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REVIEW
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Testicular Sertoli Cell Hormones in Differences in Sex Development

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The Sertoli cells of the testes play an essential role during gonadal development, in addition to supporting subsequent germ cell survival and spermatogenesis. Anti-Müllerian hormone (AMH) is a member of the TGF-β superfamily, which is secreted by immature Sertoli cells from the 8th week of fetal gestation. Inhibin B is a glycoprotein, which is produced by the Sertoli cells from early in fetal development. In people with a Difference or Disorder of Sex Development (DSD), these hormones may be useful to determine the presence of testicular tissue and potential for spermatogenesis. However, fetal Sertoli cell development and function is often dysregulated in DSD conditions and altered production of Sertoli cell hormones may be detected throughout the life course in these individuals. As such this review will consider the role of AMH and inhibin B in individuals with DSD.

Keywords: AMH, inhibin B, testes, spermatogenesis, Sertoli cell

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INTRODUCTION

Gonadal development is a complex process, whereby the genital ridge is directed to develop into testes or ovaries. The testicular Sertoli cells play an essential role during gonadal development, in addition to supporting subsequent germ cell survival and spermatogenesis (1). Sertoli cells are responsible for the production of a variety of factors including hormones, binding proteins and signalling molecules that regulate testicular development and function throughout life. Anti-Müllerian hormone (AMH) and inhibin B are hormones that play a role in sex development during fetal life, as well as regulation of spermatogenesis in adulthood. Differences/Disorders of Sex Development (DSD) are a heterogeneous group of conditions with a wide range of aetiologies and clinical features. The presence of measurable AMH and inhibin B in infants with DSD suggests the presence of testicular tissue and indicates Sertoli cell function. However, in people with a DSD, fetal Sertoli cell development and function can be dysregulated. As both of these hormones can be useful clinically, this review will focus on the role of testicular AMH and inhibin B in individuals with DSD.

PATHWAYS OF TYPICAL SEX DEVELOPMENT AND TESTICULAR CELL DIFFERENTIATION

In human embryos, gonadal precursors are present from 32 days post conception and the gonads are bipotential until 6 weeks of gestation (2). As the testicular cords are established at 6-7 weeks post fertilisation, the Sertoli and interstitial cells (including Leydig-lineage cells) originate from common gonadal progenitors and subsequently differentiate (3, 4)

Sry, sex determination region on Y chromosome is largely responsible for testis differentiation. The Sry gene is expressed in pre-Sertoli cells at 7 weeks in the XY gonad and encodes a high mobility group (HMG) box transcription factor, which binds to specific target sequences in DNA, resulting in DNA bending (5). The expression of Sry is initiated by multiple transcription factors including GATA4/FOG2/NR5A11/WT1, resulting in induction of SOX9 expression. This is further augmented by the synergistic actions of Sry and NR5A1, leading to definitive Sertoli cell differentiation (6, 7).

Much of the data relating to sex differentiation in mammals is derived from mouse studies. However, studies using human fetal tissues provide support for these mechanisms also being important for sex development in humans. Mamsen et al. undertook gene expression analysis of key genes associated with gonadal development in 67 human first trimester fetuses obtained during elective termination of pregnancy. This study demonstrated that in the bipotential gonad, WT1 and NR5A1 were highly expressed, although concentrations of WT1 decreased over time. SOX9 gene expression increased to a peak at day 48. AMH was detected in Sertoli cells from 48 days. SRY expression peaked at 44 days post conception and then decreased to basal levels at day 60 (8). That said, SRY expression has also been identified in 46,XY gonads up to 18 weeks gestation in human embryonic and fetal tissue (9).

