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Diagnosis of Male Central Hypogonadism During Childhood

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Abbreviations: ACMG: American College of Medical Genetics and Genomics; AMH: anti-Müllerian hormone; CSA, Catalytic Site Atlas; FGFR1, fibroblast growth factor receptor 1; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; INSL3, insulin-like factor 3; LH, luteinizing hormone; MAF, minor allele frequency; NGS, next-generation sequencing; SNV, single-nucleotide variant; TSH, thyrotropin.

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Abstract

The diagnosis of male central (or hypogonadotropic) hypogonadism, typically based on low luteinizing hormone (LH) and testosterone levels, is challenging during childhood since both hormones are physiologically low from the sixth month until the onset of puberty. Conversely, follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH), which show higher circulating levels during infancy and childhood, are not used as biomarkers for the condition. We report the case of a 7-year-old boy with a history of bilateral cryptorchidism who showed repeatedly low FSH and AMH serum levels during prepuberty. Unfortunately, the diagnosis could not be ascertained until he presented with delayed puberty at the age of 14 years. A gonadotropin-releasing hormone (GnRH) test showed impaired LH and FSH response. By then, his growth and bone mineralization were partially impaired. Gene panel sequencing identified a variant in exon 15 of *FGFR1*, affecting the tyrosine kinase domain of the receptor, involved in GnRH neuron migration and olfactory bulb morphogenesis. Testosterone replacement was started, which resulted in the development of secondary sexual characteristics and partial improvement of bone mineral density. This case illustrates the difficulty in making the diagnosis of central hypogonadism in boys during childhood based on classical criteria, and how serum FSH and AMH assessment may be helpful if it is suspected before the age of

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puberty, and confirm it using next-generation sequencing. The possibility of making an early diagnosis of central hypogonadism may be useful for a timely start of hormone replacement therapy, and to avoid delays that could affect growth and bone health as well as psychosocial adjustment.

Key Words: Cryptorchidism, constitutional delay of puberty, Kallmann syndrome, micro-orchidism, micropenis, hormone replacement therapy

The hypothalamic–pituitary–testicular axis is of utmost importance for many developmental and maturational processes in the male. The testis has 2 morphologically and functionally distinct compartments: the seminiferous tubules and the interstitial tissue. In the seminiferous tubules reside the germ cells, which give rise to sperm in the adolescent and adult, supported by the somatic Sertoli cells, responsible for the production of the peptide hormones inhibin B and anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance or MIS). In the interstitial tissue, Leydig cells are responsible for the secretion of the male sex steroid testosterone and the peptide insulin-like factor 3 (INSL3). While Leydig cell function is mainly regulated by pituitary luteinizing hormone (LH), and also placental human chorionic gonadotropin (hCG) in the fetus [1], Sertoli cells depend on pituitary follicle-stimulating hormone (FSH) for proliferation [2] and on the paracrine action of testosterone for maturation [3]. LH and FSH secretion by the pituitary gonadotropes is, in turn, regulated by gonadotropin-releasing hormone (GnRH) produced in the hypothalamus. Testosterone and inhibin B exert a negative feedback on LH and FSH secretion, respectively.

Male hypogonadism is characterized by a decreased function of the testes, associated with reduced production of testicular hormones, including androgens, INSL3, AMH, and/or inhibin B, and/or impaired sperm output [4, 5]. Male hypogonadism is classified as primary (or hypergonadotropic), when the testis is primarily affected, and central (secondary or hypogonadotropic), when it results from an impaired GnRH or gonadotropin secretion. More rarely, both the hypothalamic–gonadotrope axis and the testes may be concomitantly affected, and this results in a combined or dual hypogonadism [4-6]. The diagnosis of male hypogonadism has classically relied on testosterone assessment. While this is adequate in the adult and in 2 periods of development, namely neonatal activation and puberty, the assessment of circulating levels of testosterone or gonadotropins may be uninformative during childhood [7-10]. Indeed, the LH–Leydig cell axis is physiologically quiescent from the sixth month of postnatal life until the onset of puberty, which makes central ("hypogonadotropic") hypogonadism challenging to diagnose during childhood. Conversely, Sertoli cell hormones show high circulating levels during the whole prepubertal period and may represent useful biomarkers for an early identification of central hypogonadism.

