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Clinical Research Article

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FSH-stimulated Inhibin B (FSH-iB): A Novel Marker for the Accurate Prediction of Pubertal Outcome in Delayed Puberty

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Abbreviations: AUC, area under the curve; CDGP, constitutional delay in growth and puberty; FSH-iB, FSH-stimulated inhibin B; GnRHa, GnRH analogue; hCG, human chorionic gonadotropin; HH, hypogonadotropic hypogonadism; MRI, magnetic resonance imaging; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; SP, spontaneous puberty.

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Abstract

Background: Clinicians have long been struggling to find an effective tool to predict onset of puberty.

Objective: To explore stimulability of inhibin B after exogenous FSH and its potential role for prediction of onset of puberty.

Design and participants: Study subjects were enrolled into "exploratory cohort" (n = 42) and "validation cohort" (n = 19). The exploratory cohort was further divided into group 1 (healthy children with spontaneous puberty [SP], n = 26) and group 2 (patients with hypogonadotropic hypogonadism [HH], n = 16). The validation cohort included children who presented with complaints of delayed puberty.

Intervention and outcome: Participants were subjected to FSH stimulation test and GnRH analogue stimulation test. Cutoffs derived from the exploratory cohort for basal and FSH stimulated inhibin B (FSH-iB) were applied on the validation cohort. Basal LH, GnRH analogue-stimulated LH, basal inhibin B, and FSH-iB were compared with clinical outcomes on a prospective follow-up for prediction of onset of puberty.

Results: There was statistically significant increment in inhibin B after exogenous FSH in group 1 (SP) in both male (188.8 pg/mL; $P = 0.002$) and female (1065 pg/mL; $P = 0.023$) subjects. The increment was not statistically significant in group 2 (HH) in both sexes.

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FSH-iB at a cutoff of 116.14 pg/mL in males and 116.50 pg/mL in females had 100% sensitivity and specificity for labelling entry into puberty. On application of these cutoffs on the validation cohort, FSH-iB had 100% positive predictive value, negative predictive value, and diagnostic accuracy for prediction of pubertal onset.

Conclusion: Inhibin B was stimulable in both male and female subjects. FSH-iB can be considered a novel and promising investigation for prediction of onset of puberty. Future studies are required for further validation.

Key Words: inhibin B, FSH stimulated inhibin B, delayed puberty, constitutional delay in growth and puberty, hypogonadotropic hypogonadism

The onset of puberty at an appropriate time is important for optimal physiological growth and psychological wellbeing of a child. Delayed puberty is defined as the absence of signs of sexual maturation by age more than 2 to 2.5 SD values above the mean for the general population. Currently, puberty is said to be delayed if there is no thelarche by the age of 13 years in girls and no testicular enlargement by the age of 14 years in boys (1). Constitutional delay in growth and puberty (CDGP) is the most common etiology of delayed puberty, accounting for 65% of cases in boys and 30% cases in girls (2). Other etiologies include idiopathic hypogonadotropic hypogonadism, multiple pituitary hormone deficiencies, systemic illness, and hypergonadotropic hypogonadism. Systemic illness can be easily differentiated on the basis of clinical symptomatology, whereas the diagnosis of hypergonadotropic hypogonadism is evident on basal gonadotropin levels. However, differentiation between CDGP and hypogonadotropic hypogonadism (HH) poses a major clinical challenge. Although clinical and biochemical profiles of both are similar at presentation, management strategies are altogether different. Whereas CDGP warrants watchful observation to look for the onset of spontaneous puberty, HH requires timely initiation of the appropriate hormone replacement. Delay in the initiation of treatment in HH affects not only psychological wellbeing, but also impairs the acquisition of peak bone mass and fertility prospects (3). Unfortunately, there is a scarcity of reliable investigations to differentiate between CDGP and HH at the time of presentation. Therefore, presently, children are prospectively followed for the onset of puberty until the age of 18 years to differentiate between these two entities. This watchful waiting policy not only adds to the anxiety of parents and children, but also delays the timely initiation of treatment of HH.

Investigators have long sought a test that can predict the onset of puberty. Both basal levels of gonadotropins and gonadal steroids along with their response to respective exogenous stimuli have been investigated. Initially, nighttime gonadotropin pulses, basal LH, basal FSH, and basal gonadal steroids were studied. Because of the considerable overlap in basal hormone profiles in subjects with delayed puberty, dynamic tests came into existence. Various dynamic tests include the GnRH stimulation test, GnRH analogue (GnRHa) stimulation test and human chorionic gonadotropin (hCG) stimulation test. However, there is lack of uniformity in stimuli, protocols, and assays used in different studies; therefore, cutoffs presently available are variable with a wide gray zone. Evidence-based literature is still insufficient to recommend any of these tests as the "gold standard" for the routine clinical practice.

Inhibins are glycoprotein hormones belonging to the TGF-β superfamily. Inhibin B is composed of an α subunit and 1 βB subunit linked by disulphide bridge (4). It is produced by the Sertoli cells in males and granulosa cells of the small antral follicles in females (5). Inhibin B circulates at a low but measurable level in the prepubertal period and rises during late prepuberty or early puberty. Inhibin B secretion is driven by FSH in both males and females (6). The major physiological role of inhibin B is to exert negative feedback on the secretion of FSH (7). There is developmental change in physiology of inhibin B production during transition in puberty in males. In prepubertal boys, inhibin B originates from Sertoli cells only, whereas later it originates from cooperation of Sertoli cells and germ cells (8). Previous studies have shown that inhibin B rises after treatment with FSH in patients of hypogonadotropic hypogonadism over a period of 2 months to 2.8 years (9, 10).

