Spreading the clinical window for diagnosing fetal-onset hypogonadism in boys

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In early fetal development, the testis secretes – independent of pituitary gonadotropins – androgens and anti-Müllerian hormone (AMH) that are essential for male sex differentiation. In the second half of fetal life, the hypothalamic–pituitary axis gains control of testicular hormone secretion. Follicle-stimulating hormone (FSH) controls Sertoli cell proliferation, responsible for testis volume increase and AMH and inhibin B secretion, whereas luteinizing hormone (LH) regulates Leydig cell androgen and INSL3 secretion, involved in the growth and tropism of male external genitalia and in testis descent. This differential regulation of testicular function between early and late fetal periods underlies the distinct clinical presentations of fetal-onset hypogonadism in the newborn male: primary hypogonadism results in ambiguous or female genitalia when early fetal-onset, whereas it becomes clinically undistinguishable from central hypogonadism when established later in fetal life. The assessment of the hypothalamic–pituitary–gonadal axis in male has classically relied on the measurement of gonadotropin and testosterone levels in serum. These hormone levels normally decline 3–6 months after birth, thus constraining the clinical evaluation window for diagnosing male hypogonadism. The advent of new markers of gonadal function has spread this clinical window beyond the first 6 months of life. In this review, we discuss the advantages and limitations of old and new markers used for the functional assessment of the hypothalamic–pituitary–testicular axis in boys suspected of fetal-onset hypogonadism.

Keywords: hypopituitarism, cryptorchidism, micropenis, disorder of sex development, testosterone

The concept of male hypogonadism is usually associated with the adult patient, and rarely thought of as a condition in the prepubertal boy. Furthermore, male hypogonadism is most frequently equated to hypoadrogenism. Androgens are the dean of testicular hormones, and the normal testes produce very little or no testosterone during most of infancy and childhood. It is therefore easy to understand why the term hypogonadism is almost absent from the pediatrician’s terminology. However, many hypogonadal states in the male bear their origin in fetal life. With the advent of direct markers of Sertoli cell function, hypogonadism can be identified in boys beyond the early postnatal critical window of pituitary–gonadal activation (1) – called “mini-puberty” by some authors – and before pubertal age. In this review, we address the diagnostic approaches of fetal-onset male hypogonadism based on the physiology and pathophysiology of the hypothalamic–pituitary–testicular axis ontogeny.

ONTOGONY OF THE HYPOTHALAMIC–PITUITARY–TESTICULAR AXIS

FETAL LIFE: THE FIRST VERSUS THE SECOND AND THIRD TRIMESTERS

The gonadotropin-releasing hormone (GnRH) neurons derive from cells present in the nasal placode in the sixth fetal week (2), which migrate together with olfactory axons and blood vessels through the cribriform plate and arrive in the developing forebrain in the 9th–10th weeks. Several genes are involved in the development and migration of GnRH neurons, including KAL1, FGFR1, PROK2, CHD7, WDR11, and NELF, and in their homeostasis and function, including DAX1 (or NR0B1), LEP, LEPR, KISS1, KISS1R, TAC3, TACR3, and GNRH1 [reviewed in Ref. (3, 4)].

The pituitary gonadotropes develop in the Rathke’s pouch following a sequential differentiating pathway, which also includes the other pituitary cell lineages, from the oral ectoderm ancestor. Early genes, like SHH, GLI1, GLI2, LHX3, LHX4, PITX1, PITX2, OTX2, and HESX1, are involved in the differentiation of all pituitary cell lineages, whereas TBX19 (or TPII), GATA2, and SF1 (or NR5A1) are more specifically related to the gonadotrope lineage [reviewed in Ref. (5)]. Fully functional gonadotropes are present in the fetal male pituitary and secrete luteinizing hormone (LH) from week 12 and Follicle-stimulating hormone (FSH) from week 14 (6). Circulating levels of both gonadotropins increase to attain peak levels by weeks 20–25 and then decrease toward term (7–9).

The testes differentiate from the adreno-gonadal primordium by the seventh week of gestation. Interestingly, Sertoli cells actively secrete anti-Müllerian hormone (AMH), involved in the regression of the uterine anlage during the eighth and ninth weeks, i.e., before exposure to FSH. In fact, basal AMH expression is triggered by SOX9 and enhanced by SF1, GATA4, and WT1 independent of
FSH [reviewed in Ref. (10)]. Afterward, FSH increases testicular AMH output by inducing Sertoli cell proliferation and AMH transcription following the classic FSH receptor transduction pathway involving protein kinase A and cyclic AMP (10, 11). Sertoli cells also secrete inhibin B, which is present at high levels in the serum of mid-term fetuses and only slightly lower by term (8, 9). Sertoli cells are not directly regulated by androgens during fetal life since they do not express the androgen receptor [reviewed in Ref. (12)]. Approximately 1 week later than Sertoli cells do, Leydig cells differentiate in the interstitial tissue and secrete testosterone, responsible for the differentiation of the male gonaduct, the prostate, and the external genitalia, independently from fetal pituitary LH. In fact, the major regulator of testosterone production during the first trimester is chorionic gonadotropin (hCG), which circulates at high levels in fetuses with a peak at 12–17 weeks subsequently decreasing through term (7, 13). The relevance of fetal LH in Leydig cell function becomes more evident during the second and third trimesters. Both LH and hCG act on the same transmembrane receptor, the LHCG-R, present on the Leydig cell membrane and inducing cell proliferation and differentiation as well as androgen and insulin-like 3 (INSL3) synthesis and secretion. Male differentiation of internal and external genitalia is completed in the first trimester (Figure 1). Afterward, androgens induce the growth of the phallus and the trophism of the scrotum, whereas both androgens and INSL3 are important for testicular descent (14).

