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Clinical Research Article

FSH and Sertoli Cell Biomarkers Accurately Distinguish Hypogonadotropic Hypogonadism From Self-limited Delayed Puberty

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Abstract

Context: Delayed puberty is a frequent complaint in males. The differential diagnosis between self-limited delayed puberty (SLDP) and congenital hypogonadotropic hypogonadism (CHH) is challenging. Commonly used endocrine tests, focusing on stimulated levels of LH or testosterone, are not satisfactory in making a diagnosis. Because FSH action on Sertoli cells results in testis enlargement and anti-Müllerian hormone (AMH) and inhibin B increased secretion, and the FSH-Sertoli cell axis function is detectable during normal childhood and early puberty, we tested whether the assessment of serum FSH, AMH, and inhibin B would be informative to distinguish between SLDP and CHH.

Design: We performed a prospective, nested case-control study in a cohort of male adolescents presenting with delayed puberty, comparing baseline serum reproductive hormone levels to identify predictive biomarkers of CHH, after having followed all participants prospectively until a final diagnosis was ascertained based on gold-standard criteria (age 18 years or ≥4 years after testis volume reached 4 mL).

Results: Of 65 participants who completed follow-up, 33 had a final diagnosis of SLDP and 32 of CHH. Serum FSH, AMH, and inhibin B showed better diagnostic efficiency than LH and testosterone for these differential diagnoses. FSH (IU/L)×inhibin B (ng/mL) < 92 and FSH (IU/L)×AMH (pmol/L) < 537 showed high sensitivity (>93%), specificity (≥92%), predictive values (>92%), and positive likelihood ratio (>12) for CHH. The diagnostic performance remained 89.7% and 88.2% for FSH × inhibin B and FSH × AMH, respectively, when analyzed in patients without red flags (micropenis, cryptorchidism, and/or microorchidism).

Conclusion: Serum FSH combined with inhibin B or AMH is highly predictive to accurately distinguish between SLDP and CHH in adolescent males.

Key Words: AMH, congenital hypogonadotropic hypogonadism, FSH, inhibin B, LH, self-limited delayed puberty, testosterone

Abbreviations: AMH, anti-Müllerian hormone; CHH, congenital hypogonadotropic hypogonadism; MRI, magnetic resonance imaging; SLDP, self-limited delayed puberty.

Delayed puberty in males refers to the absence of signs of pubertal maturation and the persistence of testicular volume <4 mL at 14 years of age and is distinguished from hypogonadism, which is characterized by the persistence of testicular volume <4 mL by the age of 18 years. Medical history, physical examination, and general routine tests can initially rule out primary (or hypergonadotropic) hypogonadism and functional or acquired hypogonadotropic hypogonadism (1, 2). Conversely, the differential diagnosis between the 2 remaining aetiologies, self-limited delayed puberty (SLDP) and congenital hypogonadotropic hypogonadism (CHH), is challenging (1, 3). SLDP, also known as constitutional delay of puberty, is considered a transient condition where testicular volume spontaneously reaches 4 mL between the ages of 14 and 18 years and full pubertal maturation occurs within 2 to 4 years. CHH is usually due to genetic conditions affecting GnRH secretion by the hypothalamic GnRH neurons or gonadotropin secretion by the pituitary gonadotropes in response to GnRH (4). GnRH neurons originate in the olfactory placode and migrate to the hypothalamus following the developing olfactory nerve.

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Defects in this migratory process explain the coexistence of CHH associated with hyposmia/anosmia, a condition known as Kallmann syndrome (5).

Delayed puberty affects approximately 2% of adolescents and is a frequent complaint in males because their physical appearance differs from that of their peers, including lack of secondary sex characteristics and shorter stature and body size, which affects their psychosocial well-being. SLDP is a variant of pubertal development, and the mainstay approach is "watchful waiting," whereas CHH needs lifelong hormone replacement (5). Deciding whether and/or when to suggest initiating sex steroid therapy is challenging (1, 3).

Puberty is triggered by the reactivation of the hypothalamic-pituitary-gonadal axis after childhood. GnRH stimulates pituitary secretion of LH and FSH. LH induces testicular Leydig cells to secrete testosterone, which drives the development of secondary sex characteristics, whereas FSH upregulates anti-Müllerian hormone (AMH) and inhibin B secretion (6). The hypothalamic-pituitary-gonadal axis is also active during fetal life, when testosterone induces testis descent to the scrotum and penile enlargement, and FSH promotes testicular Sertoli cell proliferation resulting in testis enlargement. This explains the existence of micropenis, cryptorchidism, and/or microorchidism, considered as red flag signs in newborns with CHH (6). The LH-Leydig cell axis remains active for 3 to 6 months after birth, when it turns quiescent for the rest of infancy and childhood. Conversely, the FSH-Sertoli cell axis does not completely wane, and FSH, AMH, and inhibin B are detectable in serum during childhood (7).

The "gold standard" for distinguishing SLDP from CHH is the occurrence of progressive pubertal maturation until its completion by the age of 18 years or by 4 years after testicular volume reached 4 mL when this occurred after the age of 14 years (8). However, the uncertainty of a diagnosis requiring long "watchful waiting" is problematic for these adolescents. Hormone determinations have been used for the differential diagnosis, focused on the pituitary-Leydig cell axis. However, serum LH and testosterone levels are undetectable or very low during childhood and the initial stage of puberty, requiring the use of provocative tests to induce LH secretion (9). Unfortunately, these tests have insufficient predictive value [reviewed in (3, 8)]. Because FSH action on Sertoli cells is detectable during this period, we hypothesized that the assessment of serum FSH, AMH, and inhibin B could be informative in distinguishing between SLDP, expected to feature normal hormone levels, and CHH, which should be characterized by low hormone levels.

To test our hypothesis, we conducted a prospective, longitudinal study of a cohort of male adolescents with delayed puberty and followed them until the age of 18 years, if testicular volume did not reach 4 mL, or for at least 4 years after testicular enlargement (≥4 mL) if it occurred between the ages of 14 and 18 years. When the final, gold standard-based diagnosis was reached, subjects were classified into SLDP or CHH (complete or partial), and the predictive performance of serum FSH, AMH, and inhibin B levels at the time of initial assessment was assessed.

Methods

Study Design and Setting

This validation study adhered to the Standards for Reporting of Diagnostic Accuracy Studies initiative. This was a prospective, analytical, case-control study, nested in a cohort of male adolescents presenting with delay of puberty in whom a differential diagnosis between SLDP and CHH was proposed. We compared baseline serum reproductive hormone levels to identify predictive biomarkers of CHH, after having followed all participants prospectively until the final diagnosis was clinically ascertained using gold-standard criteria. All male subjects referred for pubertal delay to the Division of Endocrinology of Ricardo Gutiérrez Children´s Hospital, a tertiary pediatric public center in Buenos Aires, Argentina, from July 2008 to September 2019 were eligible.

Ethical Issues

Research adhered to the Declaration of Helsinki and local regulations. The study protocol was approved by the Institutional Review Board of our institution (CEI 21.03.2007). All the participants and their guardians provided informed consent.

Participants

Inclusion criteria

All male patients aged ≥13 and <18 years with a testicular volume <4 mL were eligible. Subjects were included after informed consent.

Exclusion criteria

Boys with abnormal virilization (hypospadias or ambiguous genitalia), primary hypogonadism, anorchia, or monorchism; previous treatment with testosterone for pubertal delay; or history of surgery of the central nervous system, chemotherapy or radiotherapy, or any condition suspected to potentially affect the hypothalamic-pituitary-gonadal axis, were excluded.

Selection of participants and follow-up

At baseline, a complete medical examination was performed, including weight, height, testicular volume and position, and penile length for pubertal staging. Hematocrit, hemoglobin, glycemia, urea, creatinine, albumin, bilirubin, alanine aminotransferase, and aspartate aminotransferase were determined to assess general health status. Celiac disease was excluded clinically and by antitransglutaminase antibodies determinations. Cortisol, TSH, free and total T4, IGF-1, and prolactin were determined. LH, FSH, testosterone, AMH, and inhibin B determinations were used to assess the status of the hypothalamic-pituitary-testicular axis. Medical appointments and hormone measurements were scheduled every 6 months until a final diagnosis of SLDP or CHH was made.

