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# Update on the genetics of differences of sex development (DSD)

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Human gonadal development is regulated by the temporospatial expression of many different genes with critical dosage effects. Subsequent sex steroid hormone production requires several consecutive enzymatic steps and functional hormone receptors. Disruption of this complex process can result in atypical sex development and lead to conditions referred to as differences (disorders) of sex development (DSD). With the advent of massively parallel sequencing technologies, *in silico* protein modeling and innovative tools for the generation of animal models, new genes and pathways have been implicated in the pathogenesis of these conditions. Here, we provide an overview of the currently known DSD genes and mechanisms involved in the process of gonadal and phenotypical sex development and highlight phenotypic findings that may trigger further diagnostic investigations.



## Introduction

Human gonadal development is a highly complex and intriguing process: depending on the presence or absence of a Y chromosome, and more specifically of the *Sex Determining gene on Y* (*SRY*) gene

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on this chromosome, the undifferentiated gonadal precursors are programmed to evolve to either testes or ovaries. Subsequent sex steroid hormone production by the gonads then leads to differentiation of the internal and external genitalia. Many genes that play a prominent role in these developmental processes have been identified, and mutations in these genes can result in differences (disorders) of sex development (DSD). These are a group of rare congenital conditions that affect the development of the urogenital tract and reproductive system and that lead to an atypical combination of chromosomal, gonadal or phenotypic sex. DSDs occur in approximately one in 2000–4500 births [1]. With the advent of massively parallel sequencing (MPS) techniques, new genes, pathways and pathogenetic mechanisms underlying these conditions were elucidated. Here we provide an overview of the most important genes implicated in DSD and the pathways they are involved in, and we discuss some important recent advances in the field.

## Basics concepts of gonadal development

Sex development comprises two consecutive steps. The first step, sex determination, refers to the initial developmental decision of the bipotential gonad to follow the testicular or ovarian path. This step is tightly regulated by the temporospatial expression of many genes, mostly encoding transcription factors. Subsequent sex differentiation involves the development of phenotypic sex which is mediated by hormones secreted by the developing gonads [2,3].

Sex development is initiated in the fourth week of embryonic life with the formation of the undifferentiated urogenital ridges that become colonized with primordial germ cells (week 5). Around the 6th week of embryonic development, *SRY* is expressed from the Y chromosome and starts testicular development with the stepwise formation of seminiferous tubules delineated with Sertoli cells, that drive further testicular differentiation. Afterwards Leydig cells develop, which are responsible for the production of testosterone. In the absence of *SRY*, female sex development is initiated, and the somatic compartment of the gonad will be built up by granulosa cells and steroidogenic theca cells that will together surround the germ cells and form a dynamic pool of ovarian follicles [4–6].

At the same time two duct systems co-exist: the mesonephric (Wolffian) and the paramesonephric (Müllerian) ducts. These ducts debouch in the urogenital sinus which is covered by a genital membrane. Underneath this membrane, two mesodermal swellings develop: the urethral folds and the labioscrotal swellings. These precursor structures differentiate into the internal and external genitalia under the influence of hormones secreted by the developing gonad [2]. In XY embryos, Sertoli cells produce anti-Müllerian hormone (AMH) which induces paramesonephric duct regression [7]. Testosterone, produced by the Leydig cells, supports mesonephric duct differentiation into the epididymides, vasa deferentia and the seminal vesicles and is converted into dihydrotestosterone (DHT), the latter is responsible for virilization of the external genitalia. On the other hand, in XX embryos, in the absence of AMH, the paramesonephric ducts give rise to the Fallopian tubes, the uterus and the proximal two thirds of the vagina. In the absence of (dihydro)testosterone, the urogenital sinus will develop into female external genitalia [2,5].

## Genetic pathways

### *The undifferentiated stage*

Sex development starts with the formation of the bipotential gonadal primordium from the intermediate mesoderm. Studies in mice have shown that many genes are implicated in this process; however human mutations leading to gonadal agenesis have rarely been demonstrated. Two groups of genes are associated with these earliest stages of gonadal development: homeobox genes (*Lim homeobox 9*, *LHX9*; *Empty spiracles 2*, *EMX2*; *Chromobox* 2, *CBX2*/*M33*) and other genes encoding transcription factors (*Wilms tumor 1*, *WT1*; *Nuclear receptor subfamily 5 group A member 1*, *NR5A1*). Maintenance of the urogenital ridge relies on the subtle balance between cell proliferation and apoptosis. The known and presumed interactions between these early genes are shown in Fig. 1 [2,3,8].

