in patients with impaired androgen secretion or action

Serum AMH was increased during the first year of life in 23 of 28 patients with intersex disorders caused by abnormal androgen secretion or action, including Leydig cell aplasia, steroidogenic enzyme defects, or androgen insensitivity (Fig. 1, A and B). In later childhood (1–9 yr of age), serum AMH returned to control values, yet remained significantly higher than in patients with testicular dysgenesis (Fig. 1C and Table 2). At puberty, serum AMH increased again in patients with Leydig cell aplasia and CAIS (Fig. 1D). In patients with PAIS, although AMH secretion was partially inhibited, serum AMH values remained significantly higher than in control boys when serum testosterone was higher than 7 nmol/L (Fig. 2). In our series of pubertal patients with mutations in genes encoding for steroidogenic proteins, because the androgen biosynthesis deficiency was only partial, testosterone production was sufficient to inhibit AMH; therefore, serum AMH levels were not significantly different from those observed in the normal population (Table 2).

Combined analysis of serum AMH and testosterone in intersex patients

In intersex XY patients, extremely low basal and hCG-stimulated serum testosterone levels, combined with undetectable serum AMH, were found only in patients with pure gonadal dysgenesis. Low testosterone, together with low AMH, was predictive of partial testicular dysgenesis. Low serum testosterone was associated with normal or high serum AMH only in patients with a Leydig cell-limited dysfunction, including Leydig cell aplasia/hypoplasia and steroidogenic protein defects. Normal or high serum testosterone and AMH levels were predictive of AIS.

In patients with 46,XX karyotype or sex chromosome mosaicisms including a Y-bearing cell line, serum testosterone and AMH levels were low in 23 patients (who had small, dysgenetic gonads) but attained normal levels in 10 patients (who had a significant amount of functional testicular parenchyma).

Discussion

Abnormal sexual development may result from multiple etiologies; however, the clinical presentation is rarely pathognomonic of the molecular defect responsible for the intersex condition. In XY intersex patients, the evaluation of the endocrine testicular function is one of the primary steps in the diagnostic procedure. Assessment of testicular function has classically relied on the determination of serum testosterone.

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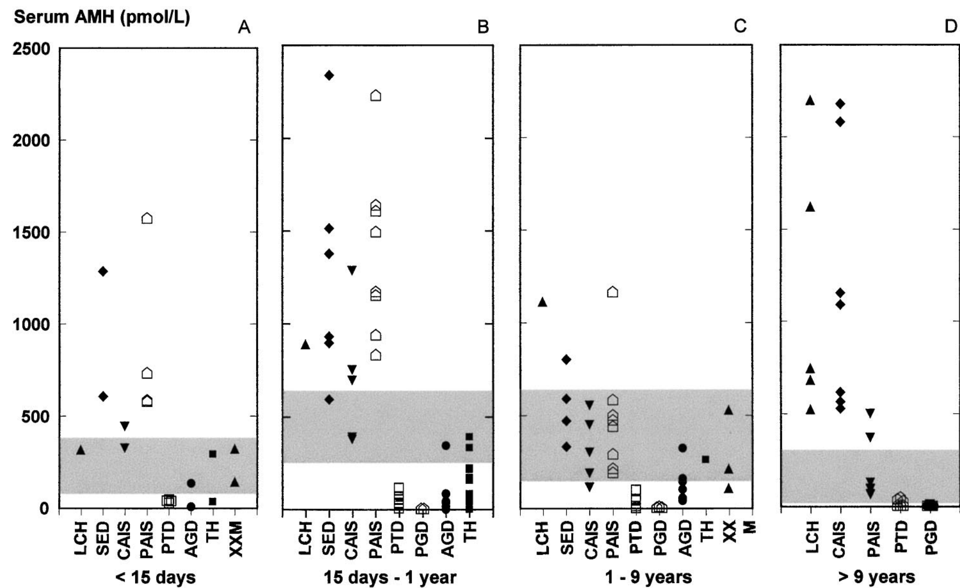

FIG. 1. Serum AMH levels in intersex patients. A, first two weeks of life; B, 15 days–1 yr; C, 1–9 yr; D, older than 9 yr. LCH: Leydig cell hypoplasia or aplasia; SED: steroidogenic enzyme defects; PTD: partial testicular dysgenesis; PGD: pure gonadal dysgenesis; AGD: asymmetrical gonadal differentiation; TH: true hermaphroditism; XXM: XX males. The shaded areas represent the normal range of serum AMH for each age group.

TABLE 2. Mean serum AMH levels (pmol/L) in intersex patients

	<15 days		15 d–1 yr		1–9 yr		>9 yr	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
Normal males	229 ± 145*a,b*	6	495 ± 202*a*	22	430 ± 242*a*	47	132 ± 128*a*	84
Pure gonadal dysgenesis (46,XY)			Undetectable	2	Undetectable	3	Undetectable	3
Partial testicular dysgenesis	51 ± 29*b*	5	46 ± 27*b*	9	40 ± 38*b*	5	17 ± 20*b*	6
Asymmetric gonadal differentiation			102 ± 122*a,b*	6	137 ± 102*b*	6		
True hermaphroditism			166 ± 35*a,b*	12			120 ± 152*a,b*	2
XX males	228 ± 126*a,b*	2			278 ± 219*a,b*	3		
Testosterone synthesis defects[a]	735 ± 498*a,c*	3	1131 ± 619*c*	7	648 ± 304*a*	5	(LCH) 1150 ± 723*d*	5
							(SED) 66 ± 28*a,b*	3
Androgen insensitivity syndrome[b]	708 ± 443*a,c*	6	1056 ± 591*c*	12	391 ± 199*a*	13	(C) 1172 ± 700*d*	7
							(P) 147 ± 75*a*	6

Comparison between all groups within each age group was performed using Dunn's multiple comparison test after an ordinary ANOVA (see *Subjects and Methods*). Different letters in italics indicate significant differences between means ($P < 0.05$) within the same column. For instance, in the <15 days column there is no significant difference between mean AMH values of normal males (*a, b*) and that of any other group, because all the other groups carry either an "*a*" or a "*b*." Mean AMH values of "Testosterone synthesis defects", and "Androgen insensitivity syndrome" (*a, c*) differ significantly only from those of "Partial testicular dysgenesis" (*b*).

