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THE ROLE OF HSD17B3 IN MALE DEVELOPMENT AND HEALTH

Disruption of Testosterone Production and Its Effects

Arttu Junnila

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1813 | MEDICA – ODONTOLOGICA | TURKU 2024

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ABSTRACT

Hydroxysteroid (17β) dehydrogenase type 3 (HSD17B3) is known to be the primary enzyme converting androstenedione (A) to testosterone (T) in human and mouse testes. It is therefore important for male development and reproductive health. Inherited HSD17B3 deficiency is a known cause of a disorder of sex development (DSD) in humans, where XY individuals are born with a female appearance due to testosterone deficiency, but are virilized during puberty as T starts to suddenly rise. Due to the rarity of the condition, many open questions remain on e.g. the compensatory source of testosterone, and the wider consequences of the T deficiency. We have established and characterized mouse lines lacking either HSD17B3 (3-KO), HSD17B1, or both (DKO). 3-KO males were born apparently phenotypically normal, but presented signs of undermasculinization at later ages, including delayed puberty, a shortened anogenital distance, subfertility with normal spermatogenesis, Leydig cell maturation defects, and lower size of many androgen-sensitive tissues. The cause of the undermasculinization was a fetal delay in testosterone production, but by birth and in adulthood 3-KO males produced significant amounts of testosterone due to massive upregulation of androstenedione production, and compensatory enzymes. In the DKO mice the fetal testosterone production was abolished almost completely, confirming HSD17B1 as the enzyme compensating for the lack of HSD17B3 in early development. The resulting undermasculinization in DKO was also much more drastic than in 3-KO. However, adult DKO testosterone production was comparable to 3-KO, indicating that yet other enzymes take over the compensation after puberty. In 3-KO, we also identified a novel developmental programming mechanism, where fetal androgen deficiency led to reduced kidney size in adult males. This mechanism resembles the effects of the so-called masculinization programming window, thus far only identified for reproductive organs. Finally, we observed that the lack of HSD17B3 led to weight gain through excess adipocity in male mice. Together, these results confirm that HSD17B3 is essential for properly functioning testosterone biosynthesis, but compensatory mechanisms exist, and that fetal androgen deficiency has long-lasting effects on later phenotype.

KEYWORDS: Steroidogenesis, testosterone, HSD17B3, male development, developmental programming

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TIIVISTELMÄ

Hydroksisteroidi-(17β)-dehydrogenaasi, tyyppi 3 (HSD17B3) on pääasiallinen androstenedionia testosteroniksi muuntava entsyymi ihmisen ja hiiren kiveksessä. Täten se on oleellinen miesten ja urosten kehitykselle ja lisääntymisterveydelle. Peritty HSD17B3-puutos tiedetään yhdeksi sukupuolen kehityksen häiriöiden aiheuttajaksi. Tällöin XY-yksilöt syntyvät ulkoisesti feminisoituneina testosteronipuutoksen vuoksi, mutta murrosiässä virilisoituvat yllättäen testosteronituotannon aktivoituessa. Harvinaisuudestaan johtuen monet HSD17B3-puutoksen yksityiskohdat, kuten korvaava testosteronin lähde ja testosteronipuutteen laajemmat vaikutukset, ovat epäselviä. Olemme luoneet ja kuvanneet hiirilinjoja, joilta puuttuu joko HSD17B3 (3-KO), HSD17B1, tai molemmat (DKO). 3-KO –urokset syntyivät ilmiasultaan normaaleina, mutta osoittivat myöhemmällä iällä merkkejä alimaskulinisaatiosta, mukaan lukien puberteetin myöhästyminen, lyhentynyt anogenitaalimitta, vähentynyt hedelmällisyys, ongelmat Leydigin solujen kypsymisessä ja androgeeniherkkien kudosten pienempi koko. Alimaskulinisaation taustalla oli sikiöaikainen viivästys testosteronituotannossa, mutta syntymään mennessä ja aikuisina 3-KO –urokset tuottivat merkittäviä määriä testosteronia korvaavien entsyymien kautta, androstenedionin tuotannon lisääntyessä huomattavasti. DKO-uroksilla taas sikiöaikainen testosteronituotanto puuttui lähes kokonaan, vahvistaen HSD17B1:n kompensoivan HSD17B3:n puutosta varhaisessa kehityksessä. Myös alimaskulinisaatio oli vakavampaa DKO:lla kuin 3-KO:lla. Aikuisilla DKO-uroksilla testosteronituotanto kuitenkin vertautui 3-KO-uroksiin, joten eri entsyymit ovat vastuussa kompensaatiosta murrosiän jälkeen. 3-KO –uroksissa kuvasimme uuden kehityksen säätelyn mekanismin, jossa sikiöaikainen androgeenipuutos johti pienempään munuaisten kokoon aikuisissa. Tämä mekanismi muistuttaa ns. maskulinisaation määräytymisen aikaikkunaa, joka on tähän mennessä osoitettu vain lisääntymiselimille. Havaitsimme myös HSD17B3:n puutoksen johtavan uroshiirten painon nousuun rasvan kertymisen kautta. Yhdessä nämä tulokset vahvistavat HSD17B3:n olevan oleellinen tehokkaalle testosteronituotannolle, mutta sen puutetta kyetään myös kompensoimaan; lisäksi sikiökautisella androgeenipuutoksella on pitkäkestoisia vaikutuksia myöhempään ilmiasuun.

AVAINSANAT: Steroidogeneesi, testosteroni, HSD17B3, sukupuolenkehitys

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Abbreviations

1-KO	*Hsd17b1* point mutation mouse line
11KA4	11-ketoandrostenedione
11KT	11-ketotestosterone
11OHA4	11-hydroxyandrostenedione
3-KO	*Hsd17b3* knockout mouse line
ACTH	Adrenocorticotropic hormone
A-dione	Androstenedione
ADX	Adrenalectomy
AIS	Androgen insensitivity syndrome
AKR1C3	Aldo-keto reductase family 1 member C3
ALC	Adult Leydig cell
AMH	Anti-Müllerian hormone
AMHR	Anti-Müllerian hormone receptor
AR	Androgen receptor
CAIS	Complete androgen insensitivity syndrome
cAMP	Cyclic adenosine monophosphate
CYP11A1	Cytochrome p450 family 11, subfamily A, polypeptide 1
CYP11B1	Cytochrome p450 11β-hydroxylase
CYP17A1	Cytochrome p450 family 17, subfamily A, polypeptide 1
CYP51	Sterol 14-demethylase
DHCR7	7-Dehydrocholesterol reductase
DHEA	Dehydroepiandrosterone
DHH	Desert hedgehog homolog
DHT	Dihydrotestosterone
DKO	*Hsd17b1-Hsd17b3* –double knockout mouse line
DSD	Disorder of sex development
E1	Estrone
E2	Estradiol
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FLC	Fetal Leydig cell

FSH	Follicle-stimulating hormone
GC-MS/MS	Gas chromatography – tandem mass spectrometry
GnRH	Gonadotropin-releasing hormone
GPR54	Kisspeptin receptor
hCG	Human chorionic gonadotropin
HE stain	Hematoxylin and eosin stain
HPA axis	Hypothalamic-pituitary-adrenal axis
HPG axis	Hypothalamic-pituitary-gonadal axis
HSD	Hydroxysteroid dehydrogenase
HSD17B1/3	Hydroxysteroid (17β) dehydrogenase type 1/type 3
HSD3B1/2	3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase type 1/type 2
HSL	Hormone-sensitive lipase
IF	Immunofluorescense
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
INSL3	Insulin-like factor 3
KAP	Kidney androgen-regulated protein
KISS1	Kisspeptin
KO	Knockout
LH	Luteinizing hormone
LHCGR	Luteinizing hormone/choriogonadotropin receptor
MPW	Masculinization programming window
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
PAIS	Partial androgen insensitivity syndrome
PKA	Protein kinase A
PTCH1	Patched 1
PTM cell	Peritubular myoid cell
SHBG	Sex-hormone binding globulin
SDR	Short-chain dehydrogenase/reductase
SF1	Steroidogenic factor 1
SOX9	SRY-related HGM-box containing gene 9
SRY	Sex-determining region Y
StAR	Steroidogenic acute regulatory protein
SULT2A1	Sulfotransferase family 2A member 1
T	Testosterone
T/A ratio	Testosterone/androstenedione ratio
WNT4	Wnt family member 4
WT	Wild-type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Petra Sipilä, **Arttu Junnila**, Janne Hakkarainen, Riikka Huhtaniemi, Laura Mairinoja, Fu-Ping Zhang, Leena Strauss, Claes Ohlsson, Noora Kotaja, Ilpo Huhtaniemi, Matti Poutanen. The lack of Hsd17b3 in male mice results in disturbed Leydig cell maturation and endocrine imbalance akin to humans with HSD17B3 deficiency. *FASEB Journal*. 2020; 34(5):6111-6128.

II **Arttu Junnila**, Fu-Ping Zhang, Guillermo Martinez Nieto, Janne Hakkarainen, Juho-Antti Mäkelä, Claes Ohlsson, Petra Sipilä, Matti Poutanen. HSD17B1 compensates for HSD17B3 deficiency in fetal mouse testis but not in adults. *Endocrinology*. 2024; 165(6)

III **Arttu Junnila**, Kalle Rytkönen, Guillermo Martinez Nieto, Mats Perk, Hao Li, Oliver Mehtovuori, Asta Laiho, Claes Ohlsson, Laura Elo, Satu Kuure, Matti Poutanen, Petra Sipilä. Testosterone exposure during fetal masculinization programming window determines the kidney size in adult mice. *Manuscript*.

1 Introduction

The phenotypic sex – the external and functional form of a male or a female – is a combination of many features, and its development a sum of many steps. It is determined first by genetic factors during early development. However, during later fetal development, after birth, at puberty and in adulthood, it is further developed, matured and maintained by hormonal factors. These hormonal factors –chiefly the sex hormones – are essential for reproduction, but also have effects on many non-reproductive tissues. The developmental factors of sex in general are highly conserved in mammals, including humans and mice (Hughes, 2001).

The proper and timely function of all the genetic and hormonal steps is required for undisturbed sexual differentiation, and the sexually dimorphic balance of hormonal regulation is important for health in later life, not just from a reproductive point of view but also as a whole. Many of the developmental checkpoints and pathways favor female development as a default, and an extra factor is needed for male development (Grumbach et al., 2003).

The factor with the widest and most visible effects in male development is the male sex hormone testosterone. Testosterone is a steroid hormone, produced mainly in the testes, from where it diffuses to circulation to reach its targets across the body. After the testis formation during the fetal period, testosterone takes over the role of driving male fetal development (Hiort, 2013). It is again involved in the reproductive maturation and development of secondary sex characteristics in puberty, and the maintenance of the male phenotype in adulthood until it gradually declines in old age (Kaufman and Vermeulen, 2005; Koskenniemi et al., 2017). It also has effects on body composition and many non-reproductive tissues (Piras et al., 2020; Zhu et al., 2014). Without testosterone effects – due to disruption of its production or its target, the androgen receptor – the male phenotype does not develop in a typical fashion. Depending on the precise cause of the disorder, the resulting phenotype can range from slight undermasculinization to a completely female appearance (Mota et al., 2015).

Testosterone biosynthesis in the Leydig cells of the testes in a multi-step process, beginning with cholesterol and culminating in the conversion of inactive male sex hormone androstenedione to the active testosterone (Miller and Auchus, 2011). This

final step is mainly catalyzed by the hydroxysteroid (17β) dehydrogenase type 3 (HSD17B3) enzyme (Luu-The et al., 1995). Lack of HSD17B3 activity due to mutations of the enzyme is one of the known causes of disorders of male development (Geissler et al., 1994). Due to developmental androgen deficiency, XY-individuals with fully developed testes nevertheless have a feminized appearance at birth and are often raised in female gender. However, at puberty they are suddenly affected by progressive virilization due to activation of testosterone production. The patients then have to undergo hormonal treatment and surgery to either retain their gender of rearing, or transition to males (Andersson et al., 1996; Bertelloni et al., 2009). The testosterone production after puberty, although often reaching the lower range of normal male values, is inefficient, as the concentration of the precursor androgen androstenedione is greatly elevated (Faisal Ahmed et al., 2000a).

The publications on HSD17B3 deficiency are mainly rare case reports of the patients at the time of their diagnosis and treatment, with no detailed follow-up. Thus, there is little data available on the possible long-term consequences of either the developmental defects or the hormonal imbalance present in the patients who retain their testes and undergo the transition to male gender. The endocrine physiology of the disorder, including the specific mechanisms and wider results of the incomplete virilization during development, and the compensating sources of testosterone, remain unclear.

In the present studies, we generated several mouse models to better understand the role of HSD17B3 in male development. Firstly, we generated a knockout mouse model of HSD17B3 and characterized it, aiming to determine the wider effects of the HSD17B3 deficiency on the phenotype, to clarify the role of HSD17B3 in steroidogenesis, and to validate the usefulness of the mouse line as a model of the human condition. Secondly, to determine whether another enzyme, HSD17B1, was involved in the remaining testosterone production capacity of the HSD17B3 knockout mice, we generated an HSD17B1 inactivating point mutant mouse line, and crossed it with the HSD17B3 knockout line to obtain mice lacking both HSD17B1 of HSD17B3 activity. Thirdly, we further studied the effects of the lack of HSD17B3 and the resulting disruption of testosterone production on the phenotype of the male mice. In this work, we focused in developmental programming mechanisms creating a persisting phenotype of androgen insensitivity in the kidney and adipose tissue.

2 Review of the Literature

2.1 Male development

2.1.1 Sexual differentiation

Human sex characteristics are determined in three interconnected and progressive steps. At the chromosomal level, sex is determined at fertilization by the combination of sex chromosomes in the fusing female and male gametes, in particular whether the fertilizing sperm cell carries an X or a Y chromosome (Lukusa et al., 1992). The resulting activation or absence of certain genes, and their interactions, cause the differentiation of male or female gonads, giving rise to gonadal sex (Hughes, 2001). Finally, the specific hormones secreted by the gonads drive the development of other tissues and organs, including the reproductive tissues and the brain, creating the characteristics that define the phenotypic sex (Gardiner and Swain, 2014).

In humans and mice, the gonads of both sexes arise from a thickening of the ventromedial surface of the embryonal mesonephros. This thickening continues to develop as the genital ridge, composed of cells from the mesonephros and also primordial germ cells migrating from the yolk sac (Eggers and Sinclair, 2012; Hughes, 2001). The genital ridge in turn gives rise to the bipotential gonads, at this stage identical in features and gene expression regardless of the sex chromosomes (Munger et al., 2013). The further development of the early bipotential gonads to testes, and the further development of other male reproductive organs, requires the presence of several factors acting in sequence. The absence of these genetic and hormonal factors leads to precursor structures developing into corresponding female organs. Defects in these steps in males can cause different forms of disorders of sex development (DSD) (Grumbach et al., 2003).

2.1.2 Genetic regulation of testis development

The development of testes initiates with the activation of a single gene in the Y chromosome, sex-determining region Y (SRY), in the somatic cells of the gonad (Lukusa et al., 1992). In the absence of SRY, or if it is dysfunctional, the gonad will go on to develop into an ovary (Fig. 1) (Grumbach et al., 2003). The expression of

SRY, which codes for a DNA-binding protein, is transient (Sekido et al., 2004). However, together with steroidogenic factor 1 (SF1) it leads to the activation of SRY-related HGM-box containing gene 9 (SOX9). A positive regulatory loop then forms between *SOX9* expression and genes such as fibroblast growth factor 9 (FGF9) to maintain their mutual expression while *SRY* expression disappears (Gardiner and Swain, 2014).

The expression of *SOX9* is the first factor in a cascade that leads the supporting cell precursors to proliferate and differentiate to Sertoli cells (Nef and Parada, 2000; Schmahl et al., 2004). Without the initial SRY activation, the supporting cells would instead differentiate to ovarian granulosa cells (Fig. 1) (Albrecht and Eicher, 2001). The developing Sertoli cells in turn produce factors that direct the formation of cords, through the aggregation of Sertoli cells, germ cells and peritubular myoid cells, that then become the seminiferous tubules (Combes et al., 2009). Outside the tubules, the differentiation of interstitial Leydig cells is also activated by Sertoli-cell produced signaling molecule Desert hedgehog homolog (DHH), activating Patched 1 (PTCH1) signaling (Fig. 1) (Gardiner and Swain, 2014; Yao et al., 2002). While ovarian development is often considered to be the default path of differentiation, it too requires active promotion, and therefore the inhibition of the ovarian differentiation driving Wnt family member 4 (WNT4) by FGF9 is also essential for testis differentiation (Fig. 1) (Jameson et al., 2012). The differentiation path of the developing gonads is determined at around 6 weeks of gestation in humans and around the embryonic day 10.5 in mice (Eggers and Sinclair, 2012).

Leydig cells develop in two main populations at different stages of life, with morphological and functional differences. Fetal Leydig cells (FLCs) develop in the fetal testis and are a separate population from the adult Leydig cells (ALCs) that develop before and during puberty (Teerds and Huhtaniemi, 2015; Ye et al., 2017). The number of FLCs increases greatly during the second trimester, then gradually declines toward birth (O'Shaughnessy, 2017). SF1 is needed for both *DHH* expression in Sertoli cells and the induction of steroidogenic enzyme expression, necessary for mature FLCs (Teerds and Huhtaniemi, 2015).

2.1.3 Hormonal regulation of male development

After testis formation, the continued development of the reproductive organs towards the male phenotype is mainly controlled by two types of hormones secreted from the newly differentiated testicular cell populations: Anti-Müllerian hormone (AMH) produced by Sertoli cells and androgens produced by Leydig cells (Fig. 1) (Nef and Parada, 2000).

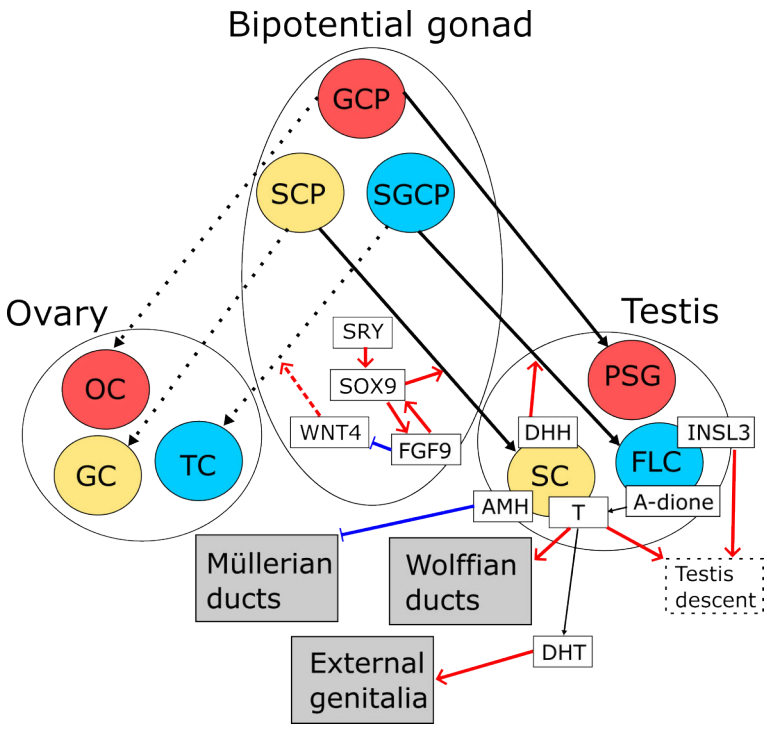

Figure 1. The differentiation of the bipotential gonad to testes. SRY and SOX9 activation drives the supporting cell precursors (SCP) to differentiate into Sertoli cells (SC) instead of granulosa cells (GC) of the ovary, while FGF9 inhibits WNT4. SC derived DHH promotes the differentiation of steroidogenic cell precursors to fetal Leydig cells (FLC) instead of theca cells (TC). In the testes, germ cell precursors (GCP) form prospermatogonia (PSG) instead of oocytes (OC). SC derived AMH leads to the regression of Müllerian ducts, while FLCs and SCs co-operate to produce testosterone, which stimulates Wolffian duct differentiation. DHT, converted from T, drives the differentiation of male external genitalia from bipotential precursor structures. Finally, testosterone and INSL3 from FLCs result in testis descent. Stimulation is presented by red arrows, inhibition by blue. Adapted from (Koopman, 1999).

AMH is a glycoprotein of the TGF-β superfamily. AMH secretion from the testis to surrounding tissues causes the regression of the paramesonephric, or Müllerian ducts, which would otherwise go on to develop into the structures of the female internal genitalia: fallopian tubes, uterus and a part of the vagina (Hughes, 2001). AMH acts through the Anti-Müllerian hormone receptor (AMHR), which is expressed in the mesenchymal cells around the Müllerian ducts. The expression is present in a gradient that parallels the direction of the regression, directly causing apoptosis in the epithelial cells of the ducts (Allard et al., 2000). Disruption of AMH action leads to persisting Müllerian derivatives in males, which continue to develop without further hormonal support and in turn can interfere with testis descent (Loeff et al., 1994).

The FLCs, on the other hand, are required for the production of several hormones that are essential for male development. The one with widest effects on the phenotype is the male sex hormone testosterone, which directly or indirectly *via* conversion to dihydrotestosterone (DHT) regulates the differentiation and development of internal and external genitalia, and later the other male characteristics. The production and effects of testosterone will be reviewed in more detail in the following chapters.

A third, more local factor required for the proper development of phenotypical male sex is the peptide hormone insulin-like factor 3 (INSL3), also produced by the FLCs. Although testosterone is needed for the regression of the suspensory ligaments initially holding the gonads in place near the kidneys, INSL3 is necessary for the transabdominal descent of the testis, mediating the contraction of the gubernaculum that draws the testes to the base of the abdomen (Nef and Parada, 1999). INSL3 is also produced in the ALCs, but its role in the adult testis is still unclear, although it may be involved in controlling male germ cell survival and the meiotic maturation of oocytes in females (Assis et al., 2016).