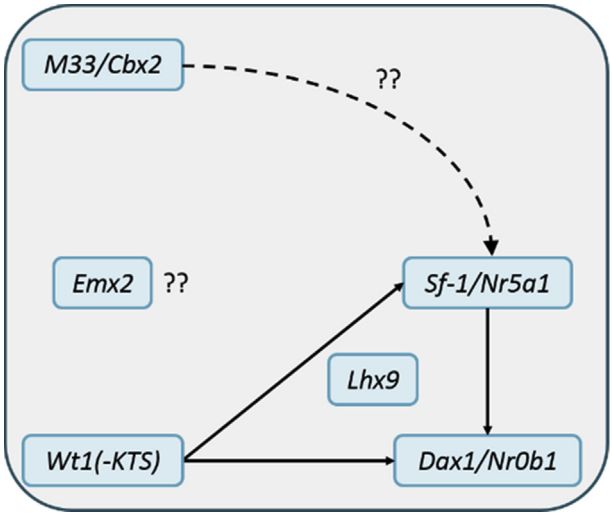


**Fig. 1.** Overview of the early regulators of sex development. The depicted genes are implicated in the development of the bipotential urogenital ridge. The Wt1-KTS isoform activates Nr5a1 expression together with Lhx9. Both Wt1 and Nr5a1 influence Dax1 expression. It is currently unclear where Cbx2 and Emx2 fit in this model and what their interaction partners are. Adapted from Wilhelm et al., (2007).

The expected phenotype is complete gonadal dysgenesis. The diagnosis can be made prenatally due to discordance between the karyotype and phenotype but affected individuals will mostly present in early adolescence due to lack of pubertal development, including breast development.

## *Male sex determination*

### *SRY: the master switch and how to turn it on*

The first difference in expression patterns between human 46,XX and 46,XY embryos occurs at embryonic day 41 with the upregulation of *SRY* expression. This single-exon gene encodes a transcription factor with a highly conserved DNA-binding domain; the high mobility group (HMG) box [9,10]. Despite intensive research efforts, little is known about SRY's function and target genes: some reports suggest a role in transcriptional activation, others in repression [11,12]. Studies of knock out (KO) mouse models have unraveled important parts of the pathways regulating *SRY* expression and many of these findings were validated by the identification of human mutations in individuals who have a 46,XY gonadal dysgenesis (GD) phenotype (Fig. 2).

A second isoform of the previously mentioned *WT1* gene (WT1+KTS) is involved in *SRY* upregulation. These isoforms are the result of alternative splicing at the end of exon 9 and only differ by the absence (-KTS) or presence (+KTS) of three amino acids. However, they exert different functions in the developing gonads, resulting in different phenotypes in transcript specific KO mice [13]. This resembles the human situation where *WT1* mutations can cause either the Frasier or Denys-Drash syndromes. Additional research revealed that the WT1+KTS isoform is not a direct activator of *SRY* transcription, but that it preferentially binds to *SRY* mRNA, functioning as a post-transcriptional stabilizer (Fig. 2B) [14].

A second transcription factor acting on *SRY* activation is GATA-binding protein 4 (GATA4) and its co-factor Friend of GATA-2 (FOG2 or ZFPM2). *Gata4*$^{-/-}$ mice die during early embryonic development because of cardiac abnormalities, hampering conclusions about their involvement in early gonadal development [15,16]. *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ mouse models demonstrate that deficiency of these genes results in the failure to form testicular cords due to reduced *Sry* expression (Fig. 2C) [17,18] and a less abundant expression of genes encoding testosterone biosynthesis enzymes. Later on in development, the GATA4/FOG2 complex is also involved in regulation of *SRY-Box 9* (*SOX9*) and *AMH* [15,19].