**POST-NATAL LIFE: INFANCY, CHILDHOOD, AND PUBERTY**

The decreasing trend in the whole hypothalamic–pituitary–testicular axis activity is reflected in low perinatal levels of all hormones (Figure 2) (9). Thereafter, an increase in circulating levels is observed in the neonate already by the end of the first week for gonadotropins, and from the second to fourth weeks for AMH, inhibin B, and testosterone (15, 16). It should be noted for testosterone that serum samples must be extracted to avoid interferences that artificially overestimate results (Figure 2). LH drives testosterone and INSL3 to peak levels during the third month; thereafter, they all decline and attain very low or undetectable levels after the sixth month (Figure 1) (16–18). Assays for INSL3 are now commercially available, with sufficient sensitivity to be used in patients during childhood (19), although an hCG test may be needed.

On the other hand, FSH continues to induce Sertoli cell proliferation resulting in a continuous increase in testis volume. Androgens may also exert a proliferative effect on Sertoli cells (20), but the effect should be indirect since the androgen receptor is still not expressed in Sertoli cells during early infancy [reviewed in Ref. (12)]. It should be noted that the absolute volume increment described in this period of life is modest (<1.5 mL) and cannot be clinically evidenced by palpation (21). AMH and inhibin B secretion is also enhanced: the levels of both hormones increase progressively through infancy (Figure 1).
The increase observed during the first months of life may be linked to the marked proliferation of Sertoli cells that occurs after mid-gestation with a further increment after birth (24), probably enhanced by the post-natal gonadotrophic surge. Serum inhibin B levels are as high as those observed in pubertal boys during the first 6 months of age; thereafter, a progressive fall occurs until the age of 4–6 years, but serum concentrations remain considerable, since they are above the lowest limit of normal adult range (23), and can be readily detected with the commercially available new generation assays (25). Serum AMH peaks during the second year and then remains fairly stable during childhood (22, 26). Altogether, these data clearly indicate that Sertoli cells are functionally active during infancy and childhood.

Testosterone and inhibin B are the most relevant physiological factors involved in gonadotropin negative feedback in the adult. A possible role for inhibin B in FSH negative feedback before puberty is still a matter of debate. Higher FSH than LH levels observed in boys with no functional gonadal tissue (27–29), the inverse correlation between FSH and inhibin B levels observed in cryptorchid boys (30), and the suppression of FSH secretion observed in pre-pubertal patients with Sertoli cell neoplastic proliferations and increased inhibin B (31) support the hypothesis of the active role that inhibin B has in regulating FSH. However, the decrease in LH and FSH levels during normal male childhood is not fully dependent on these testicular hormones, since it also occurs in a considerable proportion of boys with gonadal dysgenesis (27) or anorchia (Figure 3) (29).

A progressive increase in gonadotropin pulse amplitude and frequency occurring between 9 and 14 years of age triggers testicular pubertal maturation. LH induces Leydig cells androgen production again: intratesticular testosterone concentration increases and acts on Sertoli cells, which now express the androgen receptor. Consequently, they acquire a mature phenotype characterized by the development of the blood–testis barrier and a down-regulation of AMH production [reviewed in Ref. (12)]. The rise in serum testosterone occurs 1–2 years later (32, 33). Germ cells, hitherto limited to spermatogonia, enter meiosis and go through the complete spermatogenic process giving rise to spermatozoa. Spermatogenic development is responsible for the remarkable increase of testis volume during puberty. FSH and germ cells induce an increase in inhibin B. Serum levels of inhibin B increase concomitantly with testicular volume, and attains adult levels as early as pubertal stage II (23, 34, 35). INSL3 secretion also increases during puberty (36); in adult, the production and secretion of INSL3 is maintained by the long-term trophic effect of LH on Leydig cell structure and function and independent of the acute steroidogenic effect of LH (16).

**DEFINITION AND CLASSIFICATION OF CONGENITAL MALE HYPOGONADISM**

From the comprehension of the changes occurring in the normal physiology of the pituitary–testicular axis during pre- and postnatal life, it emerges clearly that male hypogonadism cannot be limited to hypoandrogenism. The definition should be extended to all situations characterized by a decreased testicular function, as compared to what is expected for age, involving an impaired hormone secretion by Leydig cells (androgens, INSL3) and/or Sertoli cells (AMH, inhibin B) and/or a disorder of spermatogenesis (Table 1).

It should also be considered that the clinical manifestations of male hypogonadism will vary according to: (a) the level of the hypothalamic–pituitary–testicular axis primarily affected, (b) the testicular cell population initially impaired, and (c) the period of life when the condition is established (37).

**LEVEL OF THE AXIS PRIMARILY AFFECTED: CENTRAL, PRIMARY, OR COMBINED HYPOGONADISM**

In central (or hypothalamic–pituitary) hypogonadism, testicular failure is secondary to a disorder affecting the secretion of GnRH
In primary hypogonadism, the testis is the primarily affected organ. This may lead to an impaired production of testicular hormones and a disruption of the negative feedback to the hypothalamic–gonadotrope axis, which results in an elevation of FSH and/or LH. In adult endocrinology, primary hypogonadism is usually identified as hypergonadotropic; however, as discussed in the previous section, during childhood, primary hypogonadism – or even agonadism – may present with normal gonadotropin levels (27–29).

In certain disorders, both the hypothalamic–gonadotrope axis and the testis are affected concomitantly, e.g., in DAX1 mutations or in oncologic patients exposed to cranial radiotherapy and chemotherapy. These “dual” conditions are characterized by a lack of gonadotropin elevation during puberty or adulthood in spite of the low testicular secretion of androgens and/or inhibin B [reviewed in Ref. (37)].

### WHOLE TESTICULAR VERSUS SPERMATOGENIC, LEYDIG CELL-SPECIFIC OR SERTOLI CELL-SPECIFIC FAILURE

Whole testicular failure or hypogonadism reflects the concomitant impairment of all testicular cell populations. On the contrary, the disorder may primarily involve only one testicular cell population; for instance, spermatogenic-specific failure results from Yq chromosome deletions, steroidogenic failure from defects in LH, its receptor or steroidogenic enzymes, and Sertoli cell-specific hypogonadism from defects in FSH or its receptor or in the AMH gene, as we discuss more in detail below.