2.2 Male sex hormones

2.2.1 Androgens and the AR receptor

Testosterone, as the primary circulating male sex hormone, has the central role in regulating male development in most vertebrates. It is a member of the androgenic hormones, which are more broadly categorized as sex steroids or gonadal steroids together with their feminizing counterpart estrogens, and female reproduction related progestogens. Together with adrenal corticosteroids (glucocorticoids and mineralocorticoids) they make up the steroid hormones (Fig. 2). All steroid hormones share a common backbone of fused carbon rings with varying functional groups, which is ultimately derived from their common precursor cholesterol through a network of biosynthetic pathways (Miller and Auchus, 2011). The proper functionality of these pathways in totality is essential for steroid hormone production, including sufficient androgen production. That in turn is essential for sexual development, maturation and reproductive health in men (Griffin and Wilson, 2003).

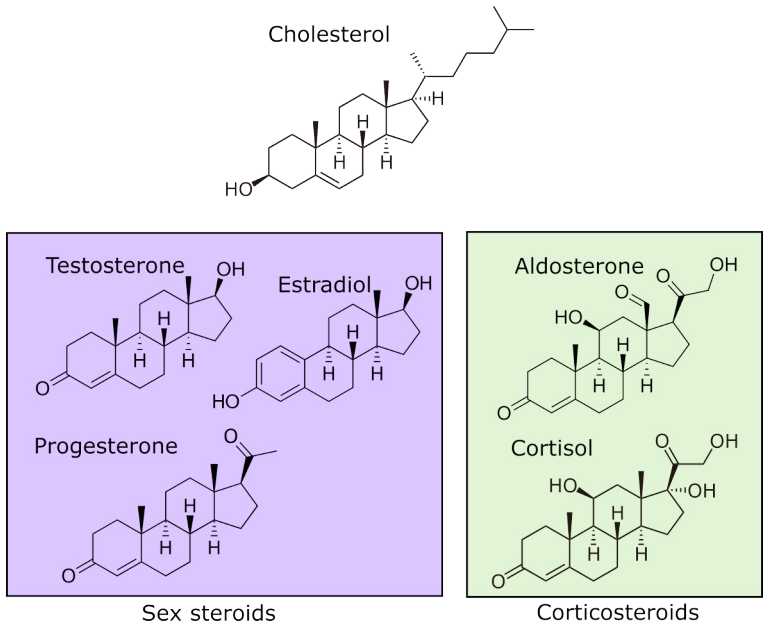

Figure 2. Cholesterol and the steroid hormones. The sex steroids – here represented by the androgen testosterone, estrogen estradiol and progestogen progesterone – and corticosteroids – mineralocorticoid aldosterone and glucocorticoid cortisol – all share a similar structure ultimately derived from cholesterol.

Approximately 95% of male testosterone is produced by the testes, where it has local effects in proper testis function (Winters, 2000). The testosterone content of human testis is 50 µg/g on average, adding to a total of 1.5 mg, and it is produced constantly at a rate of 5-7 mg/day, so majority of the testosterone is released to circulation (Southren et al., 1967; Takahashi et al., 1983). In humans, circulating testosterone is mostly bound to carriers. 44% is bound by sex hormone –binding globulin (SHBG), produced by the liver, and 54% by albumin and other proteins. Only around 2% of testosterone is transported unbound (Griffin and Wilson, 2003). In mice, the homologous androgen-binding protein is only expressed in the testes of adult animals, with low amounts reaching the circulation. This likely results in more fluctuation in the concentration of free testosterone in mice compared to human (Laurent et al., 2016). Only the unbound testosterone fraction is biologically active, entering cells through passive diffusion (O'Shaughnessy, 2017). The adrenals are also a significant source of androgen precursors, androstenedione and dehydroepiandrosterone (DHEA). These are then converted to active androgens in periphery, in both androgen target and non-target tissues. The steroidogenic pathways in males are explained in more detail in chapter 2.2.3 and 2.2.4.

The effects of androgens include the differentiation (chapter 2.4.1) and pubertal development (chapter 2.4.3) of the male phenotype, the initiation and maintenance

of spermatogenesis (chapter 2.4.4) and the regulation of gonadotropin secretion (Griffin and Wilson, 2003). It also has effects on other, non-reproductive tissues and organs, such as the musculoskeletal system, kidneys (chapter 2.4.5) and adipose tissue (chapter 2.4.6).

Androgens act by binding the androgen receptor (AR). AR is a nuclear receptor that in turn functions as a transcription factor (El Kharraz et al., 2021). Binding of the ligand – testosterone or DHT – causes a conformational change in the receptor protein (Aranda and Pascual, 2001). This allows the receptor-ligand complexes to translocate to the nucleus and bind specific androgen response element DNA sequences as homodimers, promoting the transcription of androgen target genes (El Kharraz et al., 2021; McKenna, 2014). In addition to the expression patterns of AR across tissues, tissue-specific transcription factors are then behind the differential transcriptional effects of androgen action in different tissues (Pihlajamaa et al., 2014). Androgens can also have effects through faster, non-transcription related signaling, via membrane and cytosolic androgen receptors (Toocheck et al., 2016).

Testosterone can activate the AR directly, but in many tissues the main physiological actions are mediated by the testosterone metabolite DHT which binds the AR with even greater affinity (Davey and Grossmann, 2016). Testosterone can also be aromatized to estrogens, which activate the estrogen receptors α and β (ERα and ERβ), similar nuclear receptors as AR. In these cases, the conversion is done by enzymes – 5α-reductase and aromatase, respectively – present locally in the peripheral tissues such as the reproductive tract, skin, brain, and adipose tissue (Griffin and Wilson, 2003).

More recently, a novel group of active androgens has been identified: 11-oxygenated androgens, mainly produced by the adrenal cortex (Swart et al., 2013). The most abundant forms in circulation are 11-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT) (Davio et al., 2020). 11KT has been shown to have androgenic activity equal to testosterone through the AR receptor (Pretorius et al., 2016). Nevertheless, in normal adult male the serum concentration of 11KT is less than $1/10^{th}$ of testosterone. However, in prostate cancer patients undergoing androgen deprivation therapy, 11KT has been identified as the predominant circulating active androgen (Snaterse et al., 2021).

2.2.2 The HPG axis

The main regulator of sex steroid production in mammals is the hypothalamic-pituitary-gonadal axis, which comprises of several interlinked hormonal stimulation and feedback mechanisms. In both humans and mice, hypothalamic neurons release gonadotropin-releasing hormone (GnRH) to the hypophyseal portal circulation (Kaprara and Huhtaniemi, 2018). GnRH stimulates the anterior pituitary to release

gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Fig. 3). These in turn have sex-specific – although functionally similar – effects in male and female gonads (Anne Finlayson et al., 2016).

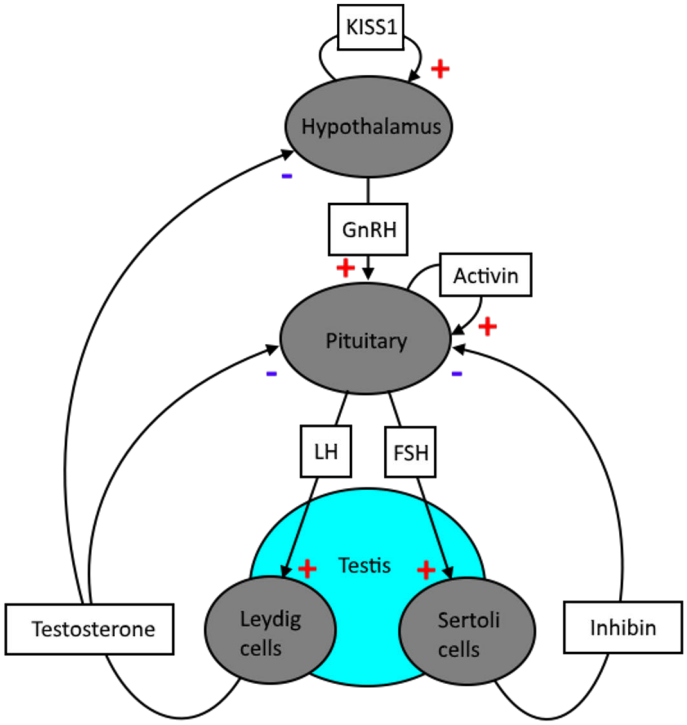

Figure 3. The main feedback loops of the male HPG axis. Hypothalamic GnRH stimulates (+) the release of LH and FSH from the pituitary. LH stimulates testosterone synthesis in the Leydig cells, which in turn inhibits (-) both GnRH and LH release. Sertoli cells are stimulated by FSH and secrete inhibin, inhibiting further FSH release. Local stimulating factors also act in hypothalamus (Kisspeptin, KISS1) and pituitary (activin).

In males, LH stimulates the production of testosterone in the testes and FSH promotes the Sertoli cell functions that support spermatogenesis (Fig. 3) (Handa and Weiser, 2014; Jin and Yang, 2014). GnRH secretion occurs episodically, once every 1-2 hours in humans. It is controlled at the hypothalamus by the so-called GnRH pulse generator. The controlled frequency and amplitude of these pulses is required for the pituitary to maintain its responsiveness to GnRH, and release LH and FSH in a controlled fashion (Maeda et al., 2010). GnRH release is controlled by kisspeptin (KISS1), which is released from several groups of hypothalamic neurons. Kisspeptin acts by activating the Kisspeptin receptor (GPR54) of the GnRH releasing neurons (Kaprara and Huhtaniemi, 2018; Oakley et al., 2009).

The release of LH is controlled by a feedback mechanism. After LH stimulates testosterone production, the increased circulating testosterone concentration suppresses the release of both GnRH at the hypothalamic level and gonadotropins at the pituitary level (Fig. 3). The likely upstream targets of sex steroids in this control mechanism are the kisspeptin neurons of the hypothalamus, expressing both AR and ER (Oakley et al., 2009). Testosterone has also been seen to have a direct regulating effect on the GnRH response in the pituitary (Tobin et al., 1997). Another regulator of the HPG axis is Sertoli-cell derived inhibin in circulation, counteracting the FSH-secretion stimulating action of locally produced activin at the pituitary (Jin and Yang, 2014; Ling et al., 1986).

The effect of LH on the Leydig cells is mediated through the LH receptor (luteinizing hormone/choriogonadotropin receptor, LHCGR, or just LHR in mouse, which lacks chorionic gonadotropins), which is a G-protein coupled transmembrane receptor (Huhtaniemi et al., 2002). LH binding at the receptor activates the cAMP signaling pathway and protein kinase A, (PKA) in turn leading to phosphorylation and activation of proteins like the hormone-sensitive lipase (HSL) (Casarini and Simoni, 2021). HSL releases cholesterol from intracellular stores for steroidogenic enzymes to process, in addition to cholesterol acquired from circulation. Protein kinase A also increases the expression of these enzymes through activating transcription factors, such as DAX-1 (Manna et al., 2007). LH receptors can also activate phospholipase C - protein kinase C –pathway, which has been shown to increase steroidogenesis, although to a lesser extent (Jo et al., 2005). Hormones other than LH, such as IGF-1, have also been found to moderately increase Leydig cell steroidogenesis and support the action of LH (Lee B. Smith and Walker, 2014; Stocco et al., 2005). In humans, steroidogenesis is also stimulated by LHCGR activation by human chorionic gonadotropin (hCG), a product of the placenta during the first half of fetal development. As stated, CG is not found in mice (Scott et al., 2009).

An additional factor in the regulation of steroidogenesis in Leydig cells is the desensitization to LH, where exposure leads to a decrease in the number of LH receptors on the cell surface, receptor mRNA expression, and likely also inhibition of the downstream signaling pathways. This in turn results in a lower response to further LH doses (Griffin and Wilson, 2003).

The production of 11-oxygenated androgens in the adrenals is not under HPG control, but instead the hypothalamic-pituitary-adrenal (HPA) axis via adrenocorticotropic hormone (ACTH) (Rege et al., 2013).

2.2.3 Sites of steroidogenesis

As previously stated, steroid hormones derive from a common precursor, cholesterol. All steroid hormone biosynthesis begins with the cleaving of the side chain of cholesterol to form pregnenolone, but from there the pathway branches. It has many alternative routes, with different enzymes catalyzing each step, and several possible end products with different functions (Fig.2, Fig. 4). However, no single cell type has the potential to produce all functional steroid hormones. Thus, the locally available enzymes determine the available pathways and thus the forms of steroid hormones produced (Miller and Auchus, 2011).

In humans, the *zona reticularis* layer of the adrenal cortex and the Leydig cells of the testes are the main sites of androgen biosynthesis, with some production also in the ovarian theca and granulosa cells. These cell types are the only ones known to express the key steroidogenic enzymes that catalyze the first steps of all steroidogenic pathways in normal conditions (Auchus, 2014). However, capacity for *de novo* testosterone production has also been detected in prostate cancer cells through expression of all the required enzymes (Cai et al., 2011). In mice, adrenals are normally not a major source of androgens, but have been shown to be capable of producing a significant amount following castration (Huhtaniemi et al., 2018).

Human adrenal glands mainly produce dehydroepiandrosterone through a pathway that is similar to the one employed in testes, which will be handled in more detail below. In adrenal glands, DHEA is largely converted further to DHEA-sulfate by sulfotransferase SULT2A1, and to a lesser extent into androstenedione. Adrenal androstenedione can be converted to testosterone in small amounts locally, likely by aldo-keto-reductase AKR1C3, but adrenals lack the enzyme activity to catalyze this step in a significant scale. Most DHEA-sulfate, DHEA and androstenedione is therefore released to circulation and converted further in the gonads or peripheral tissues (Labrie et al., 1997; Miller and Auchus, 2011).

A portion of the adrenal androstenedione is also converted to 11OHA4 by cytochrome P450 11β-hydroxylase (CYP11B1). 11KA4 and 11KT are then mainly converted from 11OHA4 in peripheral tissues (Storbeck, 2022).

Although peripheral conversion of these precursors may be a significant source of intra-tissue androgens locally, castration has been found to lower serum testosterone levels by 90-95%, proving that most circulating testosterone is produced in the testes (Labrie et al., 2000a).

2.2.4 The steroidogenic pathway in testes

The testicular Leydig cells require a constant cholesterol supply to maintain steroidogenesis. This cholesterol is acquired both from circulating lipoproteins and from *de novo* synthesis locally in the Leydig cells, and stored as cholesteryl esters in

membrane-bound lipid droplets (Hou et al., 1990). The produced steroids, on the other hand, are not stored; they diffuse out from the cells and into the spermatic venous system passively. Therefore a quick induction of synthesis is needed in response to LH stimulation (Griffin and Wilson, 2003; Miller and Auchus, 2011).

As described previously (chapter 2.2.2), stimulation by LH mainly acts through protein kinase A activation. Among other targets, PKA phosphorylates HSL, which then releases stored cholesterol for use. However, the main regulating and rate-limiting step of steroidogenesis is the first one of the pathway proper, the steroidogenic acute regulatory protein (StAR), also activated by PKA (Stocco et al., 2005). StAR transports cholesterol from the outer to the inner mitochondrial membrane. There, the cholesterol undergoes conversion to pregnenolone via several steps catalyzed by the cholesterol side-chain cleavage enzyme (CYP11A1, P450scc) (Fig. 4) (Auchus, 2014; Scott et al., 2009).

Pregnenolone passes out from the mitochondrion and reaches the smooth endoplasmic reticulum where the next enzymes of the pathway are located (Payne and O'Shaughnessy, 1996). The steroidogenesis in human Leydig cells then mostly proceeds through biosynthesis of Δ^5 steroids. First, 17α-hydroxylase (CYP17A1; P450c17; 17,20-lyase) converts pregnenolone to 17OH-pregnenolone, and then catalyses its conversion again to DHEA. The Δ^4 -route, with hydroxysteroid (3β) dehydrogenase type II (HSD3B2) first catalysing the conversion of pregnenolone to progesterone, then CYP17A1 further converting it to 17OH-progesterone and again to androstenedione, is a less significant contributor in human testis (Miller and Auchus, 2011; Payne and O'Shaughnessy, 1996). This is likely both because human CYP17A1 binds pregnenolone with a higher affinity than HSD3B, and also because human CYP17A1 has lesser 17,20-lyase activity with 17OH-progesterone as substrate than with 17OH-pregnenolone (Mak et al., 2018; O'Shaughnessy, 2017). In contrast, murine testosterone production tends to favor the Δ^4-route due to differences in the enzyme affinities (Fig. 4) (Scott et al., 2009).

SF1, previously involved in the differentiation and development of the testis, is essential for the Leydig cell expression of CYP11A1 and CYP17A1, as well as HSD3B2. The precise level of expression, however, is mainly regulated by LH (Scott et al., 2009).

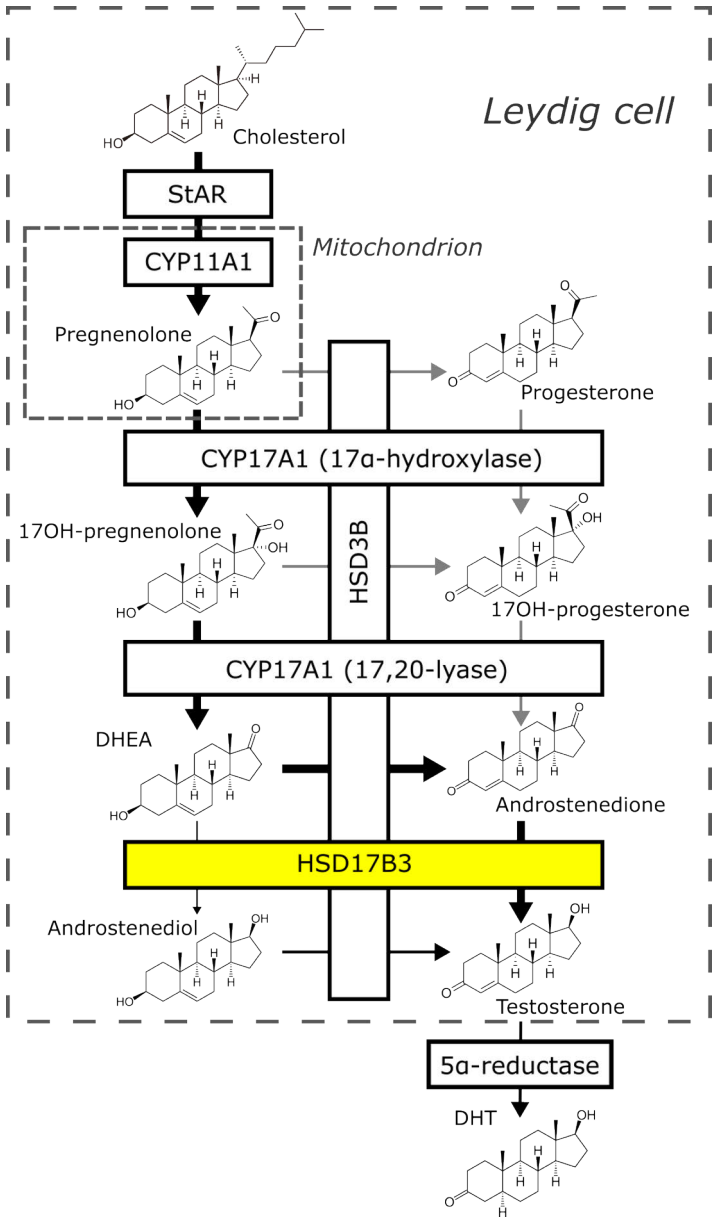

Figure 4. The steroidogenic pathway in the Leydig cell. The pathway in humans is presented with black arrows, bold arrows representing the preferred path. The preferred murine pathway through progesterone is presented with grey arrows. StAR transports cholesterol to the inner mitochondrial membrane, where its side chain is cleaved by CYP11A1 to form pregnenolone, which then passes out to be further processed. HSD17B3, catalyzing the final step from androstenedione to testosterone, is highlighted. Testosterone then diffuses out of the cell and is further converted to DHT in some target tissues. Modified from (Scott et al., 2009).

DHEA is further converted to androstenedione by HSD3B2. The DHEA transported from the adrenal glands by circulation also undergoes the same reaction in the testes. Finally, androstenedione is converted to testosterone by hydroxysteroid (17β) dehydrogenase type 3 (HSD17B3) (Fig. 4) (Miller and Auchus, 2011). These reactions can also happen in reverse order, with androstenediol as the intermediate instead of androstenedione; however, the first pathway is again more common in humans as HSD17B3 prefers androstenedione to DHEA as a substrate (Luu-The, 2001). HSD17B3 and the other enzymes of the HSD17B family will be introduced in more detail in chapters (2.3.1-2.3.3).

In the classical pathway, DHT is produced from testosterone in target tissues by 5α-reductase (Fig. 4). However, a backdoor pathway from 17OH-progesterone to DHT in several steps starting with 5α-reduction – bypassing androstenedione and testosterone – has been described in several species and is also present in humans (Auchus, 2004; Flück et al., 2011).

2.3 HSD17B enzymes

2.3.1 The HSD17B enzyme family

The main enzymes responsible for one of the final steps in the synthesis of active sex steroid hormones belong to the hydroxysteroid (17β) dehydrogenase (HSD17B) family. They convert 17-ketosteroids – i.e. DHEA, androstenedione and estrone – to 17β-hydroxylated forms – androstenediol, testosterone and estradiol, respectively – or vice versa. The hydroxylated forms, in particular testosterone and estradiol, have more potent effects, binding with their respective receptors with greater affinity (Labrie et al., 2000b). Thus, regulation of the enzyme activity can act as a pre-receptor control mechanism for the effects of sex hormones by affecting the relative levels of the different steroids, both systemically and at the level of individual tissues where the enzymes are expressed (Lukacik et al., 2006; Peltoketo et al., 1999; Saloniemi et al., 2012).