Outcome Measures and Definitions

Reference standard

SLDP was clinically ascertained when the volume of both testes was ≥4 mL at ≤18 years and attained ≥15 mL within 4 years of testicular volume 4 mL. A diagnosis of CHH was made when testicular volume was <4 mL at age 18 years (complete CHH) or when testicular volume attained ≥4 mL between ages 14 and 18 years but did not reach 15 mL within 4 years (partial CHH). After complete follow-up of the cohort, patients with a final diagnosis of CHH were considered "cases" in this nested case-control analysis, while those with a final diagnosis of SLDP were considered "controls,"

given its characteristic of being a benign variant of pubertal development.

Index tests

The main outcomes were the serum levels of FSH, AMH, inhibin B, LH, and testosterone at the time of referral for pubertal delay. Hormone assays were performed using fresh samples, except for inhibin B, which was performed on frozen samples. Due to the prospective study design, the professionals responsible for hormone measurements at baseline (index tests) could not know the participant's final diagnosis (reference standard).

Secondary outcomes

Micropenis was defined as a penis length or width 2 SD below the mean for age, according to Argentine references (10). Cryptorchidism was the absence of at least 1 testis from the scrotum, and microorchidism was at least 1 testis with volume ≤1 mL. We measured testicular volume by comparison with Prader's orchidometer. Stature was determined using a wall-mounted stadiometer and weight using a calibrated scale. Body mass index was calculated as the weight in kilograms divided by the square of the stature in meters. Skeletal maturation was appraised by a left hand and wrist X-ray to estimate bone age as described by Greulich and Pyle (11). The existence of anosmia or hyposmia was self-reported by the participants. The presence of abnormal features in the olfactory system was assessed by magnetic resonance imaging (MRI) from the frontal sinus to the sphenoid sinus, with a slice thickness of 3 mm. MRI was performed at any time of follow-up when anosmia or hyposmia was self-reported, when there was a high suspicion or confirmation of hypogonadotropic hypogonadism, or when there was a diagnosis of other pituitary hormone deficiencies. Combined pituitary hormone deficiency was defined by the presence of 2 or more pituitary hormone deficiencies, including ACTH, gonadotropins, GH, or TSH, as previously described (12). When available, a genomic analysis was performed in patients with a diagnosis of CHH. Next-generation sequencing was performed using a NextSeq 500® system (Illumina) at the Translational Medicine Unit of the Buenos Aires Children's Hospital (Unidad de Medicina Traslacional, Hospital de Niños Ricardo Gutiérrez, Buenos Aires), as previously reported (13), or at the Broad Institute of MIT and Harvard (Cambridge, MA, USA), as previously published (14). For the processing of sequencing data, we followed the best practices recommendations from the Broad Institute using the Genome Analysis Toolkit. Variant filtering and prioritization were performed using the B_platform (https://www.bitgenia.com/b-platform/). Candidate variants were selected when minor allele frequency was <1% in gnomAD exomes and genomes and in 1000 Genomes. For further analysis, single nucleotide variants and indels with a read depth ≥10× and Genotype Quality score ≥45 and variants with high and moderate impact on protein were filtered. We classified the variants according to their potential pathogenicity using the American College of Medical Genetics and Genomics/Association for Molecular Pathology guidelines for variant interpretation (15) and following the ClinGen Sequence Variant Interpretation Working Group recommendations (https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation). Additionally, applying the copy number variation prediction tool from next-generation sequencing-derived data, detection of exon copy number variants, we screened for potential copy number variation-type variants in phenotype-related genes (16).

Other variables

Exposure to general health conditions that could induce transient or functional hypogonadism was ruled out through history, physical exam, and routine clinical chemistry studies. We also assessed stature, weight, body mass index, and skeletal maturation during follow-up.

Laboratory Measurements

AMH

Serum AMH was determined using an enzyme-linked immunoassay specific for human AMH (AMH/MIS EIA or AMH Gen II, Beckman-Coulter Co., Marseilles, France, RRID:AB_2800500), as previously validated (9, 17). Intra- and interassay coefficients of variation were, respectively, 10.5% and 9.4% for a serum AMH concentration of 700 pmol/L (98 ng/mL) and 11.1% and 12.8% for a serum AMH concentration of 7 pmol/L (0.98 ng/mL). When serum AMH levels were undetectable, a value of 1 pmol/L (0.14 ng/mL), corresponding to the limit of quantification (functional sensitivity), was attributed.

Inhibin B

Serum inhibin B was determined using an enzyme-linked immunoassay specific for human inhibin B (Inhibin B Gen II ELISA®, Beckman-Coulter Co., Prague, Czech Republic, RRID:AB_2827405), as previously validated (18). Intra-assay coefficients of variation were 14% for a serum inhibin B concentration of 111 pg/mL and 9.8% for 479 pg/mL. Interassay coefficients of variation were 14% for a serum inhibin B concentration of 12 pg/mL and 7.7% for 210 pg/mL. When serum inhibin B levels were undetectable, a value of 7.2 pg/mL, corresponding to the limit of quantification (functional sensitivity), was attributed.

Testosterone

Testosterone was determined in serum using an electrochemiluminescent immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) as described (9). Intra- and interassay coefficients of variation were 2.4% and 2.6%, respectively, for a serum testosterone concentration of 176 ng/dL (6.10 nmol/L) and 1.2% and 2.3% for a serum testosterone concentration of 455 ng/dL (15.78 nmol/L). When serum testosterone levels were undetectable, a value of 10 ng/dL (0.35 nmol/L), corresponding to the limit of quantification (functional sensitivity), was attributed.

Gonadotropins

LH and FSH were determined using electro-chemiluminescent immunoassays (Roche Diagnostics GmbH) as described (17). The limits of quantification of both LH and FSH assays were 0.10 IU/L, according to the Second National Institute for Biological Standards and Control International Standard 80/552 for LH and the Second World Health Organization International Reference Preparation 78/549 for FSH. Intra- and interassay coefficients of variation were 1.1% and 1.8%, respectively, for a serum LH concentration of 2.8 IU/L

and 1.4% and 1.5% for a serum LH concentration of 16.9 IU/L. Intra- and interassay coefficients of variation were 1.0% and 4.2%, respectively, for a serum FSH concentration of 14.8 IU/L and 1.1% and 4.1% for a serum FSH concentration of 23.4 IU/L. When serum LH or FSH levels were undetectable, the value of the limit of quantification (functional sensitivity = 0.1 IU/L) was attributed.

Statistical Analyses

All statistical procedures were performed using GraphPad Prism version 10.2.1 for Windows (GraphPad Software, San Diego, CA, USA). Data distribution was assessed for normality using the Shapiro-Wilks test. For comparison of continuous variables with normal data distribution between the 2 groups (SLDP and CHH), we used the parametric *t*-test and, in the case of nonnormal data distribution, the nonparametric Mann Whitney U test. For the analysis of categorical variables, we performed a chi-square test. For all the comparisons, the level of significance was set at $P < .05$.