Recently, basal inhibin B has emerged as a new test for the diagnosis of pubertal disorders. Individual studies have shown basal inhibin B to have good accuracy to predict the onset of puberty and hence differentiation of CDGP from HH. However, diagnostic thresholds given by different studies are variable and overlapping (11-17). Consequently, a single diagnostic cutoff for routine clinical practice is still not available. Unlike GnRH and GnRHa-stimulated LH, no studies have investigated for the stimulability of inhibin B from the gonads as a predictor for the onset of puberty. Therefore, the present study was undertaken to look for the stimulability of inhibin B by FSH, and, if found stimulable,

the potential role of FSH-stimulated inhibin B (FSH-iB) as a predictor of the onset of puberty.

This is a prospective interventional study conducted in the department of Endocrinology and Paediatrics, PGIMER Chandigarh, India. The study was approved by institutional ethics committee and the trial was registered under CTRI (registration no. CTRI/2019/10/021570).

Patients and controls

Healthy children with spontaneous onset of puberty, patients with hypogonadotropic hypogonadism, and children with delayed puberty were enrolled in the study after informed consent/assent. Patients of hypogonadotropic hypogonadism on treatment with hCG, acquired multiple pituitary hormone deficiencies (ie, after pituitary surgery), and hypergonadotropic hypogonadism were excluded from the study.

Study subjects were enrolled into either the "exploratory cohort" or "validation cohort."

Exploratory cohort

The exploratory cohort comprised healthy children with spontaneous puberty and patients of HH.

Group 1 (healthy children with spontaneous puberty [SP]) included healthy children with no known comorbidities and aged between 9 and 18 years (male) and 8 and 18 years (female) who were either clinically pubertal (ie, Tanner stage 2 or higher) or biochemically pubertal (ie, Tanner stage 1 but with triptorelin stimulated LH ≥14 IU/L) were enrolled.

Group 2 included patients with HH ≥18 years of age who were gonadotropin treatment naïve. Patients who had received sex steroids were included in the study only if they were off treatment for at least 3 months.

Validation cohort

Subjects who came with complaints of nondevelopment of secondary sexual characteristics between the age of 14 and 18 years (male) and 13 and 18 years (female) were enrolled in the validation cohort. Subjects with multiple pituitary hormone deficiencies (congenital) with delayed puberty were also included in the study for the evaluation of gonadotropin deficiency. Prospective follow-up of children in the validation cohort was done until age 18 years or the entry of the patient into puberty, whichever was earlier.

Relevant history pertaining to perinatal history, delayed milestones, radiation exposure, drug intake, family history of delayed puberty, and features suggestive of other hormonal deficiencies was recorded. Age, sex, height, weight, body mass index, height age, bone age, weight age, height SD score, weight SD score, upper segment, lower segment, upper segment/lower segment ratio, and Tanner stage (18, 19) were documented. Height was measured using a wall-mounted Harpenden stadiometer in Frankfurt plane. X-ray of the left forearm and hand was done for the bone age assessment at baseline by using modified the Greulich-Pyle hand and wrist radiographic atlas (20). Testicular volume was assessed clinically using the Prader orchidometer (21). A baseline hormone profile (pooled samples) including thyroid function test, prolactin, cortisol, LH, FSH, testosterone, and estradiol was done in all the patients. In addition, a complete blood count, biochemistry, bicarbonate, pH (if indicated), and celiac serology (if indicated) was done. Magnetic resonance imaging (MRI) of the brain focusing on the pituitary, hypothalamus, and olfactory placode was performed at baseline if clinically indicated. Ultrasound scan of the scrotum (for testicular volume) in boys and ultrasound scan of the ovaries in girls was done by a dedicated radiologist. All patients underwent an FSH stimulation test and triptorelin stimulation test per study protocol.

Cutoffs were derived for basal inhibin B, FSH-iB, and delta change in inhibin B from the exploratory cohort. Delta change in inhibin B was calculated by subtracting basal inhibin B value from FSH-stimulated inhibin B value (FSH stimulated inhibin B – basal inhibin B). Cutoff values derived from the exploratory cohort were applied on the subjects in the validation cohort at the time of enrollment into the study for the prediction of onset of puberty. Cutoffs used to predict the onset of puberty for basal LH and post-GnRH agonist-stimulated LH were ≥0.3 IU/L and ≥14 IU/L, respectively (12, 22). Tanner staging, LH, FSH, and testosterone/estradiol was documented at each visit. The clinical outcome of the validation cohort was evaluated at the end of the study and compared with the prediction of pubertal outcome at the time of enrollment. Tests used for the prediction of puberty were basal LH, GnRH agonist-stimulated LH, basal inhibin B, FSH-iB, and delta change in inhibin B.

Study protocols

FSH stimulation test

Injection FSH (lyophilized highly purified urofollitropin) 150 IU was given intramuscularly on 3 consecutive days (D1, D2, and D3) in female subjects between 8:00 AM and 9:00 AM. In male subjects, injection FSH 300 IU was given IM on alternate days (D1, D3, and D5). Pooled sample (2 samples taken 20 minutes apart) for LH, FSH, testosterone, estradiol, and inhibin B were drawn on D1 before FSH injection between 8:00 AM and 9:00 AM. A post-FSH stimulation sample was drawn 24 hours after the last injection (ie,

on D4 in females and D6 in males) between 8:00 AM and 9:00 AM. Samples were analyzed for LH, FSH, testosterone, estradiol, and FSH-iB. Dosage and schedule of FSH injection were decided according to the results of the pilot study (Supplementary Table 1) (23) in which 150 IU of injection FSH was given for 3 consecutive days in 2 males and 2 females. There was an increment in inhibin B post-FSH injection in all the subjects. However, the increment was lower in male subjects; therefore, the dose of injection FSH was increased to 300 IU and the stimulus was staggered over 5 days by giving alternate-day injections in male subjects.

