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The Role of Prior Pubertal Development, Biochemical Markers of Testicular Maturation, and Genetics in Elucidating the Phenotypic Heterogeneity of Idiopathic Hypogonadotropic Hypogonadism

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As our knowledge of the molecular mechanisms underlying idiopathic hypogonadotropic hypogonadism (IHH) expands, it becomes increasingly important to define the phenotypic spectrum of IHH. In this study we examined historical, clinical, biochemical, histological, and genetic features in 78 men with IHH to gain further insight into the phenotypic heterogeneity of the syndrome. We hypothesized that at least some of the spectrum of phenotypes could be explained by placing the disorder into a developmental and genetic context.

Thirty-eight percent of the population had Kallmann syndrome (KS; IHH with anosmia), 54% had normosmic IHH, and 8% had acquired IHH after completion of puberty. Phenotypically, KS represented the most severe subtype (87% with complete absence of any history or signs of spontaneous pubertal development), normosmic IHH displayed the most heterogeneity (41% with some evidence of spontaneous puberty), and acquired IHH after completion of puberty clustered at the mildest end (all had complete puberty).

Classification based on historical or clinical evidence of prior pubertal development, rather than the presence or absence of sense of smell, served to distinguish the population more clearly with respect to other clinical and biochemical features. Comparing IHH patients according to the absence (68%) or presence (24%) of some prior pubertal development revealed significant differences in testicular size (3.3 ± 0.2 *vs.* 11.8 ± 1.2 ml; $P < 0.001$), incidence of cryptorchidism (40% *vs.* 5%; $P < 0.05$), microphallus (21% *vs.* 0%; $P < 0.05$), inhibin B levels (45 ± 4 *vs.* 144 ± 20 pg/ml; $P < 0.0001$), and Mullerian inhibitory substance levels (9.8 ± 1.4 *vs.* 2 ± 0.5 ng/ml). Most familial cases had no pubertal development (95% *vs.* 5%; $P < 0.001$); males with mutations in the KAL gene displayed the most severe phenotype.

Mean gonadotropins levels (LH, 1.8 ± 0.1 *vs.* 2.9 ± 0.4 IU/liter; FSH, 2.2 ± 0.2 *vs.* 3.3 ± 0.3 IU/liter; $P < 0.05$) and the finding of apulsatile LH secretion based on frequent sampling (80% *vs.* 55%; $P < 0.05$) were statistically different between patients lacking and those exhibiting partial pubertal development, but the overlap was extensive.

The use of clinical parameters (presence or absence of some evidence of prior pubertal development, cryptorchidism, and microphallus), biochemical markers of testicular growth and differentiation (inhibin B and Mullerian inhibitory substance), and genetic evidence provides insight into the time of onset and the severity of GnRH deficiency. Viewing IHH in the full context of its developmental, genetic, and biochemical complexity permits greatest insight into its phenotypic variability. (*J Clin Endocrinol Metab* 87: 152–160, 2002)

THE EPISODIC SECRETION of GnRH from the hypothalamus is a key requirement for the initiation and maintenance of normal reproductive function in the human. During late fetal and early neonatal development, pulsatile hypothalamic secretion of GnRH stimulates gonadotropin biosynthesis and secretion that, in turn, initiates gonadal sex steroid production (1–3). Childhood is then marked by low amplitude GnRH secretion as mirrored by LH secretion (1) and pubertal reactivation of the hypothalamic-pituitary-gonadal (HPG) axis subsequently triggers the onset of sexual maturation initially in a sleep-entrained pattern (2, 3).

Parallel development occurs in the testes before any clinical evidence of sexual maturation. The initial activation of the HPG axis during the fetal/neonatal period triggers an elevation in T levels, which is required for completion of the inguino-scrotal phase of testicular descent (4–7) and further growth of the phallus. In addition, two main waves of Sertoli and germ cell proliferation occur during the late fetal/early neonatal period and then again at early puberty, coinciding with the two periods of increased activity of the neuroendocrine axis. Shortly after the initiation of puberty, the second wave of Sertoli cell proliferation ceases concurrent with initiation of Leydig cell secretion of T, terminal Sertoli cell differentiation, and consequent pubertal development (8–11). Disruption of any one of these cumulative events from fetal to adult life can lead to abnormal reproductive function.

A selective failure of this normal developmental sequence of GnRH secretion or function results in the clinical syndrome of idiopathic hypogonadotropic hypogonadism (IHH) (12, 13). The diagnosis of IHH is traditionally established by the absence of pubertal development by age 18 yr and prepubertal sex steroid and low/normal gonadotropin

Abbreviations: AHH, Acquired idiopathic hypogonadotropic hypogonadism after completion of puberty; BMI, body mass index; CV, coefficient of variation; HPG, hypothalamic-pituitary-gonadal; I_B, inhibin B; IHH, idiopathic hypogonadotropic hypogonadism; 2nd IRP, Second International Reference Preparation; KS, Kallmann syndrome; MIS, Mullerian inhibitory substance; nIHH, normosmic idiopathic hypogonadotropic hypogonadism; TV, testicular volume; X-KS, X-linked form of Kallmann syndrome.

levels in the absence of an anatomical cause. However, phenotypic descriptions of IHH in the literature highlight the diversity of this syndrome and suggest that a variety of defects may underlie the variability of clinical presentations.