### ONSET OF MALE HYPOGONADISM: FETAL VERSUS POST-NATAL LIFE

Male hypogonadism can be congenital, i.e., fetal-onset hypogonadism, or result from a condition acquired during post-natal life. The clinical presentation depends on the period of life in which testicular failure is established. In adulthood, androgen deficiency leads to decreased libido, impotence, fatigue, loss of bone and muscle mass, increased fat mass and metabolic disorders, and spermatogenic failure results in oligo- or azoospermia. At pubertal age, male hypogonadism results in the absence or the arrest...
of pubertal development. Because the hypothalamic–pituitary–
steroidogenic function is normally low during childhood – as
explained above – male hypogonadism remains clinically unap-
parent when established in this period of life unless suspected and
actively sought for by measuring serum AMH or inhibit B in basal
conditions, or testosterone or INSL3 after stimulation with hCG
[reviewed in Ref. (37)]. Fetal-onset hypogonadism may lead to
a variety of clinical presentations, which are discussed in detail
below.

**PATHOPHYSIOLOGY OF FETAL-ONSET MALE
HYPOGONADISM**

The clinical consequences of fetal-onset male hypogonadism can be
deduced from the understanding of the normal ontogeny of the
male reproductive axis during fetal life described above. When
established in the first trimester, the lack or insufficient levels of
testis hormones during the critical window of male sex differenti-
tation (weeks 8–13) lead to disorders of sex development (DSD)
preventing with female or ambiguous genitalia. Because Leydig
cell androgen production is essentially under placental hCG – not
fetal LH – control in the first trimester, central hypogonadism
do not result in DSD. Primary hypogonadism established in the
second half of gestation and central hypogonadism lead to a
decreased number of Sertoli cells and also to an impaired testicu-
lar output of androgens and INSL3. The clinical consequences are
microorchidism, micropenis, and cryptorchidism.

**PRIMARY HYPOGONADISM ESTABLISHED IN THE FIRST TRIMESTER
Whole testicular dysfunction: gonadal dysgenesis**

Gonadal dysgenesis may result from chromosomal aberrations or
mutations affecting genes controlling testicular differentiation
(Table 2). Chromosome aberrations involving the short arm of the
Y chromosome cause gonadal dysgenesis affecting all cell pop-
ulations. Similarly, deletions of the short arm of chromosome
9 – where *DMRT1* and *DMRT2* map (38) – and duplications of
Xp21.3-p21.2 – where *DAX1* gene maps (39) and of 1p31-p35 –
where *WNT4* maps (40) – result in testicular dysgenesis. 46,XY
patients with mutations in *SRY* (41) or *MAML1* (42) also present
with gonadal dysgenesis. Mutations in other genes associated tes-
ticular dysfunction with dysfunctions of other organs (Table 2).
*SFI* mutations may associate gonadal dysgenesis with adrenal fail-
ure (43), yet isolated testicular dysfunction can be observed (44).
Mutations in *WT1* result in gonadal dysgenesis associated with
degenerative renal disease, resulting in Denys–Drash syndrome
or in Frasier syndrome (45). Haplinsufficiency of *SOX9* leads
to a polymorphic syndrome including gonadal dysgenesis, bowing
and angulation of long bones (known as campomelic dys-
plasia), hip dislocation, hypoplastic scapula, small thoracic cage,
macrocephaly, facial dysmorphism, and cardiac and renal defects
(40, 46). Homozygous mutations of *DHH* gene result in the asso-
ciation of gonadal dysgenesis and minifascicular neuropathy (47,
48). Mutations in *XI2* gene cause the ATRX syndrome, character-
ized by α-thalassemia, mental retardation, facial dysmorphism and
gonal dysgenesis (49). Recently, *MAP3K1* mutations have been
identified as another cause of partial or complete gonadal dysgen-
esis (50). Finally, mutations in *TSPYL1* have been found in patients
with gonadal dysgenesis and sudden death (51). However, the
vast majority of dysgenetic DSD cases remain unexplained, which
suggests that several other gene defects may be the underlying
cause.

Exposure to environmental disruptors *in utero* has also been
implicated as the underlying cause for interlinked reproductive
disorders like cryptorchidism, hypospadias, infertility and tes-
ticular cancer, which seem to show an increasing trend. This
association is known as the testicular dysgenesis syndrome (52).

When the gonadal dysgenesis is complete, internal and external
genitalia differentiate along the female pathway since the streak
gonads do not secrete any androgens or AMH. These 46,XY girls
are apparently normal and do not seek medical attention until
pubertal age when they present with absence of telarche and
menarche. Only in the case of contradiction between a karyotype
performed during gestation and the lack of virilization, does the
case present to the specialist immediately after birth.

In partial forms of testicular dysgenesis, the degree of undervir-
ilization depends on the amount of functional gonadal tissue the
patient has. The external genitalia may be more or less ambigu-
ous, testes do not descend and Wolffian derivatives are more or
less atrophic as signs of insufficient androgen secretion, reflect-
ing Leydig cell dysfunction. The persistence of müllerian deri-
atives reflects defective AMH production as a sign of Sertoli cell
dysfunction.

In both complete and partial forms, the androgen and inhibit
B feedback mechanisms are insufficient and the gonadotrope
secretion of gonadotropin is exaggerated.

**Leydig cell-specific dysfunction: isolated fetal hypoandro-
genism**

When only Leydig cell development and/or function are primarily
disturbed in the first trimester of fetal life, insufficient androgen
production results in undervirilisation and cryptorchidism. On
the contrary, Sertoli cells are normally active and secrete AMH
which induces full regression of Müllerian ducts. Therefore, this
apparently normal girl has no uterus and a short blind-end vagina.
Similar to complete gonadal dysgenesis, these patients seek medical
attention at pubertal age because of the absence of telarche and
primary amenorrhea. In the cases of a partial defect, androgen
secretion is insufficient to virilize the fetus adequately: the new-
born has ambiguous external genitalia and hypotrophic Wolffian
duct derivatives. The degree of virilization is commensurate with
the residual steroidogenic activity of the gonads. The gonadotrope
secretes excessive gonadotropins with an increased LH:FSH ratio,
because FSH is negatively regulated by inhibit B.