Once formed, Sertoli cells induce the development of fetal Leydig cells, *via* a *hedgehog* signalling pathway (10). At 8-9 weeks of development, the Leydig cells start to produce androgens and insulin-like factor 3 (INSL3) (11). Masculinisation of the indifferent external genitalia is induced by testosterone produced by the Leydig cells between weeks 9-20 of gestation (12, 13). Testicular descent is primarily under the control of the Leydig cells *via* the actions of INSL3 and testosterone. INSL3 regulates the first phase of testicular descent, acting *via* cyclic AMP with downstream effects *via* Wnt, β-catenin and BMP, causing the gubernaculum to swell, dilating the future inguinal canal and holding the testis close to the groin as the fetal abdomen enlarges between weeks 8-15 of gestation. The second stage of testis descent from the abdomen to the inguino-scrotal region occurs from approximately 25 weeks of gestation due to shortening of the gubernaculum cord and is dependent on the presence of sufficient exposure to androgen (14).

AMH – SECRETION AND REGULATION

AMH, previously known as Müllerian Inhibiting Substance (MIS), is a member of the TGF-β superfamily. It is a 140-kDa dimer glycoprotein, which is secreted by immature Sertoli cells from the 8th week of fetal gestation (15).

The AMH gene is located on chromosome 19 (16). Gonadotrophin-independent transcription is upregulated by SOX9, NR5A1, GATA4, WT1, AP-1 and AP-2. Late in fetal life and after birth, AMH transcription falls under the control of FSH *via* the adenyl-cyclase cyclic AMP (cAMP) pathway (17). Increased testicular AMH production in response to FSH activates protein kinase A (PKA)-mediated induction of SOX9, SF1, NFkB and AP-2, which bind to specific response elements on the AMH promoter (17, 18). FSH stimulation upregulates AMH transcription by phosphorylating the transcription factors binding to the promoter (19), an effect which is downregulated by testosterone (15).

Immunohistochemical labelling of testes has shown that the AR is expressed weakly in 2-15% of Sertoli cells from approximately the age of 5 months until the age of 4 years resulting in a physiological Sertoli cell androgen insensitivity during fetal and early postnatal life, which may protect the testes from premature Sertoli cell maturation (20). Expression progressively increases thereafter such that 90% of boys had high levels of AR expression from the age of 8 years (20). However, the AMH promoter does not have androgen response elements, and as such the androgen receptor must signal indirectly through SF-1 response elements (21).

INHIBIN B – SECRETION AND REGULATION

lnhibin B is a glycoprotein, secreted by Sertoli cells, which consists of α- and β-subunits. Most of its mechanism of action is *via* antagonism of activins on the activin type I and II receptors but there are some cells with specific inhibin-binding molecules, such as betaglycan (22). Recent genome wide association studies (GWAS) have demonstrated that LRRIQ1 and TSPAN19, two genes located on chromosome 12 may affect inhibin B production (23). Plasma inhibin B measurements reflect both Sertoli cell number and status of spermatogenesis (24). Inhibin B has a complex association with the hypothalamic-pituitary-gonadal (HPG) axis and FSH, with an initially positive association at around 3-6 months of age and prior to the onset of puberty, followed by a negative feedback loop (25).

TESTICULAR HORMONE PRODUCTION IN FETAL AND EARLY POSTNATAL LIFE

Testosterone production by the human fetal testis begins around 8 weeks gestation, with a peak between 14-17 weeks and then a sharp decline, so that in late pregnancy the serum concentration of testosterone is similar in male and female foetuses (**Figure 1**) (2, 26). Gonadotrophins are not required to initiate steroid