IHH was first reported in association with anosmia and termed Kallmann's syndrome (KS) (14). However, it was subsequently appreciated that several patients with IHH lack any evidence of an olfactory defect and thus have a normosmic form of IHH (nIHH). The degree of prior sexual development in IHH men, as mirrored by gonadal size, may range from complete absence of sexual maturation to partial puberty to near-normal testicular size with a eunuchoidal body habitus, *i.e.* the "fertile eunuch" variant (15, 16). We also described an acquired form of IHH that characteristically presents as a secondary reproductive failure occurring after completion of full puberty (AHH) (17). Other phenotypic anomalies, such as midline defects, renal agenesis, synkinesia, and cryptorchidism, have been reported as variable features of this syndrome (18–23). Associated with this clinical spectrum of IHH are a range of anomalies in the neuroendocrine pattern of GnRH secretion that can vary from a complete absence of GnRH-induced LH pulses to disorders of LH pulse amplitude, frequency, and bioactivity (3, 16, 24). Genetic heterogeneity also underlies some of the variability in IHH. To date, defects in the KAL gene (25, 26), GnRH receptor ($GnRH_R$) gene (27–32), and DAX-1 gene (33–35) have been identified as a basis for some patients with IHH. However, a genetic basis for IHH has been established in less than 20% of cases, leaving several autosomal and X-linked genes to await description.

Given that the molecular and genetic bases of IHH remain largely unknown, we have focused on refining the clinical and biochemical features of a large cohort of men with IHH in an attempt to gain further insight into the phenotypic spectrum of this syndrome. We compared the clinical and biochemical parameters of the classical subsets of IHH: KS, nIHH, and AHH. In so doing, we hypothesized that at least some of the spectrum of phenotypes could be explained in part by two critical factors: the time of onset and the degree of GnRH deficiency. Accordingly, we characterized 78 men with IHH in whom a detailed family history, clinical examination, baseline biochemical profiling, and neuroendocrine evaluation were obtained. Recently, the ability to measure circulating levels of the Sertoli cell products, inhibin B (I_B) (36) and Mullerian inhibitory substance (MIS) (37), allowed us to add biochemical assessments of their gonadal status and correlate them with testicular morphology. Viewing IHH in the full context of its developmental, genetic, and biochemical complexity permits some further insight into its phenotypic variability.

Materials and Methods

Patient population

Men with IHH. The cohort was comprised of 78 males with IHH recruited from the Reproductive Endocrine Unit of Massachusetts General Hospital between 1979 and 1999. Criteria for the diagnosis of IHH uniformly included 1) age greater than 18 yr; 2) clinical signs or symptoms of hypogonadism; 3) T levels in the hypogonadal range (<3.5 nmol/liter) in the presence of low or normal gonadotropins; 4) normal thyroid, adrenal, and GH axes as assessed by TRH and insulin tolerance tests and normal serum PRL and ferritin concentrations; and 5) normal radiological imaging of the hypothalamic and pituitary areas. Reproductive hormone therapy (T, hCG, or hCG and FSH therapy) was discontinued for at least 3 months before the study.

Normal men. Thirty-six normal males between 18–35 yr of age were chosen for evaluation of gonadotropin and gonadal steroid secretion on the basis of the following criteria: normal history and physical examination, including testicular volume (TV) greater than 15 ml by Prader orchidometer; normal serum concentrations of LH, FSH, and T; and normal semen analysis (>20 million sperm/ml, > 50% motility, >2 ml volume). The protocol was approved by the institutional review board of the Massachusetts General Hospital, and all subjects provided written informed consent.

Protocols

Clinical assessment of IHH men. A complete family history was recorded for each individual with specific reference to the occurrence of delayed puberty, hypogonadism, and anosmia. All patients were specifically questioned about any historical evidence of the spontaneous occurrence of puberty such as 1) a marked increased in the number of erections, ejaculations and nocturnal emissions; 2) the occurrence of a growth spurt; 3) initiation of shaving; and 4) marked increase in libido. The presence of at least two of these historical points or a TV greater than 4 ml at presentation in the absence of prior gonadotropin therapy was interpreted as evidence of partial spontaneous sexual maturation. The subjective report of anosmia was noted, as few had formal smell testing. Previous therapy with androgens or gonadotropins was noted. A complete physical examination was performed, including determination of TV by Prader orchidometer, measurement of stretched penile length [<2.5 cm defined as microphallus (38)], classification of pubic and axillary hair by Tanner stages, and assessment of the presence of gynecomastia according to Tanner stages. Based on this initial assessment, the population was then divided into three groups: 1) KS 2) nIHH, and 3) AHH. Those patients with KS and nIHH were further divided into those with and those without some pubertal development.

Modes of transmission. The subject's family history was used to establish the likely mode of disease transmission. As surrogate genetic markers for the syndrome, anosmia and delayed puberty were employed in this analysis as previously validated (19). Delayed puberty was defined as menarche attained after age 15 yr in females and the onset of puberty after age 16 yr in males. A family was classified as X-linked by the predominance of affected males, an absence of male to male transmission, and the presence of an unaffected female carrier. Of note, prior genetic analysis of all X-linked patients confirmed a mutation in the KAL gene (39, 40). A family was classified as autosomal dominant if direct transmission was demonstrated from generation to generation, even in the presence of incomplete expressivity. Male to male transmission was considered definitive evidence for dominant inheritance, although these pedigrees may have included both affected males and females. A family was classified as autosomal recessive if affected individuals were members of the same generation and included at least one female; the presence of consanguinity provided additional support for this mode of inheritance. An indeterminate mode of transmission was noted if two brothers were affected, as X-linkage could not be distinguished from autosomal inheritance in these pedigrees.