Leydig cell aplasia is a rare form of isolated fetal hypoandro-
genism leading to a DSD due to inactivating mutations of the
LHCG-R (Table 3) [reviewed in Ref. (53)]. Defective androgen
production by the testis can also result from mutations in one of
the five enzymatic activities necessary for the synthesis of testos-
terone from cholesterol (Table 3). Three of these are common to
adrenal and gonadal steroidogenesis: cholesterol side-chain cleav-
age (*P450scC*), 3β-hydroxysteroid dehydrogenase (3β-HSD), and
17α-hydroxylase (*P450c17*). A deficiency in any of these in 46,XY
individuals results in testicular hypoandrogenism leading to geni-
tal ambiguity and adrenal insufficiency leading to congenital
adrenal hyperplasia. Two steroidogenic steps – 17,20-lyase (activ-
ity contained in *P450c17*) and 17β-hydroxysteroid dehydrogenase
Table 2 | Clinical features in male patients with fetal-onset primary hypogonadism with whole gonadal dysfunction.

| Affected chromosome | Gene | OMIM | Associated clinical features |
|---------------------|------|------|------------------------------|
| 9p24 deletion       | DMRT1 and DMRT2 | #154230 | Dysgenetic DSD |
|                     |       |      | Mental retardation, microcephaly, facial malformations, short stature | |
|                     |       |      | Digestive or bronchial malformations | |
| Xp21 duplication    | DAX1 = NR0B1 and other genes | #300018 | Dysgenetic DSD |
| 1p31-p35 duplication| WNT4 and other genes | *603490 | Dysgenetic DSD |
| Yp11.31             | SRY   | *48000 | Dysgenetic DSD |
| Xq28                | MAML1D1 | *300120 | Dysgenetic DSD |
| 9q33.3               | SF1 = NR5A1 | +184757 | Dysgenetic DSD |
|                     |       |      | Adrenal insufficiency | |
| 11p13               | WT1   | #136680 | Dysgenetic DSD |
|                     |       | #194072 | Renal dysgenesis/tumor (Denys-Drash, Frasier and WAGR syndromes) |
|                     |       | #194080 | | |
| 17q24.3             | SOX9  | #114290 | Dysgenetic DSD |
|                     |       |       | Campomelic dysplasia | |
| 12q13.12            | DHH   | #233420 | Dysgenetic DSD |
|                     |       |       | Minifascicular neuropathy | |
| Xq21.1              | ATRX = XH2 | #301040 | Dysgenetic DSD |
|                     |       |       | Mental retardation, α-thalassemia | |
| 5q11.2              | MAP2K1 | #613762 | Dysgenetic DSD |
| 6q22.1              | TSPYL1 | #608800 | Dysgenetic DSD |
|                     |       |       | Sudden infant death | |

DSD, disorder of sex development; OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

(17β-HSD) – are required only for gonadal steroidogenesis; therefore, their defects result only in hypovirilization without adrenal insufficiency [reviewed in Ref. (53)].

**Sertoli cell-specific dysfunction: AMH deficiency**

The persistent Müllerian duct syndrome (PMDS) is a rare form of DSD characterized by persistence of Müllerian derivatives in otherwise normally virilized 46,XY individuals. Regression of Müllerian ducts normally occurs between 8 and 10 weeks of fetal development, under the influence of AMH produced by fetal Sertoli cells. If active AMH is not produced, owing to AMH gene mutations, Müllerian ducts develop into uterus, fallopian tubes, and upper vagina notwithstanding normal virilization of external genitalia and urogenital sinus. PMDS can also be consecutive to mutations of the AMH receptor type II gene (AMHR2), but in this case testicular function is normal [reviewed in Ref. (54)]. PMDS should not be considered in patients with defects in the virilization of external genitalia. Gonadotrope activity is not affected during fetal life.

**PRIMARY HYPOGONADISM ESTABLISHED IN THE SECOND AND THIRD TRIMESTERS**

**Whole testicular dysfunction: testicular regression syndrome**

The existence of fully virilized external genitalia, i.e., completely fused scrotum and a urethral opening at the tip of the penis, is indicative of the existence of functional testes in the first trimester of gestation. However, the gonads may undergo regression (vanishing testes) due to torsion of the spermatic cord or to other unknown situations, resulting in a deficient or completely absent exposure to testicular hormones until the end of fetal life. The hypoandrogenism leads to scrotal hypotrophy and micropenis. Androgen and inhibin B insufficiency results in an exaggerated gonadotrope activity.

**Leydig cell-specific dysfunction: INSL3 deficiency**

Mutations in INSL3 lead to a rare form of Leydig cell-specific dysfunction without hypoandrogenism. Newborns are normally virilized but present with cryptorchidism, reflecting the defect in testicular descent [reviewed in Ref. (55)]. Because INSL3 has no effect on the gonadotrope, LH and FSH secretion are not disturbed in these individuals during fetal life.