Triptorelin stimulation test

Pooled sample (2 samples taken 20 minutes apart) for LH, FSH, testosterone, and estradiol were drawn at baseline between 8:00 AM and 9:00 AM. Injection triptorelin 0.1 mg/body surface area (maximum, 100 µg) was given subcutaneously, and samples were drawn at 4 hours for LH and FSH and at 24 hours for estradiol and testosterone. A triptorelin stimulation test was performed 5 days after the FSH stimulation test.

All patients were given a written schedule regarding the day of injection, timing of injection, and timing of sampling. Samples were centrifuged and serum was stored at -80°C for analysis.

Hormone assays

LH, FSH, testosterone, and estradiol were measured using an electrochemiluminescence immunoassay on a fully automated analyzer (E-801, Roche Diagnostics, Switzerland). The intra-assay and inter-assay coefficients of variation were 1.6% and 2.2% for LH and 2.8% and 4.5% for FSH. The lower limit of detection for both LH and FSH was 0.1 IU/L. The intra-assay and inter-assay coefficients of variation for testosterone were 4.7% and 8.8%. The lower limit of detection was 0.087 nmol/L. The intra-assay and inter-assay coefficients of variation for estradiol were 2.6% and 4.2%. The lower limit of detection of estradiol was 5 pg/mL. Inhibin B levels were measured using a quantitative 3-step sandwich immunoassay (Inhibin B ELISA, AL-107-i, RRID: AB_2783661; Ansh Labs) (24) per the manufacturer instructions. The inter-assay coefficient of variation was 3.05% to 6.32%. The intra-assay coefficient of variation in our laboratory was 4.99%. The lower limit of detection of the assay was 1.6 pg/mL.

Procedure

For determination of inhibin B, samples and controls were incubated in microtitration wells coated with anti-inhibin β B subunit antibody for 2 hours. After incubation, washing was done. Biotinylated inhibin B antibody was added, and the wells were incubated for 1 hour followed by washing. Subsequently streptavidin horseradish peroxidase conjugate was added, and wells were incubated for 30 minutes. After the washing step, substrate tetramethyl benzidine was added and incubation was done for 8 to 12 minutes. An acidic stopping solution was added to stop further enzymatic reaction. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurement at 450 nm as the primary test filter and 630 nm as the reference filter. The optical density thus determined was plotted along y-axis versus inhibin B concentration(log) along x-axis. The concentrations in the samples were then calculated from this calibration curve.

Statistical analysis

Data obtained was analyzed by SPSS version 25. Variables were first analyzed for normality by Kolmogorov-Smirnov test. Data were specified as mean ± SD with range if normally distributed or median with range in case of non-normal distribution. Paired *t* test was used for comparison of basal and stimulated values of inhibin B with normal distribution. In the case of non-normal distribution, nonparametric tests were used. The inter-group analysis was done using independent Student *t* test for continuous variables with normal distribution; Mann-Whitney *U* test was used for continuous variables without normal distribution. Receiver operating characteristic (ROC) curves were plotted and cutoffs were derived. A P value < 0.05 was considered significant. The χ^2 method was used to compare predicted outcomes by various diagnostic tests with clinical outcomes. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were calculated.

Results

A total of 61 subjects who fulfilled the eligibility criteria were enrolled into the study. There were 42 subjects in the exploratory cohort and 19 in the validation cohort.

Exploratory cohort

Baseline clinical characteristics of male subjects

In group 1 (healthy males with SP, n = 18); of 18 subjects, 15 were clinically and biochemically pubertal (Tanner stage 2 [n = 13], Tanner stage 3 [n = 1], and Tanner stage 4 [n = 1]), whereas 3 were biochemically pubertal only (Tanner stage 1 with GnRHa-stimulated LH ≥14 IU/L). None had a history of anosmia, synkinesia, or delayed puberty. In group 2 (HH males, n = 8); 1 patient had synkinesia and none had anosmia/hyposmia. Six patients had received testosterone

injections before their visit to our center and were partially virilized. None of the patients had received gonadotropin treatment. All patients in group 2 had a prepubertal gonadal stage. Gynecomastia was present in 5 patients. Baseline characteristics of group 1 (SP-male) and group 2 (HH-male) subjects are shown in Table 1.

Baseline clinical characteristics of female subjects

In group 1 (healthy females with SP, n = 8); of 8 subjects, 6 were clinically and biochemically pubertal (Tanner stage 2 [n = 4], Tanner stage 3 [n = 1], and Tanner stage 4 [n = 1]), whereas 2 were biochemically pubertal (Tanner stage 1 with GnRHa-stimulated LH ≥14 IU/L). None had anosmia or synkinesia. In group 2, (HH females, n = 8); 2 patients had synkinesia and 1 each had anosmia and cleft lip. Seven patients had received estrogen and progesterone treatment before their visit to our center but were off treatment for more than 3 months. Baseline characteristics of group 1 (SP-female) and group 2 (HH-female) subjects are shown in Table 2.