Biochemical assessment of IHH and normal men

Baseline gonadotropin secretion. Frequent blood sampling was performed every 10 min overnight for 12 h with each sample assayed for LH. FSH, T, E2, and I_B were determined from a study pool comprised of equal aliquots of each individual sample. MIS was measured from a study pool in only 18 patients due to the instability of samples stored for more than 2 yr. Pulsatile hormone secretion was analyzed using a modification of the Santen and Bardin method (41). Each subject received a neuroendocrine classification based on the pattern of LH secretion: 1) apulsatile, defined as the complete absence of LH pulses; 2) low amplitude, defined as a normal LH pulse frequency, but a mean amplitude greater than 2 SD below the mean for normal controls; 3) low frequency defined as a normal mean LH pulse amplitude, but an LH pulse frequency greater

than 2 SD below the mean for normal controls; 4) low frequency and low amplitude; and 5) normal LH pulse pattern.

Testicular morphology of IHH. In a subset of patients (n = 26), a testicular biopsy was undertaken to assess the morphology of the testis and exclude Sertoli cell only syndrome. Twenty-two of 26 patients had a TV less than 3 ml. The degree of maturation of Sertoli cells and germ cells as well as the presence of Leydig cells were recorded.

Assays

Because this study spanned a 20-yr experience, two immunoassay systems were used for the measurements of FSH and LH. The majority of the data were obtained using in-house RIAs that have been reported previously (42, 43). More recent FSH and LH results reported in these studies were obtained using an automated microparticle enzyme immunoassay performed on the AxSYM system manufactured by Abbott Laboratories (Chicago, IL). The newer methods are two-site immunoassays that were carefully validated for our studies and, when calibrated with the same reference preparations used in the predicate RIAs, produce results that are directly comparable across methods.

AxSYM FSH calibrators ranged from 1–150 IU/liter [Second International Reference Preparation (2nd IRP) 78/549, a pituitary-derived international reference preparation], which was equivalent to 2.2–263 IU/liter in terms of 2nd IRP-71/223 (an international human menopausal gonadotropin reference preparation) used previously in the in-house RIA. Throughout the remainder of this manuscript, units for FSH will be expressed in terms of 2nd IRP-71/223. The limit of detection of the method (minimum detectable dose or sensitivity) was better than 1.1 IU/liter (2nd IRP-71/223); however, the limit of quantitation [at ~20% coefficient of variation (CV)] was 1.6 IU/liter. Thus, the reportable range of the method was established as 1.6–250 IU/liter (2nd IRP-71/223), which is higher than that of the RIA (0.8 IU/liter). For this reason, the data presented herein obtained using either RIA or AxSYM are reported based upon a limit of 1.6 IU/liter. The intraassay CV for FSH AxSYM was 2.8%. The between-assay precision was 4–9%. The two FSH methods measured virtually the same amount of FSH in serum specimens. Results obtained using the AxSYM system were highly correlated (r = 0.987; slope = 1.08; I = −0.09; n = 157) with those obtained by RIA specimens drawn from normal men.

AxSYM LH calibrators range from 1–150 IU/liter (2nd IRP-80/552), which is equivalent to 6–611 IU/liter in terms of the 2nd IRP-71/223, the same human menopausal gonadotropin reference preparation used to calibrate the RIA. Throughout the remainder of this manuscript units for LH will be expressed in terms of the 2nd IRP-71/223. The limit of detection of the AxSYM LH method was better than 1.2 IU/liter; however, the limit of quantitation at approximately a 20% CV was 1.6 IU/liter. Thus, the reportable range for the AxSYM LH assay was from 1.6–600 IU/liter. The intraassay precision of the AxSYM LH assay was 4.5%. The interassay precision was 7.5–12%. Results obtained using the AxSYM system to measure serum LH in 181 individual patient specimens were directly comparable (r = 0.987; slope = 1.08; intercept = −0.09) with those obtained by the in-house RIA.

Serum T concentrations were measured using the Coat-A-Count RIA kit (Diagnostic Products, Los Angeles, CA), which has intra- and interassay CVs of less than 10% for all samples. I_B was measured using a commercially available (Serotec, Oxford, UK) double EMSA as previously described (36). In our use, the clinical detection limit of this assay is 15.6 pg/ml, with a CV of 4–6% within plate and 15–18% between plates. Serum MIS was measured by an ELISA, as previously described (44). The limit of sensitivity of the assay is 0.5 ng/ml. The intra- and interassay CVs are 9% and 15%, respectively. Because of instability of the samples after 2 yr, samples for MIS could only been analyzed in 18 IHH men.

Statistical methods

The data are expressed as the mean ± SE unless otherwise indicated. Data from different subsets were evaluated by unpaired *t* test. Multiple means were compared by ANOVA. Qualitative differences between groups were tested by Mann-Whitney rank-sum test as well as χ^2 test. $P < 0.05$ was considered significant.

Results

Normal men

Between 1997 and 1999, 36 normal men were studied. The mean ± 1 SD values were 9.9 ± 2.5 IU/liter for LH, and 5.6 ± 2 IU/liter for FSH. The mean LH pulse amplitude was 6.7 ± 2.5 IU/liter, and LH pulse frequency was 5 pulses/12 h (range, 2–8 pulses). Pooled serum T was 18 ± 2 nmol/liter. Pooled serum I_B was 170 ± 46 pg/ml. I_B was negatively correlated with FSH (r = −0.40; $P = 0.01$). No significant correlation was observed between serum I_B levels and sperm counts.

IHH men

Historical and physical characteristics of men with IHH

*Clinical classification.*Thirty patients (38%) were diagnosed with KS, including two presenting with hyposmia. Forty-two patients (54%) with a normal sense of smell were defined as nIHH. The remaining six members of the cohort (8%) were classified as having AHH, as they had undergone complete puberty before the failure of the HPG axis and were all normosmic (17). The clinical characteristics of the entire population are summarized in Table 1.