**Sertoli cell-specific dysfunction: FSH receptor mutations**

As already discussed, Sertoli cell differentiation in early fetal life is not dependent on FSH; therefore, male fetuses with FSH receptor mutations secrete sufficient amounts of AMH to induce Müllerian duct regression. On the contrary, since FSH is an important Sertoli cell mitogen, FSH-R mutations lead to Sertoli cell hypoplasia and small testes. Adults have low sperm count, low inhibin B, and moderately elevated FSH (56).
Although variable, there are a few clinical signs that may help in the identification of the underlying cause (Table 4) [reviewed in Ref. (58, 59)]. For instance, the association of congenital multiple pituitary hormone deficiency with septo-optic dysplasia (midline neural defects and optic nerve hypoplasia) has been observed in patients with mutations in HESX1, SOX2 and SOX3. Midline defects, coloboma and polydactyly are also present in HESX1 patients, anophthalmia or microphthalmia and esophageal atresia in SOX2 cases, and X-linked mental retardation in SOX3 mutations. LH3 defects are present in patients with rigid and short cervical spine; LHX4 mutations can be found in individuals with abnormalities in the central skull base; GLI2 in patients with holoprosencephaly; PITX2 in patients with Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism, and protuberant umbilicus); SIX6 in patients with absent optic chiasm and brain cortical atrophy, and OTX2 in patients with microphthalmia. Defects in late development factors, like PROPI are present in non-syndromic patients with multiple pituitary hormone deficiencies. Currently, only <15% of the etiologies of congenital hypopituitarism have been identified (60).

Isolated hypogonadotropic hypogonadism. Congenital isolated central hypogonadism can present as the only manifestation of the disorder (normosmic hypogonadotropic hypogonadism), or be associated with partial or complete loss of olfaction (Kallmann syndrome or anosmic hypogonadotropic hypogonadism), usually associated with other anatomical and/or neurological defects [reviewed in Ref. (61)].

Hyposmic/anosmic hypogonadotropic hypogonadism with or without other syndromic features results from mutations in the genes involved in the development and migration of the GnRH primordium. Mutations in genes involved in early pituitary differentiation and development usually result in multiple pituitary hormone deficiency including hypogonadotropic hypogonadism, usually due to pituitary hypoplasia. Although variable, there are a few clinical signs that may help in the identification of the underlying cause (Table 4) [reviewed in Ref. (58, 59)]. For instance, the association of congenital multiple pituitary hormone deficiency with septo-optic dysplasia (midline neural defects and optic nerve hypoplasia) has been observed in patients with mutations in HESX1, SOX2 and SOX3. Midline defects, coloboma and polydactyly are also present in HESX1 patients, anophthalmia or microphthalmia and esophageal atresia in SOX2 cases, and X-linked mental retardation in SOX3 mutations. LH3 defects are present in patients with rigid and short cervical spine; LHX4 mutations can be found in individuals with abnormalities in the central skull base; GLI2 in patients with holoprosencephaly; PITX2 in patients with Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism, and protuberant umbilicus); SIX6 in patients with absent optic chiasm and brain cortical atrophy, and OTX2 in patients with microphthalmia. Defects in late development factors, like PROPI are present in non-syndromic patients with multiple pituitary hormone deficiencies. Currently, only <15% of the etiologies of congenital hypopituitarism have been identified (60).

### Table 3 | Clinical features in male patients with fetal-onset primary hypogonadism with Leydig cell-specific (steroidogenic) dysfunction.

| Gene       | Protein                        | OMIM         | Hormone levels                  | Associated clinical features            |
|------------|--------------------------------|--------------|---------------------------------|----------------------------------------|
| LHCG-R     | LH/CG receptor                 | #238320      | ↓↓ All steroids                 | None                                   |
| STAR       | StAR                           | #201710      | ↓↓ All steroids                 | Lipoid congenital adrenal hyperplasia   |
| CYP11A1    | P450sc                          | #613743      | ↓↓ All steroids                 | Adrenal insufficiency                  |
| CYP17A1    | P450c17 (17α-hydroxylase activity) | #202110    | ↑ Pregnenolone                  | Adrenal insufficiency                  |
|            | P450c17 (17,20-lyase activity)  | #202110      | ↑ 17OH-pregnenolone             | Adrenal insufficiency                  |
|            |                                |              | ↑ 17OH-progesterone             | Hypertension                           |
|            |                                |              | ↓↓ Pregnenolone                 |                                       |
|            |                                |              | ↑ Progesterone                  |                                       |
| POR        | P450 oxidoreductase             | #613571      | ↑ Progesterone                  | Antley–Bixler syndrome                 |
| HSD3B2     | 3β-HSD type 2                   | #201810      | ↑ DHEA                          | Adrenal insufficiency                  |
|            |                                |              | ↑ 17OH-pregnenolone             |                                       |
| HSD17B3    | 17β-HSD type 3                  | #264300      | ↑ Androstenedione               | None                                   |
|            |                                |              | ↑ DHEA                          |                                       |
|            |                                |              | ↑ 17OH-progesterone             |                                       |
|            |                                |              | ↑ 17OH-pregnenolone             |                                       |

OMIM: Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

### Central hypogonadism established in the second and third trimesters

**Whole testicular dysfunction: hypogonadotropic hypogonadism**

As already discussed, deficient LH and FSH production by the fetal pituitary has no effect on sexual differentiation occurring in the ninth to thirteenth weeks of gestation, but do impact on genital development dependent on testicular function in the second and third trimesters of fetal life. Gonadotropin deficiency may result from an impaired differentiation of the gonadotrope in the context of a defective development of the pituitary primordium, and is therefore associated with multiple pituitary hormone deficiency. Alternatively, the defect may be restricted to the gonadotrope axis as a consequence of an impaired development, migration or function of the GnRH neurons, or of an impaired function of the gonadotrope. The lack of gonadotropin stimulus in this period of fetal development may result in small testes due to FSH deficiency, micropenis reflecting hypoandrogenism due to LH deficiency, and cryptorchidism as a sign of androgen and INSL3 insufficiency secondary to LH deficiency.