Currently 14 types have been identified across all vertebrates, 12 of which are found in humans (Lukacik et al., 2006; Mindnich et al., 2004). Most HSD17B enzymes belong to the short-chain dehydrogenase/reductase (SDR) family, with the sole exception of HSD17B5, which is an aldo-keto reductase, also commonly known by the name AKR1C3 in humans (Peltoketo et al., 1999). The different enzymes have been named simply by numbering in the order of identification. Orthologous HSD enzymes are identified by the same number across different species (Mindnich et al., 2004).

The enzymes of the family generally have a low sequence homology of 15-30% and often share more similarity of their primary structure with other members of the

SDR enzyme family than with each other. The family then is defined more by the similarities of their enzymatic actions discovered mainly *in vitro,* than common ancestry, or indeed even their physiological role (Oppermann et al., 2003). However, HSD17B enzymes do share some characteristic motifs, most importantly in the structure of the cofactor binding region and active center, as well as a more general similarity in protein secondary structure (Peltoketo et al., 1999). These shared motifs include the amino acid sequence TGxxxGxG in the cofactor binding Rossmann fold, and the YxxSK sequence of the active catalytic center (Mindnich et al., 2004; Oppermann et al., 2003).

2.3.2 HSD17B enzyme activity and distribution

The different HSD17B enzymes can mostly catalyse their reactions in a reversible fashion *in vitro*. However, in the physiological conditions of intact cells the reactions are essentially unidirectional (Andersson and Moghrabi, 1997). *In vivo*, types 1, 3, 5 and 7 have been shown to preferably catalyze NADPH-dependent reduction reactions, e.g. activating ketosteroids to hydroxysteroids. On the other hand, types 2, 4, 6, 8, 9, 10, 11 and 14 work in the opposite direction by catalyzing NAD+ dependent dehydrogenation and can thus function as steroid-inactivating enzymes (Labrie et al., 2000a). This is due to structural cofactor preference, but also relative levels of cofactors present in the cells, as cofactor concentrations tend to greatly exceed steroid concentrations locally (Miller and Auchus, 2011; Ning et al., 2017).

The HSD17B types also differ in whether they prefer C_{19} –steroids (androgens), C_{18} –steroids (estrogens), other steroids, or even e.g. fatty acids as substrates. In humans, HSD17B1 is mostly estrogenic, meaning it prefers the conversion of estrone to estradiol over other substrates, and HSD17B3 is likewise androgenic, preferring to convert androstenedione to testosterone. Of the inactivating enzymes, HSD17B2 can be considered to be both antiandrogenic and antiestrogenic, accepting testosterone and estradiol equally as substrates for dehydrogenation (Labrie et al., 1997; Lukacik et al., 2006; Luu-The, 2001).

The enzyme functions can differ between species. Mouse HSD17B1, for example, has equal potential for androgen and estrogen conversion (P Nokelainen et al., 1996). Some HSD17B enzymes also have 3β-dehydrogenase activity, or may mostly catalyze other types of reactions. HSD17B4 is known to be involved in fatty acid β-oxidation and bile acid metabolism, HSD17B7 in cholesterol synthesis, and HSD17B12 is considered to be involved in fatty acid elongation and is crucial for lipid homeostasis (Heikelä et al., 2020; Mindnich et al., 2004; Saloniemi et al., 2012).

The expression patterns of the HSD17B enzymes vary considerably. In humans, HSD17B1 is mainly found in female and HSD17B3 in male reproductive tissues, but

in mice HSD17B1 is also significantly expressed in the testes, namely the Sertoli cells during the fetal period (Hakkarainen et al., 2018). Other types are expressed more widely: some, like HSD17B2 and HSD17B5 are found in a variety of tissues, and still others, like HSD17B4 and HSD17B12 are expressed ubiquitously throughout the body (Luu-The, 2001; Mustonen et al., 1997; P Nokelainen et al., 1996; Saloniemi et al., 2012)

2.3.3 HSD17B3

The human *HSD17B3* gene localizes in chromosome 9q22. It is roughly 66 kb in length, consisting of 11 exons that range from 35 bp to over 260 bp. The coded protein has a predicted length of 310 amino acids and predicted molecular weight of 34 513 Da. HSD17B3 shares only approximately 20% identity with HSD17B1 and HSD17B2, the first two enzymes of the same family identified earlier (Geissler et al., 1994).

Mouse *Hsd17b3* gene localizes in chromosome 13 and codes for a protein of 305 amino acids, with overall sequence identity of 72,5% and similarity of 94,8% with the human protein (Mindnich et al., 2004; Sha et al., 1997a). Human and mouse HSD17B3 both share the homologous regions found in all members of the SDR enzyme superfamily mentioned previously (Geissler et al., 1994; Sha et al., 1997). The three-dimensional structure of HSD17B3 has not yet been determined in detail experimentally, as its purification and crystallization for x-ray crystallography is difficult due to it being a membrane-bound hydrophobic enzyme (Lukacik et al., 2006). A predicted protein structure of mostly very high confidence can be found in the AlphaFold database ("AlphaFold Protein Structure Database," n.d.).

The gene is mainly expressed in the testes in both humans and mice, although some expression has also been detected in the brain, platelets, genital skin and adipose tissue (Corbould et al., 1998; Geissler et al., 1994; Gnatenko et al., 2005; Hoppe et al., 2006; Sha et al., 1997a; Steckelbroeck et al., 1999). The translated protein is also seen to be very testis-specific (Uhlen et al., 2015). The protein is a microsomal enzyme, localized and bound to the endoplasmic reticulum with an N-terminal transmembrane domain.

The localization and level of *HSD17B3* expression in the testis changes with development**.** In both humans and rodents the expression is seen to rise drastically during puberty, concurrently with the development of the adult Leydig cell population, and in adults it is undoubtedly localized in the Leydig cells alongside the other steroidogenic enzymes (Ge and Hardy, 1998; O'Shaughnessy et al., 2002a). However, it has been shown in mice that in fetal and neonatal testes the expression of *Hsd17b3* is localized exclusively in the seminiferous tubules, specifically in the fetal Sertoli cells (Shima et al., 2013). As the testicular cell populations mature

postnatally, the expression in tubules is first silenced and then reappears exclusively in the adult Leydig cells at puberty (O'Shaughnessy et al., 2000). Evidence for a similar pattern of expression in humans has recently been described in individuals with defective androgen signaling and in scRNAseq datasets (Al-Sharkawi et al., 2023; O'Donnell et al., 2022).

It has also been observed in rodents that the expression of *Hsd17b3* is linked to the differentiation state of the ALCs. Progenitor cells do not express the enzyme and immature cells express some, but the expression reaches adult levels only when the Leydig cells have fully matured (Wu et al., 2010; Ye et al., 2017).

Unlike the enzymes catalysing the first steps of steroidogenesis under LH control, the regulation of HSD17B3 expression and activity is not well known. Whether the known steroidogenic regulator SF1 has an effect on HSD17B3 is unclear (Scott et al., 2009). Early expression of mouse *Hsd17b3* has been shown to be LH-independent, but become dependent on LH after puberty (Baker et al., 1997). LH stimulation has also been shown to upregulate *HSD17B3* in prostate cancer cells *in vitro* (Pinski et al., 2011). Auto- and paracrine androgen action on Leydig cells is needed for optimal *HSD17B3* expression (O'Hara et al., 2015). At least in mouse fetal testis, activin A also controls *Hsd17b3* expression and steroidogenesis (Whiley et al., 2020).

HSD17B3 favors C_{19} 17-ketosteroids – DHEA, androstenedione, 5α-androstanedione and androsterone, i.e. androgens – as substrate. It preferentially catalyzes a reductive reaction resulting in 17β-hydroxylated C_{19} steroids – androstenediol, testosterone, DHT and 5α-androstanediol, respectively – with NADPH as a cofactor (Labrie et al., 1997; Miller and Auchus, 2011; Payne and Hales, 2004). HSD17B3 converts C_{18} steroids, i.e. estrogens, poorly in comparison (Luu-The et al., 1995).

The changing pattern of HSD17B3 expression during Leydig cell differentiation naturally determines the unequal steroidogenic potential of fetal, progenitor, immature and adult Leydig cells. Mouse FLCs, expressing all the steroidogenic enzymes except HSD17B3, mainly produce androstenedione, which is only converted to testosterone in the seminiferous tubules, and the same is likely true in humans (Al-Sharkawi et al., 2023; O'Shaughnessy et al., 2000). As the ALCs start to develop, at the progenitor stage they cannot yet convert androstenedione to testosterone, whereas immature LCs have the capacity to do so only at a limited scale. However, what testosterone is produced is quickly metabolized into androstanediol by other enzymes. Only in mature rodent ALCs the high levels of HSD17B3 and the silencing of the enzymes responsible for further metabolism allow for significant testosterone production (Wu et al., 2010; Ye et al., 2017).

In any case, only as HSD17B3 expression first appears in the fetal Sertoli cells, and later when it reappears in the adult Leydig cells during puberty, the testes reach their full steroidogenic potential.

2.4 Production and effects of androgens

2.4.1 Fetal development

In their earliest developmental role, the paracrine and endocrine action of testosterone, produced by the newly formed testes, is essential for the differentiation and development of male urogenital structures. The development of the FLC population and the early fetal production of androgens appears to be independent of gonadotropin control (O'Shaughnessy et al., 1998a; Word et al., 1989; Zhang et al., 2001). In addition, the early phases of fetal LH and FSH secretion seem to be independent from GnRH control (Kuiri-Hänninen et al., 2014). Thus, testosterone production and the process of virilization begin before the establishment of the HPG feedback axis. However, in humans the fetal Leydig cells soon become dependent first on placental hCG and later pituitary LH (O'Shaughnessy and Fowler, 2011).

In humans, the gonadal testosterone production begins around week 8 of gestation, peaks between weeks 11-14 coinciding with the establishment of the HPG axis, and then decreases for the rest of the gestation until birth at week 40 (Scott et al., 2009). In contrast, mouse testosterone production begins around embryonic day 13 and peaks at day 18, much later than in humans compared to the gestational period of only approximately 20 days (O'Shaughnessy et al., 1998b). In general, it seems that due to the absence of hCG and the establishment of the HPG axis only shortly before birth, most of the fetal testosterone production in rodents is gonadotropin-independent (O'Shaughnessy and Fowler, 2011). As described before, mouse FLCs do not produce testosterone directly, as HSD17B3 is instead expressed only in Sertoli cells, and this seems to also be the case for humans (Al-Sharkawi et al., 2023; Shima et al., 2013).

The Wolffian ducts require androgens to stabilize and grow, through AR induction of gene expression (Hannema et al., 2006). As Wolffian structures lack 5α-reductase to produce DHT, the main androgen responsible is testosterone itself (Grumbach et al., 2003). In the absence of testosterone or functional AR, the Wolffian ducts regress (Hughes, 2001). AR is expressed in the mesenchyme surrounding the Wolffian ducts before the epithelium of the ducts themselves, so the effects of androgens are likely mediated through the mesenchyme, as are the effects of AMH on the Müllerian ducts during the same period. Seminal vesicle mesenchymal cells have been shown to promote the growth of AR-negative Wolffian epithelium *in vitro* (Cunha and Young, 1991). In mice it has been directly shown that

epithelial AR is not needed first for Wolffian stabilization, but is important for their further differentiation to the derivative structures (Murashima et al., 2011).

Influenced by testosterone, the Wolffian ducts develop into the epididymides, *vasa deferentia* and the seminal vesicles. However, in other tissues testosterone has to be further converted to DHT by 5α-reductase locally to achieve the needed effects. DHT is required for the development of the external genitalia: urogenital sinus into the prostate, the genital tubercle and the urethral folds to penis, and the urogenital swellings to scrotum (Hiort, 2013). In conditions of impaired DHT conversion, the latter develop into their female counterparts – clitoris, *labia minora* and *labia majora*, respectively – even if the internal Wolffian structures form normally under testosterone regulation (Auchus and Miller, 2012).

As mentioned previously, testosterone is required for the initiation of testicular descent, mainly controlled by INSL3. However, the final, inguino-scrotal phase of testis descent is wholly androgen-dependent (Lim et al., 2001). The sexual differentiation of the central nervous system is also affected by the androgens already during fetal development; these effects are mediated through AR activation, but also ER activation following aromatization (Gardiner and Swain, 2014).

It has been shown in rodents by blocking androgen action during different stages of fetal development, that the masculinization of the reproductive system is not dependent so much on androgen action during the identifiable morphological differentiation of the tissues, but a preceding time period between embryonic days 15.5-18.5 termed the 'masculinization programming window' (MPW) (Welsh et al., 2008). If androgen action is blocked during this window, later androgen exposure cannot rescue the development of reproductive organs. Furthermore, although late fetal and postnatal androgen action is needed for the maturation of the tissues, their capacity to grow and develop is largely determined during this early programming window (MacLeod et al., 2010; van den Driesche et al., 2017). Similar testicular features as are seen in the animal models, inversely related to anogenital distance, have been identified in human testicular dysgenesis patients, and testicular dysgenesis has been hypothesized to be due to androgen deficiency in MPW (van den Driesche et al., 2017). Based on the timing of the window in rodents in relation to development, it is theorized that the human equivalent would be between 8-14 weeks of gestation (Welsh et al., 2008). In both mice and humans, anogenital distance is considered to be a sensitive and persisting indicator of fetal androgen exposure (Dean and Sharpe, 2013).

2.4.2 Minipuberty

The gonadotropins peak at midgestation in humans, before gradually decreasing to low levels at birth. The cause for the decrease is considered to be the suppressive

effect of increasing placental estrogen production on the HPG axis (Troisi et al., 2003). At birth, this inhibitory regulation ceases, and likely due to this the male HPG axis undergoes a period of increased activity during the first months postnatally, resulting in a transient surge of LH and FSH secretion. The LH surge leads to a correspondingly transient Leydig cell activity and a rise in the testosterone levels. This period is known as the hormonal minipuberty (Bay et al., 2007; Kuiri-Hänninen et al., 2014; Lanciotti et al., 2018). Although mice and rats are not considered to have an equivalent minipuberty, a much shorter surge, lasting only hours, can be seen in their newborn pups (Corbier et al., 1992).

Gonadotropin and testosterone peak between the ages of 1 week to 3 months in humans, reaching pubertal concentrations in circulation. The rise in testosterone is caused by both increased activity and number of Leydig cells, seen also as increased INSL3 secretion (Kuiri-Hänninen et al., 2014). The Leydig cells that emerge and proliferate during this stage are sometimes identified as a specific population of neonatal Leydig cells, which then regress afterwards (Ye et al., 2017). The testosterone surge is associated with e.g. penile growth and development of male-type behavior in infants (Kuiri-Hänninen et al., 2014). It also seems to have importance for the number of spermatogonia and Sertoli cells, as well as spermatogenesis in adulthood (Becker et al., 2015). Although the number of Leydig cells, Sertoli cells and germ cells all increase during the minipuberty, spermatogenesis is not yet initiated by the increase in testosterone, as the Sertoli cells do not express AR during infancy (Lanciotti et al., 2018).

After the peak at the age of 1-3 months testosterone level declines to the typical minimal level of infancy (Kuiri-Hänninen et al., 2014). The suppression of the HPG axis during childhood happens at the level of hypothalamic GnRH release and is likely not affected by gonadal feedback. It has therefore been suggested that the suppression affects the GnRH pulse generator mechanism and kisspeptin neurons, but the cause still remains unknown (Lanciotti et al., 2018; Plant et al., 2014a).

2.4.3 Puberty

Puberty is the period of final sexual maturation and development of gonadal function, leading to fertility. It is also the period when individuals mostly develop the more pronounced sexual dimorphism seen in adults. Puberty is initiated by the re-activation of the HPG-axis, resulting in increased gonadotropin secretion, and increased sex steroid production in the gonads. In males, this in turn drives the maturation of testes (gonadarche) and the initiation of spermatogenesis, further development of the internal and external genitalia towards their adult form, and the development of secondary sex characteristics (Plant et al., 2014a). Additionally, the whole body undergoes a spurt in growth and changes in body composition, with

males having a greater increase in fat free and skeletal mass, and females in fat mass (Loomba-Albrecht and Styne, 2009). In boys, the onset of puberty is considered normal if secondary sex characteristics appear between the ages of 9 and 13.5 years (Richmond and Rogol, 2007).

Like the prepubertal HPG suppression, the underlying cause for its release with the activation of the HPG axis, and the onset of puberty is still unclear (Kaprara and Huhtaniemi, 2018). The loss of kisspeptin function has been shown to prevent the progression of puberty in humans, the expression of *Kiss-1* increases in the relevant neurons in rats and rhesus monkeys at the onset of puberty, and administration of kisspeptin leads to early activation of the HPG axis and precocious puberty in rats (Smith and Clarke, 2007).

In humans, gonadarche is preceded by increased secretion of adrenal androgens (adrenarche) by several years. Adrenarche occurs independently of the HPG axis, and does not seem to be essential for fertility, but has some role in prepubertal development. The maturing adrenal cortex produces DHEA and androstenedione, which are probably converted to active androgens in periphery to some extent, influencing early pubic hair growth, development of body odor and acne (Plant et al., 2014b). Adrenal 11OHA4, through local and peripheral conversion to 11KT, has also been suggested as a significant driver of adrenarche (Rege et al., 2018).

The frequency and amplitude of GnRH and gonadotropin pulses start to increase from prepubertal low levels during early puberty. They mainly occur nocturnally at first, but also during wakefulness with the progression of puberty (Clark et al., 1997; Waal et al., 1991). In males, LH first stimulates the growth of Leydig cells and testicular androgen production. Androgens and FSH promote the maturation of the testes, which is associated with a dramatic, 10-fold increase in volume. This is mostly caused by the increase of seminiferous tubule diameter as mature AR-expressing Sertoli cells appear and germ cells start to proliferate. Eventually spermatogenesis is initiated (Koskenniemi et al., 2017; Plant et al., 2014b).

Whereas the FLC population was responsible for androgen production during the fetal development, the pubertal rise in androgens is associated with the development of the adult Leydig cell population. ALCs develop from stem Leydig cells, which may be a different population from the one that originates the FLCs (Shima, 2019). However, at least one study suggests that adult stem Leydig cells might be dedifferentiated FLCs (Shima et al., 2018). In rodents, the stem Leydig cells have been shown to not have any steroidogenic activity, but the expression of the various steroidogenic enzymes gradually increases as the cells differentiate through several stages: progenitor, immature and mature ALCs (Teerds and Huhtaniemi, 2015; Ye et al., 2017).

As daytime concentration of circulating testosterone begins to rise above the low prepubertal level sometime after the initial increase in intratesticular concentration

and reach an adult level at 14-15 years of age, other androgen-sensitive tissues and organs also mature (Plant et al., 2014b). Penis, epididymides, prostate, seminal vesicles and *vasa deferentia* all reach their adult size during this stage of puberty. The most outwardly obvious effect of puberty is the gradual androgen-dependent development of secondary sex characteristics such as pubic hair, facial and body hair, deepening of the voice as the larynx enlarges, as well an increase in skeletal growth and muscle mass. Androgens also further affect the brain, leading to full initiation of sexual behavior and libido (Richmond and Rogol, 2007; Sharpe, 2010).

Mouse puberty, too, is dependent on the activation of kisspeptin neurons, followed by HPG activation and gonadarche (Kumar and Boehm, 2013). In male mice, the precise timing of puberty is dependent on the strain, but an early androgen-dependent sign of the onset of puberty is the balanopreputial separation, occurring at around 22-25 days of age (Korenbrot et al., 1977; McGee and Narayan, 2013). Their genitalia develop further under the control of androgens, until they reach sexual maturity after the age of 6 weeks (McGee and Narayan, 2013; Qiu et al., 2013).

After the end of puberty, the fully developed HPG axis and gonads work together to maintain androgen synthesis and spermatogenesis to ensure fertility. The timeline of the hormonal changes in human male sexual development and maturation is overviewed in figure 5.

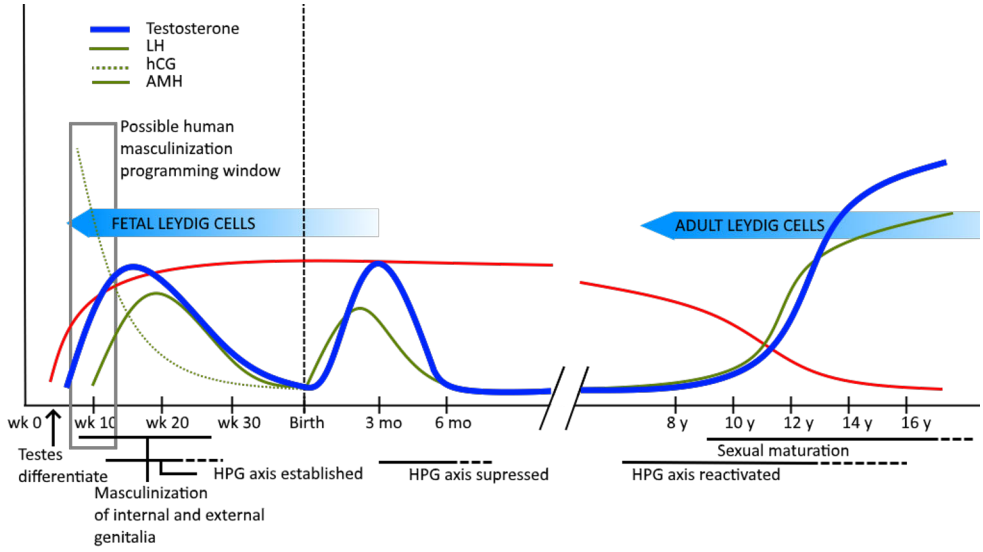

Figure 5. A graph of the changes in gonadotropin, testosterone and AMH levels during the human fetal period, minipuberty and puberty, and the related developmental phases. Adapted from (Kuiri-Hänninen et al., 2014; Scott et al., 2009)

2.4.4 Androgens and spermatogenesis

The basis of male fertility is the functioning of spermatogenesis, which depends on testosterone. Spermatogenesis is the process of germ cell development from spermatogonia through several steps to spermatozoa inside the seminiferous tubules of the testes. The seminiferous tubules are formed by two other major cell types in addition to germ cells: peritubular myoid cells that surround the tubules and provide contraction to move the sperm, and Sertoli cells that at this stage mainly function to nurture the developing sperm cells (Lee B Smith and Walker, 2014a). The major cell populations of the testes and the testosterone effects are summarized in figure 6.