Age and body mass index (BMI) at presentation. As a tertiary care center, many patients were referred to the Reproductive Endocrine Unit after having received either T therapy to induce secondary sexual characteristics or exogenous gonadotropins to stimulate testicular growth and/or induce fertility. The mean age at evaluation was 27 yr (range, 18–44; Table 1). The mean age for those presenting with no therapy was 24 yr (range, 20–30), well after normal puberty should have

TABLE 1. Baseline clinical characteristics of 78 men with IHH

	KS (n = 30)	nIHH (n = 42)	AHH (n = 6)	Total (n = 78)
Age at evaluation (yr)	28 ± 1.1	27 ± 0.7	34 ± 1.9	27 ± 0.7
BMI (kg/m^2)	25 ± 0.9	25 ± 0.7	28 ± 1.6	25 ± 0.5
Prior therapy (%)				
Gonadotropin	40	34	0	34
T	37	36	17	35
None	23	30	83	31
Puberty (%)				
Complete	0	0	100	8
Partial	13	41[a]	0	21
No	87	59[a]	0	71
Cryptorchidism (%)				
Unilateral	24	9[a]	1	18
Bilateral	24	7[a]	0	11
Microphallus (%)				
Yes	26	8[a]	0	15
Gynecomastia (%)	35	34	17	33
On hCG	16	12	0	13
TV (ml)	3.6 ± 0.5	6.6 ± 0.9[a]	19 ± 1.9	6.1 ± 0.7
Inheritance (%)				
Sporadic	60	83[a]	100	75
Familial	40	17[a]	0	25
AD	33	71		46
AR	0	29		11
X-Linked	50			31
Indeterminate	17			11

Gn, Gonadotropins; AD, autosomal dominant; AR, autosomal recessive.

[a] $P < 0.05$, KS *vs.* nIHH.

been achieved. Thirty-three percent of the IHH population were overweight, and 13% were frankly obese (BMI, >30). The mean BMIs for the different subgroups are shown in Table 1.

Sexual development. As evident in Table 1, nearly two thirds of the nIHH and KS population had neither signs nor symptoms of puberty. Patients with KS were less likely to have evidence of partial puberty than those with nIHH (Table 1). Cryptorchidism occurred in a third of the entire cohort and again was more common in the KS than the nIHH population (Table 1). Within the KS patients, cryptorchidism was most prevalent in those with an X-linked mode of inheritance (83%), all of whom were bilaterally cryptorchid. The subset of patients without any historical or clinical evidence of prior pubertal development had a higher incidence of cryptorchidism than those with some pubertal development (Table 2). Surgical repair was performed in the majority of patients with cryptorchidism (86%), with a mean age at surgery of 13 ± 2 yr (range, 4–35 yr).

Microphallus (15%) was less common than cryptorchidism (29%) and again occurred exclusively in those patients lacking any pubertal development whether nIHH or KS (Tables 1 and 2). As with cryptorchidism, the prevalence of microphallus was greater in KS than in nIHH (Table 1), particularly those with X-linked transmission (66%). Gynecomastia was noted in one third of the cohort, the majority of whom had had pretreatment with either gonadotropins or T, with only 10% having had no prior therapy. The cause of gynecomastia in the untreated subset was unclear because levels of mammotropic hormones (PRL and E2) were normal.

Testicular volumes in this heterogeneous population spanned a broad range, with measurements from 1–25 ml (Table 1). Normal TVs were observed in two subgroups, patients with AHH and those with the fertile eunuch syndrome (45). The diagnosis of fertile eunuch syndrome was given to a subgroup of six IHH men (all normosmic) with a mean TV of 19 ml despite decreased virilization, eunuchoidal proportions, and hypogonadal T levels. Patients with KS had a significantly lower TV than that of the nIHH subgroup (Table 1) even when the analysis was confined to those with no prior gonadotropin therapy (3.6 ± 0.5 *vs.* 5.7± 0.8 ml; $P < 0.05$). Those with a prior history of some pubertal development had a TV that greatly exceeded that in patients without sexual maturation (Table 2). Once again, the difference in mean TV of patients with KS and nIHH was eliminated if one first considered whether there had been any history of spontaneous puberty.

TABLE 2. IHH according to presence or absence of prior pubertal development

	No puberty (n = 53)	Partial puberty (n = 19)	*P* value
Clinical			
Cryptorchidism (%)	40	5	<0.01
Unilateral	23	5	
Bilateral	17	0	
Microphallus (%)	21	0	<0.05
TV (ml)	3.3 ± 0.2	11.8 ± 1.2	<0.0001
Inheritance			
Familial (%)	34	5	<0.01
Chemistry			
T (nmol/liter)	1.9 ± 0.1	2.1 ± 0.2	NS
LH (IU/liter)	1.85 ± 0.1	2.9 ± 0.4	<0.05
FSH (IU/liter)	2.2 ± 0.2	3.3 ± 0.3	<0.05
I_B (pg/ml)	45 ± 4	144 ± 20	<0.0001
MIS (ng/ml)	9.8 ± 1.4	2 ± 0.5	<0.001
LH pulse pattern			
Apulsatile (%)	80	55	<0.05

Pattern of inheritance. Considerable genetic heterogeneity has been found to underlie IHH. Most cases were sporadic (75%), indicating either that the frequency of spontaneous mutations is high in this syndrome or that the etiology of many cases may not be genetic. Among the 25% of familial cases, an autosomal mode of inheritance was evident in the majority (57%; Table 1) (19). A higher prevalence of familial cases was found in KS than nIHH (Table 1). The majority of familial KS cases were X-linked (X-KS; 50%). All six X-KS patients harbored a mutation in the coding sequence of the KAL-1 gene and were reported previously (39, 40). Among the familial nIHH cases, no X-linked cases existed, and an autosomal mode of inheritance accounted for all cases. All AHH patients were sporadic.