Serum concentrations of reproductive hormone levels at baseline were compared between subjects with a final diagnosis of SLDP and those with CHH. Receiver operating characteristic curves for serum levels of AMH, inhibin B, FSH, LH, and testosterone were constructed to determine the optimal cutoff points for the diagnosis of CHH. Sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios with their respective 95% confidence intervals were calculated. Similar analyses were performed for the products FSH × AMH (serum FSH in IU/L multiplied by serum AMH in pmol/L) and FSH × inhibin B (serum FSH in IU/L multiplied by serum inhibin B in pg/mL). Sensitivity was the proportion of subjects with CHH having an index test result below the cutoff; specificity was the proportion of subjects with SLDP having an index test result above the cutoff; positive predictive value was the proportion of subjects with an index test result below the cutoff who had a final diagnosis of CHH; negative predictive value was the proportion of subjects having an index test result above the cutoff who had a final diagnosis of SLDP; positive likelihood ratio was the proportion of subjects with CHH having an index test result below the cutoff divided by the proportion of subjects with SLDP having an index test result below the cutoff; negative likelihood ratio was the proportion of subjects with CHH having an index test result above the cutoff divided by the proportion of subjects with SLDP having an index test result above the cutoff; and the diagnostic efficiency (or overall accuracy) was the proportion of true findings [eg, (number of subjects with CHH having an index test result below the cutoff + number of subjects with SLDP having an index test result above the cutoff)/total number of subjects with CHH or SLDP in whom an index test result was available at baseline].

The association between secondary outcomes of the study (the proportion of subjects with a history of micropenis, cryptorchidism, and/or microorchidism) and the diagnosis of CHH was analyzed using a chi-square test and calculating the odds ratios with their 95% confidence intervals.

Sample Size

The sample size needed to calculate sensitivity with a precision of 10% and estimating a true positive rate of 95% was 61 participants, using Stata (StataCorp LLC) and applying the following formula: $[z^2 \times (TP(1 - TP))/W^2]/P$, where z = 1.96, TP is the true positive rate, W is the precision, and P is the prevalence of CHH in the sample, estimated in 0.30.

Results

Participants

Of the 99 males referred for pubertal delay, 6 boys were not eligible (Fig. 1): 3 carried chromosome anomalies, 1 had congenital adrenal hyperplasia, 1 had previous exposure to high-dose glucocorticoids, and another had been treated with testosterone for pubertal delay. One boy refused to participate. From the 92 recruited participants, a blood sample for the determination of baseline reproductive hormones could not be obtained in 3 cases. Twenty-three participants did not return for the medical visits planned in the study protocol and needed for the ascertainment of the final diagnosis, and 1 withdrew his consent. Sixty-five participants who completed up to 9.6 years of follow-up needed to reach the gold-standard diagnosis were included in the analysis (Table 1): 33 had a final diagnosis of SLDP and 32 of CHH, 28 with a complete form and 4 with a partial form (Fig. 1). Fifteen of the 32 participants with CHH had an isolated form of normosmic CHH, with no other pituitary deficiency (Table 1). Treatment with testosterone for the induction of pubertal changes was performed temporarily in 10 of 33 patients with SLDP and permanently in the 32 patients with CHH.

At baseline, testicular volume was similar in patients with SLDP and with CHH. Hyposmia/anosmia and/or abnormal findings in the olfactory tract at MRI were found in about one-third of the individuals with CHH and in none of those with SLDP. Combined pituitary hormone deficiencies were observed in 6 patients with CHH and 1 with SLDP. Genetic studies, available in 15 patients with CHH, detected pathogenic or likely pathogenic gene variants associated with CHH in 9 cases: in *FGFR1* in 2 patients, in *CHD7* and *HESX1* in 1 patient, and in *ANOS1*, *GNRHR*, *LHX4*, *PROK2*, *PROKR2*, or *TUBB3* in 1 patient each (Table 2). No pathogenic or likely pathogenic gene variants associated with CHH were found in the patients with SLDP.

Clinical Genital Features

As expected, micropenis, cryptorchidism, or microorchidism was frequent in subjects with CHH (Table 3). Of note, 56% had a history of cryptorchidism and 47% of micropenis, and 44% showed both clinical features, while small testes before pubertal age occurred in 34% of the cases. The odds of having CHH as compared to SLDP was 28.2 in individuals presenting with micropenis, 9.3 in patients with cryptorchidism, and 8.1 in boys with microorchidism. The combinations did not add any relevant effect over the single clinical signs (Table 3).

Reproductive Axis Hormone Levels

Clear differences were observed between the SLDP and the CHH groups in the trajectories of testicular volume and serum gonadotropins, testosterone, and AMH during follow-up (Fig. 2). Testicular volume reached ≥15 mL in all subjects with SLDP and did not exceed 6 mL in those with CHH. At referral, median serum LH, FSH, AMH, and inhibin B were lower in patients with CHH than in those with SLDP, while no relevant differences were observed in serum testosterone, which was below the limit of detection in most of the cases

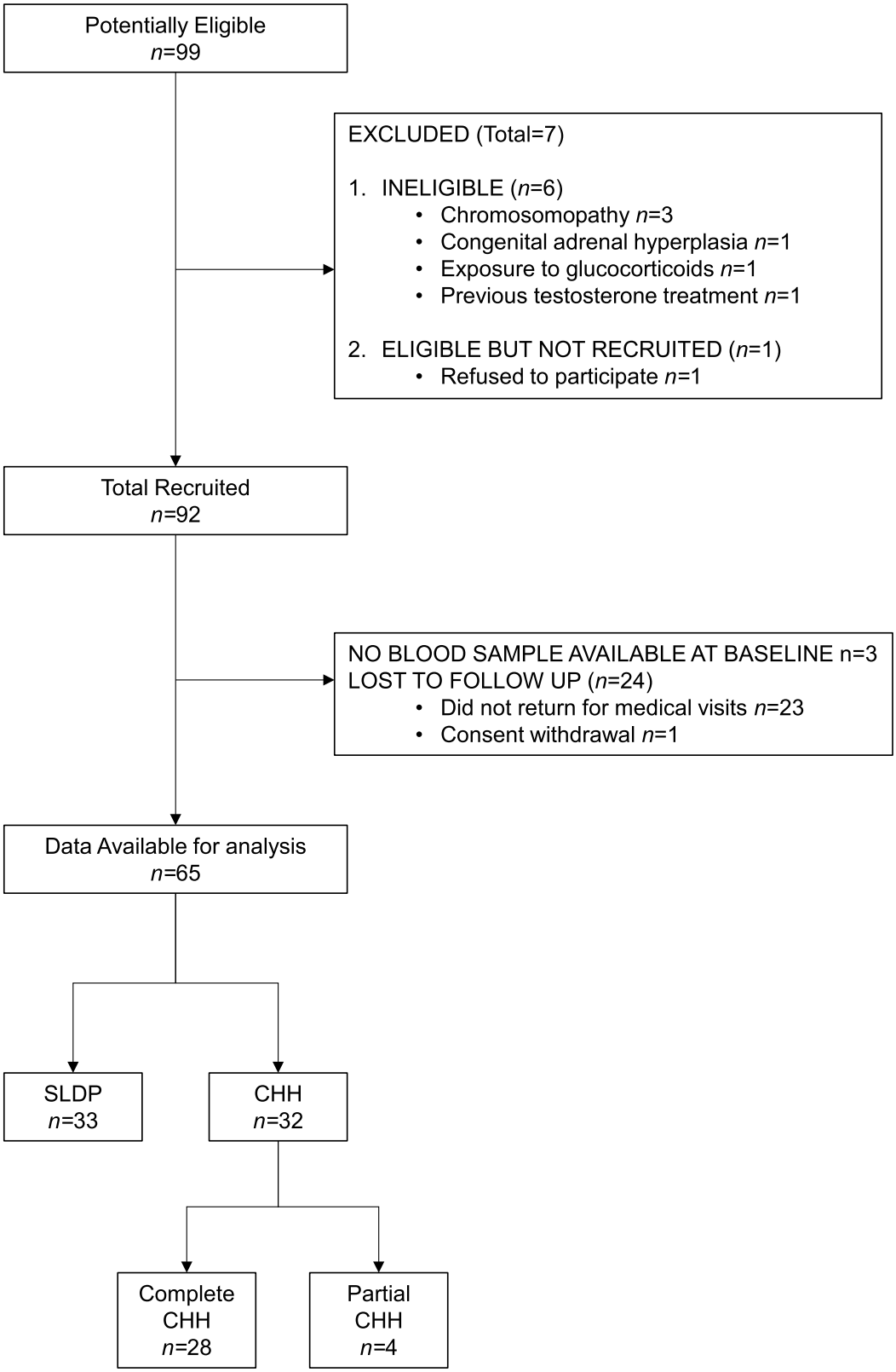

Figure 1. Flow diagram of the study.