**Multiple pituitary hormone deficiency.** Congenital hypopituitarism occurs in approximately 1:4,000–1:10,000 newborns, with a 7:3 male-to-female ratio (57), and involves multiple pituitary cell lineages in approximately 80% of the cases. Mutations in genes involved in early pituitary differentiation and development usually result in multiple pituitary hormone deficiency including hypogonadotropic hypogonadism, usually due to pituitary hypoplasia. Although variable, there are a few clinical signs that may help in the identification of the underlying cause (Table 4) [reviewed in Ref. (58, 59)]. For instance, the association of congenital multiple pituitary hormone deficiency with septo-optic dysplasia (midline neural defects and optic nerve hypoplasia) has been observed in patients with mutations in HESX1, SOX2 and SOX3. Midline defects, coloboma and polydactyly are also present in HESX1 patients, anophthalmia or microphthalmia and esophageal atresia in SOX2 cases, and X-linked mental retardation in SOX3 mutations. LH3 defects are present in patients with rigid and short cervical spine; LHX4 mutations can be found in individuals with abnormalities in the central skull base; GLI2 in patients with holoprosencephaly; PITX2 in patients with Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism, and protuberant umbilicus); SIX6 in patients with absent optic chiasm and brain cortical atrophy, and OTX2 in patients with microphthalmia. Defects in late development factors, like PROPI are present in non-syndromic patients with multiple pituitary hormone deficiencies. Currently, only <15% of the etiologies of congenital hypopituitarism have been identified (60).

| Gene       | Protein                        | OMIM         | Hormone levels                  | Associated clinical features            |
|------------|--------------------------------|--------------|---------------------------------|----------------------------------------|
| LHCG-R     | LH/CG receptor                 | #238320      | ↓↓ All steroids                 | None                                   |
| STAR       | StAR                           | #201710      | ↓↓ All steroids                 | Lipoid congenital adrenal hyperplasia   |
| CYP11A1    | P450sc                          | #613743      | ↓↓ All steroids                 | Adrenal insufficiency                  |
| CYP17A1    | P450c17 (17α-hydroxylase activity) | #202110    | ↑ Pregnenolone                  | Adrenal insufficiency                  |
|            | P450c17 (17,20-lyase activity)  | #202110      | ↑ 17OH-pregnenolone             | Adrenal insufficiency                  |
|            |                                |              | ↑ 17OH-progesterone             | Hypertension                           |
|            |                                |              | ↓↓ Pregnenolone                 |                                       |
|            |                                |              | ↑ Progesterone                  |                                       |
| POR        | P450 oxidoreductase             | #613571      | ↑ Progesterone                  | Antley–Bixler syndrome                 |
| HSD3B2     | 3β-HSD type 2                   | #201810      | ↑ DHEA                          | Adrenal insufficiency                  |
|            |                                |              | ↑ 17OH-pregnenolone             |                                       |
| HSD17B3    | 17β-HSD type 3                  | #264300      | ↑ Androstenedione               | None                                   |
|            |                                |              | ↑ DHEA                          |                                       |
|            |                                |              | ↑ 17OH-progesterone             |                                       |
|            |                                |              | ↑ 17OH-pregnenolone             |                                       |
Table 4 | Clinical features in male patients with fetal-onset central hypogonadism associated with multiple pituitary hormone deficiency.

| Gene | OMIM | Other pituitary lineages affected | Associated clinical features |
|------|------|-----------------------------------|-------------------------------|
| HESX1 | #182230 | Somatotrope, Lactotrope, Thyrotrope, Corticotrope | Septo-optic dysplasia, Midline defects, Coloboma, Polydactyly |
| SOX2 | #206900 | Somatotrope | Septo-optic dysplasia, Anophthalmia/microphthalmia, Sensorineural defects, Esophageal atresia |
| SOX3 | #312000 | Somatotrope, Thyrotrope, Corticotrope | Septo-optic dysplasia |
| LHX3 | #221750 | Somatotrope, Lactotrope, Thyrotrope | Rigid and short cervical spine, Limited head rotation |
| LHX4 | #262700 | Somatotrope, Thyrotrope, Corticotrope | Hindbrain defects, Abnormality of central skull base |
| GLI2 | #610829 | Somatotrope, Lactotrope, Thyrotrope, Corticotrope | Holoprosencephaly |
| PITX2 | #180500 | Somatotrope, Thyrotrope | Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism and protuberant umbilicus) |
| SIX6 | #212550 | Somatotrope | Anophthalmia, Brain cortical atrophy, Brachiootoenal syndrome, Oculoauriculovertebral spectrum |
| OTX2 | #613986 | Somatotrope, Thyrotrope, Corticotrope | Microphthalmia/anophthalmia, Cleft palate, Developmental delay |
| PROP1 | #262600 | Somatotrope, Thyrotrope, Corticotrope | Intra-and extra-sellar cell mass, which may degenerate leading to empty sella later in life |

OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

neurons from the olfactory placode to the hypothalamus [reviewed in Ref. (61)]. The insufficient GnRH production is associated with olfactory bulb hypoplasia or aplasia in magnetic resonance imaging. Associated clinical manifestations may change according to the defective gene: KAL1, FGF8 and its receptor FGFR1, PROKR2 and its receptor PROKR2, CHD7, NELF, HS6ST1, WDR11, SEMA3A (Table 5) [reviewed in Ref. (4)].

Normosmic isolated hypogonadotropic hypogonadism is the consequence of defects in genes involved in the regulation and function of the GnRH neuron or the gonadotrope. Impaired GnRH production may result from mutations in the GNRH1 gene or from defective regulation of the GnRH neuron by kisspeptin, neurokinin, or leptin signaling via their respective receptors. Mutations in the GNRHR gene, encoding the GnRH receptor present in the gonadotrope, are responsible for an impaired pituitary response to GnRH. In all the cases, except for defects in the neurokinin system, the secretion of both LH and FSH is impaired.