The familial cases were characterized by a severe phenotype, with 95% having had no pubertal development, and presented with a very high incidence of cryptorchidism (71%) and microphallus (55%). Of the familial cases, the most severely affected were those with KAL mutations. The only familial case with a history of spontaneous puberty was defined as nIHH with an autosomal dominant mode of inheritance. His clinical picture was consistent with that of a fertile eunuch.

Biochemical studies of IHH

Hormonal profile. Despite variations in the phenotypic presentation of the KS, nIHH, and AHH subgroups, their biochemical profiles were very similar. By definition, all patients displayed hypogonadal T levels in the setting of normal/low gonadotropin levels (Fig. 1). However, mean T levels tended to be higher in AHH compared with the two other subsets [3.1 ± 0.5 *vs.* 2 ± 0.1 *vs.* 2.1 ± 0.1 nmol/liter (91 ± 20 *vs.* 57 ± 3 *vs.* 60 ± 4 ng/dl) for AHH, KS, and nIHH respectively; $P = 0.2$]. T levels were no different in patients with or without prior pubertal development (Fig. 1). Serum E2 levels were undetectable in most patients with KS and nIHH and were in the normal range among AHH patients (28 ± 2 pg/ml). Mean levels of LH (2.2 ± 0.2 IU/liter) and FSH (2.5 ± 0.2 IU/liter) in IHH were significantly lower than those in the normal controls ($P < 0.05$), but no difference was observed among the three subgroups (KS, nIHH, and AHH) according to LH and FSH levels. However, LH and FSH levels were both significantly lower in subjects without a history of puberty than in those with such a history (Fig. 1).

The mean I_B level in the IHH population was 70 ± 8 pg/ml, significantly lower than that in the normal controls ($P < 0.05$) (46, 47). A significant positive correlation was observed between I_B and TV ($r = 0.67$; $P < 0.0001$; Fig. 2), but not with serum FSH at baseline. Notably, I_B levels were lower in the KS subset (46 ± 6 pg/ml) compared with nIHH (85 ± 12 pg/ml) and AHH (98 ± 18 pg/ml; $P < 0.05$). There was no

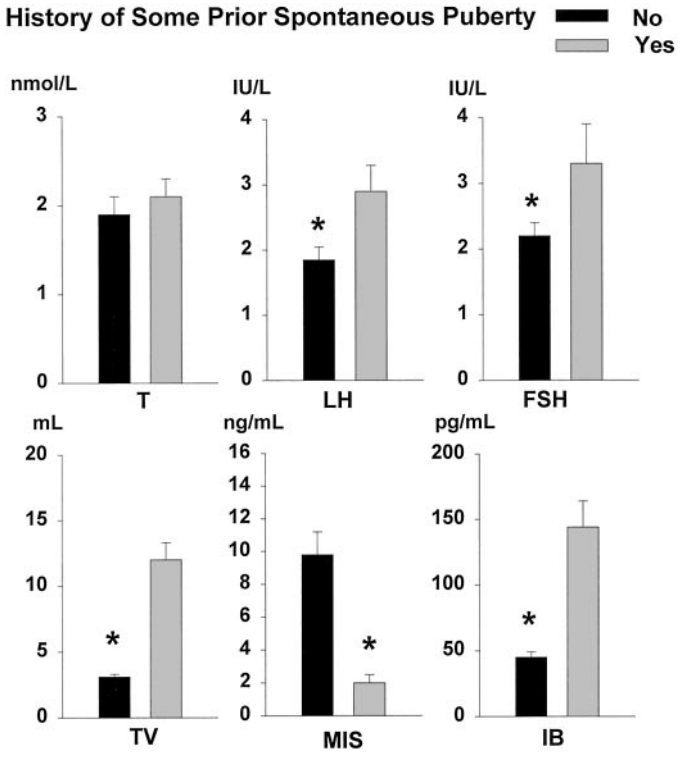


FIG. 1. T, FSH, LH, TV, MIS, and I_B at baseline in IHH men with (n = 19) and without (n = 53) a history of spontaneous puberty. AHH patients were excluded. LH, TV, and I_B are greater, and MIS levels are lower in IHH men with a history of spontaneous puberty ($P < 0.05$; mean ± SEM). To convert values of serum T from nanomoles per liter to nanograms per dl, multiply by 28.84. *, $P < 0.05$.

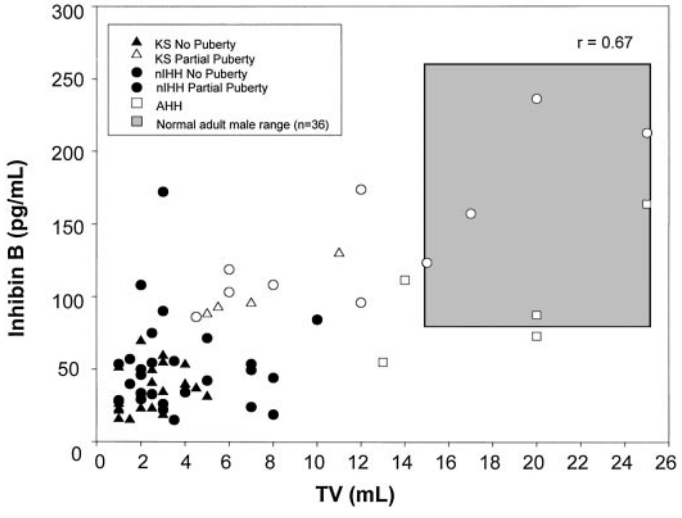


FIG. 2. Serum I_B plotted relative to TV in KS (n = 30), nIHH (n = 42), and AHH (n = 6) at baseline evaluation according to the absence or presence of puberty. The *shaded area* represents the mean (±2 SD) I_B value in 36 normal men.

