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REVIEW **Open Access**

Steroid receptors in the testis: implications in the physiology of prenatal and postnatal development and translation to clinical application

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Summary. The testes are the main source of sex steroids in the male, especially androgens and to a lesser extent estrogens. In target cells, steroid hormones typically signal after binding to intracellular receptors, which act as transcription factors. Androgens and estrogens have ubiquitous functions in peripheral organs, but also have paracrine actions within the gonads where they are far more concentrated. The levels of steroid production by the testes vary throughout fetal and postnatal development: they are high in intrauterine life and in the first months after birth, then they decline and are almost undetectable in childhood and increase again during puberty to attain adult levels. The expression of the androgen and estrogen receptors also depict specific ontogenies in the various testicular cell types. The combination of intratesticular steroid concentration with the pattern of expression of the steroid hormone receptors defines androgen and estrogen action on Sertoli, germ and Leydig cells. Here, we review the ontogeny of expression of the androgen and estrogen receptors in the testis, its impact on testicular physiology during prenatal and postnatal development, as well as its implication on the pathophysiology of different disorders affecting gonadal function throughout life.

Key words: AMH, Meiosis, Estrogens, Puberty, Spermatogenesis, Testosterone

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Introduction

The gonads are the principal source of sex steroids. In the male, the testes produce mainly androgens, but also estrogens. Like other steroid hormones, e.g. progestins and corticoids, sex steroids are synthesized from cholesterol following a series of enzymatic steps (Fig. 1) (Miller and Auchus, 2011). They passively diffuse through the membrane of target cells and typically signal after binding to intracellular receptors that act as transcription factors. Well-known sex steroid target organs are the internal and external genitalia and the musculoskeletal and the central nervous systems. However, androgens and estrogens have ubiquitous functions; amongst them, they have paracrine actions within the gonads. A particular feature of paracrine regulation of gonadal function by steroid hormones is that it takes place in a milieu where steroid concentration is largely higher (10- to 1000-fold) than in circulation and other target tissues.

Histophysiology of the developing testis

The fetal period

In humans, the testis differentiates from the sexually bipotential gonadal ridge during the 7th week post-fertilization (Mäkelä et al., 2019). Sertoli cells

Abbreviations. AMH, anti-Müllerian hormone; AR, androgen receptor; ARE: androgen response element; ATF1: activating transcription factor 1; CREB: cAMP response element-binding protein; DNA, desoxyribonucleic acid, DSD, disorders of sex development; EGF: epidermal growth factor; ER: estrogen receptor; ERE: estrogen-response element; ERK: extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GPER: G protein-coupled estrogen receptor; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MAP: mitogen-activated protein; RNA, ribonucleic acid; SCARKO: Sertoli cell specific AR knock out; Src: steroid receptor coactivator.

aggregate and surround the primordial germ cells of extragonadal origin to form the testicular cords (future seminiferous tubules). Subsequently, Leydig cells differentiate in the interstitial tissue. These initial steps of testicular differentiation occur independently of fetal pituitary gonadotropins (Fig. 2). Sertoli and Leydig cell endocrine functions play a determining role in genital differentiation. Sertoli cells secrete anti-Müllerian hormone (AMH), a glycoprotein responsible for the regression of Müllerian ducts, the anlagen of the Fallopian tubes, the uterus and the upper part of the vagina, which is hitherto present in both XX and XY embryos (Josso, 2019). Leydig cells secrete androgens, especially testosterone. These steroid hormones act through the androgen receptor and are responsible for the differentiation of the Wolffian duct into the epididymis, the vas deferens and the seminal vesicle, as well as for the virilization of the urogenital sinus and the external genitalia (Rey and Grinspon, 2011). During the first trimester of embryonic and fetal life, Leydig cell differentiation and androgen production is stimulated by placental human chorionic gonadotropin (hCG). Virilization of the internal and external genitalia is completed during the first trimester. Disorders of testicular differentiation, i.e. testicular dysgenesis, in these early stages of development result in insufficient hormone secretion and, thus, in incomplete virilization or feminization of the genitalia, depending on the degree of the abnormality (Fig. 2). Alternatively, the testis may differentiate but androgens are not produced or do not act on their target organs. All these conditions are known as 46, XY disorders of sex development (DSD) (Wisniewski et al., 2019; Grinspon et al., 2020).

From the second trimester onwards, fetal pituitary gonadotropins take over the regulation of gonadal function. Luteinizing hormone (LH) progressively replaces hCG activity on Leydig cells. In this period, androgens are necessary for testicular descent to the scrotum and for penile enlargement (Fig. 2). On the other hand, follicle-stimulating hormone (FSH) induces Sertoli cell proliferation (Meroni et al., 2019) and further upregulates AMH production (Grinspon et al., 2018; Mäkelä et al., 2019). Germ cells differentiate into gonocytes and subsequently into spermatogonia, the male stem germ cells (Diao et al., 2022), which undergo proliferation by mitosis but do not enter meiosis until puberty (Fang et al., 2022). This means that there is no sperm production during fetal life and childhood. Impairment of testicular function during the second and third trimesters of fetal life -whether primary or secondary to hypothalamic-pituitary dysfunction- usually results in small male genitalia (e.g. micropenis), testis maldescent (cryptorchidism) and low circulating levels of testosterone and AMH (Braslavsky et al., 2015; Lambert and Bougnères, 2016; Grinspon et al., 2021).

The postnatal active period

After birth, gonadotropin and androgen secretion remains active during 3 to 6 months in the male (Fig. 3). This period is usually referred to as “minipuberty”

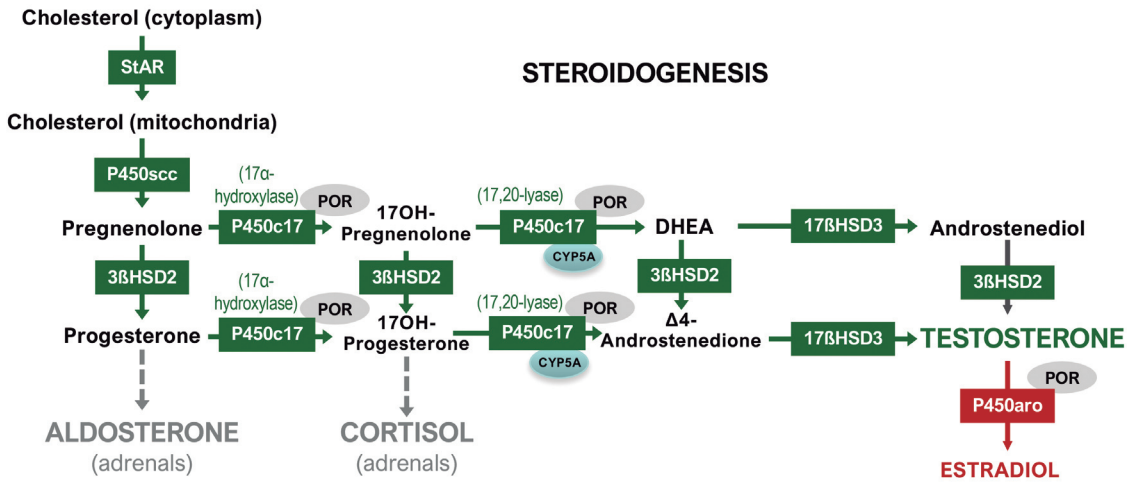

Fig. 1. Steroidogenesis. Steroids are synthesized from cholesterol. The initial steps are identical in the gonads and the adrenals. Cholesterol enters the mitochondria due to the action of Steroidogenic Acute Regulatory (StAR) protein. Subsequently, Cytochrome P450 side chain cleavage (P450scc) enzyme catalyzes the synthesis of pregnenolone, which may be converted to progesterone due to the action of 3β-hydroxysteroid dehydrogenase (HSD). Cytochrome P450c17 converts pregnenolone and progesterone to 17-hydroxypregnenolone (17OH-Pregnenolone) and 17-hydroxyprogesterone (17OH-Progesterone), through its 17α-hydroxylase activity, and subsequently to dehydroepiandrosterone (DHEA) and Δ4-androstenedione through its 17,20-lyase activity, which is facilitated by cytochrome b5 (CYP5A). Gonadal-specific 17β-HSD type 3 converts DHEA to androstenediol and androstenedione to testosterone. Cytochrome P450 aromatase (P450aro) converts testosterone to estradiol. The activity of many of these enzymes is induced by the cytochrome P450 oxidoreductase (POR). In the adrenal cortex, progesterone is the substrate for several steps yielding to the mineralocorticoid aldosterone, whereas 17OH-Progesterone is the substate for the synthesis of the glucocorticoid cortisol. Reproduced with modifications from: Rey RA, Grinspon RP. Normal male sexual differentiation and aetiology of disorders of sex development. Best Practice & Research Clinical Endocrinology & Metabolism (2011) 25:221-238. doi: 10.1016/j.beem.2010.08.013. Copyright © 2010 Elsevier Ltd (Rey and Grinspon, 2011).

because circulating levels of LH, FSH and testosterone resemble those of puberty (Kuiri-Hanninen et al., 2019; Busch et al., 2022). However, other typical features of pubertal development, such as germ cell entry to meiosis and adult spermatogenesis, do not take place (Rey, 2014). AMH production is also high during this period of life and represents an emblematic biomarker of the immature Sertoli cell population (Josso et al., 2013).