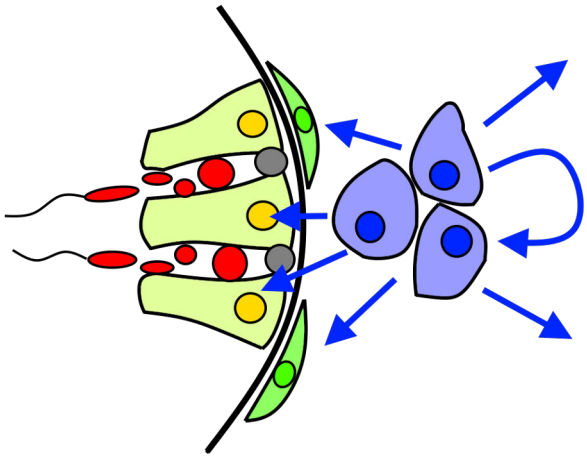

Figure 6. The cell populations of the testis. Inside the seminiferous tubule, Sertoli cells (yellow) nurture the different stages of sperm cell (red) development, starting from spermatogonia (grey). The tubule is surrounded by peritubular myoid cells (green). Leydig cells (blue) reside in the interstitial space between the tubules. The Leydig cells release testosterone (blue arrows) that affects the Sertoli cells, PTM cells and themselves in a paracrine manner, and a portion is released to circulation.

The paracrine action of intratesticular testosterone from the Leydig cells and circulating FSH from the pituitary are the main regulators of spermatogenesis. Testosterone, present in the testes in nearly 200-fold higher concentration than in circulation, diffuses to the surrounding cells (Roth et al., 2010). However, the germ cells themselves do not directly require testosterone nor FSH to develop (Lee B Smith and Walker, 2014b). Instead the targets of testosterone are the somatic cells that express AR, initiating the responses that support and promote spermatogenesis (O'Shaughnessy, 2014; Roth et al., 2010). In addition to Sertoli cells, AR is also expressed in PTM cells, as well as interstitial arteriole smooth muscle cells and vascular endothelial cells, and the Leydig cells themselves. FSH acts directly on the Sertoli cells (O'Shaughnessy, 2014; Sharpe, 2010; Lee B Smith and Walker, 2014a).

PTM cells express AR at constant levels throughout life, as do adult Leydig cells. However, Sertoli cells do not express AR during the fetal period and expression is only detected in humans from the age of 5 months, after testosterone production has been suppressed. In mature Sertoli cells, AR expression is cyclical and related to the cycle stage of the seminiferous epithelium (Lee B Smith and Walker, 2014a).

One of the major functions of the Sertoli cells is the maintenance of the blood-testis barrier, which separates the tubules to a basal and luminal component. The barrier provides a protected microenvironment for the germ cells, which pass the barrier as a part of their development. On the other hand, the low permeability of the barrier also makes the germ cells dependent on the nourishment, growth factors, as well as survival- and development-supporting signals provided by the Sertoli cells (Mruk and Cheng, 2004).

Spermatogenesis is severely disrupted in the absence of either intratesticular testosterone or Sertoli cell AR activation, as has been demonstrated in many mouse models. The blood-testis barrier is compromised by defective cell-cell junctions of Sertoli cells (Meng et al., 2005). Spermatogenesis is mostly halted during meiosis (De Gendt et al., 2004). The development of germ cells to sperm is inhibited by premature detachment from Sertoli cells due to defects in cell adhesion, but mature sperm cells are not released from the Sertoli cells and are instead phagocytosed (Holdcraft and Braun, 2003). In testosterone deficient LH receptor knockout mice, the arrested spermatogenesis can be rescued simply by testosterone replacement therapy (Pakarainen et al., 2005). Of note is that the minimum testosterone concentration required for the maintenance of spermatogenesis, although higher than the normal serum concentration, is still much lower than the normal intratesticular concentration (Coviello et al., 2004; Zirkin et al., 1989). However, in mice the testosterone threshold for the initiation of spermatogenesis has been shown to be higher than is required for the maintenance afterwards (Handelsman et al., 1999).

2.4.5 Androgens and the kidney

Kidney is one of the non-reproductive organs known to be sexually dimorphic and acutely sensitive to androgens. Male kidneys are larger in both humans and rodents, and decrease in size concurrently with the aging-related decrease in testosterone (Piras et al., 2020; Silbiger and Neugarten, 1995). Male and female kidneys have physiological differences in e.g. saline and ammonia excretion, which function at a greater capacity in females (Harris et al., 2020; Veiras et al., 2017). These differences are mainly a product of androgen regulation, causing changes in gene expression, although their physiological purpose is not yet understood (Catterall et al., 1986; Xiong et al., 2023).

The whole tissue, however, is not androgen sensitive. AR is only expressed in the proximal tubule section of the nephrons (Fig. 7) (Harris et al., 2020). The segment is also the only one with sex differences in gene expression, detected by single-cell RNA sequencing (Ransick et al., 2019; Xiong et al., 2023). The proximal tubule is mainly responsible for the differential growth of the kidney at puberty, with the cells responding to androgens first by proliferating and then with hypertrophy (Laouari et al., 2022).

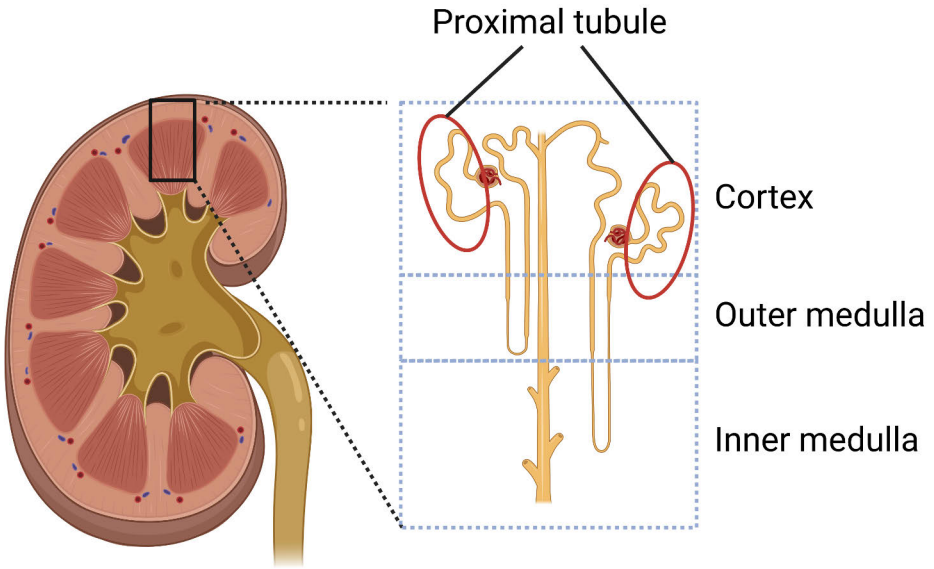

Figure 7. The anatomy of the nephron in relation to human kidney, with the androgen-sensitive proximal tubule segment highlited. Adapted from Ransick *et al.*, 2019. Created with biorender.com.

Interestingly, the fully-grown kidneys of adults are also sensitive to androgens, and male kidneys in particular require androgen exposure to maintain their size. In adult male mice, castration leads to a rapid decrease in kidney size, which is then rescued by androgen replacement with DHT (Laouari et al., 2022). A similar mechanism is also seen in human kidney transplants, where the sex of the recipient, not the donor, determined the size of the kidney 3 months after transplant. The male recipients had larger kidneys than the female recipients, whether the donor was male or female, and larger tubular epithelial area due to larger epithelial cells (Laouari et al., 2022).

In humans, fetal kidneys have been seen to grow in direct proportion to body weight without sex differences in size – male kidneys being larger but proportionate to body weight – or gross histology throughout the latter half of pregnancy, but some

sex differences have been detected in glomerular growth (Ryan et al., 2018). In mice, the postnatal kidney transcriptomes are only seen to diverge at puberty (Xiong et al., 2023). However, male kidneys are already under androgen exposure throughout the late pregnancy. The possible role of androgens specifically in kidney development, instead of just active size maintenance in adult males, is still unknown.

A final connection of androgens and the kidney is through the kidney androgen-regulated protein (KAP), expressed abundantly in the proximal tubule epithelial cells (Meseguer and Catterall, 1990). AR regulates the expression of the protein, but its function is largely unknown. However, KAP overexpression has been shown to protect mice from obesity in response to diet, and related inflammation (de Quixano et al., 2017).

2.4.6 Androgens and body composition

Body composition differs between men and women largely due to sex hormone influence. Men have greater bone and muscle mass, and the anabolic effects of testosterone on muscle are well known (Sinha-Hikim et al., 2002; Zhu et al., 2014). Adipose tissue is also sexually dimorphic in post-pubertal humans and rodents. The most obvious difference is the distribution. Both women and female mice store more fat subcutaneously, whereas males have more visceral fat (Brown and Clegg, 2010). Ovariectomized female mice gain visceral fat, while estradiol administration increases subcutaneous fat and leptin sensitivity in both males and females, demonstrating the role of estrogens in the development of the female fat distribution (Clegg et al., 2006). Women are also protected from metabolic syndrome, the risk of which increases after menopause (Lee et al., 2009).

Whereas the role of estrogens in adipose tissue is more widely studied, androgens also have their own effects. However, they are not straightforward. Visceral adipocity increases in men with age as testosterone declines and is reversible with testosterone, and hypogonadism is associated with obesity, but in women, excess androgen exposure is often seen to lead to an increase of visceral fat and metabolic disorder (Allan et al., 2008; Puder et al., 2005). AR knockout male mice develop late-onset obesity, and male mice with disrupted AR dimerization are comparable to females in body composition, but early exposure of female mice and rats to testosterone leads to weight gain and dysfunction of adipose tissue in adult animals (El Kharraz et al., 2021; Fan et al., 2005; Gulan et al., 2019; Nohara et al., 2013). Therefore, it would seem that the effects of the sex steroids on adipose tissue are also sex specific, and the sex-typical body composition may already be influenced by early developmental factors.

The gene expression in adipose tissues also differs between male and female mice, but also between depots in a sex-specific manner (Grove et al., 2010).

Differences are seen in the manner of growth, with e.g. gonadal adipose tissue seen to grow through hyperplasia and hypertrophy in female mice, but hypertrophy only in male mice, and the difference may be related to the number of adipocyte precursor cells in the depots (Wu et al., 2017). Finally, androgens have been shown to directly inhibit adipogenesis of human adipose stem cells through AR *in vitro*, demonstrating a possible mechanism behind the differential development (Chazenbalk et al., 2013).

2.5 Disorders of male development

2.5.1 46,XY disorders of sex development

As described previously, male sexual differentiation has many steps, many of which require active promoting factors: activating genes or hormones. The lack or dysfunction of these factors can easily result in the differentiation taking the 'default' route of female development, or in only partial male development, despite the nominal 'male' karyotype of XY. These cases are termed 46,XY disorders of sex development (DSD).

46,XY DSDs are characterized by the lack of or incomplete fetal masculinization. At birth, the individuals present with atypical or female external genitalia. They can have persisting Müllerian structures, and male gonads or no developed gonadal structures, depending on the cause of the DSD (Mota et al., 2015). The different causes of 46,XY DSDs are overviewed in Table 1.

Gonadal agenesis and dysgenesis, which can have various genetic causes leading to abnormal gonadal development, such as impaired function of the previously introduced factors SRY, SOX9, FGF9, SF1, GATA4, DHH etc., can be a cause of 46,XY DSDs. Defects of AMH production and action, leading to persistent Müllerian ducts, are another (Domenice et al., 2000). However, the largest group of possible causes are defects of testosterone production, metabolism, or androgen action (Globa et al., 2022; Mota et al., 2015).

Table 1. Possible causes of 46,XY DSDs. Modified from (Kohva et al., 2021).

Testicular dysgenesis	Androgen insensitivity	Disorders of androgen synthesis	Persistent Müllerian duct syndrome	Others
Partial or complete testis dysgenesis Testicular regression Ovotesticular DSD Syndrome-linked forms of DSDs	Complete (CAIS) or partial (PAIS) androgen insensitivity syndrome due to AR mutations	Disorders of regulation or function of enzymes in androgen synthesis Placental dysfunction or hormonal disruptors Disorders of cholesterol biosynthesis	*AMH* and *AMHR2* mutations	Hypospadias of unknown aetiology etc.

DSDs affect some 1:5000 people worldwide (George et al., 2010). In 46,XY individuals with normal gonadal development, impairment of virilization during development is most commonly caused by partial or complete inactivation of the androgen receptor, which is usually termed androgen insensitivity syndrome (AIS*)* (Globa et al., 2022; Hughes, 2001). Complete AIS leads to the development of female external genitalia and lack of male internal genitalia at birth, although testes form normally and AMH leads to the regression of Müllerian structures. Partial or mild AIS can have wider variety in the degree of masculinization/feminization of the phenotype depending on the degree of mutant AR activation (Hughes, 2001).

A group of possible causes that have been drawing more attention recently are those that are external, non-genetic. A major concern are endocrine disruptors present in the environment the mother is exposed to during pregnancy. Many anti-androgenic compounds in regular use, such as fungicides and plasticizers, have been identified, and suggested to act as endocrine disruptors of male development (Gray et al., 2001; Skakkebæk et al., 2001; Vinggaard et al., 2005).

Finally, defects of testosterone production are more rare, but a diverse group. They can be caused by general impairment of Leydig cell development, but also by mutations in single enzymes of the steroidogenic pathway. Defects are possible at every step, including StAR, CYP11A1, CYP17A1 and HSD3B2 (Abdulhadi-Atwan et al., 2007; Bongiovanni, 1962; Geller et al., 1999; New, 1970). The most common mutations of steroidogenic enzymes affect 5a-reductase activity and result in DHT deficiency that has major effects on the development of male phenotype, despite testosterone being produced normally (Berglund et al., 2016; Imperato-McGinley et

al., 1974). Defects in testosterone biosynthesis are rarer, accounting for some 5% of a cohort of 250 46,XY DSD patients in one study, but out of these HSD17B3 deficiency is the most common cause (Mendonca et al., 2017).

2.5.2 HSD17B3 deficiency

Among the HSD17B enzymes, HSD17B3 is so far the only one that has its role in humans clearly established by a genetic deficiency syndrome, as HSD17B3 deficiency is one of the known causes of 46,XY disorders of sex development. The disorder has historically been referred to as male pseudohermaphroditism and was first identified and characterized in the 1970s, with the responsible gene identified and cloned in 1994 (Geissler et al., 1994; Goebelsmann et al., 1973; Saez, 1971).

The incidence of the HSD17B3 deficiency varies geographically. It is most common in populations of European and Middle Eastern roots (Yang et al., 2017a). In Netherlands, the incidence has been estimated to be 1:470,000 newborns, whereas in the United States only a handful of cases in total have been reported. However, the deficiency is very common in the Arab population of the Gaza strip, where the incidence is extremely high: 1:100-300 newborns, all sharing the same mutation (Bertelloni et al., 2009; Mendonca et al., 2017).

HSD17B3 deficiency is commonly a result of a homozygous or compound heterozygous inherited mutation in the *HSD17B3* gene. Males who are heterozygous for known mutations, e.g. the fathers of affected homozygous males, do not present any symptoms of deficiency (Boehmer et al., 1999). At least 70 different mutations have been described worldwide to date, in 239 patients from 187 families (Gonçalves et al., 2022). Most of these are missense mutations (55%), frequently in the exons 3, 8, 9 and 10. Others include several instances of splice-site mutations (29%), and deletions or insertions (7%), some leading to frame shift and premature stop codons (5%) (Bertelloni et al., 2009; Gonçalves et al., 2022). Many mutations enriched in different populations can be traced to common founders (Boehmer et al., 1999; George et al., 2010).

Although the enzyme structure remains to be determined experimentally, the mutations found in patients and the corresponding effects on the function have shed some light on the structure-function relationship of the HSD17B3. Most common missense mutations that lead to complete eradication of enzyme activity affect exon 9, and impair substrate binding or the catalytic residues of the binding pocket (Payne and Hales, 2004). Several identified mutations in exon 3 affect the binding affinity of the cofactor NADPH, impairing enzyme activity to various degrees. In particular, two common missense mutations affecting the same codon result in an enzyme that is still functional but with greatly reduced activity (Mckeever et al., 2002).

2.5.3 Patient phenotype at birth

At birth, XY individuals with complete HSD17B3 deficiency typically present with mostly female appearing external genitalia, with a normal or slightly enlarged clitoris, separated labial-scrotal folds, separate urethral and vaginal openings and a blind ending vaginal pouch (Bertelloni et al., 2009; Geissler et al., 1994; Mendonca et al., 2017). As there are often no obvious indications of the condition at birth, they are undiagnosed and reared according to a female gender; one review of case reports found 78.5% of the patients assigned as female at birth (Yang et al., 2017b). In cases of mutations with some residual enzyme activity the external genitalia may be more ambiguous or even predominantly male with micropenis and hypospadias (Boehmer et al., 1999; Lee et al., 2007). In the latter cases, the patients may be assigned male sex from birth. The prostate is often absent or severely hypoplastic (Bertelloni et al., 2009). Patients sharing the same deficiency genotype, like members of the same family, can still present a fair degree of variability in their phenotype (Lee et al., 2007; B. Mendonca et al., 2000).

The testes of the patients differentiate normally and complete their transabdominal descent, but likely due to both androgen deficiency and the absence of a scrotum are usually found in the inguinal canal**.** Sometimes this manifests as inguinal hernia, which when identified can lead to an early diagnosis (Boehmer et al., 1999; Mendonca et al., 2017). The Müllerian duct derived structures of the patients have regressed completely; instead their Wolffian ducts have differentiated into male internal genitalia – epididymides, *vasa deferentia* and seminal vesicles – ranging in development from hypoplastic to normal (Andersson et al., 1996; Bertelloni et al., 2009). The absence of Müllerian duct derivatives implies that the differentiated testes produce AMH normally.

Several possible explanations for the presence of Wolffian duct derivatives have been proposed. Some amounts of testosterone may be produced by other enzymes with HSD17B activity, enough for paracrine action and the differentiation of the internal genitalia, but not enough to reach the external genitalia to be significantly converted to DHT (Mendonca et al., 2017). HSD17B1 has been shown to be expressed in the fetal mouse testis, and is known to have the capacity to convert androstenedione to testosterone, but would likely result in lower than normal level of testosterone in humans due to its lower affinity for C_{19}-steroids (Sha et al., 1997a). HSD17B5 is also expressed in the human testis and capable of catalysing the same reaction (Werner et al., 2012a). The activation of AR by the excess androstenedione, which can act as a weak androgen itself, has also been suggested to drive the differentiation of the Wolffian derivatives (Hiort et al., 2017).

2.5.4 Postnatal development in HSD17B3 deficiency

If the deficiency is not diagnosed in infancy, it normally becomes very apparent at the age of puberty, as the patients reared as females present with primary amenorrhea, lack of breast development and, most strikingly, progressive virilization (Andersson et al., 1996; Geissler et al., 1994). They develop various degrees of masculinized characteristics, like the enlargement of clitoris, deepening of the voice, increased body and facial hair, and overall male body appearance. This pubertal virilization is caused by an increase in circulating T concentration, which rises to a level similar to the early phase of normal male puberty or even into low adult male range (Auchus and Miller, 2012). Although the absence of breast development is one of the features alerting to the condition, some patients do feature prominent gynecomastia, likely due to the conversion of excess androstenedione to estrogens (Bertelloni et al., 2009; Saez, 1971).

The cause of the increase in circulating testosterone is most likely the normal pubertal activation of the HPG axis, which drives steroidogenesis. As its conversion to testosterone is disrupted, androstenedione is produced in excess. Similarly to fetal development, the accumulating androstenedione is presumably converted to testosterone eventually in significant amounts in the testes or peripherally by other HSD17B enzymes, whose contribution would be negligible when HSD17B3 is functioning normally (Andersson and Moghrabi, 1997; Boehmer et al., 1999). It has been shown in a patient that gonadectomy results in a greater drop in circulating testosterone than in androstenedione, implying that testes is still the main source of testosterone, assumed to be converted by HSD17B5 (Werner et al., 2012a).

However, other studies have demonstrated spermatic venous blood to have a comparatively lower testosterone concentration than is seen in periphery, placing more importance on peripheral conversion (Rosler et al., 1992). In some specific mutations, the remaining testosterone conversion can be attributed to residual HSD17B3 activity. It has also been suggested that the efficient aromatization activity of the placenta clears excess circulating androstenedione during the fetal period, resulting in less efficient early virilization despite the activity of the HPG axis (Andersson and Moghrabi, 1997).