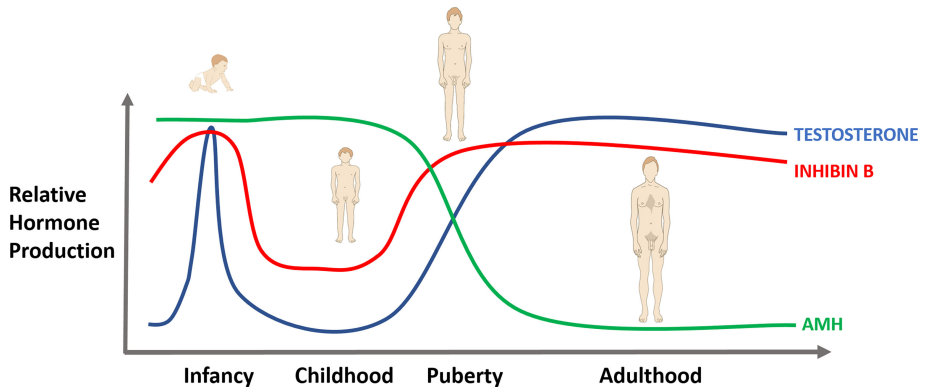

FIGURE 1 | Levels of testosterone, AMH and inhibin B from fetal life to adulthood.

synthesis during this time, but levels of testosterone are closely correlated with human chorionic gonadotrophin (hCG) levels during the early gestational period (2). Levels of testosterone and gonadotrophins are low towards the end of pregnancy and at birth before increasing in the early postnatal period, the so-called 'mini-puberty', and can be used as a window to assess the activity of the HPG axis in the first few months of postnatal life. However, they are of limited use to assess testicular function in the prepubertal boy, because of the relative inactivity of the axis during childhood.

AMH is produced at high levels in early fetal life, but is not measurable in amniotic fluid (27, 28). As the testes differentiate during the 7th week of gestation, Sertoli cells start producing AMH, which binds to the specific AMH Receptor Type II (AMHR2) on the Müllerian ducts, resulting in their regression before week 10 (29). AMH levels rise progressively from this point and then decline in the second year of life (**Figure 1**). AMH then remains stable until puberty, at which point it declines to adult levels, which are 3-4% of those in infancy (30). AMH is therefore an excellent marker of Sertoli cell function in infancy and early childhood, but its use becomes more difficult to interpret in adolescents with lower levels, as at this stage, reduction in AMH is an indicator of pubertal terminal differentiation and failure of the Sertoli cells (31). Serum levels of AMH are 50-fold lower in girls than boys at birth (32) and as such, it is a useful marker to confirm the presence of testicular tissue. No extragonadal sites of AMH production have been reported to date. Given that levels do vary with age, care must be taken with interpretation of AMH to ensure the correct reference range is used.

Inhibin B has been detected from 14-16 weeks gestation (33). and has been detected in umbilical cord samples in boys but not girls (34). Between 3-6 months of age, inhibin B rises in line with FSH and a concurrent increase in Sertoli cell number (35) (**Figure 1**). Levels persist after testosterone, LH and FSH start to fall (25). Levels then remain consistent until approximately 8 years of age, peaking at around age 17 before a slow decline to adult levels (36). Inhibin B is therefore a useful indicator of spermatogenesis and Sertoli cell function in adults, when AMH is no longer measurable.

THE ROLE OF FETAL SERTOLI CELL HORMONES IN DSD

Differences in AMH and inhibin B can be seen in many forms of DSD. These changes have been reviewed extensively elsewhere (37) but this review will discuss some specific DSD conditions.

Sex Chromosome DSD

Klinefelter syndrome, 47,XXY, is the most common sex chromosome anomaly, affecting around 1 in 660 live male births (38). Men with this condition have hypergonadotrophic hypogonadism, as well as being 6 times more likely to have cryptorchidism, which will also impact on Sertoli cell function (39). A study by Aksglaede et al., measured AMH levels in a cohort of 95 men with non-mosaic Klinefelter syndrome and demonstrated that levels remained within the population reference range in infancy, childhood and into adolescence (40). In 47,XXY infants, levels of inhibin B have also been shown to remain in the normal reference range for age (41). Based on these findings, it is presumed that fetal levels of AMH and inhibin B will also be similar between boys with Klinefelter syndrome and controls. However, levels of inhibin B do reduce significantly with age once adulthood is attained, in addition to low AMH levels reflecting the progressive degeneration of the seminiferous tubules (40).