Childhood

The active phase of the hypothalamic-gonadotrope axis progressively decreases from the fourth month in the boy, and by the seventh month almost all boys have very low or undetectable LH and testosterone levels (Grinspon et al., 2019; Kuiri-Hanninen et al., 2019; Busch et al., 2022). Typical Leydig cells are no longer present in the interstitial tissue, but Sertoli cell endocrine function remains active throughout childhood, as reflected by the high levels of serum AMH (Fig. 3) (Aksglæde et al., 2010; Grinspon et al., 2011). As already mentioned, the germ cell population is represented by spermatogonia that proliferate by mitosis but do not enter meiosis, such that spermatocytes, spermatids and spermatozoa are not present in the prepubertal testis.

Puberty

The hypothalamic-gonadotrope axis is reactivated between 9 and 14 years of age in the boy (Fig. 2 and 3). FSH induces immature Sertoli cell proliferation, resulting in a modest increase in testicular volume (Grinspon and Urrutia, 2020), and aromatase activity, responsible for the transformation of androgens into estrogens (Dorrington and Armstrong, 1975). LH provokes the differentiation of the adult Leydig cells and their steroidogenic capacity, resulting in an increase of intratesticular testosterone concentration that leads to the maturation of Sertoli cells. Typical features of Sertoli cell maturation are the decrease in AMH expression (Edelsztein et al., 2016, 2018), the development of cell-cell junctions involved in the formation of the blood-testis barrier (Willems et al., 2010b; Edelsztein and Rey, 2019), and their capacity to support adult spermatogenesis resulting in sperm production (Christin-Maitre and Young, 2022). The dramatic germ cell proliferation occurring during spermatogenesis underlies the major increase of testicular volume observed during puberty. Concomitantly with gonadal size enlargement, testicular androgen output increases resulting in progressively higher serum testosterone levels, which impact on secondary sex characteristics, linear growth

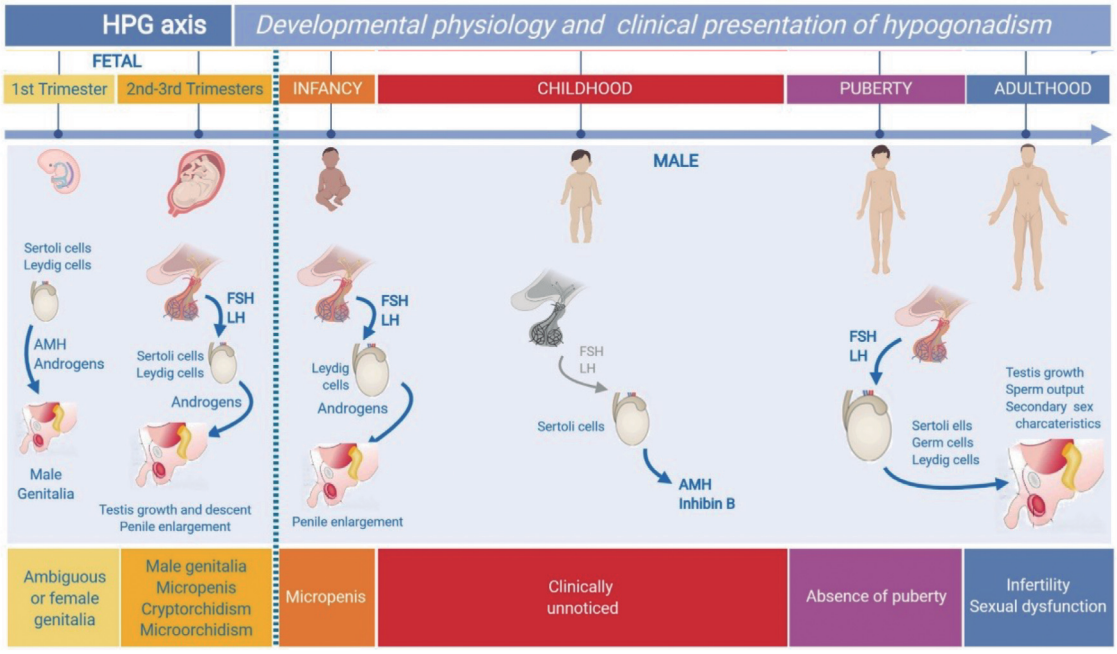

Fig. 2. Ontogeny of the hypothalamic-pituitary-gonadal (HPG) axis in males and its impact on clinical presentation of hypogonadism. The testis differentiates in the first trimester of fetal life, independently of pituitary gonadotropins. Androgens and AMH provoke male differentiation of the genitalia, whereas their absence leads to female differentiation. Hypogonadal states in this period lead to ambiguous or female genitalia in XY individuals. In the second and third trimesters, the testis increases in size and androgens provoke testicular descent and penile enlargement. Primary and central hypogonadisms result in micropenis, micro-orchidism and/or cryptorchidism in a newborn with male genitalia. In early infancy, gonadotropin and steroid secretion is active; hypogonadism precludes penile enlargement in boys. During childhood, gonadotropins and steroids are normally low or even undetectable; hypogonadism established in this period does not result in clinically evident signs. During puberty, the HPG axis is reactivated and results in the typical development of secondary sex characteristics; hypogonadism may result in absent or incomplete pubertal development, or later in infertility and/or sexual dysfunction. Reproduced with modifications from: Grinspon RP, Freire AV, Rey RA. Hypogonadism in Pediatric Health: Adult Medicine Concepts Fail. Trends Endocrinol Metab 2019;30(12):879-890. © 2019 Elsevier Ltd. (Grinspon et al., 2019). This fig. was created using BioRender (https://biorender.com/).

and bone mass acquisition.

Androgen and estrogen receptors during prenatal and postnatal testicular development

Androgen receptors

Androgen receptor function

In the classical (or genomic) pathway of androgen signaling, testosterone acts through an intracellular receptor known as the androgen receptor (AR), a member of the nuclear receptor superfamily that includes receptors for estrogens, progestins, mineralo- and gluco-corticoids, thyroid hormones, vitamin D and retinoic acid. Monomers of the AR are bound to heat-shock proteins in the cytoplasm, in an inactive state (Fig. 4). Testosterone binding induces conformational changes in the AR releasing the monomers from the heat-shock proteins and resulting in AR translocation to the cell nucleus, homodimer formation and interaction with the DNA of target genes (Quigley et al., 1995; Li and Al-Azzawi, 2009; Tan et al., 2015), usually with sequences known as androgen response elements (ARE) (Khorasanizadeh and Rastinejad, 2001; Denayer et al., 2010). The classical pathway involves a relatively slow mechanism, requiring between 30 and 45 minutes for transcriptional regulation (Shang et al., 2002). The AR dimers recruit co-activators or co-repressors, proteins that promote or inhibit androgen-mediated transcriptional activity (Chaturvedi and Dehm, 2019). These co-regulators include coordinators of transcription, histone modifiers and modifiers of DNA structure (Chmelar et al., 2007). Alternatively, instead of binding to ARE sequences, the androgen-bound AR can interact with other transcription factors having their specific binding sites on target gene promoters (Keri et al., 1994; Kallio et al., 1995; Heckert et al., 1997; Edelsztein et al., 2018). In these cases, ligand-bound AR action does not require the existence of canonical ARE sequences (Fig. 4).