FSH stimulation test in male

Mean FSH levels following exogenous FSH injection was 11.22 ± 6.28 IU/L (3.43-26.87) in male subjects. Mean basal inhibin B in group 1 (SP-male) was 219 ± 125.50 pg/mL (61.93-476.31); mean ± SD (range), whereas mean FSH-iB (FSH-stimulated inhibin B) was 408.51 ± 244.55 pg/mL (141.69-969.36). The mean delta change was 188.8 pg/mL and the difference between basal inhibin B and FSH-iB was statistically significant ($P = 0.002$). Mean basal and FSH-iB in group 2 (HH male) was 29.32 ± 35.31 pg/mL (1.6-94.6) and 45.96 ± 34.47 pg/mL (1.6-90.58), respectively. Mean delta change was 16.64 pg/mL and the difference between the means was not statistically significant ($P = 0.076$).

FSH stimulation test in female

Mean FSH levels following exogenous FSH injection were 8.44 ± 4.88 IU/L (4.23-24.46) in female subjects. Mean basal inhibin B in group 1 (SP-female) was 100.35 ± 55.79 pg/mL (39.71-180) mean ± SD (range), whereas mean FSH-iB was 1165.75 ± 1053.07 pg/mL (166.13-2574.5). Mean delta change was 1065 pg/mL and the difference between mean basal inhibin B and FSH-iB was statistically significant ($P = 0.023$). The median basal and FSH-iB in group 2 (HP-female) was 36.38 pg/mL (34.66-41.84) and 45.16 pg/mL (34.60-66.86), respectively. Delta change was 9.8 pg/mL and the difference between basal inhibin B and FSH-iB was not statistically significant ($P = 0.128$). Basal inhibin B and FSH-stimulated inhibin B in group 1 (SP) and group 2 (HH) in males and females is shown in Table 3.

Table 1. Baseline characteristics of group 1 (healthy children with spontaneous puberty) and group 2 (hypogonadotropic hypogonadism) in male subjects (exploratory cohort)

	Group 1 (male) (n = 18)	Group 2 (male) (n = 8)	*P* value
Age, y	13.80 ± 1.31 (10-15.3)	23.51 ± 2.4 (20.4-26.4)	0.000
Height, cm	143.76 ± 14.57 (119-168)	169.91 ± 5.1 (163-177.5)	0.00
Body mass index, kg/m^2	21.15 ± 5.08 (14.17-29)	24.39 ± 4.36 (17.67-29.40)	0.131
Upper segment/lower segment	0.92 ± 0.11 (0.80-1.30)	0.83 ± 0.05 (0.74-0.90)	0.043
Testicular volume, mL, right	4.55 ± 2.99	1.62 ± 0.74	0.001
Prader orchidometer	(2-15)	(1-3)	
Testicular volume, mL, right	3.0 ± 1.83	1.06 ± 0.33	0.002
Ultrasonography	(0.8-6.9)	(0.62-1.6)	
Testicular volume, mL, left	4.61 ± 3.01	1.5 ± 0.53	0.000
Prader orchidometer	(2-15)	(1-2)	
Testicular volume, mL, left	2.70 ± 1.70	0.90 ± 0.46	0.003
Ultrasonography	(0.9-7.4)	(0.3-1.56)	
Bone age, y	13 (10-14)	15 (11-18)	0.004
Basal LH, IU/L	1.03 (0.12-3.87)	0.10 (0.1-0.6)	0.002
GnRH analogue-stimulated LH, IU/L	20.4 (11.68-42.80)	0.68 (0.1-8.78)	0.000
Basal FSH, IU/L	1.82 (0.79-7.92)	0.22 (0.1-1.88)	0.000
Basal testosterone, nmol/L	0.43 (0.09-19.07)	0.27 (0.09-0.96)	0.910
Post-GnRHa testosterone, nmol/L	4.31 (0.14-32.64)	0.44 (0.08-1.76)	0.009

Data are shown as mean ± SD with range for continuous variables with normal distribution and median with range for continuous variables without normal distribution. *P* values were calculated as follows: independent Student *t* test for continuous variables with normal distribution or Mann-Whitney *U* test for continuous variables without normal distribution.

Sexual dimorphism of basal and FSH stimulated inhibin B (FSH-iB)

On comparison of male and female healthy subjects with SP (group 1), mean basal inhibin B was found to be higher in male subjects, whereas FSH-iB was higher in female subjects. The difference between the two means was statistically significant for both basal ($P = 0.033$) as well as FSH-iB ($P = 0.007$). For HH patients (group 2), no significant difference was found in the mean value of

Table 2. Baseline characteristics of group 1 (healthy children with spontaneous puberty) and group 2 (hypogonadotropic hypogonadism) in female subjects (exploratory cohort)