A recent study by Spaziani et al. (10.1007/s40618-020-01281-x) has also found that inhibin B and AMH can be high in early childhood and mini-puberty, suggesting that further research is required in this cohort of patients to confirm expected biochemical findings.

46,XX DSD

Ovotesticular DSD

About 65% of those with ovotesticular DSD have a 46,XX chromosome complement and the ability of the ovotestes to function will be variable. As such, AMH and inhibin B levels in fetal life will vary from being high for female but low for male to normal for male, depending on the clinical phenotype and assays used. Generally, in fully virilised 46,XX males, AMH, inhibin B and testosterone will be within the normal male range in childhood but the germ cells will fail to undergo complete meiosis and undergo apoptosis at puberty, resulting in low testicular volumes (15). In 46,XX children with atypical genitalia, AMH levels above the normal female range are highly suggestive of ovotesticular DSD and exclude the differential diagnoses of congenital adrenal hyperplasia, aromatase defects or virilising tumours.

SOX9 gene variants have been associated with ovotesticular DSD and skeletal dysplasias (42). The SRY gene upregulates SOX9 expression and once levels of SOX9 have reached a critical threshold, several positive regulatory loops are initiated, including autoregulation of SOX9 expression and formation of feed-forward loops *via* FGF9 or PGD2 signalling, which are required for the maintenance and sustained function of Sertoli cells (43). During testicular development, SOX9 functions by regulating the production of AMH from Sertoli cells, and possibly by repressing genes involved in ovarian development such as *Wnt4* and *Foxl2* (44). Studies regarding boys with SOX9 variants, report AMH levels, which are low to low normal (45, 46), with insufficient data regarding inhibin B levels to date.

46,XY DSD

Disorders of AMH and AMH Receptor Defects

From around 7 weeks gestation, the AMH gene is activated by SF1 in Sertoli cells. This leads to the regression of Müllerian structures in the developing male fetus (47). Where there is a mutation in the AMH gene, or in AMHR2, Persistent Müllerian Duct Syndrome (PMDS) occurs. Boys most commonly present with cryptorchidism, inguinal herniae and later infertility. Orchidopexy can be challenging, likely because the testes often have an excessively elongated gubernacular cord, potentially due to the mechanical effects of the retained uterus, or because AMH has an effect on shortening the gubernacular cord (14). A longitudinal study of 157 men with PMDS demonstrated that testicular malignant transformation occurs in 33% of individuals with PMDS (48). Malignancy has been reported in cases of PMDS with cryptorchidism and transverse testicular ectopia but has also been seen in normal testis in patients with PMDS, suggesting that the mechanism for malignancy is not just related to mechanical cryptorchidism (49). In approximately 8% of cases, malignant transformation of the Müllerian remnants can also occur, particularly after puberty, although again the mechanism for this is unknown (50). Treatment is primarily surgical, with excision of Müllerian remnants to allow for orchidopexy. Leydig cell function is typically normal but AMH levels will be low or undetectable in those with an AMH gene variant. In contrast, those with a mutation in AMHR2 will have normal-for-age AMH levels (48).

Disorders of Gonadal Development

Approximately 15% of all cases of 46,XY complete gonadal dysgenesis (CGD) result from a deletion in SRY, with the majority being located within the HMG domain (51). AMH and inhibin B levels will be low/undetectable in individuals with CGD, with the absolute level correlating to the amount of gonadal tissue and number of functioning Sertoli cells present. As such individuals with partial gonadal dysgenesis (PGD) tend to have higher levels than those with CGD (52).

Disorders of Androgen Action

Complete and partial androgen insensitivity syndrome (CAIS and PAIS respectively) are characterised by mutations in the AR gene. Testis differentiation and development, as well as gene expression patterns of AMH and AMHR are independent of AR action up to the second trimester of pregnancy. This has been confirmed by post-mortem examinations of fetuses with AR defects and expression of AMH, AMH2 and testicular differentiation markers (53). During childhood, AMH levels are usually within the normal range in boys with PAIS. By puberty, testicular AMH increases in young people with AIS, in tandem with FSH and oestradiol levels. The expression of Oestrogen Receptor α (ERα) has been confirmed in Sertoli cells from patients with CAIS (54). AMH may be a useful tool to distinguish CAIS and PAIS. A recent study of 29 AIS patients under the age of 11 years reported lower AMH levels in individuals with CAIS compared to PAIS, although still within the normal range for men (55).