The non-classical (non-genomic) pathway for

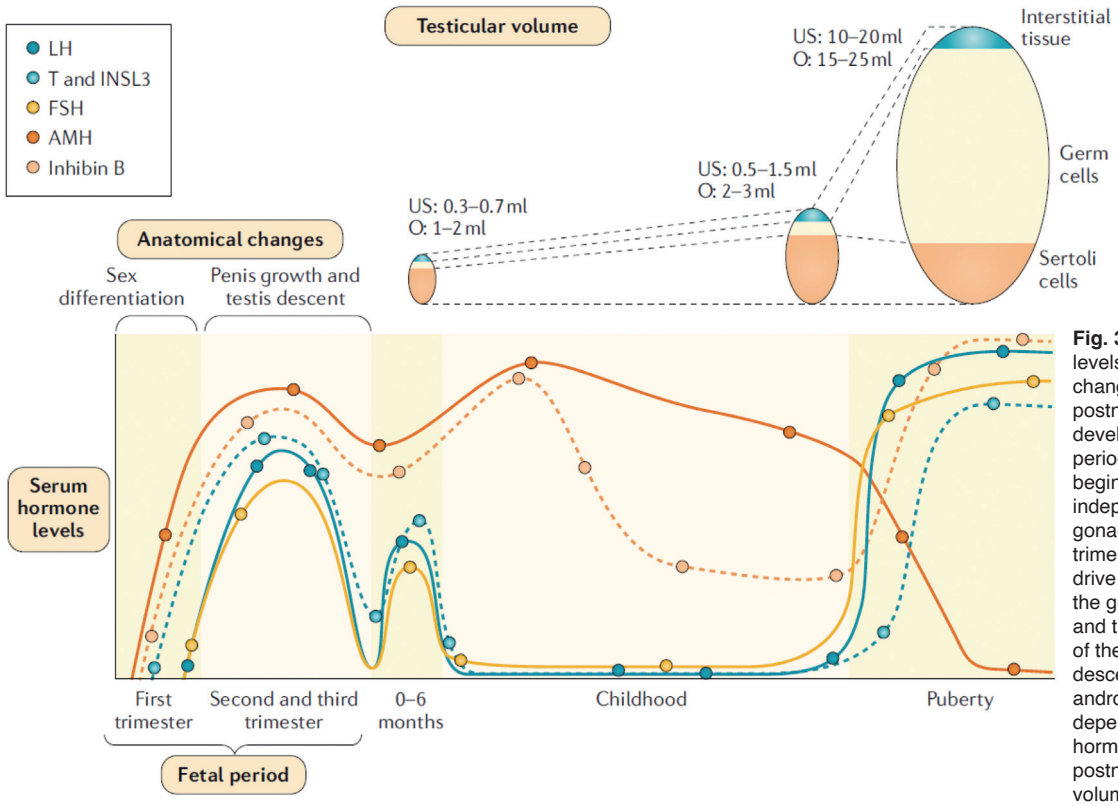

Fig. 3. Serum hormone levels and anatomical changes during fetal and postnatal male sex development. In the fetal period, testicular hormones begin to be secreted independently of pituitary gonadotropins in the first trimester of fetal life and drive fetal differentiation of the genitalia. In the second and third trimesters, growth of the genitalia and testicular descent are stimulated by androgen secretion dependent on fetal luteinizing hormone (LH). In the postnatal period, testicular volume increases during childhood due essentially to FSH-dependent Sertoli cell proliferation. When the postnatal activation period (usually called 'mini- puberty') wanes between the 3rd and 6th months of life, serum levels of gonadotropins and testosterone (T) decline, but those of the Sertoli cell markers anti- Müllerian hormone (AMH) and inhibin B persist at clearly detectable levels. During puberty, testicular volume increases dramatically owing to spermatogenic development, secondary to gonadotropin and T action. Sertoli cell markers show opposite profiles: AMH is inhibited by T whereas inhibin B is upregulated by follicle- stimulating hormone (FSH) and germ cells. INSL3, insulin- like factor 3; O, testicular volume measured by comparison to Prader's orchidometer; US, testicular volume measured by ultrasonography. Reprinted, with permission, from Salonia A., Rastrelli G., Hackett G., Seminara S.B., Huhtaniemi I.T., Rey R.A., Hellstrom W.J.G., Palmert M.R., Corona G., Dohle G.R., Khera M., Chan Y.-M. and Maggi M. (2019). Paediatric and adult-onset male hypogonadism. Nature Reviews Disease Primers 5, 38. (Salonia et al., 2019) © 2019 Springer Nature Limited.

androgen action induces rapid responses, within seconds of AR stimulation, explained by AR association with the tyrosine kinase Src (Migliaccio et al., 2000; Cheng et al., 2007) that leads to the phosphorylation of the EGF receptor (Cheng et al., 2007) and subsequent activation of MAP kinase signaling and phosphorylation of transcription factors (Fig. 4) (Fix et al., 2004; Edelsztein and Rey, 2019). More recently, a membrane-bound AR has been described to be involved in Sertoli cell physiology through ERK1/2-mediated phosphorylation of transcription factors CREB and ATF1 (Bulldan et al., 2016).

Androgen receptor localization in the developing testis

The ontogeny of AR expression within the testis has been described in several mammalian species. The pattern is very similar in rodents (Bremner et al., 1994; Majdic et al., 1995; Al-Attar et al., 1997; Edelsztein et al., 2018) and primates (McKinnell et al., 2001; Sharpe et al., 2003) including humans (Shapiro et al., 2005; Berensztein et al., 2006; Chemes et al., 2008; Boukari et al., 2009; Vija et al., 2013). Peritubular and Leydig cells express AR in their nuclei from early fetal life through the postnatal active period (Fig. 5). During childhood in humans or its equivalent in other mammals, typical Leydig cells are no longer present, but AR expression can be observed in up to 50% of interstitial mesenchymal cells. With the onset of puberty, Leydig cells reappear and show positive AR expression in their nuclei. Peritubular cells persist as AR-positive throughout life.

Contrary to peritubular and interstitial cells, the seminiferous cord cells do not express AR during fetal life and the first stages of postnatal development. Sertoli

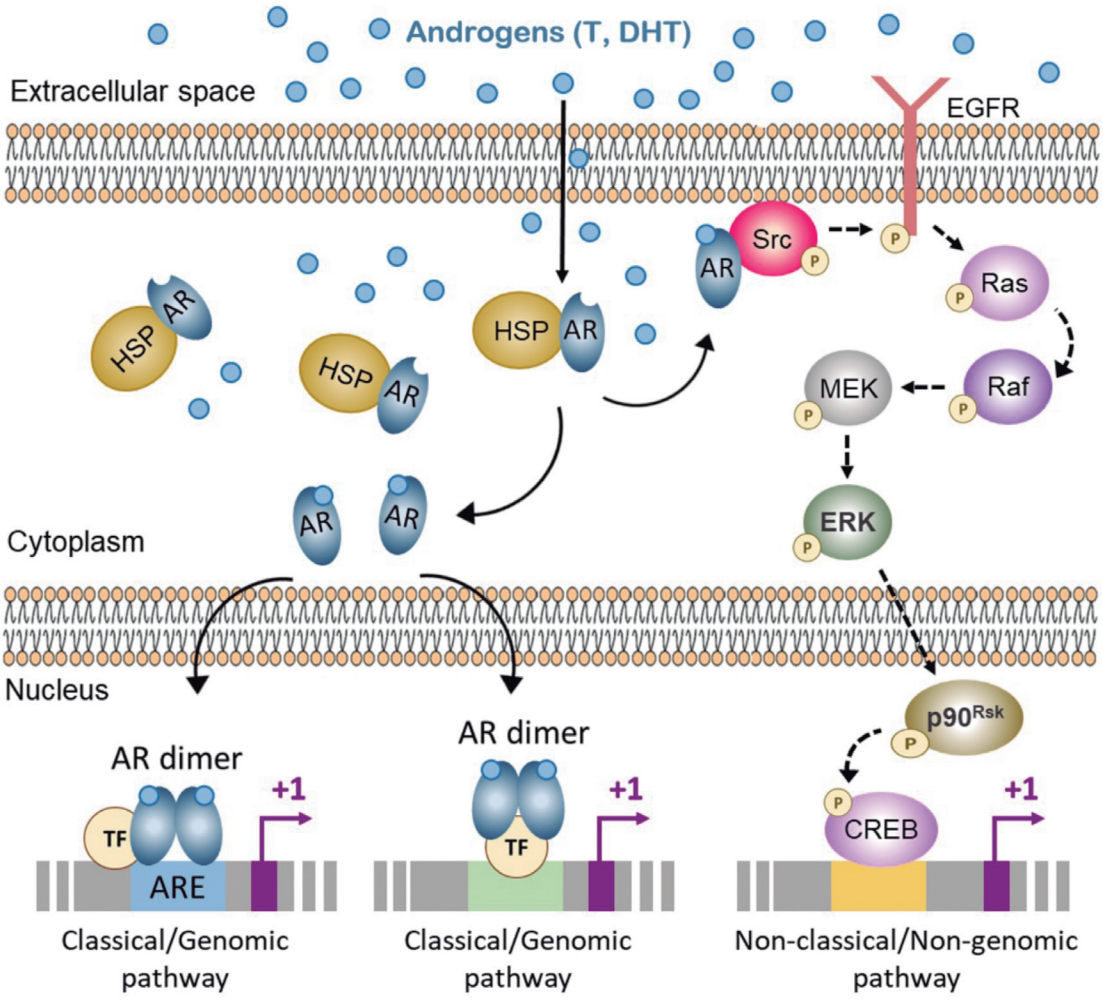

Fig. 4. Pathways of androgen signaling. Androgens, such as testosterone (T) or dihydrotestosterone (DHT) represented as blue circles, cross the cell membrane and bind to the androgen receptor (AR) in target cells, displacing the heat shock proteins (HSP). In the "classical" or "genomic" pathway, the ligand-bound AR translocates to the nucleus and forms homodimers that interact with androgen response elements (ARE) in target gene promoters or with other transcription factors (TF), finally regulating gene expression. In the "non-classical" or "non-genomic" pathway, the ligand-bound AR migrates to the inner side of the cell membrane and interacts with the Steroid receptor coactivator (Src) and activates the epidermal growth factor receptor (EGFR) signaling cascade involving e.g. the mitogen-activated protein kinase (MEK), the extracellular signal-regulated kinase (ERK), and the cAMP response element binding protein (CREB). Modified from: Edelsztein NY, Rey RA. Importance of the androgen receptor signaling in gene transactivation and transrepression for pubertal maturation of the testis. Cells. 2019;8:1-17 (Edelsztein and Rey, 2019), with permission from the authors © 2019, licensee MDPI, Basel, Switzerland (open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license).

cells begin to show a light positive staining for AR in their nuclei by the end of the first year after birth in humans (Fig. 5) and around 4-5 postnatal days in the mouse (Fig. 6). A gradual increase is observed in boys, between 4 and 8 years of age. By the onset of puberty, i.e. 9 to 14 years in humans and 7 to 9 days in mice, all Sertoli cells show high AR expression, which is maintained throughout adulthood.