	Group 1 (female)	Group 2 (female)	*P* value
	(n = 8)	(n = 8)	
Age, y	12.39 ± 2.97 (9.2-17.8)	22.74 ± 5.11 (19-34)	0.000
Height, cm	133.98 ± 17.41 (98.4-150.5)	159.78 ± 2.91 (157.50-166)	0.004
Body mass index, kg/m^2	16.84 ± 3.27 (13.7-23.40)	22.33 ± 2.71 (18.93-28.36)	0.003
Upper segment/ lower segment	0.91 ± 0.13 (0.73-1.1)	0.82 ± 0.03 (0.72-0.87)	0.093
Bone age, y	11.28 ± 2.49 (7-14)	15.28 ± 2.05 (12-18)	0.005
Basal LH, IU/L	0.71 (0.10-14.50)	0.16 (0.10-1.07)	0.065
GnRH analogue-stimulated LH, IU/L IU/L	34.19 ± 33.69 (2.78-101)	2.89 ± 2.36 (0.39-7.95)	0.025
Basal FSH, IU/L	3.41 ± 2.50 (0.61-8.79)	1.21 ± 0.60 (0.20-1.88)	0.031
Basal estradiol, pg/mL	18.25 (5-63.5)	14.75 (5-31.16)	0.442
GnRH analogue-stimulated estradiol, pg/mL	169.85 (5-1506)	21.34 (5-32)	0.065

Data are shown as mean ± SD with range for continuous variables with normal distribution and median with range for continuous variables without normal distribution. *P* values were calculated as follows: independent Student *t* test for continuous variables with normal distribution or Mann-Whitney *U* test for continuous variables without normal distribution.

basal (P = 0.582) and FSH-iB (P = 0.941) in both male and female subjects.

Diagnostic cutoff for basal and FSH-iB in males

ROC curve analysis was done among healthy children with SP (group 1 SP) and patients with HH (group 2 HH) to derive cutoff of basal inhibin B, FSH-iB, and delta change in inhibin B for denoting the onset of puberty. For male subjects, basal inhibin B of ≤50.03 pg/mL had 100% specificity for diagnosis of HH and ≥97.21 had 100% specificity for marking the onset of puberty (area under the curve [AUC] 0.965 [0.9-1]). FSH-iB at a cutoff of 116.14 pg/mL had 100% sensitivity and specificity to denote the onset of puberty (AUC-1 [1]). Delta change in inhibin B (FSH-iB – basal inhibin B) of 60.27 pg/mL had sensitivity of 83.3% with 100% specificity (AUC 0.931 [0.82-1]).

Diagnostic cutoff for basal and FSH-iB in females

Similarly, for female subjects, the basal inhibin B level of ≤38.44 pg/mL had 100% specificity for diagnosis of HH and ≥51.47 pg/mL had 100% specificity (AUC 0.964 [0.87-1]) for marking the onset of puberty. FSH-iB at a cutoff of 116.50 pg/mL had 100% sensitivity and specificity for denoting the onset of puberty (AUC 1 [1-1]). ROC analysis for delta change yielded a cutoff of 72.30 pg/mL with 100% sensitivity and specificity (AUC 1 [1-1]).

Table 3. Basal and FSH-iB in healthy children with SP and patients with HH

	Basal inhibin B (pg/mL)	FSH-iB (pg/mL)	Delta change (pg/mL)	*P* value
SP-Male	219 ± 125.50 (61.93-476.31)	408.51 ± 244.55 (141.69-969.36)	188.80	0.002
HH-Male	29.32 ± 35.31 (1.6-94.6)	45.96 ± 12.18 (1.6-90.58)	16.64	0.076
SP-Female	100.35 ± 55.79 (39.71-180)	1165.75 ± 1053.07 (166.13-2574.5)	1065	0.023
HH-Female	36.38 (18.25-43.25)	45.16 (34.60-66.86)	9.8	0.128

Abbreviations: HH-male, hypogonadotropic hypogonadism male; HH-female, hypogonadotropic hypogonadism female; SP-male, healthy male with spontaneous puberty; SP-female, healthy female with spontaneous puberty.

ROC curve analysis for basal and FSH-iB for males and females is depicted in Fig. 1A and 1B, respectively. The diagnostic cutoffs for basal inhibin B, FSH-iB, and delta change in inhibin B in male and female subjects are shown in Supplementary Table 2 and Table 3, respectively (23).

Validation cohort

A total of 19 subjects presented with delayed puberty. Out of these, 11 were males and 8 were females.

Baseline characteristics of male subjects with delayed puberty

There were 11 male subjects who presented between the age of 14 and 18 years (age range: 14 years, 1 month, to 17 years, 6 months) with delayed puberty. A family history of delayed puberty was present in 7 patients. One patient had anosmia and two had synkinesia. All patients were prepubertal according to gonadal stage. One patient (patient 10) had undescended testes on the left side. Four patients had appearance of pubic hair (Tanner stage P2). One patient (patient 4) had GH deficiency and secondary hypocortisolism. He was on supplementation for both. MRI scans of the sella and olfactory tract were normal in all but one (patient 10), who had an absent olfactory bulb and olfactory sulcus.