Inhibin B levels have been measured in different cohorts of boys with PAIS, with median levels being lower in these boys compared to controls at all ages but usually still within the normal range and higher than in other forms of XY DSD (56, 57). No statistically significant differences are reported in inhibin B between individuals with CAIS and PAIS (55).

Disorders of Androgen Synthesis

Studies of inhibin B and AMH in boys with 5α-reductase type 2 deficiency (5ARD2) compared to other DSD conditions and controls have found that boys with 5ARD2 had lower levels of both hormones compared to controls (56).

Non-Specific Disorders of Undermasculinisation

Cryptorchidism occurs due to failure of descent of the testes and is a common congenital disorder, reported to affect up to 9% of male infants in some populations (58). Boys with both unilateral and bilateral cryptorchidism have been demonstrated to have lower AMH levels than control boys, indicating testicular dysfunction in childhood. Postnatal maturation of Sertoli cells is altered in cryptorchidism and a study of 40 infants with cryptorchidism aged 4-35 months showed strong positive correlations between inhibin B, LH and FSH with Sertoli cell number (59)

A recent retrospective study of 310 prepubertal boys with cryptorchidism confirmed that whilst low AMH was prevalent in boys with both unilateral and bilateral cryptorchidism, lower levels were seen in boys with bilateral undescended testes (60). In addition, in terms of treatment, testicular descent was more likely to be successful in response to treatment with hCG in those with a higher AMH at baseline, suggesting this may be a useful predictive marker when counselling families regarding the advantages and disadvantages of hormonal versus surgical management (60).

In boys with undermasculinisation, hCG stimulation may be used to assess Leydig cell function in tandem with an AMH measurement to assess Sertoli cell function. In 138 children with a non-specific XY DSD, a normal AMH was predictive of a normal testosterone response to hCG, suggesting that where Sertoli cell function is preserved, Leydig cell function is also likely to be (61). Of the 138 boys in the study cohort, 53 (38%) had combined genital anomalies; 47 (34%) had isolated bilateral undescended testes and 29 (21%) had isolated proximal hypospadias. Boys with isolated hypospadias had a higher AMH and higher testosterone after stimulation with human chorionic gonadotrophin (hCG) compared to children with isolated bilateral undescended testes ($p=0.0001$) or children with combined anomalies including undescended testes ($p<0.0001$) (61). Children with undescended testes but no other genital anomalies had the lowest AMH and amongst those with bilateral undescended testes, children with impalpable testes had a lower median AMH than children with inguinal testes (470 (1.5, 1926) vs 832 (72, 2280) pmol/l ($p=0.04$) (61).

Testicular dysgenesis syndrome (TDS) is a term used to describe a group of associated male reproductive disorders that arise as a result of impaired androgen production or action during a critical period of fetal testicular development (62). The reduced androgen exposure is associated with cryptorchidism, hypospadias, testicular germ cell tumours and subfertility. Dysgenesis (often focal) within the testis is a frequent finding in men with these disorders, which includes undifferentiated Sertoli cells (63, 64). Animal models have been used to study Sertoli cell development and maturation in this cohort. Pregnancy exposure to di(n-Butyl) phthtalate in rats produces a similar phenotype and demonstrate altered Sertoli cell maturation, with a worse phenotype seen in cryptorchid testes compared to scrotal testes (65).

In anorchia, AMH and inhibin B levels are both undetectable, usually in combination with raised FSH levels (66).