Estrogen receptors

Estrogen receptor functions

Estrogens are synthesized from androgens by the cytochrome P450 enzyme aromatase, expressed in the postnatal testis (Berensztein et al., 2006) and stimulated by FSH in Sertoli cells (Dorrington and Armstrong, 1975). Estrogen signaling may follow 2 different pathways. The classical pathway involves the nuclear estrogen receptors α (ERα) or β (ERβ), which bind to estrogen response elements (ERE) on the regulatory sequences of target genes. The DNA-binding domains of ERα and ERβ have a 97% identity, while ligand specificity is conferred by their ligand-binding domains even though both ERs bind to estrogens with a similar affinity (Nilsson et al., 2001). The non-classical pathway involves a membrane-bound ER, known as GPER (also called GPR30 or GPER1). It is a seven-transmembrane domain, G protein-coupled receptor, predominantly localized in the endoplasmic reticulum. GPER mediates rapid cellular responses involving second messengers, ion channels and kinase activities (Prossnitz and Barton, 2014).

Estrogen receptor localization in the developing testis

The ontogeny and localization of ERα and ERβ in testicular tissue is a controversial issue in non-mammalian and mammalian species, and this may be due to the diversity of antibodies used for immunohistochemistry (Cooke et al., 2017) or by the existence of different ER isoforms (Hirata et al., 2003).

ERα mRNA and protein expression has not been detected in the testis in some studies, whereas other have reported strongly positive results. A developmental decrease in ERα was shown during mid- to late puberty in the mouse testis (Jefferson et al., 2000). While most antibodies detected ERα in peritubular and Leydig cells (Mäkinen et al., 2001; Saunders et al., 2001), other

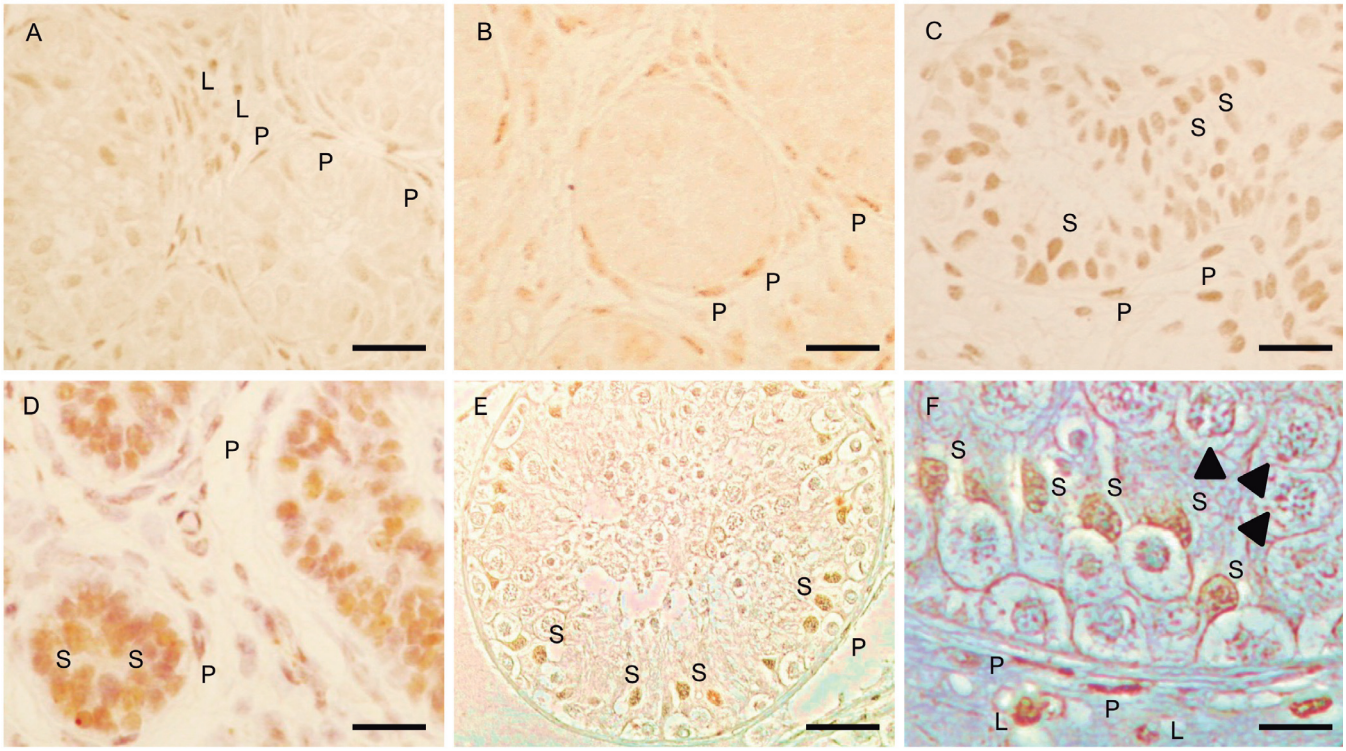

Fig. 5. Androgen receptor expression in the human testis. Immunohistochemistry for the androgen receptor in normal testicular tissue. **A.** 28 week-old fetus. **B.** 3 month-old infant ("mini-puberty"). **C.** 2 year-old boy. **D.** 4 year-old boy. **E, F.** adult testis. L: Leydig cell nuclei; P: peritubular cell nuclei; S: Sertoli cell nuclei. Arrowheads. meiotic spermatocytes. Based on images from: Chemes H.E., Rey R.A., Nistal M., Regadera J., Musse M., González-Peramato P. and Serrano A. (2008). Physiological androgen insensitivity of the fetal, neonatal, and early infantile testis is explained by the ontogeny of the androgen receptor expression in Sertoli cells. Journal of Clinical Endocrinology and Metabolism 93, 4408-4412 (Chemes et al., 2008), with permission © 2008, The Endocrine Society. Scale bars: A-D, 20 μm; E, 40 μm; F, 10 μm.

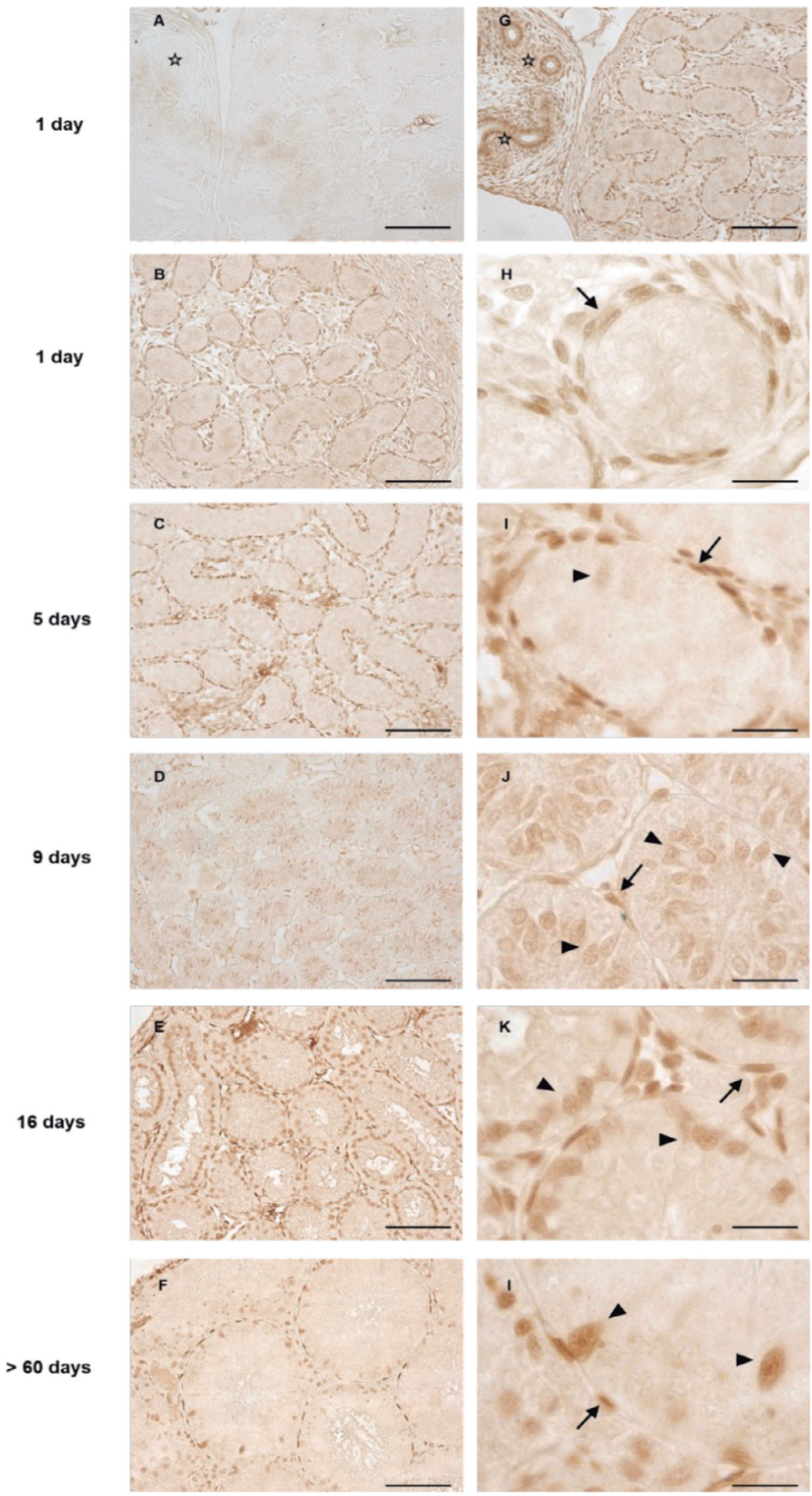

Fig. 6. Androgen receptor expression in the mouse testis. Immunohistochemistry for the androgen receptor in the postnatal mouse testis, including negative control **(A)** and positive control **(G)**. Arrows: peritubular myoid cells, arrowheads: Sertoli cells, stars: epididymal tissue. Reprinted with permission from Edelsztein N.Y., Racine C., di Clemente N., Schteingart H.F. and Rey R.A. (2018). Androgens downregulate anti-Mullerian hormone promoter activity in the Sertoli cell through the androgen receptor and intact SF1 sites. Biol Reprod 99, 1303-1312 (Edelsztein et al., 2018) © The Authors, 2018, published by Oxford University Press on behalf of Society for the Study of Reproduction. Scale bars: A-G, 100 μm; H-L, 20 μm.

antibodies as well as mRNA *in situ* hybridization studies revealed its expression also in Sertoli cells (Gunawan et al., 2011; Lin et al., 2014; Lucas et al., 2014). In human samples, we found ERα expression in Sertoli cells during childhood and puberty (Fig. 7) (Valeri et al., 2020), and Cavaco and colleagues (2009) found similar results in adult testes using the same antibody.