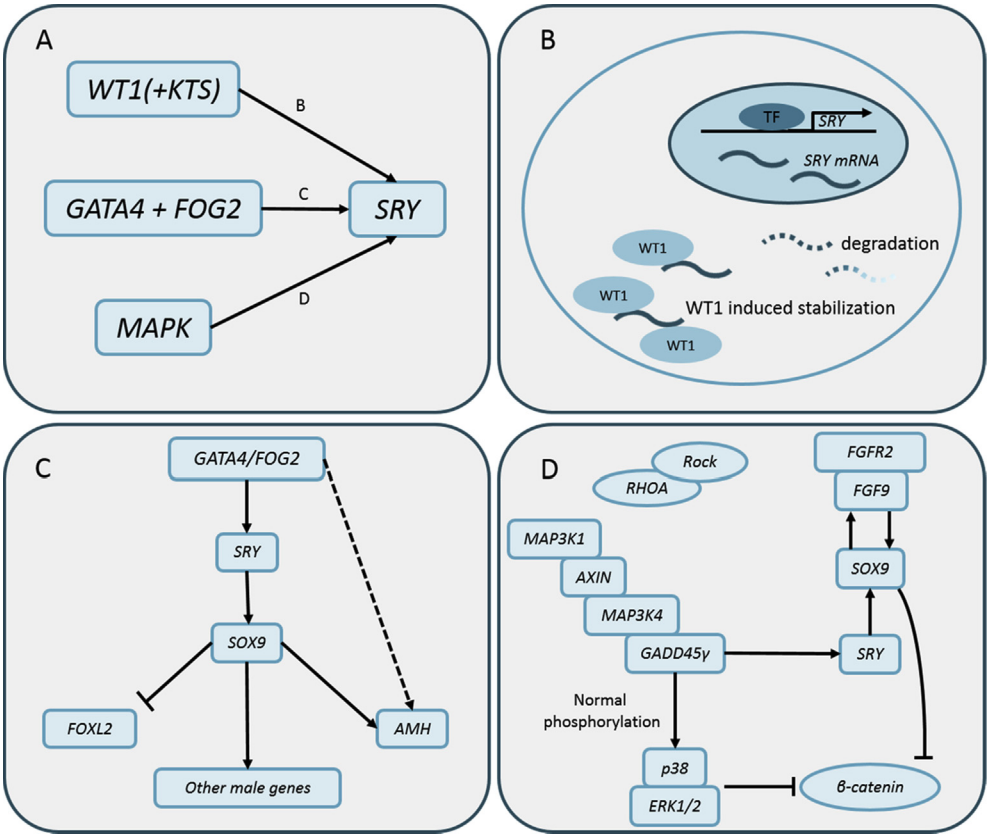


**Fig. 2.** Overview of the most important regulators of SRY expression. (A) *SRY* expressionis regulated by WT1, more specifically the +KTS isoform, the GATA4/FOG2 transcription complex and the MAPK pathway. (B) The WT1+KTS isoform is involved in *SRY* activation by binding and stabilizing the *SRY* mRNA. (C) GATA4 together with its co-factor FOG2 upregulates *SRY* expression. Besides its role in testicular determination, the complex is also involved in upregulation of *AMH* later. (D) Overview of the mechanism of MAP3K1 action during normal male development. *MAP3K1* gain-of-function mutations will lead to increased binding of RHOA and ROCK, two female specific factors that can inhibit *SOX9* and to increased phosphorylation of p38 and ERK1/2. Hyperphosphorylation will stabilize the β-catenin and initiate female developmental pathways. Furthermore, these gain-of-function mutations will sequestrate AXIN and MAP3K4, thereby abolishing *SRY* activation.

Thirdly, the mitogen-activated protein kinase (MAPK) pathway has been implicated in these early stages of testicular development (Fig. 2D). Bogani et al. identified a homozygous mutation in the *Map3k4* gene leading to male-to-female sex reversal and resulting in a dramatic reduction of *Sox9* and *Sry* expression [20]. One year later, this pathway was also linked to human sex development by the identification of *MAP3K1* mutations in two families and 11 sporadic cases with 46,XY DSD [21]. The molecular mechanism behind these mutations however, differs between mice and human (Fig. 2D) [22]. The human gain-of-function mutations alter co-factor binding and result in increased phosphorylation of downstream MAPK targets. Dysregulation of the MAPK pathway results in increased β-catenin, Wingless type 4 (WNT4) and Forkhead box protein L2 (FOXL2) expression and decreased SRY and SOX9 expression. Recently, it was shown that the effects on protein binding depend on the domain in which the mutations occur, adding new mechanistic insights in to MAPK related DSD pathogenesis [23].

Once *SRY* is activated, a male specific gene expression cascade is initiated, resulting in Sertoli and Leydig cell differentiation (Fig. 3). Important genes in these processes are *NR5A1* and the *SOX* genes.

*NR5A1: a gene with many faces*

Besides its role in the development of the undifferentiated gonad, *NR5A1* is crucial in several subsequent steps of gonadal development and functioning such as Sertoli cell differentiation and steroidogenesis. Furthermore, *NR5A1* plays a role in the regulation of the hypothalamic pituitary axis.