Case Report

Clinical and Laboratory Data

A 7-year-old boy was referred to the Division of Endocrinology of Ricardo Gutiérrez Children's Hospital, a tertiary pediatric Hospital in Buenos Aires, for endocrine assessment after bilateral orchidopexy. He was born at term by cesarean section due to breech presentation. His birth weight (2780 g) and length (48 cm) were adequate for gestational age. He was the first child of healthy, nonconsanguineous parents of Argentine origin, both with no remarkable medical history. Adjusted midparental height was 166.2 cm (25th centile for Argentine male population), and maternal menarche occurred at 12 years, adequate for Argentine girls [11]. The proband had a normal, uneventful medical history, except for bilateral cryptorchidism operated at 5 and 6 years of age (1 testis at a time) in the small city where he lived, 250 km away from Buenos Aires. On the initial physical examination, his height was 127 cm (75th centile for age) and his weight was 33.7 kg (90th centile) (Fig. 1), his genital stage was G1 according to Marshall and Tanner [12]. The right testis was high scrotal, of small size (<1 mL), and the left testis was not palpable; the scrotum was hypoplastic. The penis was of normal size for age. The remainder of the examination was normal. Gonadotropins and testicular hormones were measured in serum to assess the hypothalamic–pituitary–testicular axis: LH, FSH, and testosterone were uninformative (Table 1 and Fig. 2), while AMH was extremely low compared with the normal range for age [13], suggesting an impaired Sertoli cell hormone output. Karyotype was normal: 46,XY in 20 metaphases. Testicular ultrasonography identified both testes in low inguinal position; the length of the right and left testes was 15 mm and 13 mm respectively, slightly smaller than the mean for age (ie, 17 ± 3 mm) [14]. The patient grew along the 75th centile for height and 97th centile for weight (Fig. 1), with nothing else to highlight in his medical history.

At 12 years 2 months, physical examination showed Tanner stage G1 and PH3. Testicular ultrasonography showed prepubertal size (right testis 18 × 5 mm; left testis 15 × 5 mm). In the following 2 laboratory assessments, LH and testosterone were uninformative, while FSH and AMH were low for age and Tanner stage G1 (Table 1 and Fig. 2). No changes in the genital examination were noticed at 13 years 10 months of age, with a bone age of 14 years. Penile length was 4 cm, within the normal range for the Argentine population [15]. Growth velocity was prepubertal, which resulted in a decline in height centile (Fig. 1). School performance was adequate and relationship with his peers was not affected. His nutrition habits suggested a moderately hypercaloric diet. Routine laboratory analyses showed normal blood cell counts, hemoglobin concentration, and liver and renal functions, with a moderate elevation of triglycerides (252 mg/dL). Thyrotropin (TSH), free thyroxine, prolactin, and cortisol serum levels were normal. Bone mineral density of the lumbar spine was 0.735 g/cm^3, with a z-score at –1.7 for age, compared with the Argentine reference (16).

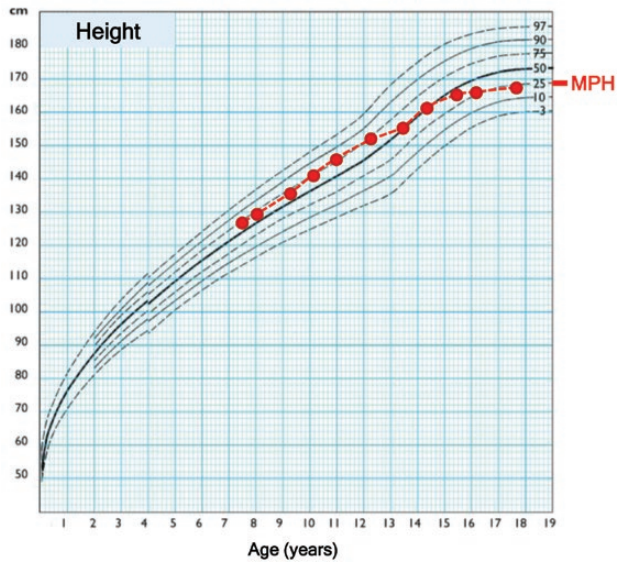

Figure 1. Growth chart of the patient, according to Argentine standards.