Most studies have reported ubiquitous expression of the ERβ mRNA and protein in reproductive organs, with differences according to species and age. In the mouse testis, ERβ is strongly expressed between postnatal days 1 and 5, but is almost absent after day 12 (Cooke et al., 2017). In human samples, we found ERβ in Sertoli cells of boys and adolescents, although with an apparently lower level of expression as compared to ERα (Fig. 7) (Valeri et al., 2020). In adult testicular tissue, ERβ could not be detected in Sertoli cells although it was present in Leydig and germ cells (Cavaco et al., 2009).

The membrane-bound GPER, predominantly localized in the endoplasmic reticulum (Revankar et al., 2005; Barton et al., 2018), is also expressed in Sertoli cells from the time of pubertal onset (Lucas et al., 2010)

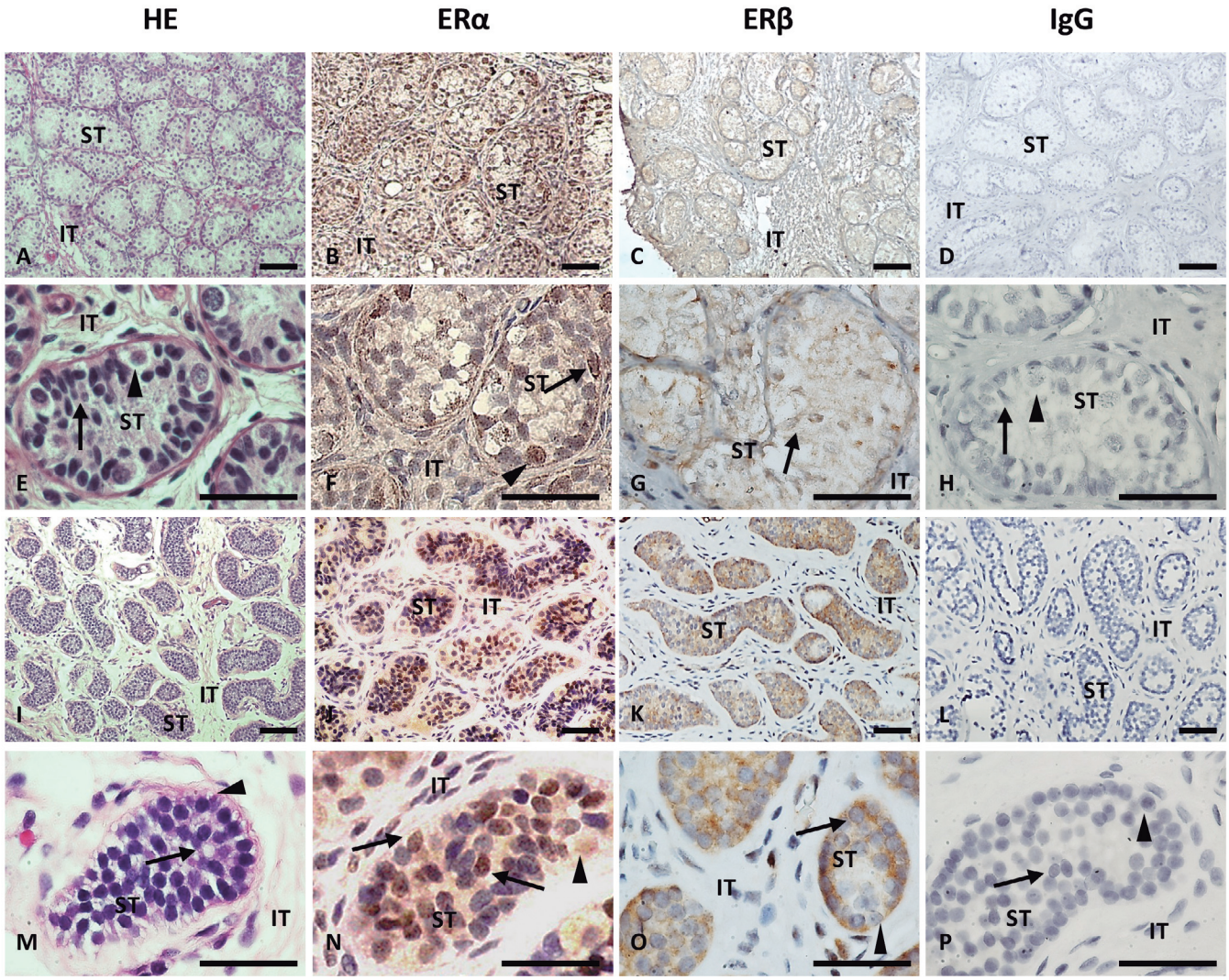

Fig. 7. Estrogen receptor expression in the human testis. Immunohistochemistry for ERα and ERβ in normal testicular tissue and in testes obtained from gonadectomy in patients with complete androgen insensitivity syndrome (CAIS). A-H: Normal prepubertal tissue in biopsies from a 7-year-old boy with acute lymphoblastic leukemia). I-P: Testis from a 6-year-old patient with CAIS. HE: hematoxylin-eosin; IgG: primary antibody was replaced by IgG from nonimmune serum (negative control); IT: interstitial tissue; ST: seminiferous tubule; arrows: Sertoli cells (ovoid or elongated nuclei); arrowheads: germ cells (spermatogonia, round nuclei and abundant pale cytoplasm). The bars represent 60 μm. Reproduced with permission from: Valeri C., Lovaisa M.M., Racine C., Edelsztein N.Y., Riggio M., Giulianelli S., Venara M., Bedecarrás P., Ballerini M.G., di Clemente N., Lamb C.A., Schteingart H.F. and Rey R.A. (2020). Molecular mechanisms underlying AMH elevation in hyperoestrogenic states in males. Sci. Rep. 10, 15062 (Valeri et al., 2020) © The Authors, 2020.

and in the adult testis (Chevalier et al., 2012).

Clinical implications of androgen receptor signaling in the developing testis

In the adult male, androgen action within the testis is essential for full spermatogenesis, i.e. the progression of germ cells from spermatogonia through meiosis (primary and secondary spermatocytes) and spermiogenesis (spermatids) into mature sperm (Fig. 8) (Rey, 2003; Wang et al., 2022). However, germ cells do not express AR, and spermatogenic development is supported via Leydig, peritubular and Sertoli cells that express the AR (Walker et al., 2015). Especially, the presence of AR in Sertoli cells is essential for intratesticular testosterone to indirectly signal to the adjacent germ cells, as shown by experiments in Sertoli cell specific AR knock out (SCARKO) mice (Wang et al., 2009).

In rodents, intratesticular testosterone concentration is elevated from birth; as soon as the AR appears in Sertoli cells (e.g. between days 4 to 9 in the mouse, Fig. 6), AMH production decreases and meiosis is initiated (Fig. 9). In the monkey, there is also a negative correlation between AR and AMH expression in Sertoli cells (McKinnell et al., 2001). In humans, the prepubertal period including infancy and childhood is much longer; intratesticular testosterone is extremely low and does not impact on Sertoli cells. During pubertal development, the increase in Leydig cell testosterone production results in androgen signaling through the Sertoli cell AR, which induces Sertoli cell maturation, characterized by the downregulation of AMH expression (Edelsztein et al., 2018), cell polarization and the development of inter-Sertoli cell junctions that form the blood-testis barrier that contributes to testicular homeostasis that protects meiotic and post-meiotic germ cells from immune attack (Fig. 10) (Willems et al., 2010b; Edelsztein and Rey, 2019; Lustig et al., 2020; Wang et al., 2022).