In human embryos, NR5A1 is expressed from embryonic day 32–33, and its expression is maintained in the somatic cells of the early testis [24]. Sekido et al. showed that Nr5a1, together with Sry, can bind on a *Sox9* gonad-specific enhancer, the so-called testis-specific enhancer of Sox9 core (TESCO) enhancer element. Their work is also in favor of a feed-forward, self-reinforcing signaling loop in which Sox9 together with Nr5a1 binds TESCO to maintain *Sox9* expression [25]. In humans, *SOX9* expression is critical for Sertoli cell differentiation (see below). Once Sertoli cells are functional, a second role for *NR5A1* emerges, being the activation of *AMH* expression, which will result in regression of the paramesonephric ducts in male fetuses. Furthermore, *NR5A1* is a trigger for virilization of the external genitalia, as it is an activator of the steroidogenic enzymes [26]. In addition, NR5A1 is expressed in the ventromedial hypothalamus and the pituitary, where it contributes to the reproductive axis by interacting with the genes encoding gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) [27]. Despite extensive knowledge on NR5A1 target genes, far less is known about regulation of *NR5A1* itself.

*Nr5a1*$^{-/-}$ mice exhibit complete gonadal and adrenal agenesis resulting in severe adrenal failure and a female phenotype with persistent paramesonephric ducts in male mice. Moreover, these animals have an abnormal spleen and ventromedial hypothalamus, and display reduced gonadotropin secretion. Heterozygous KO mice show a less severe phenotype including hypoplastic adrenals and gonads [28–30]. This broad functional spectrum of *NR5A1* is mirrored in the plethora of phenotypes seen in individuals with *NR5A1* mutations, ranging from 46,XY and 46,XX DSD to male infertility and primary ovarian insufficiency (POI) [31].

*SOX genes*

To date, *SOX9* is the only genuinely known SRY target gene. In the undifferentiated gonad, *Sox9* is expressed at low levels in both sexes, but it is upregulated immediately after *Sry* expression in Sertoli cells [32]. The importance of *Sox9* in testis development has been confirmed by the phenotype of *Sox9* KO mice; gonads of XY KO mice are characterized by ovarian differentiation. *Sox9* overexpression in XX mice revealed that *Sox9* is not only indispensable but also sufficient for testicular development [33].

Once SOX9 is expressed, it initiates multiple feed forward loops to maintain its own expression. A first loop starts with Sox9-induced upregulation of *Fibroblast growth factor 9* (*Fgf9*) which in turn activates the *FGF receptor 2* (*Fgfr2*); the latter is known to further enhance *Sox9* expression [34,35]. A second loop acts via the *prostaglandin D synthase* (*Ptgds*) gene, resulting in increased prostaglandin D2 secretion, which in a paracrine and autocrine way promotes the nuclear translocation of Sox9. Importantly, both feed forward loops can also induce *Sox9* expression in neighboring cells without endogenous *Sry*, showing that not *Sry* but *Sox9* expression is a prerequisite for Sertoli cell differentiation [36,37].

Although not involved in typical sex development, other *SOX* genes can functionally mimic the gonadal activities of *SOX9*. Ectopic gonadal expression of *Sox3* in transgenic mice results in a XX DSD phenotype. A similar phenotype is seen in humans (Table 1) [38]. Ectopic gonadal *Sox10* expression in XX mice is reported to induce Sry-independent female-to-male sex reversal. *Sox10* KO mice on the other hand do not have a particular gonadal phenotype, suggesting that *Sox10* is not necessary for testis development but that it can function as a testis-determining gene when aberrantly expressed [39]. A developmental function was also suggested for *Sox8*; double *Sox8*/*Sox9* KO models confirm a role for this gene as a reinforcer of *Sox9*, indeed, both genes are functionally redundant [40]. Very recently, *SOX8* variants were associated with a range of reproductive phenotypes (see below) [41].

Depending on the degree of testicular differentiation, disruption of the male sex determining pathway will lead to a phenotype of complete or partial gonadal dysgenesis in 46,XY individuals. Girls with complete gonadal dysgenesis will typically seek medical advice for lack of pubertal development. Newborns with partial gonadal dysgenesis will present at birth with atypical genitalia, immediately compelling further diagnostic investigations.