Central hypogonadism was suspected, and a GnRH infusion test showed an impaired response in both LH and FSH (Table 2) compared with validated cutoffs [17], confirming the diagnosis. Testosterone replacement was started with intramuscular testosterone enanthate 50 mg every 28 days, with progressive increases up to 250 mg every 28 days

Table 1. Serum levels of gonadotropins, testosterone, and AMH in the reported case at different ages

	7 yr 8 mo.	Ref. 2-8 yr G1	12 yr 2 mo.	13 yr 1 mo.	Ref. ≥9 yr G1
		Mean (range)[a]			Mean (range)[a]
LH (IU/L)	0.16	0.10 (0.10-0.18)	<0.10	<0.10	0.10 (0.10-2.78)
FSH (IU/L)	0.66	0.75 (0.24-1.70)	0.39	0.26	1.70 (0.58-2.54)
Testosterone (nmol/L)	<0.34	<0.34 (<0.34-0.34)	0.81	<0.34	0.34 (<0.34-3.74)
(ng/dL)	<10	<10 (<10-10)	24	<10	10 (<10-108)
AMH (pmol/L)	108	684 (236-1831)	66	136	713 (257-1371)
(ng/mL)	15.1	95.8 (33.1-256.4)	9.2	19.0	99.9 (36.0-192.0)

[a]References for normal means and ranges for Tanner stage G1 are from Grinspon et al. [13]

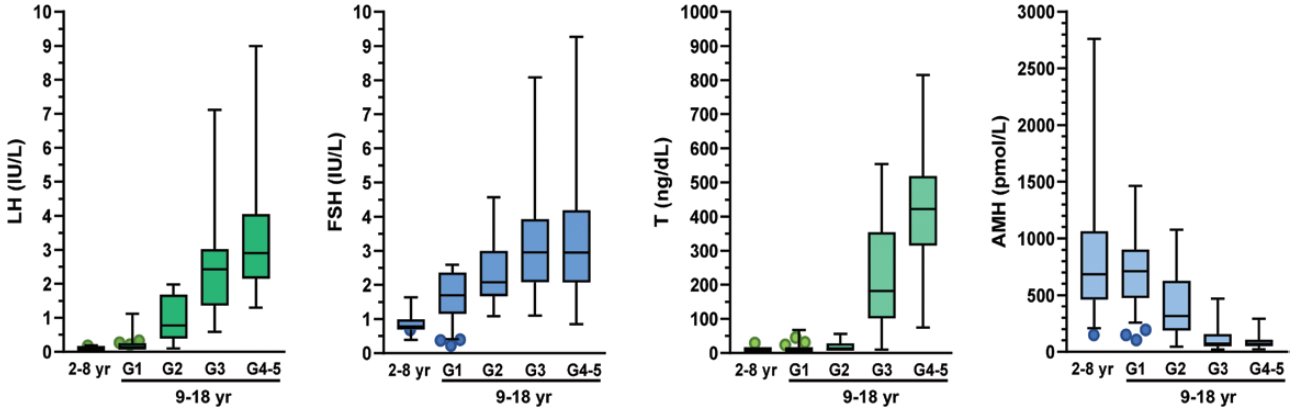

Figure 2. Serum levels of LH, FSH, testosterone (T), and anti-Müllerian hormone (AMH) in the reported case (dots) at the age of 7 years and ≥ 9 years and Tanner stage G1. Bars and whiskers represent the median, interquartile range and 3rd and 97th centiles in the normal population, as previously reported [13].