difference in I_B levels between nIHH and AHH subgroups despite a significant difference in mean TV. Despite having a similar normal TV, AHH and the fertile eunuch subset of nIHH had markedly discordant I_B levels (98 ± 18 *vs.* 226 ± 48 pg/ml, respectively; $P < 0.05$). Among patients with a complete absence of puberty (n = 53), baseline I_B levels were significantly lower than those in patients with a history of some pubertal development (Fig. 1 and Table 2). Finally, among those lacking pubertal development, I_B levels were even lower for KS than for nIHH (36 ± 3 *vs.* 53 ± 7 pg/ml; $P = 0.02$).

The mean MIS level, which was available in only 18 patients (5 KS and 13 nIHH), was 6.4 ± 1.2 ng/ml, higher than that in normal adult men (48). A significant difference among those with and without pubertal development was demonstrated (2 ± 0.5 *vs.* 9.8 ± 1.4 ng/ml; $P < 0.001$; Fig. 1). MIS correlated negatively with TV ($r = -0.55$; $P < 0.02$; Fig. 3).

Neuroendocrine studies. The majority (75%) of IHH patients failed to exhibit any detectable LH pulses, indicating lack of GnRH pulse activity at the time of assessment, whereas the remainder (25%) demonstrated abnormal, but detectable, LH pulse activity. Among the 25% with pulsatile LH activity, 13 had normal LH pulse frequency with low amplitude, 3 had low amplitude and low frequency, 1 had a normal amplitude but low frequency, and 2 had a pattern of LH secretion that, although within 2 SD of the normal range, was deemed pathological in the setting of hypogonadal T levels. Among the AHH subgroup, 5 of 6 displayed an apulsatile pattern of LH secretion. LH secretory activity was more frequently undetectable in KS than in nIHH (87% *vs.* 69%, respectively; $P < 0.05$; Fig. 4).

Eighty percent of men with an absence of puberty demonstrated an apulsatile LH pattern, whereas the remaining 20% demonstrated discernable LH activity. Among those with some degree of pubertal development, only 45% displayed detectable LH activity, whereas the remaining 55% exhibited apulsatile LH secretion (Table 2 and Fig. 4). In patients lacking pubertal development, the prevalence of an apulsatile LH pattern was similar in KS and nIHH.

Ninety-five percent of the familial cases had apulsatile LH patterns and no signs of puberty. Notably, the only familial case with some pubertal development demonstrated a detectable LH pulse pattern with low amplitude and normal frequency. All KS patients with KAL mutations had complete absence of GnRH secretion as exhibited by apulsatile patterns of LH secretion and, with the exception of one indi-

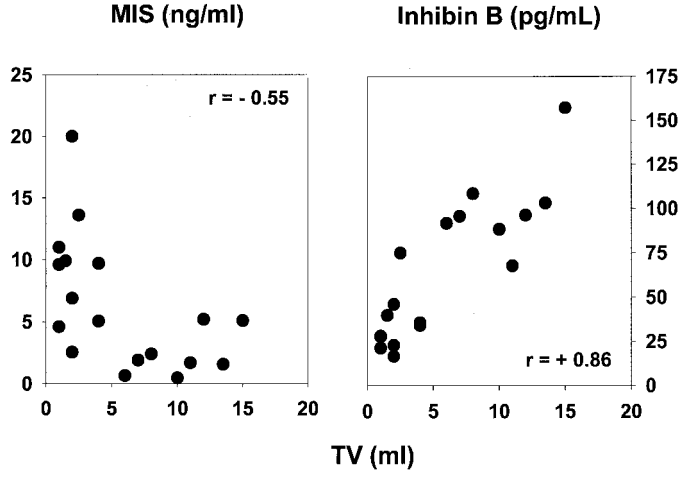
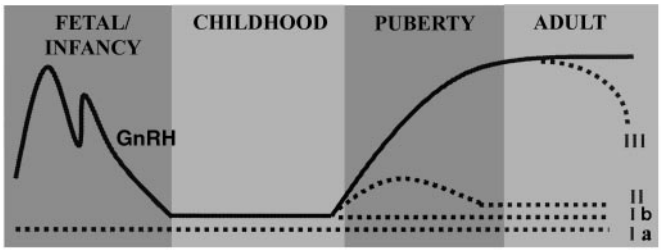


FIG. 3. Serum MIS and serum I_B plotted relative to TV in a subset of 18 IHH men with a spectrum of TV ranging from 1–15 ml.



GnRH deficiency	Complete		Partial	Adult-onset
	Ia	Ib	II	III
Testicular development	-	-	+	+ + +
Cryptorchidism	+/-	-	-	-
Microphallus	+/-	-	-	-
Inhibin B level	+/-	+	+ + +	++
MIS level	+ + +	+ + +	-	-

FIG. 4. Schematic of the activity of the HPG axis across the life cycle among normal males (*black line*) and IHH patients (*hashed line*). Ia, IHH patients who never activate the HPG axis; Ib, IHH patients who fail to reinitiate the program at puberty; II, IHH patients who display a failure of HPG axis during pubertal development; III, AHH patients with completion of puberty before HH.

vidual, undetectable gonadotropins. Of interest, this subject had a missense mutation Phe^{517}Leu in the third fibronectin type III-like repeat, whereas the other X-linked KS patient harbored deletions with alteration of splicing, frameshift, or stop codons resulting in synthesis of a truncated anosmin protein (39, 40).