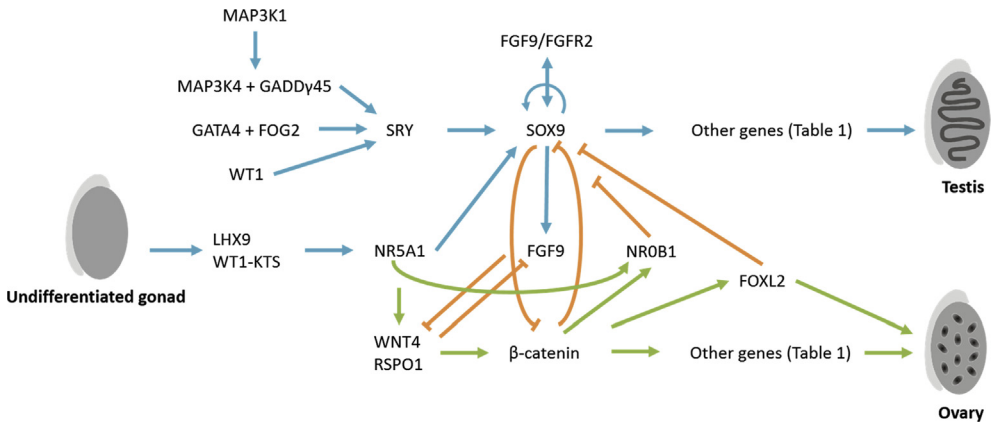


**Fig. 3.** Overview of the most important genes for male and female sex development and their most established interactions. In blue: interactions important for male sex development. In green: interactions important for female sex development. In orange: genes and interactions involved in the mutual antagonism between male and female pathways.

*Female sex determination*

For a long time, ovarian development has been seen as the default pathway in the absence of *SRY*. Several lines of evidence contradict this view and are in favor of the existence of ovary-specific genes and pathways. Two different mechanisms that may trigger ovarian development have been proposed [42]. The first mechanism hypothesizes the existence of an ovary-determining gene, acting as the female counterpart of *SRY*. However such a gene has not yet been identified [43]. The Z-factor model on the other hand may explain those cases in which 46,XX individuals develop testes in the absence of *SRY*. The Z-factor is a hypothetical, hitherto unidentified gene expressed in the XX gonad that is supposed to repress testis development, thereby allowing ovarian differentiation. In XY gonads, the Z-factor is repressed by SRY action. Inactivation of the Z-factor in XX individuals would then abrogate inhibition of the male pathway, thereby causing female-to-male sex reversal [12]. Below, we elaborate on some ovarian pathways that have been studied in detail (Fig. 3) [42].

*The WNT/RSPO1/β-catenin pathway*

WNT signaling pathways are widely used during development; WNT ligands bind with the membrane associated Frizzled/LRP5/6 receptor complexes, after which the complexes dimerize. Subsequently, β-catenin is detached from a destruction complex, allowing its nuclear translocation and interaction with target genes. In the absence of Wnt ligand, β-catenin is retained in the destruction complex and cannot act as a transcriptional activator [44]. *Wnt4*$^{-/-}$ mice display partial female-to-male sex reversal, suggesting that Wnt4 promotes ovarian development. Transgenic XY mice overexpressing Wnt4 do not display male-to-female sex reversal, suggesting that Wnt cannot repress *Sry* activity [45–47].

Another factor involved in β-catenin upregulation is R-spondin 1 (RSPO1). Homozygous *Rspo1* KO mice fail to activate *Wnt4* and have a masculinized phenotype. Expression analysis in *Wnt4*$^{-/-}$ and *Rspo1*$^{-/-}$ mice prove that *Rspo1* is necessary for robust *Wnt4* activation in the developing gonad and that it functions as an upstream regulator of β-catenin stabilization.

Conditional β-catenin KO mice confirm its importance in ovarian development, as they display ovarian defects comparable to those observed in *Wnt4* and *Rspo1* KO mice. Overexpression of β-catenin in the somatic cells of developing XY gonads results in male-to-female sex reversal. These data underscore the importance of β-catenin as a key ovary-promoting and anti-testis gene. So far no human β-catenin mutations have been identified [48].

*FOXL2*

The *Forkhead box L2* (*FOXL2*) gene was first implicated in sex development by the identification of a deletion in the proximity of *Foxl2* in the polled intersex (PIS) goat. These goats are characterized by

polledness in both sexes and female-to-male sex reversal in XX goats [49]. Human *FOXL2* mutations are associated with the blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES), consisting of eyelid malformations associated or not with POI [50–52]. To date, no *FOXL2* mutations have been identified in 46,XX DSD cases. The BPES phenotype is mimicked in *Foxl2*$^{-/-}$ mice, displaying no sex reversal. *Foxl2*$^{-/-}$, *Wnt4*$^{-/-}$ double KO mice do display testicular differentiation, underscoring the relevance of a synergistic action of the two genes for ovarian development and inhibition of the testicular pathways [53].

Lack of ovarian development in XX individuals is expected to result in complete gonadal dysgenesis, and hence absent puberty in otherwise typical girls. This phenotype cannot be distinguished from early-onset POI on clinical grounds. Rarely, testicular development has been observed in XX individuals who have *RSPO1, NR5A1* or *WT1* mutations [54–59]. Such individuals may present with variable degrees of virilisation at birth. A distinct phenotype of ovarian hyperandrogenism and absence of uterine development has been found in women with *WNT4* mutations. These phenotypes are thought to result from incomplete suppression of the male sex determining pathway or male sex steroid biosynthesis respectively (see below) [60].