Physiological androgen insensitivity of Sertoli cells during fetal and early postnatal periods

Absence of Sertoli cell maturation and of full spermatogenesis

One intriguing observation is that Sertoli cells keep an immature aspect, both morphologically and functionally, and germ cells do not enter meiosis to develop full spermatogenesis despite being exposed to high gonadotropin and intratesticular testosterone levels during the fetal and early postnatal periods of life. For

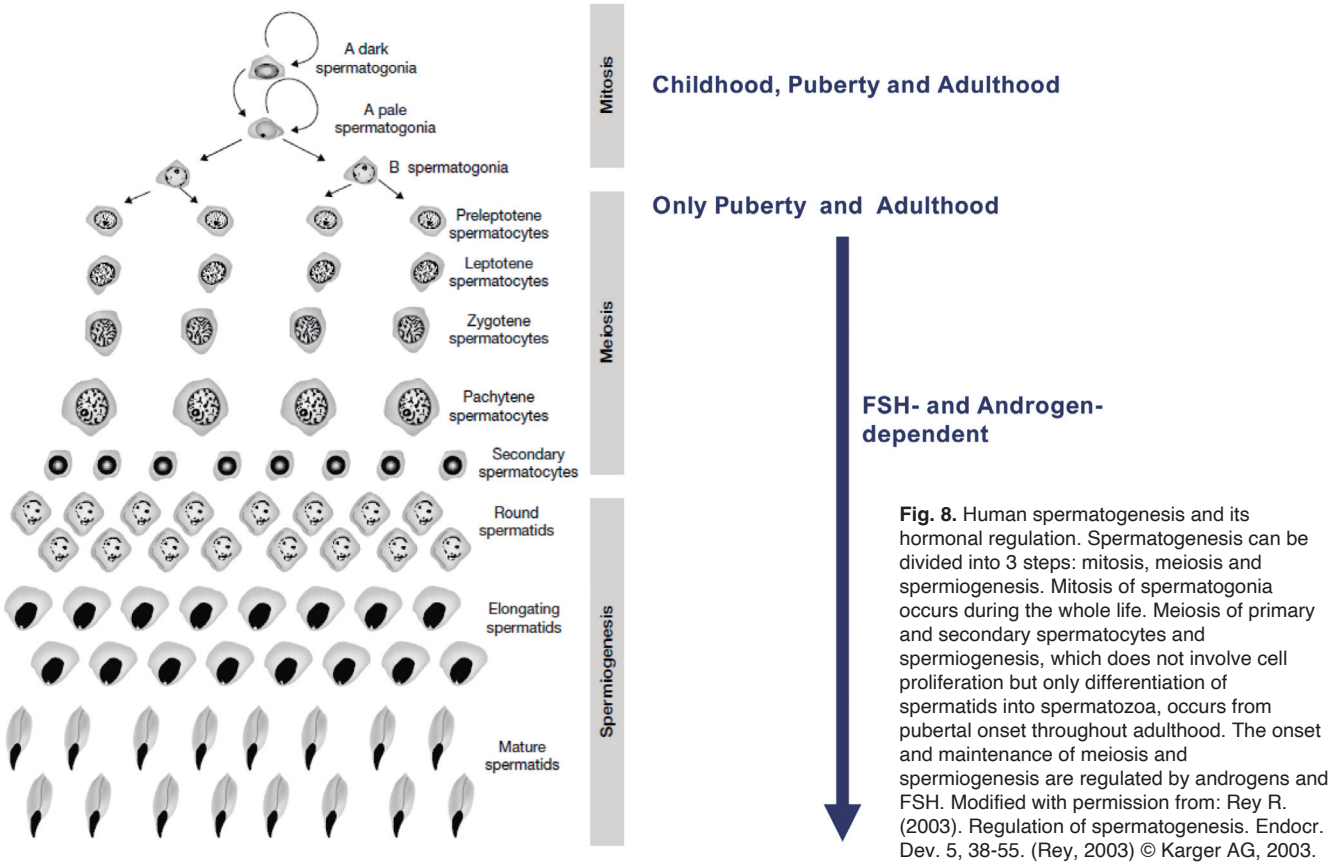

Fig. 8. Human spermatogenesis and its hormonal regulation. Spermatogenesis can be divided into 3 steps: mitosis, meiosis and spermiogenesis. Mitosis of spermatogonia occurs during the whole life. Meiosis of primary and secondary spermatocytes and spermiogenesis, which does not involve cell proliferation but only differentiation of spermatids into spermatozoa, occurs from pubertal onset throughout adulthood. The onset and maintenance of meiosis and spermiogenesis are regulated by androgens and FSH. Modified with permission from: Rey R. (2003). Regulation of spermatogenesis. Endocr. Dev. 5, 38-55. (Rey, 2003) © Karger AG, 2003.

instance in humans, gonadotropins and androgen levels similar to those observed in adults are present during approximately 12 to 15 months, from the beginning of the 2nd trimester of fetal life until the 3rd to 6th postnatal months (Fig. 3). At pubertal age, such a prolonged exposure to gonadotropins and androgens results in the acquisition of a mature phenotype of Sertoli cells and the onset of meiosis.

The lack of development of mature features of the seminiferous cords can be largely explained by the lack of AR expression within Sertoli cells (Chemes et al., 2008; Boukari et al., 2009). Leydig and peritubular cells express AR from early fetal development (Figs. 3, 4), but the expression in Sertoli cells seems absolutely required for testosterone to induce signs of Sertoli cell maturation, such as the downregulation of AMH expression (Fig. 10) (Edelsztein et al., 2018) or the upregulation of Eppin and Rhox5 (Willems et al., 2010a) and the establishment of tight junctions involved in the blood-testis barrier (Fig. 10) (Willems et al., 2010b; Edelsztein and Rey, 2019, Edelsztein et al., 2022; Wang et al., 2022).

Lack of typical changes in serum biomarkers in infants with precocious puberty

As mentioned, serum AMH is a typical biomarker of immature (prepubertal) Sertoli cells. Its levels decline during puberty in several mammalian species (Al-Attar et al., 1997; Rota et al., 2002; Almeida et al., 2013), including humans (Rey et al., 1993), as a consequence of the direct inhibition of AMH expression mediated by the steroid-bound AR (Edelsztein et al., 2018). In boys with precocious puberty, i.e. signs of pubertal development before the age of 9 years, serum AMH is low for age but adequate for pubertal maturation stage (Rey et al., 1993).

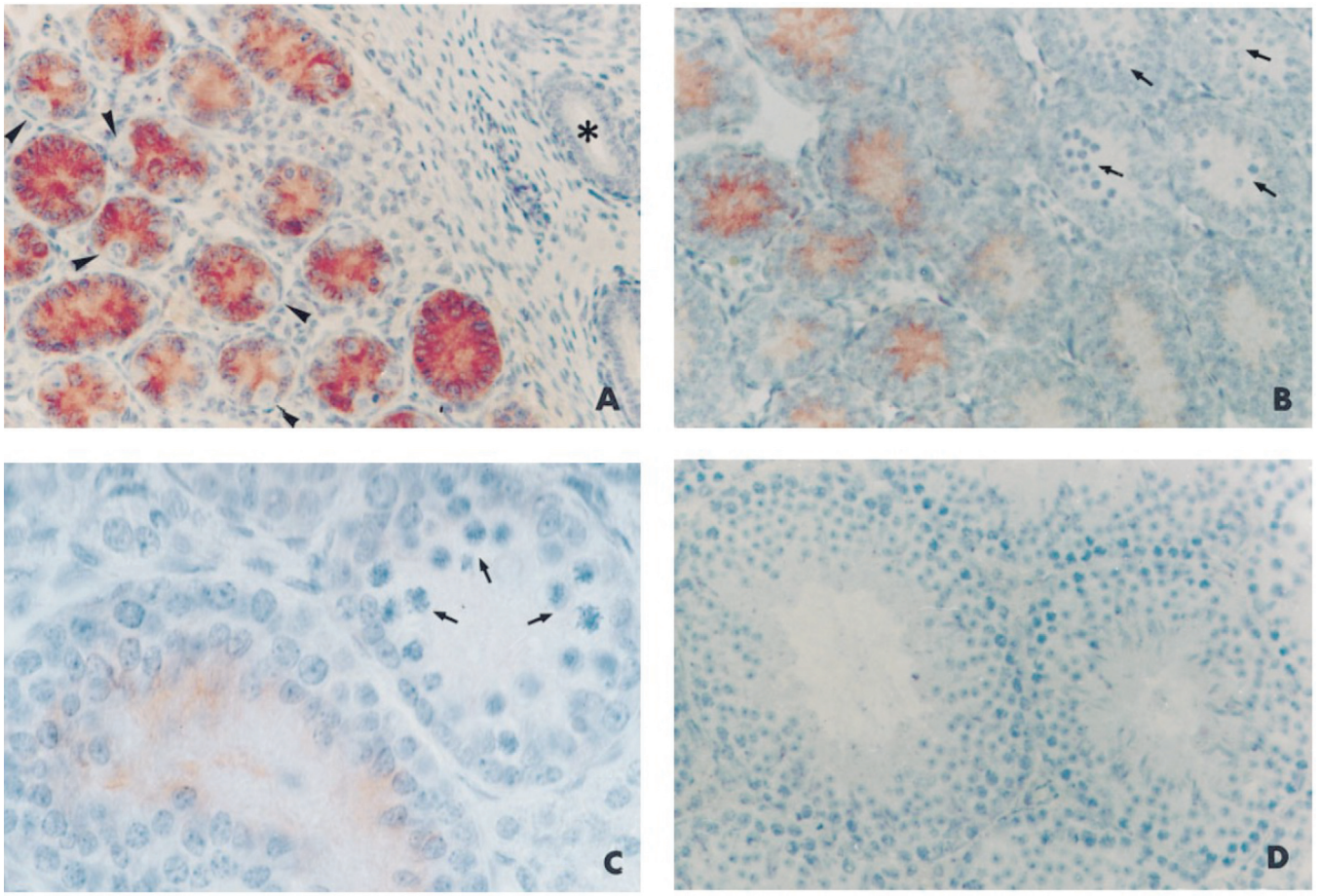

Fig. 9. AMH expression in the mouse testis. Immunohistochemistry for anti-Müllerian hormone (AMH) in the postnatal mouse testis. **A.** Postnatal day 4, a positive signal is observed in Sertoli cell cytoplasm; germ cells (arrowheads), interstitial tissue, and epididymal epithelium (*) are negative. **B, C.** Day 9, seminiferous tubules containing meiotic spermatocytes (arrows) are AMH-negative, while neighboring premeiotic tubules are positive. **D.** Adult: all tubules are AMH-negative. Reprinted with permission from: Al-Attar L., Noël K., Dutertre M., Belville C., Forest M.G., Burgoyne P.S., Josso N. and Rey R. (1997). Hormonal and cellular regulation of Sertoli cell anti-Müllerian hormone production in the postnatal mouse. J. Clin. Invest. 100, 1335-1343 (Al-Attar et al., 1997). © The American Society for Clinical Investigation, Inc., 1997.