As HSD17B3 is predominantly expressed in the testes, the deficiency has only been seen to affect XY individuals. XX women who are homozygous for mutations resulting in reduced HSD17B3 enzyme activity are asymptomatic, with typical genitalia and development as well as normal serum androgen levels for women of equivalent age (Rösler et al., 1996). Women with homozygous or compound heterozygous mutations resulting in complete lack of enzyme activity have also been shown to be developmentally and endocrinologically normal (Mendonca et al., 1999). Although some of the women in the latter study had problems with infertility,

their sisters carrying the same mutations did not. Thus, it seems unlikely that the lack of HSD17B3 disrupts female development or fertility in a significant fashion.

In males, HSD17B3 deficiency has grave consequences for fertility, likely due to both the low testosterone during development and the prolonged cryptorchidism caused by the inguinal location of the testes before intervention. Normal germ cells are often present in the testes of young individuals, but disappear with age. Sertoli cells undergo atrophy, and spermatogenesis is impaired even when the testes are surgically descended (Bertelloni et al., 2009). At least one report of germ cell neoplasia in a patient has been made, and progressing Leydig cell hyperplasia is common, implying a possibly increased risk of gonadal malignancies if the testes are retained (Folsom et al., 2018; Rösler, 1992; Yang et al., 2017a). No reports of fertile male patients could be found.

2.5.5 Endocrinology and diagnosis

The similarity of the presentation of HSD17B3 deficiency with other, more common causes of 46,XY DSD complicates its diagnosis. The neonatal and prepubertal phenotype is clinically indistinguishable from AIS, and similar DSD with pubertal virilization is also seen in 5α-reductase deficiency (Cohen-Kettenis, 2005; Lee et al., 2007). The standard differential diagnosis is based on the endocrinological profile of the patients.

The endocrine hallmark of HSD17B3 deficiency is high concentration of circulating androstenedione alongside the low or borderline normal circulating testosterone. The concentrations themselves may vary greatly and are not reliable indicators alone, but always result in an abnormally low T/A ratio (Bertelloni et al., 2009). The usual limit considered to be a reliable indication of the disorder is a T/A ratio of less than 0.8 (Faisal Ahmed et al., 2000a). However, significantly higher ratios have been reported in cases with some residual HSD17B3 activity, or when measuring steroids with LC-MS/MS methods instead of conventional immunoassays (Fujisawa et al., 2023).

Usually the basal androgen production in pubertal and postpubertal patients, as well as during the minipuberty of the first postnatal months, is high enough for diagnosis (Bertelloni et al., 2009; Demir et al., 2015). However, in the intervening period the circulating androgen concentrations tend to be too low to accurately measure by common methods. Therefore hCG stimulation is needed to transiently stimulate the androgen production and reveal the imbalance (Lee et al., 2007). Other androgens are also affected by the deficiency: DHEA is often elevated and DHT varies between low levels in some patients to high in others (George et al., 2010). The ratio of serum androstenediol to DHEA has been suggested as another diagnostic

marker for HSD17B3 deficiency due to the occasional instances of high T/A ratios (Fujisawa et al., 2023; Sato et al., 2024).

In any case, a lowered T/A ratio clearly distinguishes HSD17B3 deficiency from the common alternative causes of DSDs. AIS is associated with greatly increased testosterone levels in circulation, and 5α-reductase deficiency with a normal T/A ratio but an elevated T/DHT ratio. However, a lowered T/A ratio can also be caused by gonadal dysgenesis, which should be ruled out (George et al., 2010).

The hormonal diagnosis is not completely unambiguous, especially if hCG stimulation is needed. The protocols have been criticized for lack of standardisation, possibly resulting in inconsistent results between diagnostic sites (Bertelloni et al., 2009). The variability in the response to hCG stimulation is high between subjects and the response can be slower than expected. Therefore it is recommended that if abnormal T/A ratios are detected and testicular dysgenesis has been ruled out, the diagnosis should be confirmed with genetic testing to identify the possible *HSD17B3* mutations (Bertelloni et al., 2009; Faisal Ahmed et al., 2000a; Lee et al., 2007).

Besides the balance of androgens, the gonadotropin levels of postpubertal patients are also often abnormal. LH tends to be elevated, both at the basal state and after hCG stimulation, even if circulating testosterone reaches normal male range. This may indicate that the feedback control of the HPG axis is disrupted. FSH is also sometimes elevated but varies greatly. This could be more due to the prolonged cryptorchidism and testicular failure than any direct endocrine effects of the deficiency itself (Bertelloni et al., 2009; Hiort et al., 2017).

2.5.6 Treatment and follow up

The standard treatment of HSD17B3 deficiency is a combination of surgery and hormonal treatment. As the patients are usually raised as females if not diagnosed at an early age, they – or their parents in the case of young children – have to choose to either retain their assigned gender or transition to males. In the former case, orchiectomy is performed to prevent or halt virilization, external genitalia are surgically corrected, and estrogen treatment is administered to initialize and maintain female pubertal development. In the latter case, orchidopexy is performed, male genitalia are formed surgically and androgen treatment may be temporarily administered to support male development (Bertelloni et al., 2009; Mouriquand et al., 2016).

Previously feminization was the standard procedure, as it matched the patients' sex of rearing and required less complex surgical procedures. Currently it is preferred to perform the transition to male if the patient is diagnosed early, providing improved outcomes for sexual development. If the diagnosis is not made until puberty, it is recommended to pharmacologically halt the puberty and virilization, keeping the

testes intact until gender identity can be ascertained (Mouriquand et al., 2016). Gender reassignment has been reported in 39-64% of cases reared as females (Cohen-Kettenis, 2005).

The published reports on HSD17B3 deficiency are mostly case reports from the time of diagnosis and treatment. Little information is available on longer-term follow-up of patients after treatment. There are also few reports of the patient's development and health beyond reproductive parameters, although androgens are known to affect many aspects of the phenotype. Furthermore, the large portion of patients who retain their testes are not only affected by the developmental undermasculinization, but live the rest of their lives in a state of considerable endocrine imbalance. The long-term effects of this are poorly understood.

3 Aims

The aim of this study was to use mouse models to understand the role of HSD17B3 in testosterone biosynthesis, and the effects of HSD17B3 deficiency, which also affects humans but is poorly understood in many aspects. Understanding these factors is essential to improve the treatment of these patients, but also to better understand other conditions of androgen deficiency. The increased knowledge of the compensatory mechanisms of testosterone biosynthesis are also relevant for the treatment of androgen related diseases.

The specific aims were:

- To generate and characterize a mouse model of HSD17B3 deficiency.
- To identify whether HSD17B1 is involved in the compensation of testosterone production in the absence of HSD17B3 activity.
- To clarify the effect of the lack of HSD17B3 on phenotype, aiming to identify previously unknown mechanisms of developmental androgen programming and effects of androgen deficiency on non-reproductive tissues.

4 Materials and Methods

4.1 Animals

The HSD17B3 knockout mice (3-KO) were generated by using a targeting vector (KO first allele; **I,** Supplemental Fig. 1A) obtained from The European Conditional Mouse Mutagenesis (EUCOMM) Program that was targeted to the G4 ES cells (C57Bl76N;129 hybrid; **I**, Supplemental Fig. 1B). The Neo-LacZ cassette flanked with Frt-sites was deleted in ES cells by electroporating the pCAGGS-Flpe plasmid into the cells (**I**, Supplemental Fig. 1C). Correctly targeted ES cell clones, without the Neo cassette, were injected into C57BL/6N blastocysts, and the embryo transfer to the pseudopregnant recipients was carried out using routine techniques. The resulting chimeric male mice were bred with female C57BL/6N mice to establish germ-line transmission. Mice, with exon 3 floxed (Hsd17b3fl/fl), were further crossed with mice expressing the Cre-recombinase universally (CAG-Cre; 14), producing offspring with the deletion of the exon 3 in the germ line (**I**, Supplemental Fig. 1D). The mice were genotyped by PCR from genomic DNA extracted from ear samples, with a primer pair capable of distinguishing the wild type (WT) and deleted alleles (Hsd17b3Se; 5'-GCATTGTAGGTAAGCCCTTC-3', Hsd17b3As; 5'-CAAGGAGCAGAGCATGCACA-3') (**I,** Supplemental Fig. 1).

To generate HSD17B1/HSD17B3 double-knockout (DKO) mice, we established a mouse line with a specific Hsd17b1 Ser143Ala point mutation (1-KO), using the CRISPR/Cas9 gene editing system. This substitution has been shown to result in a near complete inactivation of the enzyme in vitro and in cell culture (25). In brief, a single guide RNA (sgRNA; 5'-GTGCTGGTGACCGCGAGTGT-3') was synthesized with GeneArt Precision gRNA Synthesis Kit (ThermoFisher Scientific, California, 92008, USA) and purified using GeneJET RNA Cleanup and Concentration Micro Kit (ThermoFisher Scientific). The quality and concentration of the sgRNA were assessed using NanoDrop spectrophotometer and Qubit RNA BR assay kit (ThermoFisher Scientific). Subsequently, we microinjected the sgRNA (25 ng/µl), Cas9 mRNA (50 ng/µl; Integrated DNA Technologies, Inc., Iowa, USA), and a repair template in the form of single-stranded DNA (ssDNA, 50 ng/µL; TCTGCCAGACATGAAGAGGCGCCACTCTGGGCGTGTGCTGGTGACCGC GGCAGTGGGAGGCTTGATGGGTGAGCGAAAGGGACAGAGTAAGAGGT

TCCGA)(IDT, Integrated DNA Technologies) into C57BL/6NCrl mouse zygotes. Zygotes were transferred to pseudopregnant recipients, the pups screened for the mutation and those positive used for further breedings.

DKO mice were obtained by mating mice heterozygous for both alleles. The mice were genotyped by PCR from genomic DNA extracted from ear biopsies, used to identify the individual mice. The primer pairs used were capable of distinguishing the wild type (WT) and knock-in allele of Hsd17b1 (Hsd17b1KiSe; 5'- GCTTGGGACCATTCGGATGC -3', Hsd17b1KiAs; 5'-GCTGGGGTTCCCAAGCTTTA -3') after Cfr42I (SacII) restriction enzyme (Thermo Fisher Scientific) digestion of the PCR products, and the WT and deleted alleles of Hsd17b3 were detected as described above.

The sex of the animals was confirmed by analyzing the presence of sex-determining region of Chr Y (Sry) from genomic DNA using primers SrySe (5'-TCTTAAACTCTGAAGAAGAGAC-3') and SryAs (5'-GTCTTGCCTGTATGTGATGG-3').

Mice were housed under a controlled environment (12h light cycle, temperature 21 ± 3°C, humidity 55% ± 15%, specific pathogen free) at the Central Animal Laboratory of the University of Turku. Soy free SDS-RM3 chow (Special Diets Service, Witham Essex, United Kingdom) and tap water were available ad libitum. All animal experiments were approved by the Finnish Animal Ethics Committee, that also fully met the requirements as defined by the U.S. National Institutes of Health guidelines on animal experimentation.

The anogenital distance of the mice was determined by measuring the distance between the external genitalia and the anus with a digital caliper (Hogetex, Germany). The measurements were done at birth, the age of 14 days, and at the time of sacrifice for adult mice.

For experiments *ex vivo*, mice were sacrificed at the predetermined age by carbon dioxide asphyxiation, followed by collection of blood via cardiac puncture and cervical dislocation. Tissues were collected, weighed, and snap frozen in liquid nitrogen for steroid measurements, HSD17B activity measurements and gene expression analysis, or fixed for histology, immunohistochemistry and in situ -hybridization. The length of the femurs of 3-month-old animals were measured with a digital caliper (Hogetex, Germany). Newborn pups were sacrificed by inducing hypothermia by indirect exposure to ice, followed by decapitation, dissection, and similar freezing or fixing of testes. To collect mice from fetal timepoints, pregnant damns from timed matings were sacrificed as above, and the uterus was dissected to ice-cold PBS. The pups were dissected from the uterus and decapitated, and the tissues were collected as above.

Adrenalectomy was performed at 3 months of age. A blood sample was collected from the saphenous vein three days before the operation. The surgical adrenalectomy

was carried out under isoflurane anaesthesia, and the adrenal glands were snap frozen for steroid measurements or fixed for histology. In order to maintain sodium balance after surgery, 0.9% sodium chloride (Baxter, Deerfield, IL, USA) was available ad libitum. Two days after the adrenalectomy the mice were sacrificed, terminal blood collected via cardiac puncture, and testes collected and frozen for steroid measurements.

4.2 Puberty onset

For female mice, the onset of puberty was determined by daily monitoring of the timing of the vaginal opening, from the age of 21 days until the age of 34 days when all females had entered puberty. For male mice, the balano-preputial separation was analyzed from the age of 25 days until the age of 32 days when all males had entered puberty.

4.3 Estrous cycles

Vaginal smears were collected daily for a period of 10 days from females at the age of 2 months. Vaginal lavage was performed with sterile PBS, which was then air-dried on microscope slides. The smears were fixed with ethanol, stained with Mayer's hematoxylin (Sigma-Aldrich), and mounted. The cytological analysis of the phases of the estrous cycles was done by observing the types and ratios of cells present in the samples via light microscopy (Byers et al., 2012).

4.4 Fertility and sperm analyses

At the age of 2 months, 3-KO and WT males were mated with WT NMRI female mice. The breeding pairs were placed in individual cages and the appearance of vaginal plugs was monitored daily for one month. After a confirmed plug or after a week if no plugs were observed, the females were removed and new females were placed with the male. The litter sizes and the sexes of the pups were recorded for each female. The fertility of female KO mice was confirmed by mating with heterozygous males as a part of the routine colony breeding.

For the analysis of sperm parameters, sperm from 3-month-old 3-KO and WT males was collected by making incisions in cauda epididymidis and incubating the tissue in KSOM +AA medium (MR-121-D-UC, Merck, USA) at 37 ºC for 15 min. To assess sperm count and motility, a dilution of the sperm suspension was loaded in a 20-mm-deep counting chamber (Leja Products, Nieuw-Vennep, The Netherlands) and submitted to computer-assisted sperm analysis (CASA, CEROS II, Hamilton Thorne, Beverly, MA, USA). Recorded parameters included the

curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement, beatcross frequency, linearity (linearity = VSL/VCL), straightness (straightness = VSL/VAP), and wobble (wobble = VAP/VCL). Minimum of 200 spermatozoa were analyzed from each sample using following parameters: motility VAP > 4 μm/s and VSL > 1 μm/s, and progressive motility VAP > 75 μm/s; straightness > 50%. For sperm staining, sperm suspensions were spread on superfrost slides, fixed and processed for Papanicolau staining.

4.5 Gonadotropin and steroid measurements

Serum LH and FSH concentrations were measured by time-resolved immunofluorometric assays. The concentrations of serum A-dione, T, DHT, progesterone, estrone (E1) and estradiol (E2) were analyzed by a validated gas chromatography tandem mass spectrometry method, with the quantification limits of 12 pg/ml, 8 pg/ml, 2.5 pg/ml, 74 pg/ml, 0.5 pg/ml and 0.5 pg/ml respectively (Nilsson et al., 2015). The method was also used to measure intra tissue steroid concentrations in testis, epididymis, prostate, liver, kidney, adrenal gland, spleen, and gonadal fat pad from 3-KO and WT mice, after homogenizing the tissues in sterile deionized water 1:10 (w/v) using an Ultra-Turrax homogenizer (IKA-Werke, Staufen im Breisgau, Germany; Wilmington, NC) as described previously (Nilsson et al., 2015).

4.6 HSD activity analyses

HSD17B activity was measured from testis tissue homogenates. The tissues were homogenized in 10 mM KH2PO4 (pH 7.4), 1 mM EDTA and 20% glycerol, with cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The protein concentrations of the homogenates were measured with the Pierce BCA Protein Assay Kit (Thermo Scientific Pierce; Thermo Fisher Scientific, Rockford, IL, USA) according to manufacturer's instructions. Five μg of protein was mixed with [H3]A-dione (adult testes), in a final concentration of 17 nM (500 000 cpm; PerkinElmer, Waltham, MA, USA), or unlabeled A-dione (Sigma-Aldrich) in a final concentration of 10 mM (neonatal testes). The cofactor (NADPH, Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 1.4 mM to initiate the reaction. For adult testes, the samples were incubated at +37°C for 1h and the reaction terminated by placing the samples into an ethanol-dry ice bath. The steroids were then extracted twice with 1 ml of tert-butyl methyl ether (Honeywell, Morris Plains, NJ, USA), the ether was evaporated to dryness, and the steroids were reconstituted in 48% acetonitrile in water. The ratio of A-dione converted to T was determined by separating the [H3] steroids by HPLC (Waters, Saint-Quentin,

France) connected to an on-line β-counter (PerkinElmer). For newborn testes, the samples were incubated at +37°C for 3 h, the reaction was terminated by snap-freezing in liquid N_2, and steroids were measured by the GC-MS/MS method described above.

HSD activity of the mutant mouse *Hsd17b1* was tested in cells. Briefly, Q5 Site directed mutagenesis kit (NE Biolabs) was used to introduce the Ser143Ala point mutation to mouse Hsd17b1-Myc-DDK plasmid (#MR222239, Origene) according to manufacturer's instructions. Mutated and WT plasmids were transformed to competent *E. coli* provided with the kit and plated on kanamycin selection plates. Selected colonies were picked for maxipreps and the plasmid purified by NucleoBond Xtra Maxi plus kit (Macherey-Nagel). Mutant and WT plasmids were transiently transfected into MCF7 cells using a TransIT-LTI transfection reagent (Mirus Bio) along with a sham transfection. HSD activity was measured with [H3] labelled E1 from 1 h, 3 h, 7 h and 24 h timepoints as described above.

4.7 Testosterone treatments

Adult heterozygous 3-KO female and male mice were mated individually, monitored daily, and separated after the appearance of a vaginal plug. On days 14.5 – 17.5 post coitum, pregnant dams were treated with testosterone propionate (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich), administered s.c. once daily at a dose of 20 mg/kg. The anogenital distance of the newborn pups was measured, and male 3-KO and WT pups were left to mature after genotyping. Males were sacrificed at the age of 3 months, and the anogenital distance as well as the weights of testes, epididymides, seminal vesicles, kidneys and *levator ani* muscle were measured.

For the collection of fetal kidneys for RNAseq, pregnant dams were treated either with testosterone propionate 20 mg/kg, or with corn oil as vehicle control, and sacrificed by carbon dioxide asphyxiation at 16.5 days post coitum, after 2 hours from the third testosterone propionate injection. The pups were sacrificed as described above, and the kidneys were collected and snap-frozen in liquid nitrogen for RNA extraction.

4.8 Histology, IHC, IF and *In situ* -hybridization

The samples were fixed in 10% neutral buffered formalin (FF-Chemicals, Haukipudas, Finland) for approximately 24 hours at room temperature. Testes were fixed in Bouin fixative (Sigma-Aldrich) for approximately 24 hours at RT. The samples were then dehydrated, embedded in paraffin, and 5 µm thick sections were prepared, deparaffinized and rehydrated, and stained with hematoxylin and eosin (HE) and analyzed via light microscopy.

For immunohistochemistry (IHC) and immunofluorescense (IF), the sections were rehydrated in serial incubations of xylene and ethanol, followed by antigen retrieval in a pressure cooker in 10 mM citrate buffer. Blocking of non-specific antibody binding was carried out with 3% bovine serum albumin (BSA) in PBST (PBS with 0.05% Tween). The primary antibody was diluted in the same blocking solution and incubated at 4°C overnight. The primary antibodies (**I:** anti-AR, -SULT1E1, -SOX9, -GATA4, -Ki67, -Tubulin; **III:** anti-Cyclin D1, -JAG1, -LEF1, -PAX2, -Calbindin, Podocalyxin, -AR, -LRP2, -PhHH3) and their dilutions are described in their respective publications. For IHC, Endogenous peroxidase activity was blocked with a 20 minute incubation with 1% H2O2. For IF, Autofluorescence was blocked with 100 mM NH4Cl for 10 minutes in RT. Secondary antibody incubation was followed by color formation with DAB and by a brief background staining with Mayer's hematoxylin, or nuclear staining with DAPI. The results were analyzed via light microscopy or fluorescent microscopy.

The area of adipocytes from HE stained sections was determined with the Adipocount software (Zhi et al., 2018).

In situ –hybridization was used to visualize the expression of Hsd17b3 mRNA in neonatal WT and 3-KO mouse testis sections, fixed in Bouin's fixative (Sigma-Aldrich), dehydrated, embedded in paraffin and sectioned. In situ –hybridization was carried out using the RNAscope 2.5 HD Assay-Brown –kit according to manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA, USA), using a probe for mouse Hsd17b3 (Mm-Hsd17b3-No-XHs, Cat # 516601), as well as Ubc as positive control and dapB as negative control.

4.9 Gene expression

Total RNA was extracted from frozen samples with Trisure (Bioline, London, UK) following manufacturer's instructions. The purified RNA samples were dissolved in water, and quality of the samples was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). One µg of RNA was treated with DNase Amplification Grade Kit (Thermo Fisher Scientific, Waltham, MA, USA) and used for cDNA synthesis (SensiFast, Bioline). The cDNA was then used to quantify gene expression by qPCR (CFX96 Real-Time PCR detection system, Bio-Rad, Hercules, CA, USA) using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). Expression relative to the WT levels was calculated from Ct values using the ΔΔCt method. The primer sequences used are presented in their respective publications (**I**, Table 1; **II**, Table 1; **III**: in text). The primers for adipose tissue analyses are presented in Table 2.

The analyses of AR chromatin binding upon testosterone activation in kidney was performed utilizing previously published ChIP-seq data (GSM1146473,

GSM1146474, GSM1146475 from GSE47192) (Pihlajamaa et al., 2014) and UCSC Genome Browser (mm9).

Table 2. Primers used for qPCR analysis.