(Table 1 and Fig. 3). Receiver operating characteristic curves were constructed to assess the diagnostic performance of gonadotropins and testicular hormones for distinguishing between SLDP and CHH (Fig. 3). FSH, AMH, and inhibin B showed higher areas under the curve than LH and testosterone, resulting in better sensitivity, specificity, predictive values, positive likelihood ratio, and overall efficiency for the diagnosis of CHH (Table 4). The most efficient cutoff values

Table 1. Characteristics of the study participants and serum hormone levels at baseline

	n	SLDP	n	CHH	p	n	iCHH	p
Age at recruitment (yr)[a]	33	14.2 (13.0-16.5)	32	15.3 (13.0-17.8)	NA	15	15.3 (13.3-17.8)	NA
Age at diagnosis ascertainment (yr)[a]	33	17.7 (15.4-20.9)	32	18.8 (18.0-22.5)	NA	15	18.6 (18.0-22.4)	NA
Follow-up (yr)[a]	33	3.3 (1.9-6.7)	32	4.1 (0.6-9.6)	NA	15	4.3 (1.8-9.6)	NA
Weight (kg)[a]	33	48.3 (25.6-87.6)	32	52.0 (30.0-78.0)	NA	15	58.0 (35.6-78.0)	NA
Height (cm)[a]	33	147.5 (133.0-162.5)	32	157.2 (128.0-170.8)	NA	15	156.0 (140.0-170.8)	NA
BMI (kg/m^2)[a]	33	21.6 (14.0-35.4)	32	20.1 (16.4-31.3)	NA	15	22.2 (16.3-31.3)	NA
Average testicular volume (mL)[a]	33	2.5 (1.0-3.0)	32	2.0 (1.0-3.0)	NA	15	2.0 (1.0-2.3)	NA
BA (yr)[a]	30	13.0 (10.0-14.5)	31	13.5 (9.0-16.0)	NA	14	13.3 (11.5-15.0)	NA
BA-CA (yr)[a]	30	−1.7 (−4.0 to −0.1)	31	−2.3 (−6.6 to −0.5)	NA	14	−1.9 (−3.7-0.5)	NA
Anosmia/hyposmia, n (%)	31	0 (0.0)	29	9 (31.1)	NA	NA	NA	NA
Abnormal olfactory system at MRI, n (%)	6	0 (0.0)	25	8 (32.0)	NA	NA	NA	NA
CPHD, n (%)	33	1 (3.1)[b]	32	6 (18.7)[c]	NA	NA	NA	NA
Confirmed genetic diagnosis (CHH genes)	29.	0 (0.0)	15	9 (60.0)	NA	6	4 (66.7)	NA
Delayed puberty in mother or father (%)	33	13 (39.4)	32	8 (26.7)	NA	15	1 (6.7)	0.0045
LH (IU/L)[a]	32	0.6 (0.1-3.3)	31	0.1 (0.1-1.5)	0.0002	15	0.1 (0.1-1.5)	0.0045
FSH (IU/L)[a]	31	2.5 (0.3-11.3)	31	0.6 (0.2-2.4)	<0.0001	15	0.6 (0.2-2.4)	<0.0001
Testosterone (ng/dL)[a]	33	10.0 (10.0-39.0)	31	10.0 (10.0-43.0)	0.12	15	10.0 (10.0-23.0)	0.46
AMH (pmol/L)[a]	33	396 (136-1115)	32	172 (26-686)	<0.0001	14	154 (26-462)	<0.0001
Inhibin B (pg/mL)[a]	30	84 (37-218)	31	21 (10-141)	<0.0001	15	21 (10-64)	<0.0001
FSH (IU/L)×AMH (pmol/L)[a]	31	1211 (93-4478)	31	101 (12-903)	<0.0001	14	120 (11-577)	<0.0001
FSH (IU/L)×inhibin B (pg/mL)[a]	27	211.6 (11.7-767.1)	30	14.6 (2.1-217.1)	<0.0001	15	14.5 (2.4-118.8)	<0.0001

P-values, calculated for the main and secondary outcome measures, represent the statistical significance after performing a Mann-Whitney test between SLDP and CHH or iCHH.
Abbreviations: AMH, anti-Müllerian hormone; BA, bone age; BMI, body mass index; CA, chronological age; CHH, congenital hypogonadotropic hypogonadism; CPHD, combined pituitary hormone deficiency; iCHH, isolated normosmic congenital hypogonadotropic hypogonadism; MRI, magnetic resonance imaging; NA, not applicable; SLDP, self-limited delayed puberty.
[a]Data are presented as median and range.
[b]GH deficiency (n = 1).
[c]GH + ACTH + TSH deficiencies (n = 4), TSH deficiency (n = 1), ACTH deficiency (n = 1).

did not change for LH, FSH, testosterone, and AMH when the whole cohort of patients with CHH or only those with complete forms were analyzed. For inhibin B, the best cutoff was lower for the complete forms of CHH, and the diagnostic performance seemed slightly better. The combinations of FSH and AMH or inhibin B improved the differential diagnosis capacity (Table 4). The diagnostic efficiencies of FSH × AMH and FSH × Inhibin B were exceptionally high, between 93.5% and 95.9% (Table 4 and Fig. 3), with very satisfactory performances for all parameters: sensitivity (>93%), specificity (≥92%), predictive values (>92%), and positive likelihood ratio (>12). As expected, cutoff values were lower for complete forms than for the whole cohort of CHH, but the diagnostic performances were overall similar. Because the differential diagnosis is more challenging between SLDP and isolated CHH, we performed all comparisons again after excluding patients with hyposmia/anosmia or associated pituitary hormone deficiencies (Table 4). The diagnostic efficacies of FSH × AMH and FSH × Inhibin B remained similarly high.

Also, considering that the high diagnostic efficacy for CHH could be overestimated by the inclusion of the subgroup of patients with red flag signs (micropenis, cryptorchidism, and/or microorchidism), we analyzed the diagnostic performance of FSH × AMH and FSH × Inhibin B in patients with or without red flag signs. The differences between CHH and SLDP were as notorious in patients with or without red flags (Supplementary Table S1) (19). Similarly, although the performance of hormone levels was better in patients with red flags, the performance of biochemical testicular markers was satisfactorily high (between 88.2% and 89.7%) in the absence of red flags and clearly higher than the performance of red flags alone (Supplementary Table S2) (19). Accordingly, the areas under the curve were very high (≥0.90) even in patients without red flags (Supplementary Figure) (19). Altogether, this means that the use of the biochemical markers FSH × AMH and FSH × Inhibin B improves the diagnostic procedure in patients with or without red flags.

Discussion

In this study of male adolescents with delayed puberty, using stringent gold-standard criteria for the diagnosis of CHH after exhaustive longitudinal follow-up, we found that biomarkers of the FSH-Sertoli cell axis are highly predictive to distinguish between SLDP and CHH with accuracy and precision at the initial consult. Prior studies focused on LH levels to attempt to differentiate between SLDP and CHH. Because serum LH is very low or undetectable in normal boys until the onset of puberty (9), random LH determination has proven ineffective for the differential diagnosis (20), and different stimulation tests with native GnRH or GnRH analogs have failed to identify a universally accepted LH level cutoff to distinguish between SLDP and CHH (8).