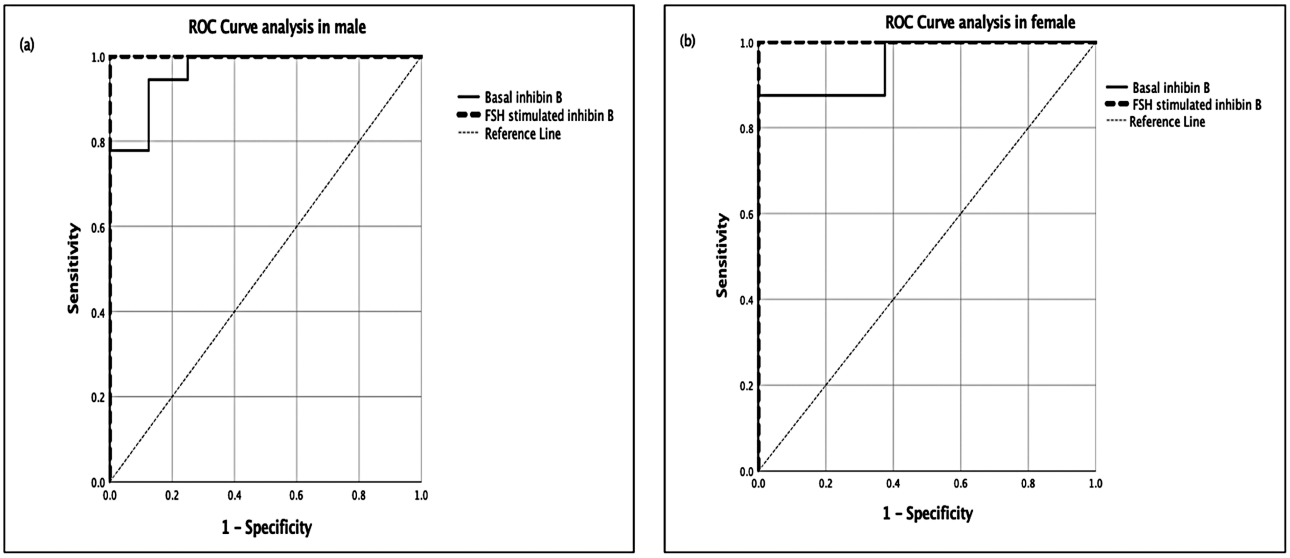

Figure 1. ROC area under curve analysis among children with spontaneous puberty (SP) and patients with hypogonadotropic hypogonadism (HH) in exploratory cohort. (A) ROC curve analysis for basal and FSH-stimulated inhibin B in males. AUC with 95% confidence interval basal inhibin B (AUC, 0.965 [0.9-1]); FSH-stimulated inhibin B (AUC, 1 [1-1]). (B) ROC curve analysis for basal and FSH-stimulated inhibin B in females. AUC with 95% confidence interval basal inhibin B (AUC, 0.964 [0.87-1]); FSH stimulated inhibin B (AUC, 1 [1, 1]). AUC, area under the curve; ROC, receiver operating characteristic.

Baseline characteristics of female patients with delayed puberty

There were 8 female subjects who presented between the age of 13 to 18 years (age range: 13 years, 2 months, to 17 years, 7 months) with delayed puberty. A family history of delayed puberty was present in one subject. None had anosmia, hyposmia, synkinesia, or any skeletal abnormality. Three patients (patients 2, 4, and 6) had GH deficiency and secondary hypocortisolism, whereas 2 patients (patients 7 and 8) had isolated GH deficiency. All were receiving supplementation for respective hormone deficiencies. Two patients (patients 2 and 6) had hypoplastic pituitary and one (patient 4) had partial empty sella. All subjects had normal MRI scans of the sella and olfactory tract. Baseline characteristics of male and female subjects with delayed puberty are shown in Table 4.

Follow-up in male subjects

On long-term prospective follow-up, 9 male subjects manifested spontaneous appearance and progression of clinical signs of puberty (range, 3-9 months). Patient 4 did not show any signs of puberty spontaneously until age 18 years. Patient 10 had anosmia, GnRHa-stimulated LH of 1.23 IU/L, and absent olfactory bulb and sulcus on MRI of the sella. Hence, diagnosis of HH was substantiated in both of them. Three subjects who had basal LH < 0.3 IU/L and 5 subjects who had GnRHa-stimulated LH < 14 IU/L entered puberty on prospective follow-up. Of the 3 subjects who had basal inhibin B in the equivocal range, 2 entered puberty, whereas one did not. For delta change in inhibin B, 3 subjects who had increment below 60.27 pg/mL entered puberty on follow-up. All subjects who had FSH-iB ≥116.14 pg/mL entered puberty, whereas those with <116.14 pg/mL did not enter puberty on prospective follow-up.

Follow-up in female subjects

Three female patients showed clinical signs of onset and progression of SP (range, 6-9 months). Four patients (patients 1, 3, 4, and 5) did not show signs of spontaneous puberty until age 18 years. Patient 6 had multiple pituitary hormone deficiencies (secondary hypocortisolism and GH deficiency) along with hypoplastic pituitary on MRI of the sella with GnRHa-stimulated LH of 1.79 IU/L. So, the clinical diagnosis of hypogonadotropic hypogonadism was substantiated in all 5 of them. One subject with basal LH <0.3 IU/L entered into puberty, whereas 2 subjects with basal LH ≥0.3 IU/L did not. One subject with GnRHa-stimulated LH <14 IU/L entered into puberty on prospective follow-up. One subject with basal inhibin B < 38 pg/mL entered into puberty, whereas one subject with basal inhibin B in equivocal range did not enter puberty on follow-up. For delta change in inhibin B, one subject with the value above cutoff did not enter puberty on follow-up. All subjects who had FSH-iB ≥116.50 pg/mL entered puberty, whereas those with < 116.50 pg/mL did not enter puberty on prospective follow-up.