Gene	Forward primer sequence	Reverse primer sequence
Irs1	TTAGGCAGCAATGAGGGCAA	CGTGAGGTCCTGGTTGTGAA
L19	GGACAGAGTCTTGATGATCTC	CTGAAGGTCAAAGGGAATGTG
Mmp3	GGGTTGGAGATGACAGGGAA	GGAGAAAGTGAGTGGGGTCA
Pck1	CTCGAGATGTGGCCAGGATC	GGGCGAGTCTGTCAGTTCAA
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT

Full names of genes: Insulin receptor substrate 1; Ribosomal protein L19; Matrix metalloproteinase 3; Phosphoenolpyruvate carboxykinase 1; Peptidylprolyl isomerase A.

4.10 Flow cytometry

Pregnant dams were sacrificed and E18.5 pups were collected and sacrificed as described above. After sex confirmation by examining the gonads, and taking a tail piece for genotyping, both legs were removed and the femur and tibia dissected to ice-cold PBS. Before antibody incubations, the bones were finely minced in the PBS with scissors, passed gently through a 1 ml pipette to obtain a single cell suspension, and the suspension passed through a 70µm cell strainer to remove debris. Cells were counted using a Bürker chamber, and pelleted by centrifuging at 1000 x g for 10 minutes at + 4°C. The pellet was resuspended to ice-cold PBS with 2% FBS at 106 cells/ml, the antibodies were added at recommended concentrations, and samples incubated in dark, on ice, for 20 minutes. Antibodies used were anti-CD31-eFluor 450 (# 48-0311-82; 1.25 µg/ml), anti-CD45-FITC (# 11-0451-82; 1.25 µg/ml), anti-CD24-PE (# 12-0242-82; 0.15 µg/ml) and anti-Sca1-APC (# 17-5981-82; 0.3 µg/ml) (Invitrogen). Then the samples were centrifuged 5 minutes at 400 x g, + 4°C, pellet washed with PBS + 2% FBS, and the wash repeated 2 more times. After final resuspension, samples were analyzed with NovoCyte flow cytometer (Agilent Technologies, Santa Clara, CA, USA) and FlowJo 10.8 software (BD Life Sciences, Franklin Lakes, NJ, USA).

4.11 RNA sequencing

For RNA sequencing, total RNA was extracted from E16.5 kidneys from the testosterone supplementation experiment as described above. The quality of the RNA samples was confirmed by Bioanalyzer. Library preparation and sequencing was performed by Novogene Co. with Illumina NovaSeq 6000 system. Data analysis was performed using a combination of programs; The quality of the raw sequencing

reads was checked with FastQC tool version 0.11.14. Further analysis was carried using R version 3.6.1 and Bioconductor version 3.9 (Huber et al., 2015). The reads were aligned to the UCSC mm10 mouse genome reference derived from Illumina iGenomes using Rsubread package 2.0.0 and its inbuilt Refseq gene annotation. Rsubread was also used for calculating the genewise read counts. Normalization was performed using calcNormFactors method of the edgeR package version 3.28.0 (McCarthy et al., 2012). Statistical testing between sample groups was carried out using ROTS version 1.14.0 and the differentially expressed genes were selected requiring false-discovery rate (FDR) below 0.05 and absolute fold-change (FC) above 1.5.

Gene ontology (GO) enrichment analysis was performed using threshold free approach with gage package (version 2.36.0). Range of genes per term required was set between 15 and 250, and the test was performed with comparison scheme 'as.group' using the function 'gs.KSTest' for the non-parametric Kolmogorov-Smirnov test. The genelists sorted based on average ranks of both statistical significance and fold-change from the differential expression testing were used as input.

Gene expression data were analyzed through the use of QIAGEN Ingenuity Pathway Analysis (IPA) (Krämer et al., 2014). Canonical pathway analysis identified the pathways from QIAGEN IPA library of canonical pathways that were most significant to the data set. Only genes from the dataset that met a P-value cutoff of 0.05 and were associated with a canonical pathway in the QIAGEN Knowledge Base were considered. The significance of the association between the data set and the canonical pathway was measured using a right-tailed Fisher's Exact Test.

The data set containing gene identifiers and their corresponding expression values was uploaded into the QIAGEN IPA software and networks were generated. Each identifier was mapped to its corresponding entity molecule in QIAGEN's Knowledge Base. These molecules were overlaid onto a global molecular network developed from information contained in the QIAGEN Knowledge Base. Networks of eligible molecules were then algorithmically generated based on their connectivity and some networks were then merged and combined to understand the underlying molecular mechanisms.

4.12 Statistics

Statistical analyses were done using GraphPad Prism 9 and 10 softwares (GraphPad Software, La Jolla, CA, USA). The normality of the data was evaluated on the basis of a Shapiro-Wilk normality test. Statistical difference between two groups was determined by two-tailed Student's t-test or Mann-Whitney test for normally and non-normally distributed data, respectively. For comparison of multiple groups, one-

way ANOVA and Tukey's multiple comparisons test, or Kruskal-Wallis test and Dunn's multiple comparisons test were used for normally and non-normally distributed data, respectively. Significance was determined as $p \leq 0.05$.

Table 3. The overview of the analyses carried out at different timepoints, in relation to the developmental stage of the mice. The original publications are indicated by roman numerals.

Fetal period	Infancy	Puberty	Adult
E14.5-17.5 • Testosterone supplementation (**III**) *E15.5* • Steroids (**III**) • Gene expression (**II**) • IHC (**III**) *E16.5* • RNAseq (**III**) *E18.5* • Steroids (**III**) • Gene expression (**II**, **III**) • IF (**III**) • Bone marrow flow cytometry	*0 d* • AG distance (**III**) • Steroids (**II**) • HSD activity (**II**) • *In situ* (**I**) • Testis histology (**II**) *14 d* • AG distance (**I**) • Testis histology and IHC (**I**) • Kidney histology and IHC/IF (**III**)	*21-34 days* • Puberty onset (**I**) *27 days* • Steroids **(I)** • Testis weights (**I**) *28 days* • Testis histology and IHC (**I**)	*2 months* • Fertility (**I**) • Estrous cycles (**I**) *3 months* • AG distance (**I, II, III**) • Body weight & composition • Femur length (**I**) • Tissue weights (**I, II, III**) • Steroids (**I, II**) • HSD activity (**I**) • Testis histology & IHC/IF (**I, II**) • Kidney histology & IHC/IF (**III**) • Gene expression (**I, II**) *6 months* • Body weight and composition *10 months* • Body weight and composition

5 Results

5.1 Generation of the *Hsd17b3* knockout and *Hsd17b1* Ser143Ala point mutation mouse lines

In WT mouse testis, HSD17B3 and HSD17B1 expression was detected at fetal age, already at E15.5. HSD17B1 expression was seen to peak at E18.5, and HSD17B3 expression at birth (**II**, Fig. 2A). Both then fell drastically during the first weeks after birth, until HSD17B3 expression rose again from 28 days forward, after puberty (**I**, Fig. 1A).

To achieve the inactivation of HSD17B3 in mice, we deleted exon 3 of the *Hsd17b3* gene (Fig 8A-D; **I**, Supp. Fig. 1). This resulted in a marked reduction of mRNA expression measured by RT-qPCR, likely due to the disruption of the open reading frame resulting in a nonsense mutation and mRNA decay (**I**, Fig. 1B). The effect on expression was also detected with *in situ* hybridization in neonatal testes, where the mRNA was seen to localize solely in the WT Sertoli cells (**I**, Fig. 1C).

As a result of the deletion, no HSD enzyme activity in conversion of A-dione to T was detected testicular lysate from 3-month-old 3-KO mice when using NADPH as cofactor (**I**, Fig. 1D). This confirmed both the success of the model generation and the importance of HSD17B3 for testosterone production.

For HSD17B1, a model generated previously by knocking out the whole gene with a lacZ cassette was found to result in a downregulation of an unrelated gene in the proximity of *Hsd17b1*, which complicated the final phenotype (Jokela et al., 2017). Therefore, a new model with inactivating Ser143Ala point mutation of HSD17B1 was generated by CRISPR/Cas gene editing, to further investigate its role in steroidogenesis (Fig. 8E-F; **II**, Fig. 1A-C;). The final, double-KO model was then generated by cross breeding the 1-KO and 3-KO mice (Fig. 8G). The point mutation had no effect on *Hsd17b1* mRNA expression in adult ovaries (**II**, Fig 1D).

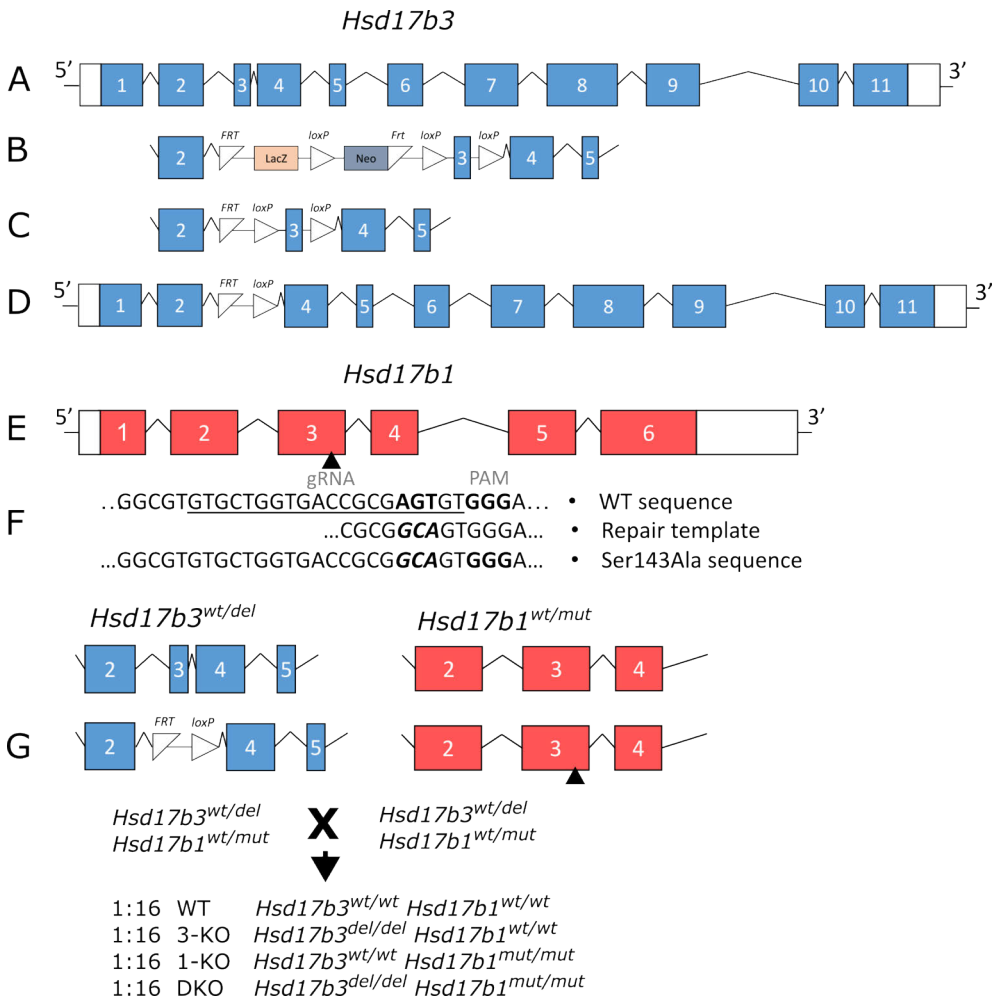

Figure 8. The generation of the mouse lines. A) Mouse WT *Hsd17b3* allele. B) Targeted *Hsd17b3* allele. C) Floxed allele in *Hsd17b3*$^{fl/fl}$ mice. D) *Hsd17b3* with deletion of exon 3 in HSD17B3KO after cre-lox recombination. E) Mouse WT *Hsd17b1* allele. F) The gRNA and repair template to generate the Ser143Ala point mutation with CRISPR/Cas. G) The crossing of the mouse lines produced double-heterozygous animals, which were mated to produce pups homozygous for either or both mutations, as well as WT control animals. Exons, introns and other elements not to scale. Modified from **I**, Supp. fig. 1; **II**, Fig. 1.

However, despite the continued expression, the point mutation was seen to be effective in abolishing HSD activity in E1 to E2 conversion, measured with the mutated enzyme expressed in MCF7 cells (Fig. 9). Finally, in the crossed mouse line, a marked reduction of A-dione to T conversion activity was detected in DKO neonatal testes lysates in comparison to WT and 1-KO, but also a further reduction from 3-KO (**II**, Fig. 2D). These results then confirmed the effect of the point

mutation in abolishing the enzyme activity, and the success of creating a mouse line without either HSD17B3 or HSD17B1 activity.

3-KO female mice were seen to be phenotypically normal in all analyzed parameters (**I**, Supp. Fig. 2). No drastic abnormalities were seen in 1-KO females either: they were fertile, and the mouse colony could be maintained through homozygous matings. Furthermore, 1-KO males did not differ from WT controls in any parameters, analyzed alongside DKO littermates.

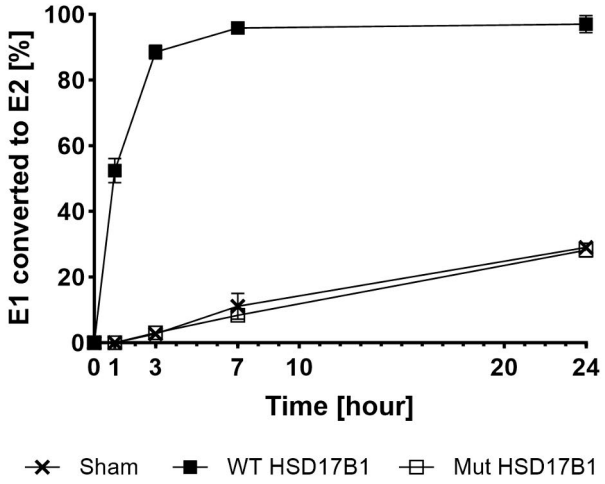

Figure 9. The conversion of E1 to E2 is impaired in MCF7 cells transfected with the mutated HSD17B1 in comparison with the WT enzyme.

5.2 Endocrine phenotype

5.2.1 The lack of HSD17B3 leads to delays in fetal and pubertal testosterone production

The effect seen on enzyme activity *in vitro* was reflected in steroid measurements *in vivo*. Intratesticular testosterone was seen to be significantly reduced in 3-KO during the fetal period, at E15.5, alongside accumulation of androstenedione (**III**, Fig. 2A). However, a clear rise closer to WT concentrations was seen already at E18.5 (**III**, Fig 2B). At birth, the intratesticular testosterone of 3-KO pups was seen to be no different from WT (**II**, Fig. 2B). Therefore, it was clear that the lack of HSD17B3 did not lead to a complete lack of fetal testosterone production, but only a delay before sufficient compensatory mechanisms were activated.

At the age of 27 days, after the onset of puberty we could see that the serum testosterone concentrations of 3-KO males did not differ from WT males, which had already reached the levels of adults at 3 months age. At the same time, androstenedione concentrations were clearly elevated in 3-KO, revealing the compensatory testosterone biosynthesis to be less efficient than HSD17B3 (**I**, Fig. 3A). This lead to a significantly decreased T/A ratio in serum (**I**, Fig. 3B).

Despite the normal serum testosterone, 3-KO intratesticular testosterone was seen to be decreased at the same 27 day timepoint (**I**, Fig. 4A). In the testes, we also saw an increase in androstenedione, progesterone and even estrone concentrations, indicating a bottleneck in the final steroidogenic step (**I**, Fig. 4A). However, as we would see in the adult males, this lack of testosterone again reflected only a delay before compensation could catch up.

5.2.2 The lack of HSD17B3 in steroidogenesis is compensated in adult male mice

It was clear from the preceding results that even in the absence of HSD17B3, some testosterone was being produced by compensatory mechanism. Even so, in adult 3-month-old males, the serum testosterone concentrations were unexpectedly seen to be 3-fold higher than in WT controls (**I**, Fig. 3A). This was accompanied by an even greater, 100-fold increase in serum androstenedione, leading to a significantly reduced T/A ratio (**I**, Fig. 3A-B). Smaller increases were also seen in serum DHT and progesterone concentrations. However, despite the high amount of testosterone in circulation, a 40-fold increase in serum LH was also seen in 3-KO males (**I**, Fig. 3C). No differences were seen in serum FSH. Thus, based on the serum results, it was clear that 3-KO males could effectively compensate for the lack of HSD17B3, but the observed great excess of androstenedione likely reflected the comparative inefficiency of the enzymes involved.

5.2.3 Testes remain the main source of testosterone in HSD17B3 deficient adult male mice

At the age of 3 months, intratesticular testosterone concentrations in 3-KO males were seen to be comparable to WT, while androstenedione saw only a 10-fold increase (**I**, Fig. 4A). Estrogens were also detected in the testes in small amounts, with an increase in E1 and decrease in E2 seen in 3-KO nevertheless demonstrating that E1->E2 conversion was also affected by the lack of HSD17B3 (**I**, Fig. 4A).

This increase in androstenedione production was driven by the observed increase in serum LH. While it remains unclear why serum LH remained high in the presence of higher-than-normal serum testosterone, the effects of the pituitary feedback

through LH on the testes were clear. *Lhr* was upregulated in the testes, as were *Star*, *Cyp11a1* and *Cyp17a1*, the first steps of steroidogenic pathway, known to be LH-regulated (**I**, Figs. 3D, 4B). Several genes related to *de novo* cholesterol synthesis – *Cyp51, Dhcr7 and Hsd17b7* – were also upregulated, implying that the compensation did not involve only the classical enzymes of steroidogenesis, but also the local production of the precursor cholesterol (**I**, Fig. 4C). In contrast, no effect was seen in the gene expression of the enzymes in the later steps of testosterone biosynthesis, *Hsd3b1* and various HSD17B enzymes. It is possible that the activity of the present enzymes could be at a sufficient level without upregulation, as mainly the expression of StAR is considered to be the rate-limiting step in normal steroidogenesis (Auchus, 2014).

To see whether peripheral conversion of the excess androstenedione to testosterone could be a factor in the testosterone conversion of 3-KO males, we measured the steroids from several tissues in comparison with the serum concentrations. Out of the tissues analyzed (testis, epididymis, prostate, adrenal gland, kidney, liver, white adipose tissue and spleen), only testes had a tissue/serum ratio over 1 (**I**, Table 2). This implies that the testosterone in the other tissues was mainly of serum origin and the testes remained the only net contributor of testosterone. Why a normal concentration of testosterone in the testes leads to an elevated concentration in serum remains to be explained.

We also investigated the role of adrenals in more detail, as they have recently been shown to contribute to testosterone production by us (Huhtaniemi et al., 2018). We performed adrenalectomies on 3-month WT and 3-KO mice, measuring serum steroids before and after. In response, we saw small decrease in serum A-dione in 3-KO, though it remained greatly elevated in comparison to WT (**I**, Fig. 4D). However, we also observed an *increase* in serum testosterone and DHT in 3-KO following the adrenalectomy, although the measured values were similar to those previously seen in intact animals (**I**, Fig. 4D). Nevertheless, this further demonstrated that the adrenals were not a significant contributor to testosterone production in our model.

5.2.4 HSD17B1 is responsible for compensating the lack of HSD17B3 in fetal testis, but not in adults

We next wanted to identify the compensatory enzymes with HSD17B activity, responsible for the remaining testosterone production capacity in 3-KO. We suspected that in the presence of high androstenedione concentrations, less efficient enzymes could also participate in A-dione to T conversion. One mouse enzyme known to be able to catalyze the reaction, although to a lesser degree than HSD17B3, is HSD17B1. The delayed rise in 3-KO testosterone during the fetal period also coincided with the rise in *Hsd17b1* expression described above. Thus we generated

the 1-KO and DKO mouse lines to clarify whether HSD17B1 was involved in the compensation seen in 3-KO.

At birth, the DKO males did indeed have drastically reduced intratesticular testosterone and elevated androstenedione, whereas by that time the 3-KO pups had reached normal intratesticular testosterone concentrations (**II**, Fig. 2B-C). Therefore, although small amounts of testosterone were still being produced, no other enzyme could effectively compensate for the lack of both HSD17B3 and HSD17B1 in fetal testis. The steroid profile of the male 1-KO animals was not seen to differ from WT, and the resulting phenotype was also normal.

However, the steroid levels of adult DKO males were nearly identical to 3-KO, with very high androstenedione in circulation and in the testes, but also high circulating testosterone and normal intratesticular testosterone (**II**, Fig. A-B, D-E). The T/A-dione ratio was equally reduced in 3-KO and DKO, and the testis/serum ratio was similar, again indicating that testes remain a significant source of testosterone (**II,** Fig. 4C, F). A slightly higher degree of upregulation of steroidogenic genes was seen in DKO in comparison with 3-KO (**II**, Fig. 4G). Together these results indicate that HSD17B1 is crucial for the compensation of HSD17B3 deficiency in fetal mouse testis, but not involved in the compensation in adult animals. Nor can it substitute for HSD17B3 fully, and likely requires a build-up of the precursor androstenedione, leading to the observed delay in fetal testosterone production in 3-KO.

5.3 Undermasculinized phenotype

5.3.1 Fetal androgen deficiency leads to undermasculinisation in adult male mice

At birth, 3-KO male mice appeared phenotypically normal and did not present the degree of feminization seen in human individuals with HSD17B3 deficiency. Their appearance and anogenital distance, which is considered to be a sensitive and permanent indicator of fetal androgen exposure, were on par with male WT littermates (**II**, Fig. 2E). Male DKO pups on the other hand had a clearly feminized anogenital distance (**II**, Fig. 2E). This demonstrates that the different degree of impairment in fetal testosterone production – a slight delay in 3-KO vs. near-complete abolishment in DKO – led to a clear difference in urogenital development, and even the reduced production in 3-KO was enough to apparently masculinize the internal and external genitalia.