Testicular morphology. Testicular biopsies were performed in 26 of 78 patients, 22 of whom showed immature testes characterized by tubules with no lumen, and small tubular diameter (0.05–0.08 μm). Variable numbers of spermatogonia with no evidence of spermatogenesis and absence of Leydig cells were also noted. This histological picture correlated completely with the clinical presentation of absent pubertal development, small TV (mean, 2.5 ml), and low I_B levels (40 pg/ml). Among these 22 patients, 5 exhibited unilateral cryptorchidism with reduction of spermatogonia within the cryptorchid testis. Of the remaining 4 patients of the 26 who had had a testicular biopsy, 2 who fulfilled criteria for the fertile eunuch subset had complete spermatogenesis on biopsy and normal I_B levels (mean, 254 pg/ml). However, few Leydig cells were present, agreeing with prior reports (45, 49). Finally, 2 patients presented with a variable degree of spermatogenic arrest. The first had KS with microphallus, unilateral cryptorchidism, and prepubertal testes (TV, 1.5 ml) and had prior therapy with T. His I_B level was 15 pg/ml, and testicular histology revealed immature Sertoli cells and maturational arrest at the level of the primary spermatocytes. The second patient had been diagnosed with nIHH, had had prior therapy with hCG, and had a TV of 7 ml, an I_B level of 70 pg/ml, and mature Sertoli cells but decreased number of germs cells, ranging from spermatogonia to primary spermatocytes with occasional presence of spermatids.

Discussion

This study further documents a remarkable diversity among IHH men on the basis of history, physical characteristics, mode of inheritance, and baseline biochemical and histological parameters (14–16, 19, 21, 23, 50). Such a detailed analysis of the classical subgroups of KS, nIHH, and AHH in a large cohort has not previously been reported. This study reveals that KS, in general, and X-KS, in particular, exhibit the most severe phenotype of GnRH deficiency, whereas nIHH spans a broader clinical spectrum. AHH, which is characterized by complete pubertal maturation with a subsequent failure of GnRH secretion (17), remains quite distinct from the other subgroups, with the exception of its similar biochemical parameters at presentation. In addition, the current data suggest that there is a definite phenotypic overlap between nIHH and KS. Therefore, stratifying this hypogonadotropic population according to the time of onset and the severity of GnRH deficiency provides a developmental perspective for viewing these syndromes and proves a more informative approach to understanding the clinical spectrum of IHH.

KS clearly represents the most complete form of GnRH deficiency. Indeed, the vast majority of KS patients in this cohort had a complete lack of sexual development, a nearly uniform lack of pulsatile GnRH-induced LH secretion, a high incidence of cryptorchidism (48%) and microphallus (26%), the lowest I_B levels, and histologically immature testes. Recent genetic, and pathological studies have shed some light on the pathophysiology of X-linked KS. Mutations in the KAL gene, encoding for a neural cell adhesion molecule, cause X-KS, the result of which is an arrest of GnRH and olfactory neuronal migration beyond the cribiform plate leading to the complete failure of activation of the HPG axis (25, 26, 51–53). However, X-KS comprises only a small percentage of KS cases (19, 40). Thus, as yet unidentified, autosomal genes are probably implicated in the complex developmental process of olfactory and GnRH neuronal migration.

In contrast, patients with nIHH display the widest clinical spectrum of IHH ranging from complete to partial GnRH deficiency. Approximately 60% of nIHH patients lie at the severe end of the phenotypic spectrum, displaying no pubertal development. Thus, with the exception of normal olfaction, they are clinically and biochemically indistinguishable from KS patients. However, 40% of men with nIHH give a history consistent with an arrest of pubertal development. Thus, this subset of nIHH have probably undergone some neonatal and pubertal activation of the HPG axis, as evidenced by partial testicular development, low prevalence of cryptorchidism, absence of microphallus, and higher I_B levels that are within the normal adult male range. At the mildest end of this spectrum lie the rare and interesting patients with the fertile eunuch variant of IHH (49) characterized by decreased virilization, eunuchoidal proportions but normal TV, preservation of spermatogenesis, and higher levels of I_B. The first autosomal gene described to cause nIHH, the GnRH-R gene, is of interest as complete resistance to GnRH was anticipated in these patients. Instead, a full clinical spectrum of nIHH has emerged depending on the degree of inactivation of the mutant receptor ranging from complete to partial forms, including the fertile eunuch variant (27–32, 54). It is expected that defects in genes regulating the biosynthesis and secretion of GnRH as well as the cascade of signaling

through the receptor may lead to varied GnRH deficiency/function and account for the large variability seen in nIHH.

By definition, AHH differs from KS and nIHH in that complete puberty and occasional fertility occur before the onset of complete HH, and therefore this group defines the milder end of the IHH spectrum. Having eliminated central nervous system neoplasms and infiltrative processes in these patients, the pathophysiology underlying AHH remains obscure except for the fact that 90% respond to exogenous GnRH (17). Thus, it is not clear whether AHH results from a genetic cause with a latent phenotype or is epigenetic. To date, no genetic defect has been demonstrated in AHH.