Table 2. Result of the GnRH test[a] in the reported case at the age of 14 years

	Basal	15 min	30 min	45 min	60 min	120 min	Reference cut-off[b]
LH (IU/L)	<0.10	1.54	1.60	1.47	1.27	1.04	≥5.80
FSH (IU/L)	0.34	1.94	2.32	2.51	2.72	3.01	≥4.60

[a]GnRH 100 µg, infused intravenously at 0.83 µg/min for 120 minutes [17].
[b]Reference levels for normal response in prepubertal boys, Tanner stage G1, aged 9-14 years [17].

2 years later. Olfaction was referred to as normal by the patient, but a magnetic resonance imaging scan showed reduced olfactory sulci and bulbs, with normal pituitary and central nervous system features. A validated test to assess olfaction could not be performed. Abdominal ultrasonography was normal. A repeated scan of the lumbar spine at 16 years 3 months of age, 2 years after start of testosterone treatment, showed a bone mineral density of 0.759 g/cm^3, z-score –1.9. In his last visit at 17 years 8 months, the patient's height was 165 cm (25th centile), having reached an adult height coincident with mid-parental height, and his weight was 78.5 kg (90th centile). He had genitalia of adult aspect (Tanner stages G5 and PH5), with testicular volume of 2 mL and penile length of 10 cm. With the diagnosis of congenital central hypogonadism, he continued on testosterone enanthate treatment at 250 mg intramuscularly every 28 days.

Genomic and Protein Structure Analyses

To search for gene variants responsible for the diagnosis, targeted next-generation sequencing (NGS) of the patient's genomic DNA obtained from peripheral blood cells was performed at the Translational Medicine Unit of Buenos Aires Children's Hospital, using the TruSight One® sequencing panel (Illumina), which provides coverage of 4813 genes, with >99% of the bases of the target regions with ≥10× coverage and ≥20 sequencing quality (QUAL) score. The initial analysis identified 25595 variants in 4506 genes. After filtering for candidate variants with minor allele frequency (MAF) <1% in gnomAD and 1000 Genomes, further analysis of single nucleotide variants (SNVs) and indels, using a read depth ≥10×, a Phred quality score ≥20, and GQ score ≥60 among the 41 candidate genes for hypogonadotropic hypogonadism available in the TrueSight One® sequencing panel (Illumina), detected 1 variant in exon 15 of *FGFR1* (Fig. 3A). The variant was NM_001174067.1 (FGFR1): c.1955A>C, p.His652Pro. Sanger sequencing confirmed the existence of the variant in heterozygosis (Fig. 3B). The variant was not reported in any of the consulted databases, and an alternative variant in the same position (Hys652Arg, named Hys621Arg according to GenBank NM_023110 sequence numbering used by the authors [18]) has been classified as pathogenic in a patient with Kallmann syndrome (hypogonadotropic hypogonadism associated with hyposmia/anosmia, OMIM # 147950).

The bioinformatic analysis of the His652Pro variant revealed relevant issues related to its potential role for protein function. Analysis of the Hidden Markov Model (HMM) domain logo [19], retrieved from the Pfam 34.0 database (http://pfam.xfam.org/), shows that His652 is a highly conserved residue belonging to the protein tyrosine and serine/threonine kinase family (PF07714, Fig. 3C). Likewise, query of the Catalytic Site Atlas (CSA) database (http://www.ebi.ac.uk/thornton-srv/databases/CSA/) informed that the residues involved in the catalytic mechanism proposed for the phosphorylation of FGFR1 substrates are Asp654, Arg658, Asn659, and Asp672. The availability of many crystals spanning this domain allows protein structural analysis, revealing that all 4 residues are in close proximity to His652, forming an active site rich in hydrogen bond interactions, surrounded by the alpha-helix elements of the protein secondary structure. Therefore, even though His652 is not tagged as an active site residue in CSA, it is likely to play a role in the structural integrity of the catalytic site and, thus, its mutation to proline could alter the active site structure in a significant way. Moreover, protein structure stability analysis using FoldX (http://foldxsuite.crg.eu/) on a set of 84 crystal chains with >99% sequence identity showed that His to Pro mutation results in a 1.16 ± 1.42 kcal/mol with a median of 1.55 kcal/mol penalty in the protein folding free energy, values which are compatible with a possible destabilization of the protein and its active site, likely leading to an altered phosphorylation of its substrates.