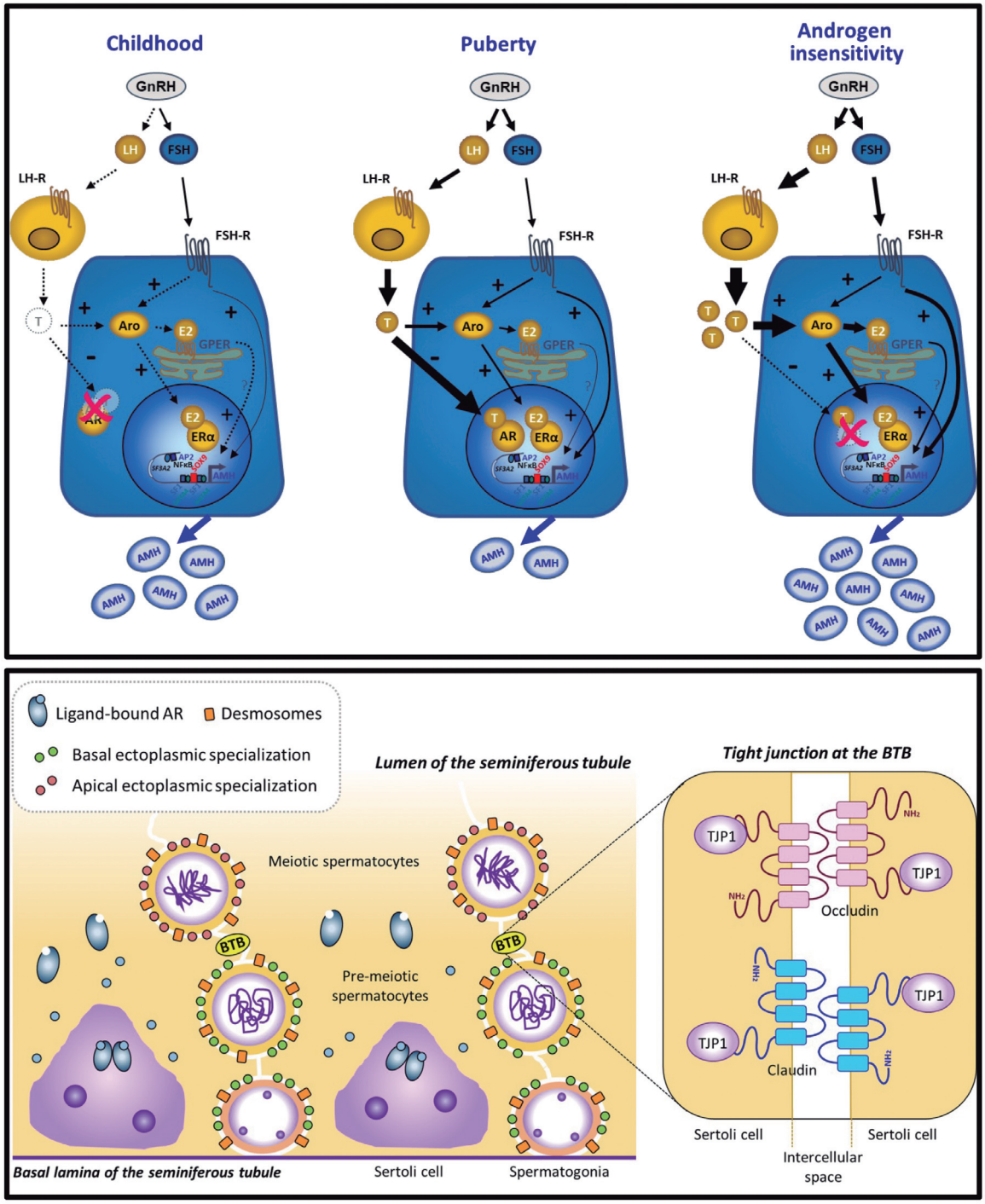

Fig. 10. Androgen and estrogen regulation of seminiferous tubule maturation at puberty. Upper panel: Interaction between androgens, estrogens and FSH on the regulation of AMH expression in Sertoli cells. During childhood, the hypothalamic-pituitary-gonadal axis is quiescent, and the extremely low steroid levels do not exert any physiological regulation on AMH production that is mostly hormone-independent. At puberty, the reactivation of the GnRH neuron and the gonadotropes results in higher LH and FSH levels. LH induces testosterone secretion by testicular Leydig cells. FSH acts on its receptor in the Sertoli cell membrane, resulting in a direct upregulation of AMH expression, through the cyclic AMP-PKA pathway involving transcription factors SOX9, SF1, AP2 and NFκB, and in an indirect upregulation of AMH by inducing aromatase expression. Aromatase converts androgens into estrogens, which can upregulate AMH directly by binding to the nuclear ERα or indirectly acting through the GPER expressed in the membrane of the endoplasmic reticulum. Nonetheless, the inhibitory effect of androgens overrides the stimulatory effect of FSH and estrogens on AMH expression, resulting in a decreased AMH secretion. In hyperestrogenic states with abrogated androgen action, such as the androgen insensitivity syndrome, the inhibitory effect of androgens does not exist, FSH and LH increase resulting in high testosterone that is converted to estradiol. Consequently, AMH production is substantially boosted. Reprinted with permission from: Edelsztein et al. Front Endocrinol (2022). Lower panel: The blood-testis barrier (BTB) is formed by intercellular unions between adjacent Sertoli cells. In the presence of androgens, AR-expressing Sertoli cells can mature and express several genes needed for BTB formation, such as Cldn3, Cldn11, Ocln and Tjp1. CLDN3, CLDN11, OCLN and TJP1, together with other proteins and components of the cytoskeleton, such as actin bundles, constitute tight junctions at the BTB. BTB: Blood-testis barrier, TJP1: Tight junction protein 1. Reproduced with permission from: Edelsztein N.Y. and Rey R.A. (2019). Importance of the Androgen Receptor Signaling in Gene Transactivation and Transrepression for Pubertal Maturation of the Testis. Cells 8, 1-17 (Edelsztein and Rey, 2019). © The Authors 2019, published by MDPI.

Interestingly, two patients with precocious puberty have been described in whom serum AMH was not downregulated (Grinspon et al., 2013). The common feature of these two patients, as opposed to all others reported, is that they were <1 year-old at the time of diagnosis (Fig. 11), an age when AR is not yet expressed in Sertoli cells. Furthermore, in one of them who did not receive immediate treatment, serum AMH declined to pubertal levels by the age of 2 years, in concordance with the known ontogeny of the AR that begins to be expressed in Sertoli cells during the second year of postnatal life in humans (Chemes et al., 2008).

Biomarkers in 46, XY patients with congenital disorders of sex development (DSD)

In 46, XY individuals, the lack of normal testosterone secretion by Leydig cells or of androgen action via the AR in target organs during the first trimester of intrauterine life results in an insufficient or a complete lack of virilization of the external genitalia (Rey and Grinspon, 2011). When the androgen secretion defect is due to testicular dysgenesis, Sertoli cells are also affected, and serum AMH is low (Grinspon et al., 2020). Conversely, when only Leydig cell function is impaired, e.g. Leydig cell aplasia/hypoplasia due to LH receptor mutations (Kremer et al., 1995) or steroidogenic enzyme defects (Auchus and Miller, 2012), the extremely low or absent androgen production in the testes results in a complete lack of maturation of Sertoli cells, as illustrated by the elevated levels of serum AMH typical of prepuberty (Rey et al., 1999) and the absence of adult spermatogenesis (Kremer et al., 1995). Similar features are observed in patients with 46, XY DSD due to AR mutations resulting in androgen insensitivity (Rey et al., 1994) and in mice with naturally occurring AR defects (Al-Attar et al., 1997) or with experimentally induced AR gene knockouts (Wang et al., 2009). Interestingly, testicular cell-specific AR knockout mouse models clearly prove that Sertoli cell AR expression is crucial for cell maturation (Chang et al., 2004), formation of the blood-testis barrier and support of adult spermatogenesis (Wang et al., 2006).