Table 2. Detailed information on the gene variants found by NGS in male patients presenting with delayed puberty in whom a final diagnosis of central hypogonadotropic hypogonadism was reached after sufficient follow-up

Data set ID	NGS method	Gene(s)	Transcript ID	HGVS or ISCN	Variant type	Zygosity	Inheritance	Familial segregation	Previous reports	ACMG/AMP classification (criteria applied)
97	WES	*ANOS1, FAM9A, VCX3B*	NA	arr[hg19] Xp22.31 (8184272x1,8235644-8878782x0,8907483x1)	CNV (643Kb deletion in Xp22.31)	Hemizygous	XLR	NA	Novel	Pathogenic
70	WES	*CHD7*	NM_017780.4	c.5405-17G>A; p.(His1801_Gly1802insAspGlyHisGlyThr)	Splice site	Heterozygous	AD	De novo	PMID: 29 255 276	Pathogenic (PS3/PM1/PM2/PM6/PP3/PP5)
		HESX1	NM_003865.3	c.350A>C; p.(Gln117Pro)	Missense	Heterozygous	AD, AR	Mother heterozygous	PMID: 17315526	Likely Pathogenic (PM1/PM2/PP2/PP3)
96	WES	*FGFR1*	NM_023110.2	c.606A>T; p.(Arg202Ser)	Missense	Heterozygous	AD	Mother heterozygous	Novel	Likely pathogenic (PM1/PM2/PP2/PP3)
86	TSO	*FGFR1*	NM_023110.2	c.2144A>T; p.(Glu715Val)	Missense	Heterozygous	AD	Mother heterozygous	Novel	Likely pathogenic (PM1/PM2/PP2/PP3)
24	TSO	*GNRHR*	NM_000406.3	c.416G>A; p.(Arg139His)	Missense	Homozygous	AR	Mother and father heterozygous	PMID: 11397871	Pathogenic (PM1/PM2/PM5/PS3/PP2/PP3)
105	smMIP Custom Panel	*LHX4*	NM_033343.4	c.611G>T; p.(Trp204Leu)	Missense	Heterozygous	AD	Father WT; mother NA	This patient in PMC8208670	Likely pathogenic (PM1/PM2/PP2/PP3)
41	TSO	*PROK2*	NM_001126128.2	c.163del; p.(Ile55Ter)	Nonsense	Heterozygous	AD	NA	PMID: 17959774	Pathogenic (PVS1/PS3/PM1)
67	WES	*PROKR2*	NM_144773.4	c.868C>T; p.(Pro290Ser)	Missense	Heterozygous	AD	Mother WT; father NA	PMID: 29161432; 18826963	Pathogenic (PM2_Supp/PS3/PP3_Strong/PM1)
103	Custom Panel (Twist)	*TUBB3*	NM_006086.4	c.1228G>A; p.(Glu410Lys)	Missense	Heterozygous	AD	Mother WT; father NA	PMID 20074521; 28299356; 23378218	Pathogenic (PM1/PM2_supp/PS2/PS3/PS4/PP2/PP3/PP5)

Abbreviations: ACMG, American College of Medical Genetics and Genomics; AD, autosomal dominant; AMP, Association for Molecular Pathology; AR, autosomal recessive; HGVS, Human Genome Variation Society; ISCN, International System for Human Cytogenomic Nomenclature; NA, not available; NGS, next-generation sequencing; TSO, TruSight One® clinical panel; WES, whole exome sequencing; WT, wild type; XLR, X-linked recessive.

Table 3. Comparison of the prevalence of genital features at baseline between subjects with SLDP (n = 33) and those with CHH (n = 32)

	SLDP n (%)	CHH n (%)	χ^2	*P*-value	OR	95% CI
Micropenis	1 (3.0)	15 (47.0)	16.8	<.0001	28.2	3.4-232.4
Cryptorchidism	4 (12.0)	18 (56.0)	14.1	.0002	9.3	2.7-32.8
Microorchidism	2 (6.0)	11 (34.0)	8.1	.0043	8.1	1.6-40.4
Micropenis + Cryptorchidism	1 (3.0)	14 (44.0)	15.2	<.0001	24.9	3.0-205.2
Micropenis + Microorchidism	0 (0.0)	7 (22.0)	8.1	.0045	19.7	1.1-361.3
Cryptorchidism + Microorchidism	1 (3.0)	7 (22.0)	5.3	.0208	9.0	1.0-77.7

Abbreviations: CHH, congenital hypogonadotropic hypogonadism; CI, confidence interval of odds ratio; OR, odds ratio; SLDP, self-limited delay of puberty.

In contrast to LH, FSH levels remain clearly detectable in males throughout the prepubertal period (9). Few previous studies suggested that FSH, AMH, and/or inhibin B could be helpful for the diagnosis of CHH (21-26). However, most were retrospective (22-25), lacked a clear operational definition or ascertainment of the reference standard (22-24), or enrolled participants with initial signs of puberty (27) or with acquired hypogonadotropic hypogonadism (25). Our prospective work provides strong evidence, based on a sufficiently large cohort longitudinally followed until a conclusive differential diagnosis was reached, that basal FSH combined with inhibin B or AMH has a high diagnostic efficacy (>93%), without the need for cumbersome and expensive provocative tests.

Congenital cryptorchidism, micropenis, and microorchidism are considered red flags for the diagnosis of CHH (20). As expected, our results show a history of cryptorchidism and micropenis in approximately half of the subjects with CHH and testes <1 mL in about one-third of the cases. A clear association was evident between any of these clinical signs and CHH, based on high odds ratios. However, the combination of 2 or more signs did not increase the probability of CHH. While cryptorchidism was the most frequently observed sign in patients with CHH, micropenis was more strongly associated. This may be due to the fact that penile length is mostly dependent on fetal and neonatal androgen levels (28), whereas testicular descent is more multifactorial (29). Smell impairment and/or imaging defects in the olfactory tract, typical of Kallmann syndrome, were found in almost one-third of the subjects with CHH. It is clear that these clinical red flags should be sought in males with delayed puberty because they are helpful for a differential diagnosis. However, although they were identified many decades ago, their predictive value may be insufficient to drive a clinical decision.

Patients with micropenis, cryptorchidism, and/or microorchidism are likely to have more severe forms of CHH. As expected, the performance of biomarkers of the FSH-Sertoli cell axis was better in patients with at least 1 red flag. However, in the absence of these, the performance of the biomarkers was satisfactorily high and clearly higher than the performance of red flags alone, underscoring the clinical value of assessing FSH and inhibin B or AMH to distinguish between CHH and SLDP.

Next-generation sequencing technologies emerged as a promising tool for the early diagnosis of patients with suspected CHH, with a progressively increasing list of candidate genes (30). In our series, DNA was available in 15 patients with CHH, and causative variants were present in 9 of them. Although the use of genomic strategies has significantly contributed to improvement in the diagnosis and the understanding of the underlying pathophysiology, their diagnostic efficacy remains below 60% (30-32).

The main strengths of our study are its prospective design and the longitudinal follow-up of participants until the gold standard for the diagnosis of SLDP or CHH was reached. Most retrospective case-control studies include patients older than 14 years of age in whom the final diagnosis was already known before the results were analyzed (21, 24, 25, 33-35). Our study recruited adolescent males from the age of 13 years, and some had to be followed every 6 months up to 9.6 years before a final diagnosis could be established on the basis of a rigorous reference standard. The clinical onset of puberty in boys occurs at a mean age of 11.5 to 11.8 years (36) and, although 14 years is the age universally used for the definition of delayed puberty, most boys are already concerned and seek medical attention when they lack pubertal signs by 12 to 13 years of age. Delaying the diagnosis and the decision to initiate hormone replacement may prove stressful for adolescents and have a negative impact on their growth spurt and bone mass accrual. Our study sample adequately represents the population usually referred for a differential diagnosis between SLDP and CHH. Furthermore, studies including only patients >14 years may have a recruitment bias toward males with CHH. On the other hand, some published studies have based their diagnosis of SLDP on the appearance of pubertal signs without follow-up to full pubertal maturation (23, 37). In contrast, we adopted a stringent requirement of testis volume ≥15 mL in order to avoid classifying patients with partial CHH as having SLDP (35). Other studies ended the follow-up at the age of 18 years (26, 27, 38); although this may seem sufficiently long, it could result in misclassification of the diagnosis in patients starting with pubertal signs after the age of 14 and in whom puberty was not complete by the age of 18.