The variant found in our patient was therefore classified as likely pathogenic for central (hypogonadotropic) hypogonadism according to the American College of Medical Genetics and Genomicscriteria, since it met the requirement of ≥3 moderate (PM1-PM6) criteria [20]. Both parents had the normal sequence, indicating that the variant in the patient was de novo.

Discussion

We report the case of a boy referred at prepubertal age for endocrine assessment due to congenital

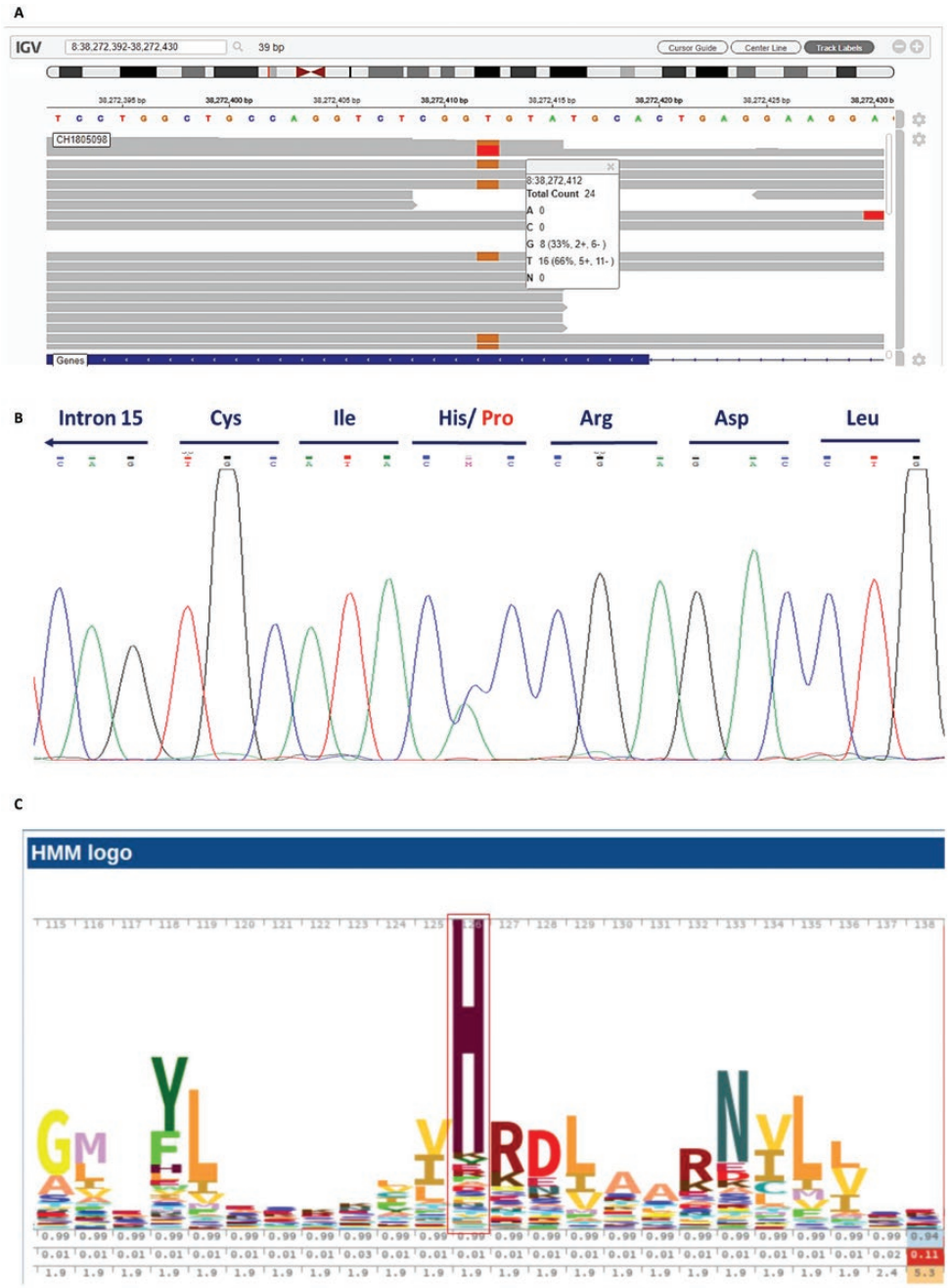

Figure 3. (A) Representative read resulting from next-generation sequencing of peripheral DNA from the reported case, focusing on exon 15 of *FGFR1*, using the Integrative Genome Viewer tool (IGV v.1.4.2, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA). (B) Sanger sequencing confirming the c.1955A>C, resulting in p.His652Pro in heterozygosis. (C) Pfam HMM logo spanning the region of interest; red box: highly conserved His652.

cryptorchidism whose final diagnosis of isolated central hypogonadism of genetic etiology was delayed until the age of approximately 14 years based on the usual criteria for assessing the pituitary–Leydig cell axis (low LH and testosterone), although it could have been ascertained during childhood if the focus had been placed on the pituitary–Sertoli cell axis (low FSH and AMH) and genetic analyses had been performed promptly. This case illustrates the difficulty in making the diagnosis of central hypogonadism in boys until pubertal delay prompts the clinical assessment, and how promising neglected biomarkers are to suspect it earlier and confirm it using increasingly available technologies such as gene panel sequencing.

Diagnosis of Central Hypogonadism at Pubertal Age

Pubertal delay in males is defined by the absence of testis enlargement (≥4 mL), the clinical milestone of Tanner stage G2 [12], at an age that is 2 SDS later than the population mean, namely 14 years of age [21]. Constitutional delay of puberty, a transient condition characterized by the persistence of the physiological prepubertal status