FSH-iB had 100% sensitivity, specificity, PPV, NPV, and diagnostic accuracy for the prediction of entry into puberty in both male and female subjects. PPV was 100% for all the tests in male, but NPV was low. In females, PPV was 100%

Table 4. Baseline characteristics of male and female subjects with delayed puberty (validation cohort)

	Gender	Age, y	Family history of delayed puberty	Anosmia	Synkinesia	Tanner stage	Bone age, y
Patient 1	M	15.4	P	A	A	A-P2TV-2/2	13
Patient 2	M	14.5	P	A	P	A-P1TV-2/2	12
Patient 3	M	17.6	P	A	A	A-P2TV-2/3	13
Patient 4	M	15.8	A	A	A	A-P1TV-1/1	11
Patient 5	M	14.1	P	A	A	A-P1TV-2/2	13
Patient 6	M	14.4	A	A	A	A-P1TV-2/2	12
Patient 7	M	15	P	A	A	A-P1TV-2/2	12
Patient 8	M	16	A	A	P	A-P2TV-1/2	13
Patient 9	M	14.2	P	A	A	A-P2TV-3/2	11
Patient 10	M	14.5	P	P	A	A-P1TV-UDT/1	13
Patient 11	M	15.2	A	A	A	A-P1TV-2/2	13
Patient 1	F	17.10	A	A	A	A + P2B1	12
Patient 2	F	13.2	A	A	A	A-P1B1	13
Patient 3	F	16.2	P	A	A	A-P1B1	14
Patient 4	F	16.11	A	A	A	A-P1B1	12
Patient 5	F	17.7	A	A	A	A-P1B1	13
Patient 6	F	13.9	A	A	A	A-P1B1	10
Patient 7	F	14.2	A	A	A	A-P1B1	12
Patient 8	F	13.11	A	A	A	A-P1B1	10

Table 5. Diagnostic performance of basal inhibin B, FSH-stimulated inhibin B, delta change in inhibin B, basal LH, and GnRH analogue-stimulated LH in male subjects (validation cohort)

	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Diagnostic accuracy, %
Basal inhibin B	77.78	100	100	50	81.83
FSH stimulated inhibin B	100	100	100	100	100
Delta change in inhibin B	66.67	100	100	40	72.73
Basal LH	66.67	100	100	40	72.73
GnRH analogue-stimulated LH	44.44	100	100	28.57	54.55

Delta change in inhibin B was calculated as FSH stimulated inhibin B – basal inhibin B.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

for GnRHa-stimulated LH and basal inhibin B. Sensitivity, specificity, PPV, NPV, and diagnostic accuracy of various diagnostic tests for male and female subjects are shown in Table 5 and Table 6, respectively. No adverse event was recorded in the study participants.

Discussion

Present study for the first time explores the stimulability of inhibin B in response to exogenous FSH for the prediction of the onset of puberty. Inhibin B was found to be stimulable after exogenous FSH in both male and female healthy children having SP, whereas it was nonstimulable in patients of hypogonadotropic hypogonadism. FSH-iB had 100% sensitivity and specificity for denoting the onset of puberty in both the sexes in the exploratory cohort. Furthermore, on application of the cutoff values derived from the exploratory cohort in children who presented with delayed puberty (ie, validation cohort), FSH-iB had 100% PPV, NPV, and diagnostic accuracy in both the sexes for the prediction of the pubertal outcome. FSH stimulation test performed better than almost all the investigations available in routine clinical practice, making it a novel and promising investigation in the armamentarium for the differential diagnosis of delayed puberty.

Both CDGP and HH have similar clinical presentation at adolescence. Currently available diagnostic investigations for the differential diagnosis of delayed puberty include basal LH, GnRH-stimulated LH, testosterone response to hCG, and basal inhibin B. These tests are helpful; however, none is fully reliable. Therefore, there is an unmet need to explore better diagnostic test to resolve the enigma in etiology of delayed puberty.

Table 6. Diagnostic performance of basal inhibin B, FSH-stimulated inhibin B, delta change in inhibin B, basal LH, and GnRH analogue-stimulated LH in female subjects (validation cohort)

	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Diagnostic accuracy, %
Basal inhibin B	66.67	100	100	83.33	87.50
FSH-stimulated inhibin B	100	100	100	100	100
Delta change in inhibin B	100	80	75	100	87.50
Basal LH	66.67	60	50	75	62.50
GnRH analogue-stimulated LH	66.67	100	100	83.33	87.50

Delta change in inhibin B was calculated as FSH stimulated inhibin B – basal inhibin B.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

Basal gonadotropins have limited utility because of the considerable overlap between CDGP and HH. GnRH and GnRHa stimulation tests have different peak LH cutoffs in different studies ranging from 5.5 IU/L to 14 IU/L (25). In the present study, basal LH had 72.7% diagnostic accuracy in males and 62.5% in females for prediction of pubertal onset. Likewise, diagnostic accuracy of GnRHa-stimulated LH was 54.5% in males and 87.5% in females in our study, reiterating the limitations of these tests.

Basal inhibin B has been recognized as an important marker for the onset of puberty. However, major limitation with basal inhibin B is the presence of varying cutoffs ranging from 28.5 pg/mL to 111 pg/mL in different studies (11-17). Diagnostic accuracy of basal inhibin B in our study was 81.8% in males and 87.5% in females, thus endorsing the limited utility of basal inhibin B as a single marker in the differential diagnosis of delayed puberty. Onset of puberty is preceded by gradual increase in GnRH pulsatility and subsequently pituitary gonadotropins. Pituitary gonadotropins act on the gonads, leading to increase in the gonadal steroids and peptides. Before the consistent detectable rise in basal hormone levels from the target glands, there is an increase in responsiveness of the glands to their respective trophic stimuli. This increase is due to priming of the target gland. The priming includes both increase in sensitivity of gland to the trophic stimulus as well as development of stimulable pool of the hormone in the target gland (26). This is the premise behind the use of exogenous pharmacological stimuli for the various dynamic tests, as evidenced by GnRH stimulation test for LH and hCG stimulation test for testosterone. Although basal inhibin B has been used to predict pubertal outcome for quite some time, the stimulability of inhibin B to its trophic hormone (ie, FSH) has not been previously explored.