Specific Gene Variants

NR5A1 (also known as Steroidogenic Factor 1 or SF1) is a nuclear receptor transcription factor whose expression commences in the coelomic epithelium and continues in steroidogenic cells. Its expression has been demonstrated in the bipotential gonad from 32 days post conception and following testis determination (around 42 days onward), its expression is maintained in the somatic cells of the early testis, which suggests it may play a role in supporting SOX9 expression (67). It is also known to activate the expression of AMH in Sertoli cells from around 7 weeks gestation, resulting in the regression of Müllerian structures in the developing male foetus and is responsible for activating the expression of steroidogenic enzymes from 8 weeks gestation, resulting in the androgenisation of the external genitalia (9). AMH and inhibin B levels in individuals with NR5A1 gene variants have been reported as normal to low-normal (68, 69).

Early studies of simulated FSH deficiency *via* administration of hCG demonstrated that FSH is required for normal spermatogenesis, although sperm production was not entirely suppressed in the absence of FSH (70). Babies born with FSH receptor mutations, have Sertoli cell hypoplasia and small testis resulting in low spermatogenesis and low inhibin B in adulthood. Müllerian structures do regress however suggesting AMH levels are likely to be normal in early fetal life (71).

GATA-binding protein 4 (GATA4) is a transcription factor which is known to be involved in the development of some forms of congenital heart disease (72). Studies with GATA4 mutations have demonstrated its likely involvement in gonadal development in conjunction with its cofactor, Friend of GATA2 (FOG2) (73). These genes are upstream of SRY and when mutated cause significant reductions in SRY expression [Sekido and Lovell-Badge, 2013]. In a family of individuals with mutations in GATA4, 46,XY DSD and congenital heart disease, AMH levels were consistently low and functional analysis demonstrated that mutations in GATA4 may reduce the action of the AMH promoter (74).

Hypogonadotrophic Hypogonadism

In cases of congenital hypogonadotrophic hypogonadism, deficient LH and FSH does not affect Müllerian regression and early sex development, but does impair genital development, which is dependent on testosterone from mid to late pregnancy. This may result in small testes as a result of FSH deficiency and micropenis +/- cryptorchidism arising from LH deficiency (75). A study of 8 men with hypogonadotrophic hypogonadism (4 with Kallmann's syndrome, 4 idiopathic), demonstrated that AMH was high for age, because serum testosterone remained low and therefore did not downregulate AMH. Treatment with recombinant human FSH increased serum AMH, Further treatment with hCG increased testosterone and reduced AMH and inhibin B (76).

Current Research Gaps

Overall, whilst many studies have considered AMH concentrations as an indicator of testicular development in children and young people with DSD, very few have focussed on inhibin B. Therefore, the use of Inhibin B as a marker of Sertoli cell function should be a research priority in future years, particularly when assessing adolescents with DSD, in whom AMH levels are more difficult to interpret.

In young children with non-specific 46,XY disorders of undermasculinisation, AMH and inhibin B levels both show

good correlation with post-hCG testosterone (61, 77), potentially obviating the need for a stimulated test. hCG stimulation tests are invasive for the child and logistically difficult for the healthcare team and family and as such, studies assessing the clinical utility of AMH or inhibin B levels as an alternative to hCG testing to assess gonadal function will be invaluable.

Summary and Conclusions

To summarize, fetal Sertoli cell hormones are crucial for normal sex development. In particular, AMH is responsible for regression of Müllerian structures. Both AMH and inhibin B represent useful biomarkers in children with DSD conditions, as they allow for confirmation of the presence of testicular tissue, as well as monitoring of gonadal function. However, their secretion is age-dependent, requiring specific reference ranges and reliable assays if they are to be used in clinical practice. Serum levels of both, in conjunction with those of androgens and gonadotrophins, can, however, be helpful in the diagnosis of DSD conditions, with specific patterns being more likely to be seen in certain disorders.

AUTHOR CONTRIBUTIONS

Both authors contributed sugnificantly to the conception and writing of this manuscript. Both authors agreed on the final version.

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