Our study has some limitations. The sample of eligible participants most probably reflects the population of male adolescents reaching pediatric endocrinologists in tertiary hospitals, rather than the general population of adolescents not starting puberty at the age of 13. This probably explains why the proportion of participants with final diagnoses of SLDP and CHH was approximately 50% each, when in the general population SLDP accounts for 63% to 69% of the causes of delayed puberty (2, 39). This does not undermine the applicability of our results. Indeed, our conclusions are pertinent to adolescents who finally undergo medical evaluation by a specialist in pediatric endocrinology. Those who can be initially diagnosed by a pediatrician or the general practitioner, and are not referred to

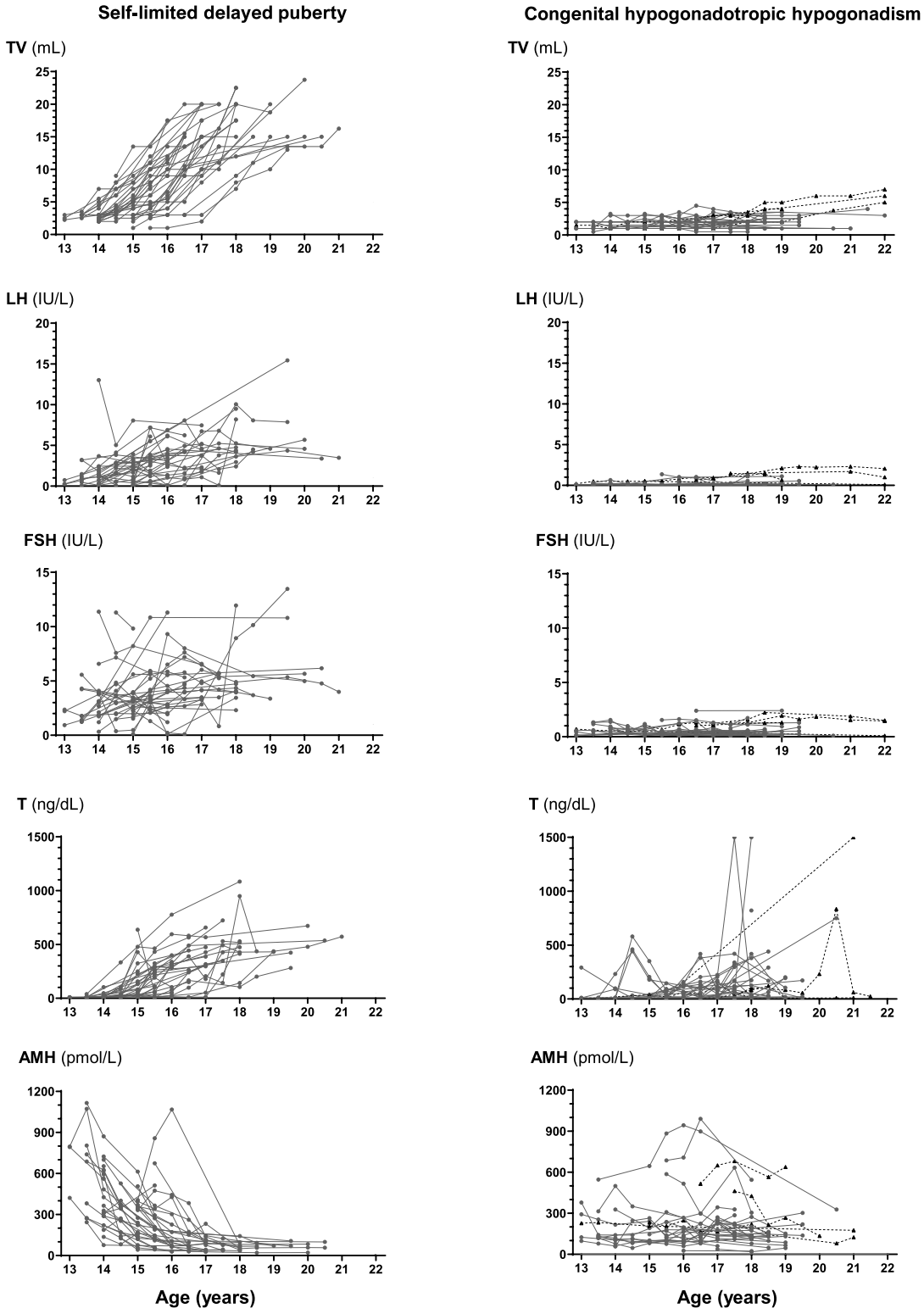

Figure 2. Trajectories of TV and serum LH, FSH, testosterone, and AMH in individuals with self-limited delay of puberty or CHH (complete CHH in grey and partial CHH in black dashed line).

Abbreviations: AMH, anti-Müllerian hormone; CHH, congenital hypogonadotropic hypogonadism; TV, testicular volume.

a specialist, do not represent the problematical population for the differential diagnosis between CHH and SLDP. Another unexpected fact was the low proportion of patients with partial forms of CHH in our cohort. The prospective design of our study with a consecutive enrollment of all boys presenting with delayed puberty should guarantee the representativity of our sample as regards the whole spectrum of males with delayed puberty. However, we cannot exclude that individuals lost during follow-up were more likely to have SLDP or milder forms of partial CHH.