Clinical features of hyperandrogenic states in boys: androgen origin

The main sources of androgens are the gonads and the adrenal glands. As mentioned, the androgen effect on target organs during normal puberty in males results in the development of adult genital sex and other male physical characteristics, linear growth and bone age acceleration as well an increase in bone and muscle mass. Maturation of the gonads is characterized by adult spermatogenesis, resulting in a massive increase of testicular volume. Indeed, the increase in the size of the testes is clinically used as one of the main signs of pubertal onset in boys (Salonia et al., 2019). Androgen excess can abnormally occur before the age of puberty, i.e. before 9 years in boys. The origin of these androgens can be the testes, the adrenal glands or, more rarely, extragonadal/extraadrenal androgen-secreting tumors or exogenous androgen administration. On the other hand, testicular size increase may occur in boys in the absence of testicular androgen production, i.e. not due to precocious pubertal maturation. The understanding of the normal and the disordered androgen-regulated functions of the seminiferous tubule compartment provides a great help for the interpretation of the underlying pathophysiology.

As previously discussed, the induction of adult spermatogenesis resulting in testicular volume increase requires very high intratesticular testosterone concentrations in order for Sertoli cells to mature and

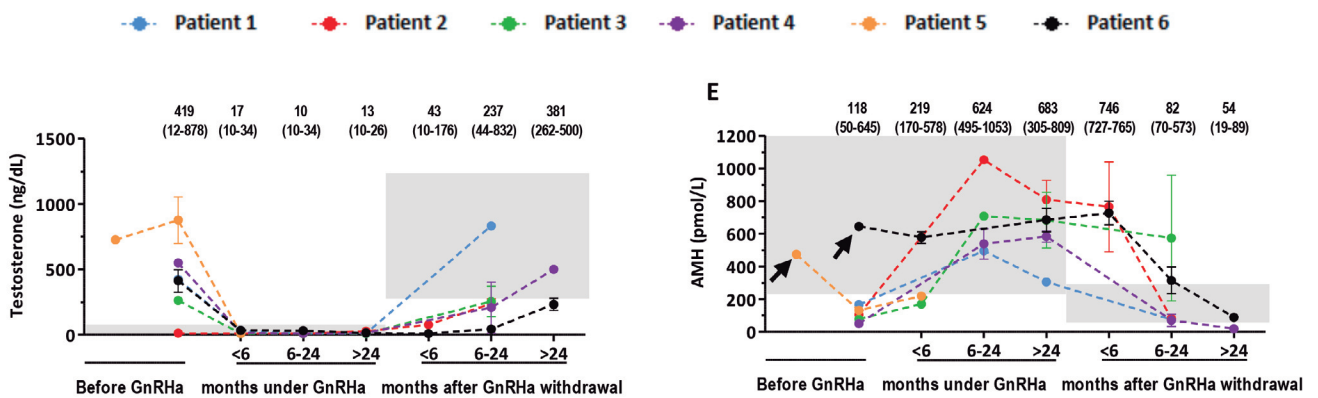

Fig. 11. Androgen regulation of AMH production in boys with central precocious puberty. In 6 patients with central precocious puberty, serum testosterone levels were high for age at diagnosis. Serum AMH was low for age in 4 of them, but not in 2 patients who were aged <1 year (arrows). When testosterone levels decreased in response to treatment with an analogue of gonadotropin releasing hormone (GnRH), serum AMH recovered high (prepubertal) levels. When treatment was discontinued, testosterone increased again and AMH was inhibited. Modified with permission from: Grinspon R.P., Andreone L., Bedecarrás P., Ropelato M.G., Rey R.A., Campo S.M. and Bergadá I. (2013). Male Central Precocious Puberty: Serum Profile of Anti-Mullerian Hormone and Inhibin B before, during, and after Treatment with GnRH Analogue. Int. J. Endocrinol. 2013, 823064. (Grinspon et al., 2013). © Grinspon et al., 2013, published by Hindawi.

transduce androgen effects to germ cells. In a boy under the age of 9 years, a concomitant increase in testicular volume and a decrease in serum AMH are clearly indicative of a testicular origin of a precocious hyperandrogenic state. This can be due to central precocious puberty (Latronico et al., 2016), gonadotropin-independent precocious puberty (also called testotoxicosis or male-limited familial precocious puberty, due to activating mutations of the LH receptor) (Kremer et al., 1995) or Leydig cell tumors/hyperplasia (Karmazyn et al., 2018). Conversely, when the clinical features of androgen action (development of secondary sex characteristics, linear growth and bone age acceleration, increase in muscle mass) are not associated with an increase in testicular volume and a decline in serum AMH, they point to an extragonadal androgen origin, e.g. congenital adrenal hyperplasia, androgen-secreting tumors, exogenous androgen sources (cosmetics, drugs, etc.). In these cases, the intratesticular concentrations are far lower than those observed in the previously mentioned conditions, even though circulating levels are similar or even higher. Finally, when a boy presents with prepubertal macroorchidism but with high (prepubertal) serum AMH and no signs of peripheral androgenization, the increase testicular volume is not a sign of pubertal maturation, but rather indicates other genetic conditions (e.g. Fragile X syndrome) (De Sanctis et al., 2014), gonadal neoplasia (Karmazyn et al., 2018), or compensatory testicular macroorchidism in the case of monorchidism or unilateral testicular atrophy (Grinspon et al., 2016).

Clinical features of hypoandrogenism in pubertal age

As discussed in the introduction, low to absent androgen testicular production is the normal state during childhood. This "hypoandrogenic" state can extend into pubertal age in two different conditions that are usually difficult to distinguish clinically: constitutional delay of puberty and central (or hypogonadotropic) hypogonadism (Raivio and Miettinen, 2019). Constitutional delay of puberty is characterized by a prolongation of the physiological prepubertal condition beyond the age of 14 years in males: gonadotropins and testosterone levels remain prepubertal (i.e. low), and AMH production by the testes also remains prepubertal (i.e. high), showing the lack of androgen-dependent down-regulation. In boys with central (hypogonadotropic) hypogonadism, while serum gonadotropins and testosterone are low like in constitutional delay of puberty, AMH is not high but low, reflecting the defective Sertoli cell function due to FSH deficiency (Rohayem et al., 2015; Grinspon et al., 2021).

Clinical implications of estrogen receptor signaling in the developing testis

Most of the evidence related to the role of estrogens in the testis is indirect, and conclusive interpretations are missing on the potential underlying mechanisms.

Clinical features of hypoestrogenic states

Aromatase deficiency is a rare condition due to loss-of-function variants in *CYP19A1* resulting in decreased or null estrogen production (Fukami and Ogata, 2022). Impaired estrogen action in target tissues, i.e. estrogen insensitivity/resistance, has been described in rare cases of individuals carrying deleterious variants in *ESR1*, encoding ERα, or ESR2, encoding ERβ (Guercio et al., 2020). Male patients with aromatase deficiency or estrogen resistance have apparently normal pubertal development, with extended linear growth resulting in tall stature due to the lack of epiphyseal fusion, which is estrogen dependent. The implication of estrogen signaling on testicular function is controversial. In a few cases, oligospermia and infertility described in adults with aromatase deficiency or ESR1 mutations suggested a role for estrogens on spermatogenesis (Hammes and Levin, 2019). AMH secretion, which is stimulated by estrogens (see "*Clinical features of hyperestrogenic states*" below), was found to be low despite elevated circulating E2 in a male with estrogen insensitivity caused by defective ERα function (Bernard et al., 2017). Yet, the patient had untreated cryptorchidism, whose contribution to the low serum AMH cannot be ruled out.

In one patient with aromatase deficiency where testicular biopsy was available, a complete germ cell depletion was observed, but the sample had been taken after testosterone treatment which has a clear impact on endogenous gonadotropin and testosterone production (Maffei et al., 2004). Conversely, macroorchidism was described in a young adult with aromatase deficiency (Morishima et al., 1995), probably reflecting Sertoli cell hyperplasia due to the persistent elevation of serum FSH and full spermatogenesis. ERβ seems to play no essential role in testicular physiology, as shown by specific mouse knockout models (Krege et al., 1998).

Clinical features of hyperestrogenic states

Aromatase excess syndrome is an extremely rare genetic disorder caused by rearrangements of chromosome 15 resulting in ectopic or excessive expression of *CYP19A1* (Fukami, 2020). The most typical feature is prepubertal or peripubertal gynecomastia and accelerated bone age progression with final short stature. External genitalia and fertility are normal, suggesting that the estrogen excess does not impair testicular function significantly.

In 46, XY individuals with DSD due to AR mutations (androgen insensitivity syndrome) entering pubertal development, testicular AMH output increases concomitantly with FSH and estradiol levels. The lack of AMH downregulation, in spite of high androgen levels, can be explained by the disruption of AR signaling. Furthermore, the increase in AMH production can be explained by a direct action of FSH on Sertoli cell

proliferation and on AMH transcription (Lukas-Croisier et al., 2003; Lasala et al., 2004, 2011). Interestingly, patients with androgen insensitivity show increased aromatization of androgens to estrogens at puberty, as shown by breast development (Quigley et al., 1995), and AMH is also elevated in boys with Peutz-Jeghers syndrome with estrogens produced by Sertoli cell proliferations leading to suppressed FSH (Venara et al., 2001). Using experimental mouse models, we have recently shown that estradiol upregulates AMH expression in Sertoli cells involving ERα binding to an estrogen-response element (ERE) present on the human AMH promoter (Fig. 10) (Valeri et al., 2020). GPER-mediated signaling may also be involved by more modestly regulating AMH transcription (Valeri et al., 2020) and Sertoli cell proliferation (Lucas et al., 2014).

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