**Cell-specific dysfunction: dissociated hypogonadism**

**Isolated LH deficiency.** Congenital isolated LH deficiency with normal or high FSH production results from mutations in the LHB gene encoding the β subunit of LH (62, 63), and from defects in the neurokinin system responsible for the regulation of GnRH pulses. Neurokinin is a neuropeptide encoded by TAC3, which signals via the neurokinin receptor encoded by TACR3 (64, 65). Micropenis and cryptorchidism may be observed, as a consequence of the fetal hypoandrogenism during the second and...
Table 5 | Associated clinical features in male patients with fetal-onset isolated central hypogonadism due to defects in the migration of the GnRH neuron.

| Gene | OMIM | Associated clinical features |
|------|------|-----------------------------|
| KAL1 | #308700 | Bimanual synkinesia, unilateral renal agenesis Less frequently: palate defects (cleft lip/palate), dental agenesis, ataxia, nystagmus, ear anomalies, hearing loss, visual defects, abnormal ocular movements |
| FGFR8/FGFR1 | #612702/#147950 | Cleft lip/palate, bone anomalies (syndactilia), dental agenesis Less frequently: hearing loss, bimanual synkinesia, ear anomalies, midline facial defects, choanal atresia, cardiac defects, coloboma |
| PROK2/PROKR2 | #610628/#244200 | Sleep disorder, high-arched palate, bimanual synkinesia, hearing loss, pectus excavatum, hypodontia, obesity, nystagmus |
| CHD7 | #612370 | Coloboma, heart defects, choanal atresia, retardation of growth, genital anomalies, and ear abnormalities |
| NELF | #614838 | None |
| HS6ST1 | #614880 | Cleft lip/palate, clinodactyly |
| WDR11 | #614858 | No |
| Sema3A | #614897 | No |

OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

third trimesters, but there is normal testes volume in the newborn and child, because FSH levels are adequate. A mild form of isolated LH deficiency is the underlying pathophysiology of the “fertile eunuch” syndrome, characterized by the absence of signs of hypoandrogenism until puberty, when eunuchoid proportions become apparent in males with normal testis volume and sperm production. Mutations in GNRHR (66) and LHB (67) genes have been described.

Isolated FSH deficiency. Male fetuses with insufficient FSH may develop small testes during the second and third trimesters owing to Sertoli cell hypoplasia. External genitalia do not show signs of hypoandrogenism since LH production is normal or elevated (68).

Combined or Dual (Primary and Central) Hypogonadism

DAX1 is a transcription factor encoded by NR0B1 mapping to the short arm of the X chromosome. It has essential functions at several levels of the pituitary–gonadal and adrenal axes. DAX1 mutations result in a disorder characterized by adrenal hypoplasia and combined hypogonadism (Table 1). Testicular Sertoli and Leydig cell function is primarily affected resulting in moderately low hormone production; however, since the hypothalamic–pituitary axis is also defective, the gonadotrope is unable to increase LH and FSH production, despite the absence of an effective negative feedback loop.

Prader–Willi syndrome is another form of combined central and primary hypogonadism. This condition results from the lack of the paternally inherited chromosome 15 region q11-q13; this can be due to deletions in the paternal chromosome, to maternal disomy of 15q11-q13, or to a defective imprinting that silences the paternal chromosome 15. Several genes expressed exclusively from the paternal chromosome are believed to be involved in this syndrome (including MAGEL2, MKRN3, NDN, SNURF-SNRPN, and the HBII genes), although their underlying mechanism is not well understood (69). Hypogonadism is reflected in signs such as microopenis, cryptorchidism, scrotal hypoplasia, and microorchidism (70). However, the pathophysiology seems to be heterogeneous, and hypogonadism may be observed earlier or later in life, with a diverse participation of the hypothalamic–pituitary axis (19,71–74).

DIAGNOSTIC ASSESSMENT OF FETAL-ONSET MALE HYPOGONADISM

The clinical and laboratory assessment of boys with suspected hypogonadism shows a wide spectrum and varies according to the etiology of the condition. Signs of hypoandrogenism are common to all; however, as already discussed, these signs will vary according to the period of fetal life in which hypogonadism is established. On the other hand, the evaluation of the other testicular hormones, and of any associated non-reproductive phenotype, may be extremely helpful in the diagnostic assessment of these boys.

PATIENTS WITH AMBIGUOUS OR UNDERVIRILIZED EXTERNAL GENITALIA

If fetal hypogonadism is the underlying cause for the existence of a DSD presenting with ambiguous or insufficiently virilized genitalia (i.e., hypospadias, bifid scrotum), the condition can only be due to primary gonadal failure. The need for a differential diagnosis between testicular dysgenesis (i.e., whole gonadal dysfunction) and a specific steroidogenic failure emerges. A few clinical signs can be helpful in certain cases: the existence of two palpable gonads >1 mL is highly indicative of non-dysgenetic DSD (75), whereas the association of syndromic phenotypes – like skeletal dysplasia, macro/microcephaly, cardiac or renal defects, thalassemia, mental retardation, or minifacicular neuropathy orientate to gonadal dysgenesis (Table 2). Skeletal dysmorphisms may be present in patients with POR deficiency associated with the Antley–Bixler syndrome. Association with adrenal insufficiency is indicative of a non-dysgenetic steroidogenic defect (StAR, P450sc, P450c17, POR, 3β-HSD), although mutations in SF1 resulting in gonadal dysgenesis are also a possible cause.

Results from hormonal laboratory assessment in the newborn and infant should be interpreted according to reference values for age. In DSD patients, this is particularly relevant in the first month of life (Figure 2) (15), when patients are studied for diagnosis. During the first 3–6 months after birth, basal hormone level determinations may be helpful (Figure 4). The existence of normal levels of testosterone, AMH, and inhibit B rule out testicular dysfunction, and other etiologies of DSD should be sought (76, 77). When all testicular hormones are low and gonadotropins are elevated, gonadal dysgenesis is most likely [reviewed in Ref. (77)].
Low testosterone (53) with normal or elevated AMH (78) is characteristic of Leydig cell-specific hypogonadism. A prolonged hCG test (six IM injections every other day) and an ACTH test are necessary to distinguish between LHCG-R, STAR, and steroidogenic enzyme defects (Table 3). Gonadotropin levels may be somewhat elevated in the first months of life but they are usually normal during childhood in patients with steroidogenic defects (53). This is another example where primary hypogonadism is not hypergonadotropic in pediatric patients.