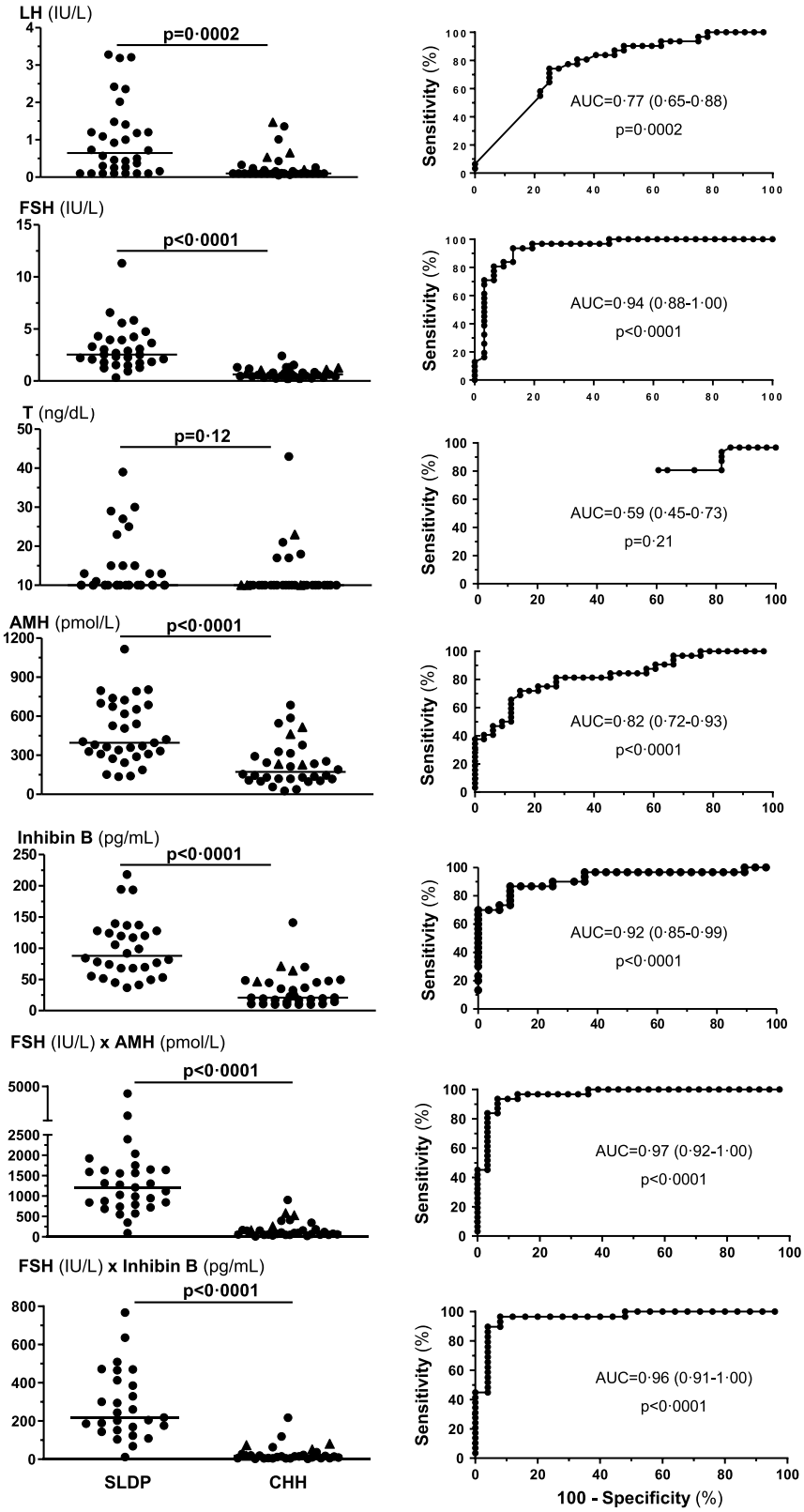

Figure 3. Serum hormone levels at baseline in participants with a final diagnosis of self-limited delayed puberty or congenital hypogonadotropic hypogonadism (circles for complete forms and triangles for partial forms) and receiver operating characteristic curves.

Abbreviations: AUC, area under the curve.

In conclusion, in male adolescents presenting with no clinical signs of pubertal onset, biomarkers reflecting a low activity of the FSH-Sertoli cell axis, such as low serum FSH combined with low inhibin B or AMH, have a high efficacy to distinguish CHH from SLDP with accuracy and precision at the time of referral, without the need for an invasive

Table 4. Diagnostic performance of serum hormone levels and its combinations

		Cutoff	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%)	NPV (%)	+ LR	Diagnostic efficacy (%)
LH (IU/L)	All CHH	<0.35	80.7 (62.5-92.6)	65.63 (46.8-81.4)	69.4	77.8	2.4	73.0
	Complete CHH		88.9 (70.8-97.7)	65.6 (46.8-81.4)	68.6	87.5	2.6	76.3
	All iCHH		73.3 (44.9-92.2)	65.6 (46.8-81.4)	50.0	84.0	2.1	68.1
	Complete iCHH		83.3 (51.6-97.9)	65.6 (46.8-81.4)	47.6	95.4	2.4	72.1
FSH (IU/L)	All CHH	<1.4	93.6 (78.6-99.2)	87.1 (70.2-96.4)	87.9	93.1	7.3	90.3
	Complete CHH		92.6 (75.7-99.1)	87.1 (70.2-96.4)	86.2	93.1	7.2	89.7
	All iCHH		93.3 (68.0-99.8)	87.1 (70.2-96.4)	77.8	96.4	7.2	89.1
	Complete iCHH		91.7 (61.5-99.8)	87.1 (70.2-96.4)	73.3	96.4	7.1	88.4
T (ng/dL)	All CHH	<22	93.6 (78.6-99.2)	18.2 (7.0-35.5)	51.8	75.0	1.1	54.7
	Complete CHH		96.3 (81.0-99.9)	18.2 (7.0-35.5)	49.1	85.7	1.2	53.3
	All iCHH		93.3 (68.0-99.8)	18.2 (7.0-35.5)	34.1	85.7	1.1	41.7
	Complete iCHH		100.0 (73.5-100.0)	18.2 (7.0-35.5)	30.8	100.0	1.2	40.0
AMH (pmol/L)	All CHH	<328	81.3 (63.6-92.8)	72.8 (54.5-86.7)	74.3	80.0	3.0	76.9
	Complete CHH		85.7 (67.3-96.0)	72.7 (54.5-86.7)	72.7	85.7	3.1	78.7
	All iCHH		86.7 (59.5-98.3)	69.7 (51.3-84.4)	59.1	92.3	2.9	77.1
	Complete iCHH		91.7 (61.5-99.8)	69.7 (51.3-84.4)	52.4	95.8	3.0	75.6
Inhibin B (ng/mL)	All CHH	<66	90.0 (73.5-97.9)	75.9 (56.4-89.7)	79.4	87.5	3.7	82.8
	Complete CHH	<49	92.3 (74.9-99.1)	89.3 (71.8-97.7)	88.9	92.3	8.6	90.6
	All iCHH	<66	100.0 (76.8-100.0)	75.9 (56.5-89.7)	66.7	100.0	4.1	83.7
	Complete iCHH	<49	100.0 (71.5-100.0)	85.7 (67.3-96.0)	78.6	100.0	7.0	92.3
FSH × AMH	All CHH	<537	93.6 (78.6-99.2)	93.6 (78.6-99.2)	93.5	93.5	14.5	93.5
	Complete CHH	<480	96.3 (81.0-99.9)	93.6 (78.6-99.2)	92.9	96.7	14.9	94.8
	All iCHH	<537	93.3 (68.0-99.8)	90.3 (74.3-98.0)	87.5	96.7	9.6	93.4
	Complete iCHH	<480	100.0 (73.5-100.0)	93.6 (78.6-99.2)	85.7	100.0	15.5	95.3
FSH × Inhibin B	All CHH	<92	96.6 (82.2-99.9)	92.3 (74.9-99.0)	93.3	95.8	12.6	94.4
	Complete CHH	<66	96.0 (79.7-99.9)	96.0 (79.7-99.9)	96.0	95.8	24.0	95.9
	All iCHH	<92	100.0 (76.8-100.0)	92.3 (74.9-99.0)	87.5	100.0	13.0	95.0
	Complete iCHH	<66	100.0 (71.5-100.0)	96.0 (79.7-99.9)	91.7	100.0	25.0	97.2

Abbreviations: +LR, positive likelihood ratio; AMH, anti-Müllerian hormone; CHH, congenital hypogonadotropic hypogonadism; CI, confidence interval; iCHH, isolated normosmic congenital hypogonadotropic hypogonadism; NPV, negative predictive value; PPV, positive predictive value; T, testosterone.

stimulating test. The diagnostic efficacy is optimal in male adolescents with a history of micropenis, cryptorchidism, and/or microorchidism and remains high even in the absence of those red flag signs.

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Author Contributions

R.A.R., R.P.G., and F.C. conceptualized the study. R.A.R. and R.P.G. designed the methodology and were responsible for obtaining funding and overall project administration. S.C., L.C.B., P.B., M.G.B., G.S., A.K., H.C., A.J.A., G.F.A., M.G.R., I.B., R.A.R., and R.P.G. provided patient data. Y.M.C. and W.H. contributed to genetic analyses. S.C., R.A.R., and R.P.G. contributed to formal analysis, interpreted the data, and wrote the original draft. S.C., L.C.B., G.S., M.G.B., M.G.R., and R.P.G. accessed and verified the data. All authors had full access to the data, approved the final version, and had final responsibility for the decision to submit for publication.

Disclosures

The authors declare no competing interests.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

References

1. Howard SR, Dunkel L. Delayed puberty-phenotypic diversity, molecular genetic mechanisms, and recent discoveries. *Endocr Rev*. 2019;40(5):1285-1317.
2. Jonsdottir-Lewis E, Feld A, Ciarlo R, Denhoff E, Feldman HA, Chan YM. Timing of pubertal onset in girls and boys with constitutional delay. *J Clin Endocrinol Metab*. 2021;106(9):e3693-e3703.
3. Harrington J, Palmert MR. An approach to the patient with delayed puberty. *J Clin Endocrinol Metab*. 2022;107(6):1739-1750.