of the hypothalamic–pituitary–testicular axis, is by far the most frequent cause of late pubertal onset in boys [22, 23]. It is, however, a diagnosis of exclusion. Other main etiologies include primary and central hypogonadism. Primary hypogonadism is easily diagnosed because low gonadal hormones are associated with elevated gonadotropins [5]. Central hypogonadism may be congenital or acquired. The latter may be due to lesions of the central nervous system or to general chronic conditions, which can be ruled out with the general clinical assessment [24]. Conversely, the differential diagnosis between constitutional delay of puberty and congenital central hypogonadism is challenging in the case of males with a prepubertal appearance after the age ≥12 years [5, 21, 23, 25].

Circulating levels of LH and testosterone physiologically remain at prepubertal levels until Tanner stages 2 or 3 [13] and, therefore, are not useful for an early diagnosis in males with no anatomic signs of pubertal development at the expected age. Several diagnostic tests based on the stimulation of gonadotropin release have been proposed, but none is universally accepted [26]. The use of genomic analyses applying high throughput technologies has increased the diagnostic efficiency in patients with suspected congenital central hypogonadism [27, 28]. Our patient carries a single nucleotide variant at position 1935 of the gene encoding the fibroblast growth factor (FGF) receptor 1. The variant results in a change from histidine to proline at position 621 of the protein, where resides the tyrosine kinase domain of the receptor, susceptible to autophosphorylation. This variant is novel, yet a histidine–arginine variant reported at the same position proved to be causative of Kallmann syndrome, in other words central hypogonadism associated with hyposmia [18, 29]. In fact, the FGF signaling pathway is clearly involved in GnRH neuron migration and olfactory bulb morphogenesis during early fetal life [30], and loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome [31].