However, already at the age of 2 weeks, the 3-KO mice began to present signs of undermasculinization, as their anogenital distance was seen to be reduced in comparison with WT controls (**I**, Fig. 2A). The difference grew more pronounced

by the age of 3 months, although the 3-KO male anogenital distance was still clearly greater than in females (**I**, Fig. 2A). The DKO males retained their female-like anogenital distance and appearance even in adulthood; many even had visible nipples (**II**, Fig. 3B).

We also analyzed other parameters that are known to be androgen sensitive. The onset of puberty was seen to be delayed in 3-KO males by an average of 5 days (**I**, Fig. 2B). This is likely a reflection of the identified delay in testosterone production at 27 days. Testis weight was also seen to be slightly reduced at 27 days (**I**, Fig. 2D).

The difference in testis weight persisted at the age of 3 months (**I**, Fig. 2D). A similar decrease was also seen in the weights of 3-KO epididymides, prostate lobes, seminal vesicles, kidney, and *levator ani* muscle, all known androgen-sensitive tissues, whereas the weight of the spleen was increased (**I**, Fig. 2E). As with the anogenital distance, the degree of undermasculinization was seen to be even greater in adult DKO testes, epididymides, seminal vesicles, kidney and *levator ani*, although the internal genitalia had still mostly differentiated properly due to some remaining testosterone production (**II**, Fig. 3A, C-D). Therefore, the degree of fetal androgen deficiency was seen to be proportionally reflected in the phenotype of the adult mice.

5.3.2 Spermatogenesis is delayed and Leydig cell maturation disturbed in 3-KO and DKO

Unlike in the commonly cryptorchid human patients, the testes of the 3-KO mice descended normally. The fertility of the 3-KO mice was, however, slightly affected: they obtained fewer litters overall, although the litter size and sex ratio were similar between 3-KO and WT males, making them subfertile (**I**, Supp. table 1). The testes of DKO mice were descended to the inguinal region, but not further due to the lack of scrotum. The fertility of the DKO males was not assessed, but it is likely that the feminized external genitalia would prevent them from mating.

Both the 3-KO and DKO testes appeared morphologically normal in histology at birth and the age of 3 months, despite the difference seen in weight, although seminiferous tubule diameter was seen to be slightly reduced in 3-KO at the ages of 4 weeks and 3 months (**I**, Fig. 5A-B, Supp. fig. 4; **II**, Fig. 2F-G, Fig. 5A). AR expression also appeared normal and was detected by IHC in Sertoli cells, Leydig cells and peritubular myoid cells in both 3-KO and WT testes (**I,** Fig. 3E-F).

A delay, however, was seen in the first wave of spermatogenesis, progressing normally at 2 weeks of age but delayed at 4 weeks in 3-KO, alongside Sertoli cell organization (**I**, Supp. fig. 3A-B). This again likely reflects the delay seen in the initiation of pubertal testosterone production, and is the probable cause for the difference in testis size at 27 days. Afterwards, however, spermatogenesis seemed to

progress normally (Supp. fig. 4A-E). No significant differences were seen in sperm parameters in adult 3-KO males, suggesting another cause for the subfertility (**I**, Supp. table 2). In DKO males too, spermatogenesis appeared to progress normally, and *cauda epididymides* were filled with mature spermatozoa (**II**, Fig. 5C). In both models, then, the active testosterone production of adults is sufficient for the initiation and maintenance of spermatogenesis despite the developmental androgen deficiency.

In HE staining of the adult 3-KO testis, Leydig cells were initially seen to have a peculiar staining pattern, with a prominent eosinophilic area in the cytoplasm, adjacent to the nucleus, which was not seen in WT Leydig cells (**I**, Fig. 5A). While electron microscopy did not clarify the identity of this stained area, we saw a similar staining in WT Leydig cells at earlier age of 4 weeks, suggesting that the staining pattern was characteristic of immature adult-type Leydig cells (**I**, Fig. 5A). A tendency towards lower number of Leydig cells per section area was also seen, although not statistically significant, while the number of Sertoli cells was similar (**I**, Fig. 5C). However, no difference was seen in the proliferation of Leydig cells at either 2 or 4 weeks of age.

To further characterize the maturation of the Leydig cells, we analyzed the expression of markers for the different stages of Leydig cell maturation at mRNA and protein level. All the analyzed markers of fully mature adult Leydig cells – insulin-like 3 (*Insl3*), nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*, also known as *Sf-1*), sulfotransferase family 1E, member 1 (*Sult1e1*), prostaglandin D2 synthase (*Ptgds*) and vascular cell adhesion molecule 1 (*Vcam1*) – were downregulated in 3-KO testes at 3 months, although not as low as WT samples from the age of 21 days, containing only immature Leydig cells (**I**, Fig. 6A). In contrast, markers for fetal Leydig cells did not differ between the genotypes. IHC stainings of SULT1E1 and VCAM1 showed a similar pattern of expression between 3-KO and WT at 4 weeks and 3 months. Adult DKO testes presented a virtually identical Leydig cell phenotype to that in 3-KO, with similar pattern of HE staining and similar downregulation of the markers of mature Leydig cells (**II**, Fig. 5A-B). Notably, the Leydig cell phenotype was not more severe in DKO, unlike the other aspects of the undermasculinization. All together, these results suggest that the development of immature Leydig cells to fully mature ones is impaired by the lack of HSD17B3, although this does not seem to have a negative effect on spermatogenesis.

5.3.3 Adult 3-KO male mice have reduced kidney size not corrected by high testosterone

Although the anogenital distance is considered to be a permanent read-out of developmental androgen exposure, many of the tissues seen to be affected by undermasculinization have been shown to respond to e.g. androgen deprivation and reintroduction by relatively rapid weight changes even in adult mice (Ayata et al., 1988; Dean and Sharpe, 2013; Laouari et al., 2022). However, in 3-KO and DKO mice the undermasculinized phenotype persists despite the high serum testosterone concentrations, as shown above. Furthermore, the degree of undermasculinization differed between the 3-KO and DKO mice despite nearly identical amounts of testosterone production in adults, the only identified difference being fetal testosterone production. Therefore, it seems clear that the adult phenotype is an irreversible result of the fetal androgen deficiency.

One of the affected tissues was kidney, which was on average 10% lighter at 3 months in 3-KO and further reduced in weight in DKO (**III**, Fig. 1A; **II**, Fig. 3D). To further understand why the adult phenotype was less responsive to androgens, we focused on analyzing kidney development in 3-KO males.

Besides the difference in size, the gross histology of the 3-KO kidneys appeared normal (**III**, Fig. 1B). No drastic changes were seen in nephron development in fetal kidneys, analyzed via immunofluorescent stainings of E18.5 WT and 3-KO kidneys (**III**, Fig. 1C). The number of glomeruli, however, was slightly reduced in postnatal 3-KO males at the age of 2 weeks (**III**, Fig. 1D). As the difference in size and number of glomeruli was not due to obvious developmental impairment, it is likely caused instead by a quantitative effect in the mechanisms of size determination.

5.3.4 Fetal androgen deficiency affects the development of proximal tubules

In WT kidneys, IHC staining of AR revealed expression already during late fetal development, at E15.5, and high expression in adult kidney at 3 months of age (**III**, Fig. 2C). Throughout development AR localized in the proximal tubule segment of the nephron.

Hepatocyte nuclear factors *Hnf1b* and *Hnf4a* have been linked to kidney size determination and development (Heidet et al., 2010; Marable et al., 2018). We saw the expression of both to be downregulated in male fetal kidneys from E18.5, after the period of low androgen production (**III**, Fig. 2D). An analysis and cross-referencing of AR ChIP-seq data from Pihlajamaa *et al*. 2014 implies that both Hnf1b and Hnf4a are likely directly regulated by AR (**III**, Fig. 2E). Their analysis also identified Hnf4a as a collaborating factor in mouse kidney AR signaling, and

observed the expression responding to testosterone treatment in adult castrated mice (fold change 1.52, p = 0.02).

Next, we aimed to identify if the delay in androgen action affected the development of the size difference by influencing cell proliferation in the AR-expressing proximal tubules. We performed double IF stainings of low-density lipoprotein receptor-related protein 2 (LRP2) as a marker of proximal tubule segments, and mitosis marker phosphohistone H3 (pHH3) from the rapidly growing kidneys from 2 week old 3-KO and WT males to identify and count the proliferating cells. Cell counting revealed that the number of proliferating cells within the proximal tubule area was smaller in 3-KO kidneys (Fig. 2F). This indicates that the fetal androgen deficiency had persisting effects on the development of the androgen-sensitive proximal tubules.

5.3.5 Fetal testosterone supplementation partially rescues the undermasculinized phenotype of 3-KO

To test our hypothesis that the persisting effect on the phenotype, including the kidney, was caused by the fetal testosterone deficiency, we performed a testosterone rescue experiment. Pregnant dams were given testosterone supplementation 14.5-17.5 days *post coitum*, coinciding with the period of testosterone deficiency in 3-KO male fetuses.

The testosterone supplementation dosage was seen to be sufficient and effective in completely masculinizing female pups. Following the development of the male pups, we saw that the anogenital distance of fetally testosterone-treated 3-KO males was fully rescued and comparable to WT controls at 3 months of age (**III**, Fig. 3A). The additional testosterone on top of endogenous production did not elevate WT anogenital distance further in comparison to untreated WT males, indicating that there is a limit on the effect of fetal androgen programming on tissue development.

Kidney weight was also seen to be rescued in adult 3-KO males (**III**, Fig. 3B). A similar effect was observed in the *levator ani* muscle (Fig. 10A). This demonstrates that the androgen-affected growth potential of the tissues is indeed programmed by exposure already during fetal development. On the other hand, 3-KO testes, epididymides and seminal vesicles still failed to reach WT size despite the testosterone treatment (Fig. 10B).

To identify the pathways affected in the kidneys by the fetal androgen deficiency and rescue in kidney development, we did RNA-seq analyses of treated and untreated kidneys of WT and HSD17B3KO male pups at E16.5, after 3 days of testosterone supplementation. Pathway analysis of genes differentially expressed between untreated HSD17B3KO and WT kidneys revealed several affected pathways

relevant for the phenotype, including gene ontology terms related to e.g. regulation of tube diameter and tube size (**III**, Fig. 3C).

Finally, we identified a set of 292 genes that were normalized in 3-KO kidneys to WT level by testosterone supplementation, which led to normalized kidney weight in later development. We compared this set of genes with previously published scRNA-seq data from post-natal 1-day-old kidney, to identify genes expressed in the proximal tubules and likely direct targets of androgen regulation. From the 292 genes, only one, insulin-like growth factor binding protein 5 (*Igfbp5*) was also in the list of proximal segment genes (**III**, Fig. 3D). In the top ten pathways in IPA pathway analysis from the genes normalized in HSD17B3 kidney by testosterone supplementation were pathways linked to regulation of the eukaryotic initiation factor eIF2 and eIF4 signaling, and hepatic fibrosis signaling pathway (**III**, Fig. 3E). In all of these, mechanistic target of rapamycin kinase (*Mtor*) gene, eukaryotic translation initiation factor 4E member 3 (*Eif4e3*, $p \leq 0.05$), and forkhead box O1 were seen to be downregulated. Further, IPA network analysis linked *Igfbp5* with mTOR signaling via AKT function (Fig. 3E).

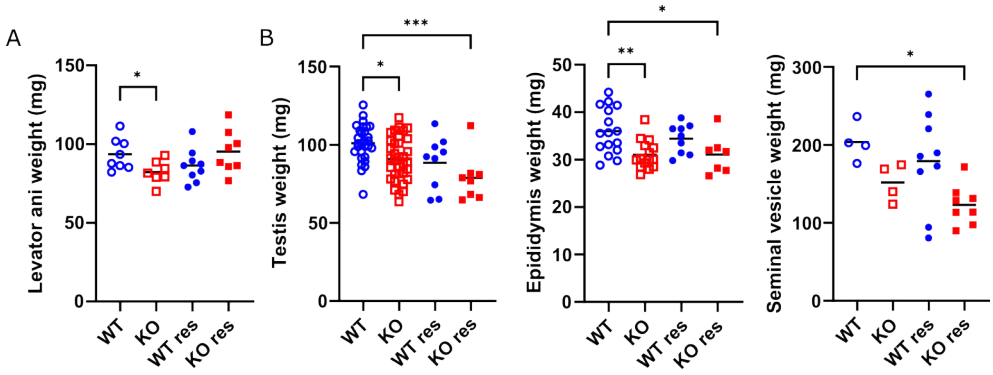

Figure 10. Effects of the fetal testosterone supplementation on other tissues A) The weight of the *levator ani* muscle was also normalized in 3-month-old 3-KO males. B) The weights of testes, epididymides and seminal vesicles, however, remained low in HSD17B3KO despite the testosterone supplementation. WT res, KO res = rescue experiment with testosterone. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$

5.3.6 3-KO male mice have increased adipocity

Besides the persisting undermasculinization, another consistent finding among all 3-KO mice was an increase in body weight at different ages (**I**, Fig 2C). This was not associated with an otherwise increased body size, measured by femur length (**I**, Fig 2C).

We performed a more thorough analysis of the weight development, and saw the difference develop already from an early age, and was most drastic at the age of 3

months, leveling out by the age of 10 months (Fig. 11A-B). No difference was seen in food consumption at 3 months (Fig. 11C).

A body composition analysis revealed that the excess weight was mostly fat mass. The fat percentage actually increased until the age of 10 months; the apparent disappearance of the weight difference was seen to be due to lower lean mass (Fig. 11D). An increasing trend was seen in all analyzed fat depots, and was greatest in the inguinal and gonadal fat pads (Fig. 11E).

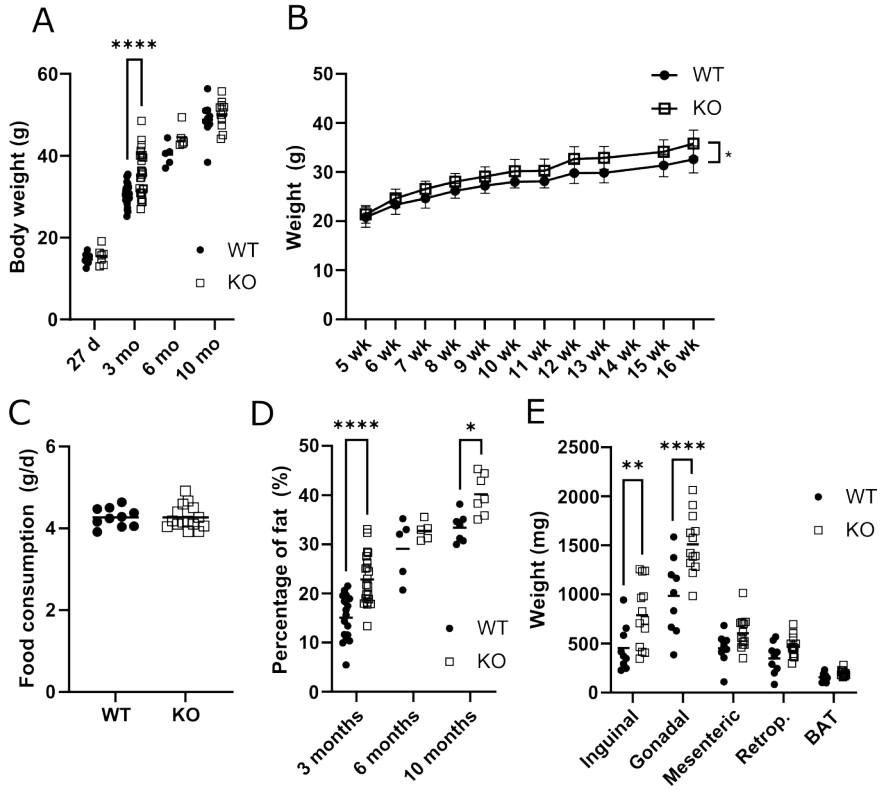

Figure 11. The lack of HSD17B3 leads to weight gain in male mice through excess fat mass. A) The Weight of the HSD17BKO mice is significantly increased at the age of 3 months, and an increasing trend is seen at 6 months, leveling out by 10 months. B) The weight difference is seen to develop already from the age of 5 weeks onwards. C) No difference was seen in food consumption between WT controls and 3-KO. D) The weight gain is due to excess fat mass seen as an increase in fat %. E) An increasing trend is seen in all fat depots, but it is greatest in inguinal and gonadal fat pads. Data presented as individual values with lines indicating means. B: Labels indicate means and whiskers SD. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$

We first suspected that the weight gain could be a result of the steroidogenic imbalance caused by the significant increase in androstenedione production in 3-KO

also leading to increased glucocorticoid production. However, measurements of corticosterone from 3-KO and WT mouse feces in basal and stressed condition, or long-term accumulation from hair samples showed no differences between the genotypes (data not shown).

5.3.7 The adipose depots of 3-KO males have feminized features due to developmental androgen deficiency

We next wanted to understand whether the weight gain was a result of the undermasculinized phenotype, as adipose tissue distribution develops in a sex-specific manner, and 3-KO mice had a significant increase in inguinal fat mass, considered characteristic of a female-type distribution.

One of the known sex differences in mouse adipose tissue is the relation of adipocyte size and number in different depots, with e.g. females having smaller cells in inguinal fat. We looked into the histology of inguinal and gonadal fat depots by automated analysis of cell size, but did not identify changes in 3-KO. However, that the average area of adipocytes was not increased in the larger depots indicated that the cell number instead must be increased (Fig. 12A).

In analysis of genes known to have sexually dimorphic expression in adipose depots, some differences hinting at feminization in 3-KO could be seen – an increase in *Irs1* and *Pck1*expression in the inguinal fat – but not at a significant level (Fig. 12B) (Grove et al., 2010). Likewise, a trend of upregulation of matrix metalloproteinase *Mmp3* was detected in 3-KO fat depots, suggesting that the depots have a larger portion of preadipocytes; a larger ratio of preadipocytes to mature adipocytes has again been seen in female depots (Fig. 12C) (Wu et al., 2017).

Together these results suggest that the differentiation of adipocytes could be altered in 3-KO, resulting in excess development and feminized distribution of adipose tissue. The likely cause would again be the fetal androgen deficiency, as the adipose tissue seems unresponsive to the high adult testosterone in the same manner as the rest of the undermasculinized phenotype, and adipocyte differentiation is known to be directly inhibited by testosterone *in vitro* (Chazenbalk et al., 2013).

Finally, we carried out flow cytometry analyses of E18.5 bone marrow to identify changes in the amount of mesenchymal stromal cell differentiation to adipocytes instead of bone forming cells while exposed to reduced testosterone concentrations in 3-KO. The results, however, indicated a smaller number of adipocyte progenitors present in 3-KO bone marrow (Fig. 12D).

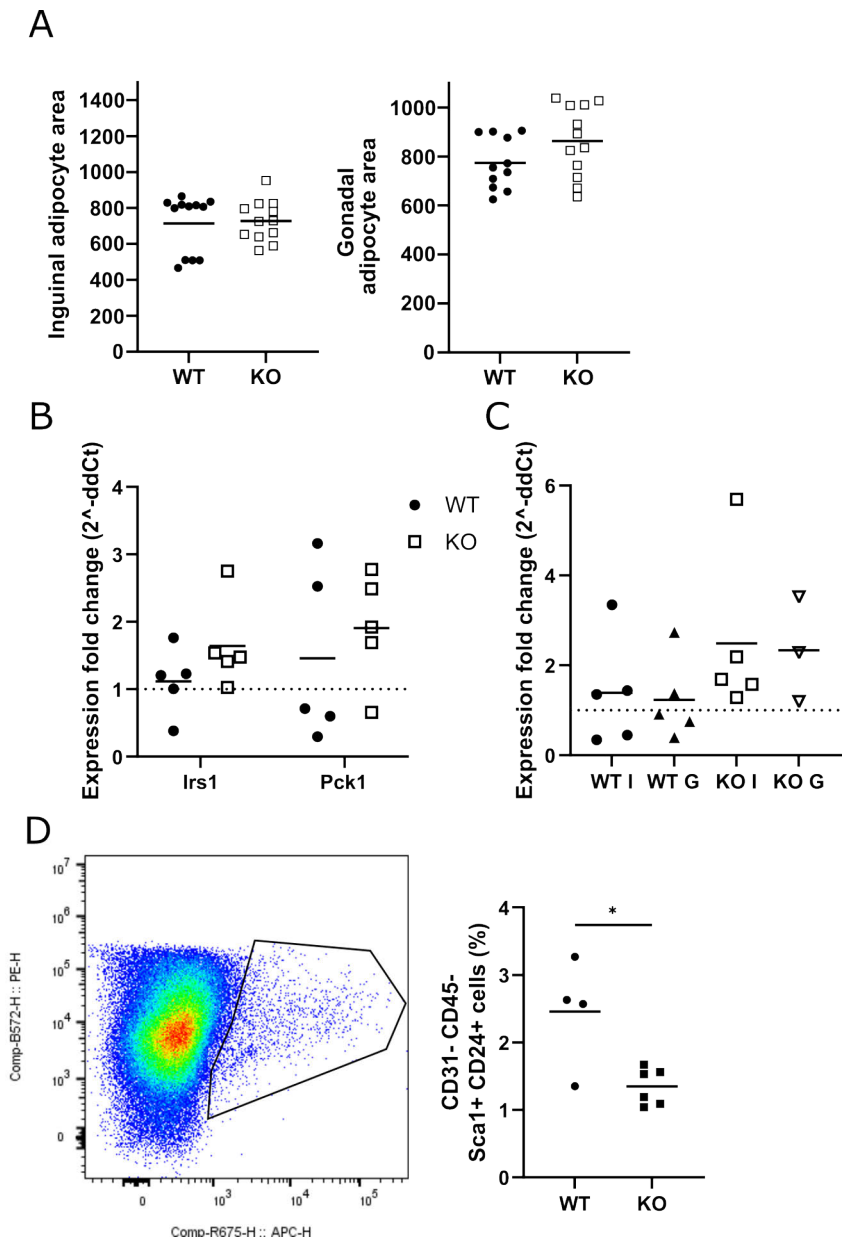

Figure 12. The increase in fat mass of 3-KO mice is mainly caused by an increased amount of adipocytes, hinting at feminization. A) The adipocyte area does not differ between 3-KO and WT controls in either inguinal or gonadal adipose tissue. B) The expression of *Irs1* and *Pck1* shows an increasing trend in the inguinal fat depot. C) The expression of *Mmp3* also shows an increasing trend in both inguinal and gonadal depots in 3-KO. D) At E18.5, 3-KO bone marrow contains a smaller portion of differentiating CD31- CD45- Sca1+ CD24+ adipocyte precursors in comparison to bone-forming cells.