4. Argente J, Dunkel L, Kaiser UB, *et al.* Molecular basis of normal and pathological puberty: from basic mechanisms to clinical implications. *Lancet Diabetes Endocrinol*. 2023;11(3):203-216.
5. Young J, Xu C, Papadakis GE, *et al.* Clinical management of congenital hypogonadotropic hypogonadism. *Endocr Rev*. 2019; 40(2):669-710.
6. Rey RA, Grinspon RP, Gottlieb S, *et al.* Male hypogonadism: an extended classification based on a developmental, endocrine physiology-based approach. *Andrology*. 2013;1(1):3-16.
7. Grinspon RP, Urrutia M. The importance of follicle-stimulating hormone in the prepubertal and pubertal testis. *Curr Opin Endocr Metab Res*. 2020;14:137-144.
8. Harrington J, Palmert MR. Clinical review: distinguishing constitutional delay of growth and puberty from isolated hypogonadotropic hypogonadism: critical appraisal of available diagnostic tests. *J Clin Endocrinol Metab*. 2012;97(9):3056-3067.
9. Grinspon RP, Bedecarrás P, Ballerini MG, *et al.* Early onset of primary hypogonadism revealed by serum anti-Müllerian hormone determination during infancy and childhood in trisomy 21. *Int J Androl*. 2011;34(5pt2):e487-e498.
10. Anigstein CR. Longitud y diámetro del pene en niños de 0 a 14 años de edad. *Arch Argent Pediatr*. 2005;103(5):401-405.
11. Greulich WW, Pyle SI. *Radiographic Atlas of Skeletal Development of the Hand and Wrist*. Stanford University Press; 1959.
12. Braslavsky D, Grinspon RP, Ballerini MG, *et al.* Hypogonadotropic hypogonadism in infants with congenital hypopituitarism: a challenge to diagnose at an early stage. *Horm Res Paediatr*. 2015; 84(5):289-297.
13. Correa Brito L, Keselman A, Villegas F, *et al.* Case report: novel SIN3A loss-of-function variant as causative for hypogonadotropic hypogonadism in Witteveen-Kolk syndrome. *Front Genet*. 2024; 15:1354715.
14. Duckett K, Williamson A, Kincaid JWR, *et al.* Prevalence of deleterious variants in MC3R in patients with constitutional delay of growth and puberty. *J Clin Endocrinol Metab*. 2023;108(12): e1580-e1587.
15. Richards S, Aziz N, Bale S, *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424.
16. Fowler A, Mahamdallie S, Ruark E, *et al.* Accurate clinical detection of exon copy number variants in a targeted NGS panel using DECoN. *Wellcome Open Res*. 2016;1:20.
17. Grinspon RP, Ropelato MG, Bedecarrás P, *et al.* Gonadotrophin secretion pattern in anorchid boys from birth to pubertal age: pathophysiological aspects and diagnostic usefulness. *Clin Endocrinol (Oxf)*. 2012;76(5):698-705.
18. Lopez Dacal J, Prada S, Correa Brito L, *et al.* Testicular dysfunction at diagnosis in children and teenagers with haematopoietic malignancies improves after initial chemotherapy. *Front Endocrinol (Lausanne)*. 2023;14:1135467.
19. Castro S, Correa Brito L, Bedecarras P, *et al. Hypogonadotropic Hypogonadism Versus Self-Limited Delayed Puberty*. Consejo Nacional de Investigaciones Científicas y Técnicas; 2024. http://hdl.handle.net/11336/243074
20. Persani L, Bonomi M, Cools M, *et al.* ENDO-ERN expert opinion on the differential diagnosis of pubertal delay. *Endocrine*. 2021; 71(3):681-688.
21. Grinspon RP, Ropelato MG, Gottlieb S, *et al.* Basal follicle-stimulating hormone and peak gonadotropin levels after gonadotropin-releasing hormone infusion show high diagnostic accuracy in boys with suspicion of hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2010;95(6):2811-2818.
22. Adan L, Lechevalier P, Couto-Silva AC, *et al.* Plasma inhibin B and antimullerian hormone concentrations in boys: discriminating between congenital hypogonadotropic hypogonadism and constitutional pubertal delay. *Med Sci Monit*. 2010;16(11): CR511-CR517.
23. Binder G, Schweizer R, Blumenstock G, Braun R. Inhibin B plus LH vs GnRH agonist test for distinguishing constitutional delay of growth and puberty from isolated hypogonadotropic hypogonadism in boys. *Clin Endocrinol (Oxf)*. 2015;82(1):100-105.
24. Rohayem J, Nieschlag E, Kliesch S, Zitzmann M, Inhibin B. AMH, but not INSL3, IGF1 or DHEAS support differentiation between constitutional delay of growth and puberty and hypogonadotropic hypogonadism. *Andrology*. 2015;3(5):882-887.
25. Varimo T, Miettinen PJ, Kansakoski J, Raivio T, Hero M. Congenital hypogonadotropic hypogonadism, functional hypogonadotropism or constitutional delay of growth and puberty? An analysis of a large patient series from a single tertiary center. *Hum Reprod*. 2017;32(1):147-153.
26. Chaudhary S, Walia R, Bhansali A, *et al.* FSH stimulated inhibin B (FSH-iB): a novel marker for the accurate prediction of pubertal outcome in delayed puberty. *J Clin Endocrinol Metab*. 2021; 106(9):e3495-e3505.
27. Coutant R, Biette-Demeneix E, Bouvattier C, *et al.* Baseline inhibin B and anti-mullerian hormone measurements for diagnosis of hypogonadotropic hypogonadism (HH) in boys with delayed puberty. *J Clin Endocrinol Metab*. 2010;95(12):5225-5232.
28. Rohayem J, Alexander EC, Heger S, Nordenstrom A, Howard SR. Mini-Puberty, physiological and disordered: consequences, and potential for therapeutic replacement. *Endocr Rev*. 2024;45:460-492.
29. Elamo HP, Virtanen HE, Toppari J. Genetics of cryptorchidism and testicular regression. *Best Pract Res Clin Endocrinol Metab*. 2022; 36:101619.
30. Vezzoli V, Hrvat F, Goggi G, *et al.* Genetic architecture of self-limited delayed puberty and congenital hypogonadotropic hypogonadism. *Front Endocrinol (Lausanne)*. 2023;13:1069741.
31. Cassatella D, Howard SR, Acierno JS, *et al.* Congenital hypogonadotropic hypogonadism and constitutional delay of growth and puberty have distinct genetic architectures. *Eur J Endocrinol*. 2018; 178(4):377-388.
32. Amato LGL, Montenegro LR, Lerario AM, *et al.* New genetic findings in a large cohort of congenital hypogonadotropic hypogonadism. *Eur J Endocrinol*. 2019;181(2):103-119.
33. Zevenhuijzen H, Kelnar CJ, Crofton PM. Diagnostic utility of a low-dose gonadotropin-releasing hormone test in the context of puberty disorders. *Horm Res*. 2004;62(4):168-176.
34. Mosbah H, Bouvattier C, Maione L, *et al.* GnRH stimulation testing and serum inhibin B in males: insufficient specificity for discriminating between congenital hypogonadotropic hypogonadism from constitutional delay of growth and puberty. *Hum Reprod*. 2020;35(10):2312-2322.
35. Aung Y, Kokotsis V, Yin KN, *et al.* Key features of puberty onset and progression can help distinguish self-limited delayed puberty from congenital hypogonadotrophic hypogonadism. *Front Endocrinol (Lausanne)*. 2023;14:1226839.
36. Mouritsen A, Aksglæde L, Soerensen K, *et al.* The pubertal transition in 179 healthy danish children: associations between pubarche, adrenarche, gonadarche, and body composition. *Eur J Endocrinol*. 2013;168(2):129-136.
37. Mishra PK, Mishra I, Choudhury AK, *et al.* Accuracy of various tests alone and in combination to differentiate IHH from CDGP. *Indian J Endocrinol Metab*. 2022;26(2):160-166.
38. Chan YM, Lippincott MF, Sales Barroso P, *et al.* Using Kisspeptin to predict pubertal outcomes for youth with pubertal delay. *J Clin Endocrinol Metab*. 2020;105(8). e2717-e2725.
39. Sedlmeyer IL, Palmert MR. Delayed puberty: analysis of a large case series from an academic center. *J Clin Endocrinol Metab*. 2002; 87(4):1613-1620.