Diagnosis of Central Hypogonadism in Prepubertal Patients: Difficulties and Potential Benefits

The term "hypogonadotropic" hypogonadism and its initial conceptual definition for the male, as low testosterone with normal to low LH, were coined for the adult patient [5, 6]. As mentioned, except for the first 3 to 6 months of postnatal life, childhood is characterized by extremely low to undetectable circulating levels of LH and testosterone. This explains why, when the window of opportunity for clinically identifying central hypogonadism in the infant is missed [32], the diagnosis is usually delayed until the age of puberty when the typical features of pubertal delay call the attention of the patient, his family and/or the pediatrician [21, 25].

For many boys with constitutional delay of puberty, reassurance and watchful waiting are sufficient. However, delayed pubertal onset may cause significant psychosocial burden and the impact of its persistence into adulthood raises concern [33]. The delay in the action of sexual steroids on the skeleton may also negatively affect the pubertal growth spurt and the achievement of an adequate bone mass, and some patients may benefit from an early diagnosis that could drive medical intervention aiming to induce a development similar to that of their peers [21, 25, 34].

It is obvious that early intervention is only possible if the patient comes to the attention of the pediatric endocrinologist before pubertal delay is suspected, before 13-14 years of age. This is not unusual in boys with cryptorchidism or micropenis [35]. However, in many cases the patient is referred to the specialist after the age of 6 months, when the pituitary–Leydig cell axis is normally quiescent. Our patient was referred at the age of 7. As expected, basal levels of serum LH and testosterone were uninformative. However, the biomarkers of the pituitary–Sertoli cell axis indicated an impaired function. FSH has been frequently neglected in the assessment of testicular function when central hypogonadism is suspected, with LH levels being the most frequently used endpoint. In our patient, FSH was repeatedly below the normal range for age and Tanner stage 1 between 7 and 12 years of age. FSH actions with major physiological and clinical relevance on Sertoli cells include cell proliferation and secretion of AMH and inhibin B [36]. Persistently low FSH might underlie the small testicular volume in this patient, since the size of the testis depends mainly on the mass of Sertoli cells before puberty [37]. Similarly, AMH production is under FSH regulation during infancy and childhood [38, 39], and low AMH has been reported in untreated neonates with central hypogonadism [35, 40-43], with an increase after FSH administration [40, 42, 43]. Our patient had persistently low AMH between 7 and 12 years of age, while being at Tanner 1 stage. Unfortunately, we could not measure inhibin B levels, another potentially useful marker [35, 40-43], and INSL3, whose probably low production could underlie the lack of testis descent, together with testosterone deficiency [1, 44]. Nonetheless, the combined use of FSH and AMH

as serum biomarkers clearly pointed to the diagnosis of central hypogonadism at an age where this diagnosis is usually overlooked.

Central hypogonadism with otherwise normal pituitary function has classically been classified as anosmic/hyposmic (Kallmann syndrome) or normosmic. In patients with Kallmann syndrome, variants in genes involved in GnRH neuron specification and migration, which also regulate the olfactory tract development in early fetal life, are causative of Kallmann syndrome [23, 30]. Congenital normosmic central hypogonadism is most frequently associated with variants in genes involved in GnRH synthesis and secretion [25]. Although our patient did not refer with anosmia, the magnetic resonance imaging scan showed reduced olfactory sulci and bulbs. Hyposmia is not always easy to ascertain [45], and this is particularly true for pediatric patients, owing to the lack of validated olfactory tests for young children.

Congenital central (or hypogonadotropic) hypogonadism is a diagnosis difficult to establish during childhood, despite its suspicion in males with a history of micropenis, cryptorchidism, and/or micro-orchidism, based on LH and testosterone determinations, classically used in adults. The assessment of FSH and Sertoli cell biomarkers, such as serum AMH and inhibin B, may be very useful to drive the diagnosis and the search for its genetic etiology. The advent of NGS coupled to bioinformatic analysis has added a powerful tool for the ascertainment of the diagnosis in an increasing number of cases. The availability of an accurate diagnosis of central hypogonadism that will need hormone replacement treatment may be useful for a timely decision to start therapy, thus avoiding unnecessary delays that could undermine the adolescent's psychosocial state and bone health.

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Data Availability: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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