[a] Includes Leydig cell hypoplasia/aplasia (LCH) and steroidogenic enzyme defects (SED), except in >9 yr group, where results are given separately.

[b] Includes complete (C) and partial (P) forms in all groups, except in >9 yr group, where results are given separately.

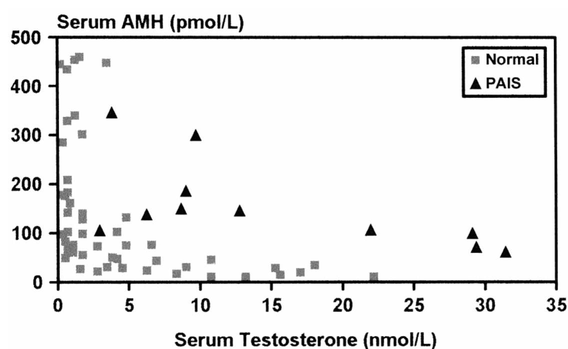

FIG. 2. Serum AMH levels plotted against serum testosterone in patients of pubertal age with PAIS.

Seminiferous tubule function is usually not studied and, only after ultrasonography, MRI, laparoscopy or laparotomy, when remnants of the müllerian ducts are detected, is Sertoli cell dysfunction considered. Disregarding the functional state of Sertoli cells may result in an incomplete understanding of the pathophysiology of certain cases of intersex states. Serum AMH is a reliable marker of Sertoli cell function and can be used to assess the functional state of the tubular component of the testes in patients with intersex conditions. Furthermore, AMH concentration in serum reflects Sertoli cell function at the moment the patient is studied, whereas the presence or absence of müllerian remnants is only an indication of testicular status in early fetal life, *i.e.* when müllerian ducts are sensitive to AMH. In this paper, we show that a simple measurement of serum AMH gives enough information to orient the diagnosis and treatment in certain groups of XY patients with ambiguous genitalia, before more cost- and time-consuming molecular or image-based studies and/or invasive surgical exploration are undertaken.

When sexual ambiguity is the expression of low androgen production, the etiology may be either gonadal dysgenesis, resulting in insufficient testosterone and AMH secretion, or

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isolated Leydig cell dysfunction. Serum AMH assay proves to be extremely useful for a differential diagnosis, because AMH concentration is normal or high in Leydig cell aplasia or steroidogenic enzyme defects but is low or undetectable when the intersex condition is caused by gonadal dysgenesis. Hence, the diagnosis of pure or partial gonadal dysgenesis can be reliably made, and surgical removal of the gonads should be considered, owing to increased risk of tumor development (23). Serum AMH thus provides unique information (not supplied by other simple biochemical studies) of great help for the therapeutic behavior.

Androgen insensitivity is another type of dissociated tubular-interstitial testicular dysfunction in which Leydig cell steroidogenesis is normal, but Sertoli cells, which are androgen target cells, are insensitive to testosterone, owing to mutations in the androgen receptor (21). In patients younger than 6 months–1 yr, this condition can be distinguished from gonadal dysgenesis, because serum androgen and AMH levels are normal or elevated. However, in later infancy and childhood, assessment of serum testosterone is informative only after the injection of hCG. Serum AMH determination can be very useful: with normal AMH levels, the diagnosis of testicular dysgenesis can be ruled out without the need for an hCG test.

In 46,XX patients, ambiguous or male external genitalia reflect androgen action. In fetuses with normal ovaries, the disorder is known as female pseudohermaphroditism and is most frequently caused by congenital adrenal hyperplasia. Alternatively, 46,XX individuals may carry testicular tissue, *e.g.* XX males and true hermaphrodites. Serum AMH levels higher than 75 nmol/L are an unequivocal proof of the existence of testicular tissue, excluding the diagnosis of female pseudohermaphroditism. Normal serum AMH either suggests the diagnosis of 46,XX male or indicates the existence of a significant amount of functional testicular parenchyma in true hermaphrodites. Serum AMH assay is particularly useful, in this respect, when the neonatal activation of androgen secretion has vanished.

Serum AMH assay has been frequently used in pediatric endocrinology seeking all/nothing responses, *e.g.* in boys with nonpalpable gonads, to determine the existence of ectopic testes (24, 25) and in patients with persistent müllerian duct syndrome, to determine whether the condition is caused by AMH synthesis defect or by AMH insensitivity (4). Here we show that assay of serum AMH is also of great interest, to quantitatively assess the function of Sertoli cells, and represents a helpful tool (in association with testosterone assay) in the diagnosis of intersex states. A promising future as a diagnostic test was recently predicted for serum AMH determination, provided that it becomes widely available (26). Using a commercial kit, we provide evidence that the evaluation of serum AMH in patients with sexual ambiguity helps to distinguish between abnormal testicular determination, dissociated tubular-interstitial testicular function, and end-organ insensitivity to sex hormones.

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