*Signaling at crossroads: interactions and transdifferentiation*

Over the years it has become increasingly clear that gonadal maintenance throughout life requires continued gene expression. This is accomplished by several inhibitory interactions between male and female specific genes [6,42,61].

The most important factor for ovarian maintenance seems to be FOXL2. Together with the estrogen receptors, FOXL2 inhibits *SOX9* expression in adult ovaries. Gene expression analysis in *Foxl2*$^{-/-}$ adult mice reveals upregulation of male specific genes like *Sox9* and ovaries of these mice contain testicular cord-like structures. The conditional *Foxl2* KO mouse model confirms these findings. Here, adult mice show transdifferentiation of granulosa and theca cells to Sertoli and Leydig cells respectively [62,63].

Similarly, testicular fate is also unstable; loss of *Doublesex and mab-3 related transcription factor 1* (*Dmrt1*) in mouse Sertoli cells reprograms them to a granulosa fate through activation of *Foxl2* [64]. These examples suggest that prolonged antagonism between male and female specific genes is required for gonadal maintenance (at least in mice).

*Androgen biosynthesis and action*

DSDs can also result from defects in steroid hormone biosynthesis or androgen action (Fig. 4). These conditions are all characterized by autosomal recessive inheritance – or X-linked inheritance in case of androgen insensitivity syndrome (AIS). Affected individuals have variable genital phenotypes, ranging from typically female to ambiguous. The most important genes involved in testosterone biosynthesis are those encoding the cytochrome P450 (CYP) enzymes, the hydroxysteroid dehydrogenase (HSD) enzymes and their cofactors, such as P450 oxidoreductase (POR) and cytochrome B6. Most of these enzymes are involved in the three major steroidogenic pathways, and therefore lead to combined sex steroid, mineralocorticoid and glucocorticoid deficiencies, resulting in various forms of congenital adrenal hyperplasia (CAH). The different enzymatic defects are reflected in slightly different phenotypes and distinct biochemical patterns. Adrenal insufficiency is usually the key finding that will ultimately lead to a hormonal or molecular genetic diagnosis [65]. Besides defects in adrenal steroidogenesis, DSDs can result from enzymatic defects in the final steps of testosterone biosynthesis; namely the conversion of androstenedione to testosterone by 17β-hydroxysteroid dehydrogenase 3 or the subsequent conversion of testosterone to DHT by 5α-reductase 2. Affected individuals have female or ambiguous external genitalia and absence of paramesonephric structures. These conditions may trigger a diagnostic trajectory either due to rapid virilization in adolescent girls, which has been attributed to the pubertal activation of iso-enzymes, or the finding of atypical sexual characteristics in neonates respectively. For more detailed information on CAH and steroid biosynthesis defects, we refer to a review by Mendonca et al. [65].

Inactivating *androgen receptor* gene (*AR*) mutations cause various forms of AIS, depending on the severity of the androgen insensitivity. In complete AIS (CAIS), AR function is completely abolished,