In the present study, stimulability of inhibin B to exogenous FSH was explored. Healthy children with spontaneous puberty showed a significant increment in inhibin B after FSH, reflecting the stimulability of inhibin B in response to exogenous FSH. It is a well-known fact that during prepubertal period, the release of FSH from pituitary is less tightly regulated compared to LH (27). The presence of FSH during the prepubertal period leads to progressive preparation of the gonads for puberty (ie, priming of gonads) in the form of proliferation of Sertoli cells in males and granulosa cells in females. This is evidenced clinically by increase in testicular volume, though modest, on ultrasonography during prepubertal period (28) in males and presence of multifollicular ovaries in late prepuberty in females (29). Priming is contributed by "minipuberty" as well. Therefore, significant increment in inhibin B after exogenous FSH represents optimal priming of gonads. Nonstimulability of inhibin B in patients of hypogonadotropic hypogonadism can be attributed to the lack of FSH during the prepubertal period and absence of minipuberty. FSH-iB at a cutoff value of 116.14 pg/mL in male subjects and 116.50 pg/mL in female subjects had 100% sensitivity and specificity for denoting the onset of puberty.

In the validation cohort, which included children with delayed puberty FSH-iB, was able to predict the onset of puberty accurately in all the subjects who presented with delayed puberty when compared with the clinical outcome on prospective follow-up. FSH-iB outperformed basal LH, GnRHa-stimulated LH, and basal inhibin B for the prediction of pubertal outcome. Furthermore, it was interesting to observe that 2 male patients (patients 2 and 8) in the validation cohort, who had synkinesia with testicular volume of <2 mL, each entered into puberty on subsequent follow-up. The clinical outcome of both was rightly predicted by FSH-iB. Likewise, among 3 female subjects with multiple pituitary hormone deficiencies, one entered into puberty on subsequent follow-up and was rightly predicted by FSH-iB. Therefore, FSH-iB performed well in the individuals who were not clearly distinguishable on clinical grounds and was also able to test hypothalamic-pituitary-gonadal axis in patients with multiple pituitary hormone deficiencies.

FSH stimulation test can be considered as a novel and promising test for the prediction of the onset of puberty. FSH-iB turned out to be a more consistent predictor of

puberty compared with other available tests because it reflects the gradual build-up of the Sertoli cell pool/granulosa cell pool in response to steady rise in FSH during the prepubertal period. In addition, FSH stimulated inhibin B response reflects the functional integrity of hypothalamic-pituitary-gonadal axis because stimulation by GnRH is required for the secretion of FSH, which in turn renders the Sertoli cells/granulosa cells responsive to exogenous FSH.

Among the available investigations, only prolonged protocol using a combination of GnRHa stimulation test and 19-day hCG stimulation test has 100% sensitivity and specificity to differentiate between CDGP and HH (30). However, this test is lengthy and cumbersome. In the quest for a better marker, recently, the kisspeptin stimulation test (31) was introduced. Besides limited availability of kisspeptin, the procedure for the test is cumbersome, requiring priming by GnRH and hospitalization. The FSH stimulation test, besides being a consistent marker for the prediction of pubertal outcome, is performed over shorter duration, requires fewer injections, and does not require priming with exogenous GnRH or hospitalization; thus, overcoming the limitations of previously mentioned protocols.

We also observed gender difference (sexual dimorphism) in the basal and stimulated levels of inhibin B. Similar to the previous studies, basal inhibin B level was found to be higher in male than female subjects (32). However, to our surprise, FSH-iB was higher in females compared with males. A likely explanation for the high basal inhibin B in males can be that proliferation of Sertoli cells is a gradual and additive phenomenon resulting in development of pool of the Sertoli cells. This results in a slow and sustained rise in basal inhibin B. In females, however, the proliferation of granulosa cells is a cyclical phenomenon. The greater increment in inhibin B in response to the exogenous FSH in females can be due to the rapid proliferation of granulosa cells in the growing follicles in female. Furthermore, Sertoli cells start maturing during the peripubertal period in male, though timing of transition from immature to mature Sertoli cells is not exactly known. The lack of capacity of mature Sertoli cells to proliferate any further may be responsible for lesser increment of inhibin B in response to the exogenous FSH in male. However, plausibly, exogenous FSH does increase the functionality of conglomeration of Sertoli cells and germ cells, leading to increment in inhibin B during peripubertal period. Nevertheless, further studies are needed for more insight into the normal physiology of basal and FSH-iB.

Strengths

Ours is the first study to demonstrate the presence of stimulability of inhibin B and explore the role of FSH-iB as a predictor of the onset of puberty in both the sexes. Application of cutoffs derived from an exploratory cohort and their validation on prospective follow-up makes our study results more robust. Moreover, subjects were enrolled with strict inclusion and exclusion criteria and there was no attrition during follow-up.

Limitations

The number of subjects in the validation cohort was fewer, but given the rarity of the condition, this number can be considered satisfactory. Nevertheless, more extensive studies are needed for further validation.

Conclusions

Inhibin B is stimulable in both male and female subjects with spontaneous puberty. FSH-iB can be considered a novel and promising diagnostic tool for the prediction of onset of puberty. Replication of results of the present study in a larger cohort is required to further affirm the results.

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Additional Information

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