**Patients with Male Genitalia**

The existence of normal male external genitalia rules out a fetal primary hypogonadism established in the first trimester, except for the rare form of Sertoli cell dysfunction due to AMH mutations leading to PMDS (54). PMDS patients were most frequently present with bilateral cryptorchidism; serum AMH is undetectable but the other reproductive hormones are within the normal range for age.

Fetal-onset central hypogonadism and primary hypogonadism established in the second or third trimester have clinical signs of hypoandrogenism as a common feature: small penis and undescended gonads. Microorchidism can be indicative of insufficient FSH stimulus – i.e., central hypogonadism – or of a testicular regression syndrome – i.e., a primary hypogonadism that can progress to anorchism. Because the hypothalamic–pituitary–testicular axis remains active for 3–6 months after birth (17, 18), this period represents a window of opportunity to establish the diagnosis of hypogonadism (1). However, the diagnosis can still be suspected and confirmed during the rest of infancy and childhood.

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**FIGURE 4** A schematic guide for the interpretation of serum hormone levels in patients with fetal-onset male hypogonadism. IHH, isolated hypogonadotropic hypogonadism; Inh B, inhibin B; MPHD, multiple pituitary hormone deficiency; Nx, normal. Levels are considered as high, normal, or low as compared to the reference levels for newborns or prepubertal boys.
In some cases, the clinical presentation with cholestasis and/or hypoglycemia in the newborn or failure to thrive in infants can orientate the diagnosis to multiple pituitary hormone deficiency. Associated malformations in cerebral and hypothalamic-pituitary regions found on magnetic resonance imaging can be of further help (Table 4). A familial history of anosmia/hyposmia is suggestive of the diagnosis of isolated central hypogonadism, which could be reinforced by some anatomical or neurodevelopmental features in the infant or child (Table 5). Associated primary adrenal failure could orientate to adrenal hypoplasia congenital due to DAX1 mutations, whereas neonatal hypotonia and developmental delay may be indicative of Prader–Willi syndrome.

In childhood, primary hypogonadism does not equate to hypergonadotrophic hypogonadism

The endocrine laboratory is necessary to certify the diagnosis of male hypogonadism. Basal gonadotropins, testosterone, and INSL3 are useful until the age of 3–6 months; thereafter dynamic stimulation tests are necessary to assess them. On the contrary, the Sertoli cell markers, AMH and inhibin B, are informative all through infancy and childhood without the need for stimulation tests. As discussed earlier, the occurrence of micropenis and non-palpable gonads prompts the differential diagnosis between central hypogonadism and testicular regression after the first trimester (Figure 4). If the patient is <3–6 months old, low levels of gonadotropins and Leydig and Sertoli cell hormones are suggestive of central hypogonadism (16, 79, 80), whereas high gonadotropins associated with low/undetectable testicular hormones are diagnostic of primary hypogonadism. After the age of 6 months, basal testosterone and INSL3 are no longer informative because they are normally low/undetectable during the rest of infancy and childhood. Low gonadotropins also lose usefulness. Undetectable AMH (81–83) and inhibin B (83, 84) are diagnostic of anorchia. The elevated levels of LH and FSH observed in these boys during the first years of life can subsequently decline to normal levels; therefore, serum gonadotropins within the reference range for age may not be informative during childhood (29). This is another clear example in pediatrics where primary hypogonadism is not hypergonadotrophic.

Central hypogonadism is not always hypergonadotrophic

The presence of micropenis, cryptorchidism, and microorchidism should prompt an early diagnosis of central hypogonadism, from which two main benefits may derive: first is to orientate the diagnosis of multiple pituitary hormone deficiency, favoring the opportune hormone replacement treatment (thyroid hormone, hydrocortisone, growth hormone). Second, as it has been posited, the occurrence of micropenis and non-palpable gonads prompts the differential diagnosis between central hypogonadism and testicular regression after the first trimester (Figure 4). If the patient is <3–6 months old, low levels of gonadotropins and Leydig and Sertoli cell hormones are suggestive of central hypogonadism (16, 79, 80), whereas high gonadotropins associated with low/undetectable testicular hormones are diagnostic of primary hypogonadism. After the age of 6 months, basal testosterone and INSL3 are no longer informative because they are normally low/undetectable during the rest of infancy and childhood. Low gonadotropins also lose usefulness. Undetectable AMH (81–83) and inhibin B (83, 84) are diagnostic of anorchia. The elevated levels of LH and FSH observed in these boys during the first years of life can subsequently decline to normal levels; therefore, serum gonadotropins within the reference range for age may not be informative during childhood (29). This is another clear example in pediatrics where primary hypogonadism is not hypergonadotrophic.

The hypoandrogenic states leading to micropenis and cryptorchidism – resulting from isolated LH deficiency due to mutations in the LHβ subunit or in the neurokinin system – are characterized by low LH and testosterone, but normal or elevated FSH. Interestingly, this central form of hypogonadism can even be hypergonadotropic, as observed in a young patient with delayed puberty, who had a functionally inactive but immunoreactive LH resulting in elevated serum levels associated with low testosterone (89).

Conversely, congenital isolated FSHβ deficiency, which presents with microorchidism but normal penile size and scrotal testes, has undetectable FSH and low inhibin B in adults with normal androgen with high LH after puberty (89, 90). No reports exist in childhood.

CONCLUDING REMARKS

Fetal hypogonadism of the first trimester is primary and results in dysgenetic or cell-specific forms of DSD. In the second and third trimesters, primary and central hypogonadism share signs of hypoandrogenism and defective INSL secretion – i.e., micropenis, hypoplastic scrotum and cryptorchidism – and of Sertoli cell hypoplasia – i.e., microorchidism. In prepubertal patients, classical serum markers, like gonadotropins and testosterone, are helpful essentially during the first 3–6 months of life. With the advent of AMH and inhibin B, a biochemical diagnosis can also be envisaged during the rest of childhood. Clinical findings may also help in the diagnosis beyond early infancy. Finally, the pediatrician should not expect elevated gonadotropin levels during childhood to foresee a primary hypogonadism.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript writing and approved the final version.

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