Given the degree of phenotypic overlap between the classical subsets of IHH, this study indicates that dividing the cohort according to the presence/absence of some evidence of prior pubertal development using the surrogate markers of testicular size and historical evidence of some degree of pubertal development allows a clearer discrimination between the subgroups. Although no significant difference in mean LH, FSH, and T levels was observed between the classical subgroups, mean gonadotropin levels were higher in subjects with some degree of spontaneous puberty, which might explain in part the spontaneous testicular growth among those with partial puberty. Although a higher frequency of apulsatile LH secretion was recorded among those who lack sexual development, discernable LH pulses (*i.e.* evidence of GnRH secretion) were evident in up to 20% of the subjects in this group. It is likely that the initiation of puberty requires a prolonged period of gonadotroph priming during which the requirements for GnRH stimulation are higher than those required to maintain the neuroendocrine axis postpubertally (55). Conversely, 50% of those IHH patients with some degree of prior spontaneous pubertal development and most patients with AHH did not exhibit any LH pulse at the time of the study. This finding suggests that in these patients, transient reactivation of the HPG axis during puberty had presumably occurred, but there was a subsequent failure of the GnRH secretory program thereafter. From these data, we conclude that mean gonadotropin levels and the current pattern of LH secretion are not reliable surrogate markers of the stage of sexual development. However, given the lack of an ultrasensitive LH assay in this study, there may have been a tendency to overestimate the apulsatile pattern of GnRH secretion.

In contrast, clinical (cryptorchidism and microphallus) and biochemical (I_B and MIS) markers of testicular growth and differentiation were able to discriminate between IHH men with and without puberty. Moreover, among those with prepubertal testes and no evidence of spontaneous puberty, these clinical markers in conjunction with serum I_B levels provide insight into the activity of the HPG axis during the feto/neonatal window. The frequency of cryptorchidism is high in IHH patients with no history of puberty, similar to that reported (50%) in some small series (18, 23, 50). In contrast, patients with some evidence of pubertal development have a very low incidence of cryptorchidism that approximates that of the general population [3–5% at birth and 1% by the age of 3 months (56)]. However, both cryptorchidism and microphallus lack sensitivity, as other factors may compensate for the lack of feto-neonatal T secretion. Indeed, in the complete androgen insensitivity syndrome, cryptorchidism is absent in 25% of patients (57). Maternal secretion of hCG may compensate for *in utero* GnRH/LH deficiency to some degree in IHH patients. In addition, the incidence of microphallus reported in IHH in this study may have been underestimated, given the potential for successful induction of phallus growth with prior androgen therapy during childhood (38) and reliance on patient recall of this event.

The Sertoli cell products, I_B and MIS, provide additional gonadal indicators of the onset and degree of GnRH deficiency. In the normal ontogeny of I_B secretion, serum levels increase during neonatal activation of the HPG axis (58, 59) and then decline, remaining easily measurable throughout childhood despite low levels of FSH stimulation. I_B levels normally increase during the early stages of puberty, reaching their peak long before adult levels of T are attained and remaining constant unless spermatogenesis is disrupted (60–62). In contrast, IHH men with absence of pubertal development display low/undetectable I_B levels (46, 47), well below those observed in normal childhood (58). These observations suggest that prior gonadotropin exposure, probably operating via stimulation of immature Sertoli cells, is a prerequisite for normal I_B secretion during the relatively hypogonadal state of childhood. Interestingly, men with X-linked KAL mutations and the most complete failure of activation of the HPG axis also exhibit the lowest I_B levels. Thus, I_B levels represent a surrogate marker of the activity of the reproductive axis during the fetal/neonatal period among those with no sexual maturation. In IHH men with some evidence of spontaneous puberty, I_B levels reach the normal range despite low gonadotropins, presumably reflecting a robust Sertoli cell proliferation initiated by activation of the HPG axis during the neonatal window and early puberty. Of note, in those men with normal TV, I_B levels are significantly lower in AHH than in the fertile eunuch variants. These data may reflect a disruption of spermatogenesis in AHH after the onset of HH (61, 62). Alternatively, the fertile eunuch variant may exhibit a larger Sertoli cell population, probably caused by unopposed FSH stimulation of immature Sertoli cells (61, 62). Finally, MIS, the first detectable secretory product of fetal Sertoli cells (37), also serves as a surrogate biochemical marker of whether puberty has occurred (48). MIS levels remain high throughout fetal and postnatal life and decline with terminal differentiation of Sertoli cells, the initiation of Leydig cell T secretion, and the onset of spermatogenesis (44). Accordingly, MIS levels in this study were highest among those with no pubertal development and low I_B levels and lowest in those with the less complete form of GnRH deficiency, as evidenced by larger testes, higher I_B levels, and probably some persistence of testicular T secretion.

In conclusion, although the classical clinical subdivisions of IHH are useful starting points for genetic inquiries, applying a developmental and genetic context to the evaluation of these patients is helpful in understanding the phenotypic variability of this heterogeneous group. Categorizing patients according to clinical parameters (prior pubertal development, cryptorchidism, and microphallus), testicular markers (I_B and MIS levels), and genetic defects has the advantage of providing insight into the time of onset and the severity

of the syndrome (Fig. 4). As such, those patients with the most severe form of GnRH deficiency will present with prepubertal testes, a high incidence of cryptorchidism and microphallus, undetectable/low I_B levels, and high MIS levels. In contrast, those with some pubertal development will present with some spontaneous testicular growth, I_B levels in the normal range, and low/undetectable MIS levels. It is likely that those IHH men with total inactivation of the HPG axis during the neonatal period will benefit from a therapy targeted at stimulating gonadal development preferentially, specifically Sertoli cell proliferation, before induction of puberty.

Acknowledgments

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