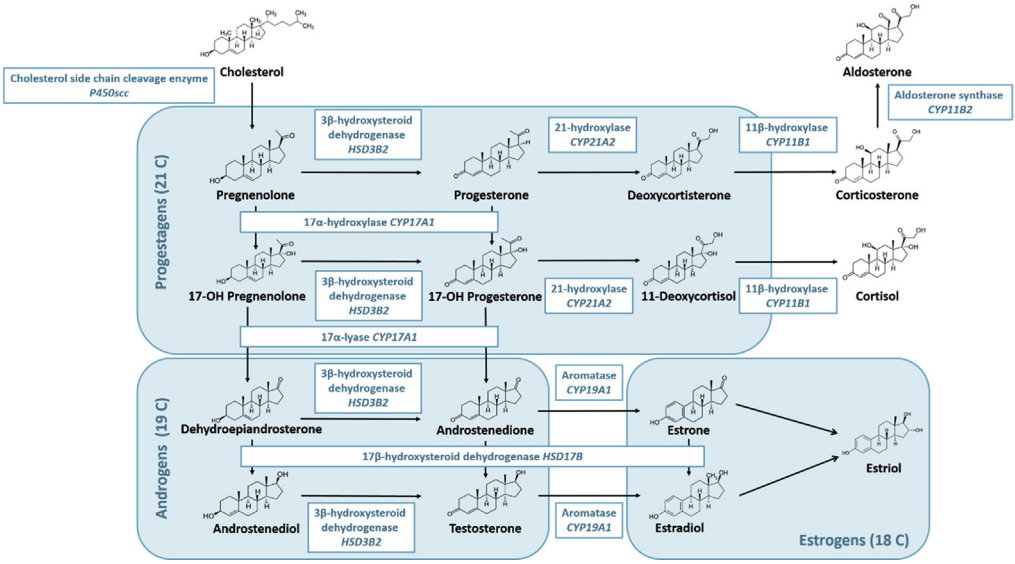


**Fig. 4.** Overview of the steroid biosynthesis pathway.

resulting in typical female external genitalia. The diagnosis is usually made prenatally, due to incongruence of the karyotype and phenotype, in childhood following surgery for inguinal hernia or inguinal masses, or in puberty, due to absence of menses. In contrast to XY girls who have complete gonadal dysgenesis, adolescents who have CAIS will experience breast development as excessive testicular androgens are aromatized to estrogens [66]. In partial AIS (PAIS) some residual AR activity exists, which may lead to the development of atypical, i.e. undermasculinized genitalia. Some men with *AR* mutations show typical male development but have impaired onset of puberty and development of secondary sexual characteristics and perturbed spermatogenesis. This is referred to as mild AIS (MAIS). So far, no clear-cut genotype–phenotype correlations have been established, however some trends can be observed. Mutations resulting in CAIS are often located in exon 1 and are mostly protein truncating (nonsense variants, frameshift variants, chromosomal rearrangements). Missense mutations in the ligand binding domain are more often identified in individuals with PAIS. Strangely, mutations resulting in MAIS are also mainly located in exon 1, more specifically between amino acid 214 and 511 [67]. In 15% of individuals who have CAIS and 65% of those who have PAIS, no *AR* mutation can be found, suggesting that unexplored non-coding mutations or mutations in other genes, like AR co-factors, are involved. Indeed, a recurring 5′UTR *AR* mutation found in CAIS illustrates the potential importance of non-coding regions in DSD pathogenesis. In such cases, an objective argument for defective AR signaling can be found by testing the responsiveness of patients' genital skin fibroblasts (GF) to DHT. In normal conditions, DHT induces upregulation of *apolipoprotein D* (*APOD*) expression. However, in approximately 1/3 of these mutation negative patients, reduced levels of *APOD* (AIS type II) are seen [68]. In some of these cases, the GFs show reduced *AR* expression as well. Most recently, an inverse correlation between *AR* expression and methylation of two CpG islands in the *AR* promoter has been observed, proposing defects in epigenetic regulation as a possible molecular mechanism for AIS [69].

## Human mutations associated with DSD

Table 1 gives an overview of currently known DSD genes. When possible both the human and the murine counterparts are given. When different phenotypes are associated to a certain gene, these are listed.

**Table 1**
Overview of the so far known human DSD genes and their associated phenotypes.

| Gene | Locus | Inheritance | Mouse Phenotype | Human Phenotype | | |
|---|---|---|---|---|---|---|
| | | | | Mutation type | DSD related | Other |
| Genes involved in 46,XY DSD | | | | | | |
| *AMH* | 19p13.3 | AR | *Amh*$^{-/-}$: males with internal male and female genitalia | Variable | Persistent Müllerian duct syndrome | / |
| *AMHR2* | 12q13.13 | AR | *Amhr2*$^{-/-}$: males with internal male and female genitalia | Variable | Persistent Müllerian duct syndrome | / |
| *AR* | Xq12 | X | Cre/lox conditional *Ar* knockout: female-like appearance, small testes | Variable | Complete and partial androgen insensitivity syndrome | / |
| *ARX* | Xp22.13 | X | *Arx*$^{-/-}$: arrest of Leydig cell differentiation, abnormal neuronal migration | Missense | GD | Lissencephaly, epilepsy, intellectual disability temperature instability |
| *ATRX* | Xq13.3 | X | *Atrx*$^{-/-}$: embryonically lethal Sertoli-cell-specific *Atrx*$^{-/-}$: hypoplastic testes and discontinuous seminiferous tubules, delayed onset of spermatogenesis | Variable | GD, absent Müllerian structures | Dysmorphic features, intellectual disability, α-thalassaemia |
| *BMP4* | 14q22.2 | AD | No gonadal phenotype observed | Missense | Hypospadias | / |
| *CBX2* | 17q25 | AR | *Cbx2*$^{-/-}$: XY male-to-female sex reversal, XX impaired ovarian development | Missense | 46,XY complete GD | / |
| *DHH* | 12q13.1 | AR | *Dhh*$^{-/-}$: disruption of testis cord formation due to abnormal peritubular tissue | Missense, frameshift variant | Complete or partial GD | Minifascicular neuropathy |
| *DMRT1* | 9p24.3 | AD | *Dmrt1*$^{-/-}$: impaired testicular development and loss of Sertoli and germ cells | | GD | Dysmorphic features, intellectual disability, microcephaly |
| *ESR2* | 14q23.2-q23.3 | ? | No gonadal phenotype observed | Missense, in-frame variant | GD, female external genitalia | Dysmorphic features, eye abnormalities, anal atresia, rectovestibular fistula |
| *FGFR2* | 10q26.13 | AD | *Fgfr2*$^{-/-}$: embryonically lethal *Fgfr2*$^{flox/+}$: XY male-to-female sex reversal | Missense | Complete GD | Crouzon-like craniosynostosis |
| *GATA4* | 8p23.1-p22 | AD | *Gata4*$^{-/-}$: embryonically lethal due to cardiac abnormalities *Gata4*$^{ki/ki}$: failure of testicular cord formation | Missense, deletion | GD, ambiguous genitalia, | Congenital heart defects (atrial septum defects, ventricular septum defects, tetralogy of Fallot), diaphragmatic hernia |