6 Discussion

6.1 HSD17B3 deficiency and the mouse model

Testosterone production and function is of utmost importance to male development. For this process, the role of HSD17B3 as the main enzyme catalyzing the final step is well established, and the dysfunction of HSD17B3 has major consequences for the development of the male phenotype (Geissler et al., 1994; Sha et al., 1996). Human HSD17B3 deficiency is a rare cause of 46,XY DSD worldwide, and the studies on the patients tend to be limited to descriptions of the condition at the time of diagnosis and treatment, and the discovered causative mutations (Gonçalves et al., 2022). While these results reveal the most obvious effects of the deficiency and the subsequent impairment of testosterone production on the reproductive phenotype, and the mutations provide insights to the functioning of the enzyme, a lot remains unknown of the disorder. This includes the specifics of the incomplete fetal virilization, the mechanism behind the activation of testosterone production at puberty, and the long-term health consequences of the DSD and the continued endocrine imbalance in patients who keep their testes.

In this work, we have aimed to answer these questions by generating an animal model for the human condition, a mouse line lacking HSD17B3 activity. The structure of HSD17B3 and its role in testosterone production in mouse is highly similar to human (Sha et al., 1997b). Therefore, we expected also the resulting phenotype to be similar.

The generation of the mouse HSD17B3 knockout mouse model was successful, and the resulting mouse line demonstrated no NADPH-dependent HSD17B activity in androstenedione-testosterone conversion in the adult testis. The phenotype of the animals was clearly undermasculinized, with reduced anogenital distance, delayed puberty, subfertility and reduced weight of many androgen sensitive tissues. However, the undermasculinization was mild, instead of the drastic DSD seen in humans. This is at least partially due to a larger remaining capacity of fetal testosterone production in 3-KO mice, as we would later demonstrate with the DKO model. Nevertheless, male mouse development also likely requires a lower level of androgen exposure. Similar difference in the consequences of hypoandrogenism is seen e.g. in a mouse KO model of 5α-reductases, where again the

undermasculinization is much milder in mice than the DSD observed in humans with 5α-reductase deficiency (Mahendroo et al., 2001).

Still, the endocrine phenotype of the 3-KO male mice was very similar to the one previously described in humans, characterized by developmental androgen deficiency, but significant amount of testosterone postpubertally. However, the testosterone production was accompanied by a massive upregulation of androstenedione production, resulting in a significantly reduced T/A ratio in circulation, a diagnostic hallmark of HSD17B3 deficiency in humans (Faisal Ahmed et al., 2000b). Like the human patients, 3-KO mice also had high LH in circulation.

6.2 Steroidogenesis in 3-KO and DKO

Unexpected, however, was the degree of compensatory testosterone production seen in adult 3-KO mice, which had 3-fold higher than normal concentrations of testosterone in circulation alongside the sky-high androstenedione. In humans with inactivating *HSD17B3* mutations the testosterone levels at adulthood tend to reach only the lower limits of reference values (B. B. Mendonca et al., 2000). It was therefore clear that surprisingly active compensation for the lack of HSD17B3 in testosterone production was taking place in the mice.

The results of the characterization of the 3-KO mice led us to try to identify the source of the compensatory testosterone production. The data on the tissue/serum ratio of testosterone in the 3-KO mice indicated that the serum T was still mainly of testicular origin. The slight increase in serum androgen levels after ADX could be due to the release of suppression of testicular androgen synthesis by corticosteroids (Bambino and Hsueh, 1981; Hardy et al., 2005). It has also been shown in a study that postpubertal testicular tissue from HSD17B3 deficiency patients converted A-dione to T to a significant extent (Eckstein et al., 1989). Furthermore, analysis of serum A-dione and T in another patient before and after gonadectomy demonstrated that the gonadectomy caused a greater proportional decrease in T than in A-dione (Werner et al., 2012). Thus, it is likely that the testes remain a significant source of the circulating T also in HSD17B3 deficiency patients.

The adult 3-KO mice reached normal intratesticular testosterone concentrations in the presence of a 10-fold increase in androstenedione. This increase in androstenedione concentration was achieved through the upregulation of genes of both cholesterol biosynthesis – *Cyp51*, *Nsdhl* and *Hsd17b7* – and the first steps of steroidogenesis – *Star*, *Cyp11a1* and *Cyp17a1*. This upregulation was driven by the increased HPG stimulation through high serum LH, and the upregulation of *Lhr* in the testes. That circulating LH remains high in the presence of high serum testosterone is puzzling, but another study on an unrelated *Hsd17b3* knockout mouse model with nearly identical endocrine profile has demonstrated that reintroducing

active HSD17B3 in testes normalized the concentrations of both androstenedione and circulating LH (Rebourcet et al., 2020). This indicates that the regulation of the HPG axis is not permanently disrupted by the lack of HSD17B3. Rather, the high LH and the resulting upregulation of androstenedione production is likely required for the compensatory testosterone production to function. This in turn demonstrates that the compensatory enzyme activity is much less efficient than that of HSD17B3.

Another enzyme capable of catalyzing the same reaction and expressed in the mouse testis is HSD17B1. Human HSD17B1 is considered to be primarily involved in ovarian estradiol production, but can also accept androgens as substrate, although with lower affinity (Puranen et al., 1997). Rodent HSD17B1, while also highly expressed in the ovary, catalyzes the conversion of estrone to estradiol and androstenedione to testosterone with similar catalytic efficacy (Nokelainen et al., 1996; Puranen et al., 1997). In mouse testis, HSD17B3 and HSD17B1 are expressed concurrently in the fetal Sertoli cells responsible for the final conversion of androstenedione to testosterone (O'Shaughnessy et al., 2002b; Shima et al., 2015). The pattern of expression is likely similar in humans (Al-Sharkawi et al., 2023; Hakkarainen et al., 2018). We also observed that the rise in HSD17B1 expression in the WT fetal testis coincided with the activation of compensation that increased the intratesticular testosterone concentration.

To clarify the role of HSD17B1 in the compensation, we generated first the 1-KO mouse line and then crossed it with 3-KO to generate the DKO mice. The inactivation of HSD17B1 was successful, as demonstrated by the effect seen in estrone to estradiol conversion capacity of cells transfected with the mutant enzyme, and more importantly, the reduced A-dione to T conversion of neonatal DKO testes in comparison to WT, but also 3-KO. This was reflected in the neonatal intratesticular testosterone, brought to very low levels in DKO, confirming that HSD17B1 was indeed crucial in compensating the lack of HSD17B3. Interestingly, although the remaining A-dione to T conversion of HSD17B1 in neonatal 3-KO testis was only 5% of conversion by HSD17B3 in WT and 1-KO, the low amount of activity still resulted in normal intratesticular testosterone concentrations without an increase in androstenedione at birth.

While the fetal testosterone production was almost completely abolished by the lack of both HSD17B3 and HSD17B1, the steroid profile of the adult DKO males was nearly identical to 3-KO, with serum testosterone higher than in WT, and comparable intratesticular testosterone. Therefore, it is clear that yet another enzyme is capable of testosterone biosynthesis in adult mouse testis. Testosterone in both 3-KO and DKO mice rises after puberty, similar to humans with HSD17B3 deficiency. Thus, the compensatory mechanism activated at puberty may be the same, although the enzymes responsible are yet to be identified. Several candidates exist in the families of short-chain dehydrogenase/reductase (SDR) and aldo-keto-reductase

(AKR) families, which include nearly a hundred enzymes, the substrate specificities of which are only superficially known (Gabrielli et al., 2022; Penning, 2015). HSD17B5 (AKR1C3) has been suggested as the compensating enzyme in human HSD17B3 deficiency, but mouse does not have a close ortholog (Werner et al., 2012b). HSD17B12 has been theorized to be the compensating enzyme by another group working with a *Hsd17b3* knockout mouse model, but the studies our group has performed on HSD17B12 has shown it to be mainly involved in lipid metabolism (Heikelä et al., 2020; Rebourcet et al., 2020).

In any case, no upregulation of *Hsd17b1*, *Hsd17b6*, *Hsd17b12*, or *Akr1c18* (the closest mouse enzyme to AKR1C3) expression was observed by us in adult 3-KO testis. It is unknown whether this means none of these enzymes are involved in the compensation, or simply that no upregulation is needed and the existing expression is enough when excess androstenedione is available. Further studies are sorely needed to probe the surprising complexities of steroidogenesis.

The difference in the degree of undermasculinization in 3-KO and human HSD17B3 deficiency is explained to a large degree by the difference in fetal androgen production, as demonstrated by the DKO. It is likely that HSD17B1 is unable to compensate for the lack of HSD17B3 to a significant degree in humans, due to its greater preference for estrone over androstenedione as a substrate (Puranen et al., 1997). Still, the capacity of human HSD17B1 to produce testosterone, indicated by the masculinization of female fetuses in transgenic mice over-expressing the human enzyme under Ubiquitin-C promoter, has been demonstrated previously (Saloniemi et al., 2009). As even the XY individuals with HSD17B3 deficiency still produce some testosterone to cause the fetal differentiation of male internal genitalia, it is thus possible that HSD17B1 is involved in this low-level testosterone biosynthesis

The excess testosterone in 3-KO and DKO serum in comparison with WT has proven hard to explain. The simplest cause would be serum androstenedione, increased 100-fold, being converted peripherally to testosterone in significant enough amounts that it would be reflected in serum T concentration. However, none of the tissues we analyzed, known to have capacity for steroid metabolism, demonstrated tissue/serum ratios that would indicate local testosterone production. Still, it is not unlikely that still another peripheral source, or combination of sources, could be responsible.

6.3 Undermasculinization in 3-KO and DKO

The undermasculinization of male mice with disrupted testosterone biosynthesis is not surprising. The degree of undermasculinization is proportional to the severity of the disruption of testosterone production, demonstrated by the difference in the

phenotype of 3-KO and DKO males. However, what is more interesting is that the undermasculinized phenotype persisted in the 3-KO and DKO male mice even in the presence of high circulating testosterone in adulthood. Many of the tissues analyzed, like the seminal vesicles, kidneys, and adipose tissue, are known to respond rapidly and reversibly to changing androgen levels in adult mice and even in humans (Ayata et al., 1988; Laouari et al., 2022). There must be some mechanism, then, that makes the 3-KO tissues in question less sensitive to, or less capable of responding to androgens.

The only period of significantly low testosterone we discovered in the 3-KO mice was during late fetal development, at E15.5. Testosterone production was delayed, but rose close to normal already by E18.5 as compensation by HSD17B1 activated. This leaves only a short window of androgen deficiency to produce the undermasculinization we see in the animals later. In DKO males, the more drastic phenotype may be due to an even lower testosterone in the same time window than is present in 3-KO. It might also be due to a longer exposure to androgen deficiency, continuing until birth when 3-KO has reached a normal concentration of testosterone.

Nevertheless, the observed time window of hypoandrogenism matches well with what is known about the masculinization programming window, identified precisely between E15.5 and E18.5 in rodents, and is therefore very likely directly responsible for e.g. the permanent reduction of the anogenital distance (Dean and Sharpe, 2013; Welsh et al., 2008). Androgen action during this programming window is required for the target tissues to reach their full size with later androgen exposure; without sufficient androgen activity during the window, the tissues remain smaller even with high androgen exposure later. On the other hand, androgen suppression immediately after the window has no such effect, and likewise, undermasculinization due to normal fetal androgen exposure but later androgen deficiency in LHR knockout mice is reversible with testosterone even in adulthood (Pakarainen et al., 2005; van den Driesche et al., 2017).

This programming window has so far been demonstrated to affect the male reproductive tissues, but not some of the other androgen-sensitive tissues we saw affected. For the first time, we have now presented evidence that adult kidney size determination in mouse is similarly affected by fetal testosterone levels. High adult testosterone does not normalize the kidney size, but it was rescued by testosterone supplementation in the fetal time window of androgen deficiency.

The effect seems to be mediated through the AR expressing cells of the proximal tubule, as has previously been shown for the responsiveness of adult kidney size to androgens, and causes a lower rate of proliferation during later kidney development. The effect then may be mediated e.g. through mechanisms that determine the amount of available stem or precursor cells at earlier development.

The pathways changed between 3-KO and WT contained relevant pathways related to tubular size determination. The number of genes seen to be normalized by testosterone in 3-KO was relatively small, 292. However, this is not surprising as the resulting phenotype is not drastic: the kidney structure and function is not obviously impaired, besides the size reduction. From the set of genes, only *Igfpb5* was found to be expressed in the proximal tubules of neonatal mouse kidneys in another study, and it is a likely androgen target in adult mouse kidney (Adam et al., 2017; Pihlajamaa et al., 2014). IGFPB5 acts by regulating IGF functions, but also independently of IGFs (Duan and Allard, 2020). The accompanying downregulation of *Mtor*, *Eif4e3*, *Foxo1* in 3-KO kidneys indicates complex changes in the network of these signalling pathways affecting cell growth and proliferation. As all were normalized by the size-rescuing testosterone supplementation, they likely contribute to the kidney growth. However, cause and effect are hard to establish in such complex systems and require further studies.

It should also be noted that the affected pathways in the established programming of the reproductive tissues are not well understood and may involve same factors, but it is also possible that they are highly tissue-specific. The success of the fetal testosterone supplementation in rescuing the adult kidney and *levator ani* weight, but failure to rescue the epididymis and seminal vesicle weight also indicate tissue-specific differences in the programming mechanisms or timing.

Other generally androgen-sensitive tissues seemed less sensitive to the fetal androgen deficiency. The testes of the 3-KO and DKO mice, although slightly decreased in size, nevertheless had active and qualitatively normal spermatogenesis in adults. A slight delay was observed in the initiation of spermatogenesis, as was also in puberty onset and testosterone production at the time, all likely connected. This demonstrates that spermatogenesis only needs testosterone for pubertal initiation and adult maintenance, and does not depend on earlier developmental priming. That patients of HSD17B3 deficiency often lose their germ cells and cannot initiate normal spermatogenesis at puberty is likely due to their prolonged cryptorchidism, which the mice are less susceptible to (Rösler, 1992).

Interestingly, spermatogenesis was seen to fail in a previous *Hsd17b1* knockout model generated by us (Hakkarainen et al., 2018). However, the deletion of the whole gene was also seen to silence the nearby *Naglu* gene, causing metabolic complications in the phenotype unrelated to HSD17B1 (Jokela et al., 2017). Although *Naglu* was ruled out as the cause of the reproductive phenotype, it is possible that the deletion also similarly affected the expression of some other gene. Nevertheless, it is also possible that HSD17B1 could possess some role in testis unrelated to its enzymatic function.

Whereas spermatogenesis and Sertoli cells were mostly unaffected in the 3-KO and DKO, the development of Leydig cells was determined to be abnormal. The

appearance of the cells and their expression of maturation markers clearly indicated that the transition from immature ALCs to fully mature ones was impaired. Whether this was an effect of androgen deficiency, or the imbalance of steroidogenesis and HPG stimulation, remains unknown. However, fetal androgen action has been shown to program ALC development, and deficiency to lead to compensated LC failure with low to normal testosterone and elevated LH in serum (Kilcoyne et al., 2014). Leydig cell hyperplasia is common in HSD17B3 deficiency, but we saw no signs of similar changes in 3-KO or DKO, so it may again rather be a result of cryptorchidism in humans (Gotoh et al., 1984). In any case, the maturation defect of the Leydig cells did not seem to have adverse effects for the other cell populations of the 3-KO testes.

The final clear effect of the lack of HSD17B3 was seen in adipose tissue. The increase in body weight seen in 3-KO due to excess fat mass, especially in inguinal and gonadal adipose depots, was significant. We ruled out excess glucocorticoids and local estrogens as causes, and finally focused on the possibility of a similar developmental programming effect as was seen in the undermasculinized phenotype, leading to a feminized adipose tissue development. Developmental programming in adipose tissue by other hormonal factors, and androgen effects on adipocyte differentiation are well established (Chazenbalk et al., 2013; Rodgers and Sferruzzi-Perri, 2021). Still, no direct connection of fetal androgen exposure and the development of normal male body composition have been discovered so far. Some of our results support the conclusion that the adipose tissue in 3-KO is feminized due to changes in differentiation caused by the fetal androgen deficiency. However, more work is needed to prove this. In addition, the possibly competing effects of low developmental and high adult testosterone may complicate the phenotype.

6.4 Results in perspective

Due to the sparsity of reports, it is unknown whether XY individuals with inactivating HSD17B3 mutations demonstrate similarities to the phenotype we have described in mice, e.g. weight gain and female-like kidney size despite their significant testosterone production. This may well be the case, and if so, can have unexpected consequences for their health. Weight gain can lead to metabolic disorders, and atypically small kidneys in relation to body size could be poorly prepared to handle infections or other challenges.

Besides just HSD17B3 deficiency, which is admittedly rare, our data on the developmental programming via hormones has broader utility in understanding other developmental sex hormone imbalances or disorders of sex development. Many more common disorders such as e.g. 5α-reductase deficiency, androgen insensitivity syndrome, or exposure to hormonal disruptors, can have similar effects on endocrinology and development.

We identified a new role for mouse HSD17B1 in testosterone biosynthesis in the absence of HSD17B3, and it is possible it has a smaller role in humans too. The mouse models also provide further possibilities in identifying the remaining compensatory enzymes. This data will potentially have broad utility to various androgen-linked diseases, such as polycystic ovary syndrome in women and prostate cancer in men, where overproduction of androgens has a vital role in disease pathogenesis. In particular, HSD17B3 has been shown to contribute to the intratumoral androgen production of castration-resistant prostate cancer, and a polymorphism in *HSD17B3* gene has been shown to be associated with an increased risk of developing prostate cancer (Margiotti et al., 2002; Montgomery et al., 2008). Several inhibitors of HSD17B3 have also been identified and suggested to be possible selective treatment options for hormone-depended prostate cancer (Ning et al., 2017). Possible compensatory mechanisms of HSD17B3 would need to be taken into account in treatment options.

In the included studies, we have used several different mouse models to better navigate the complexities of androgen biosynthesis *in vivo*. These results integrate well into the wider understanding of male steroidogenesis, development and biology obtained from previous animal studies, but also have a clear connection to a known human condition of HSD17B3 deficiency. A thorough initial characterization of the reproductive phenotype of the mouse lines allowed us to follow with in-depth analyses of different aspects of the phenotype to find novel insights to the raised questions. The sizes of the cohorts used in analyses were often large for animal studies, and main results were seen to be repeatable across cohorts, allowing us to have confidence in the findings.

However, a work of this nature has limitations. The translatability of the results to humans is not guaranteed, and we already saw what are most likely species differences both in the respective enzyme activities and the developmental mechanisms reflected in the phenotype. The practical aspects of animal work did not always allow us to have the most suitable sample sets, and the variation between animals could well mask some details of the phenotype.

The phenotype of the mice clearly demonstrated that the affected pathways and mechanism are complex, in many cases resulting in surprisingly mild or otherwise unexpected outcomes. This made thoroughly analyzing the causes and effects beyond the scope of this work. Our results provide strong leads, but given the chance, more should be done to fully follow through with the lines of research opened by the models, aiming to answer the many open questions identified in the previous chapters. Finally, the evaluation of the full translatability of our results will have to wait for further studies.

7 Summary/Conclusions

In this work, we generated and characterized mouse models lacking either HSD17B3 activity, HSD17B1 activity, or both. Our results demonstrate that HSD17B3 is required for normal androgen production in mice, as it is in humans. The lack of HSD17B3 results in an endocrine disruption, with HPG stimulation increasing testicular androstenedione production, until less efficient compensatory enzymes can produce significant amounts of testosterone, especially after puberty. These results are very similar to the endocrine phenotype seen in humans with HSD17B3 deficiency.

During fetal development, HSD17B1 is the only significant enzyme compensating for the lack of HSD17B3, as the lack of both leads to near-complete abolishment of testosterone production capacity in testis. However, in adult male mice, yet other unknown enzymes takes over the role, as both 3-KO and DKO produce high amounts of testosterone.

Despite the compensation by HSD17B1, the rise in fetal testosterone production is delayed in 3-KO. This results in an undermasculinized phenotype, at least partly due to developmental programming caused by androgen deficiency in the so-called masculinization programming window. We also identified kidney as a novel target of such developmental programming – likely a factor in the sex dimorphism seen in kidney development – as well as pathways behind the effect.

The new aspects of the phenotype, like the weight gain, and changes in organ sizes resulting from the developmental hypoandrogenism, can have relevance from a patient point of view. This extends to other developmental disruptions of androgen action.

However, the undermasculinization in 3-KO is less drastic than the DSD seen in humans with HSD17B3 deficiency. The phenotype of the DKO more closely resembles the human condition, due to more complete androgen deficiency, reflecting differences in enzymes of testosterone biosynthesis between the species. Still, the further compensating enzymes may well be similar.

In conclusion, 3-KO and DKO models have provided new insights into steroidogenesis and developmental programming in males, and demonstrated their usefulness for further research.

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