(*continued on next page*)

**Table 1** (*continued*)

| Gene | Locus | Inheritance | Mouse Phenotype | Human Phenotype | | |
|---|---|---|---|---|---|---|
| | | | | Mutation type | DSD related | Other |
| *HHAT* | 1q32.2 | AR | / | Missense | Complete GD | Dwarfism, chondrodysplasia, narrow, bell-shaped thorax, micromelia, brachydactyly, microcephaly with cerebellar vermis hypoplasia, facial anomalies, hypoplastic irides and coloboma of the optic discs |
| *HOXA13* | 7p15.2 | AD | No gonadal phenotype observed | Variable | Hypospadias in males, Müllerian duct fusion defects in females | Hand-foot-genital syndrome: limb abnormalities |
| *INSL3* | 19p13.11 | AD | *Insl3*$^{-/-}$: bilateral cryptorchidism, impaired fertility in both sexes | Missense, nonsense | Cryptorchidism | / |
| *MAMLD1* | Xq28 | X | Normal | Missense, nonsense | Hypospadias and micropenis | Myotubular myopathy |
| *MAP3K1* | 5q11.2 | AD | *Map3k1*$^{-/-}$:no gonadal phenotype observed<br>Map3k4$^{-/-}$: male-to-female sex reversal | Missense | Partial and complete GD | / |
| *NR0B1* (*DAX1*) | X21.3 | X | *Nr0b1*$^{-}$: impaired testis cord formation and spermatogenesis | Variable | GD with hypogonadotropic hypogonadism<br>Congenital adrenal hypoplasia | Congenital adrenal hypoplasia, cleft palate, intellectual disability |
| *NR5A1* | 9q33 | AD | *Nr5a1*$^{-/-}$: gonadal and adrenal agenesis<br>*Nr5a1*$^{+/-}$: impaired adrenal stress response, hypoplastic gonads | Variable | 46,XY GD<br>Hypospadias<br>Micropenis<br>Cryptorchidism | Primary adrenal insufficiency |
| *RXFP2* | 13q13.1 | AD | *Rxfp2*$^{-/-}$: cryptorchidism | Missense | Cryptorchidism | / |
| *SOX8* | 16p13.3 | AD | *Sox8*$^{-/-}$: reduced fertility<br>Sox8$^{-/-}$, Sox9$^{-/-}$: variable degree of male-to-female sex reversal | Missense, chromosomal rearrangements | GD | Male infertility, POI |
| *SOX9* | 17q24-q25 | AD | *Sox9−/−:* male-to-female sex reversal | Variable | GD | Campomelic dysplasia, Cooks syndrome, Pierre Robin sequence |
| *SRD5A2* | | | | | | |
| *SRY* | Yp11.3 | Y | *Sry*$^{-}$: male-to-female sex reversal | Missense, deletion | Complete GD | / |

| | | | | | | |
|---|---|---|---|---|---|---|
| *TSPYL1* | 6q22.1 | AR | / | Missense, insertion | GD | Sudden infant death syndrome |
| *WT1* | 11p13 | AD | *Wt1*$^{-/-}$: gonadal and adrenal apoptosis<br>*Wt1*+KTS$^{-/-}$: complete male-to-female sex reversal, renal insufficiency<br>*Wt1*+KTS$^{+/-}$: PT resembling Frasier syndrome<br>*Wt1*-KTS−/−: hypo- and dysplastic kidneys and streak gonads | Deletions<br>Missense<br>Splice site | WAGR syndrome: GD<br>Denys-Drash syndrome: GD<br>Frasier syndrome: GD | Wilm's tumor, aniridia, intellectual disability<br>Early-onset nephropathy, Wilm's tumor<br>Nephropathy in adolescence |
| *WWOX* | 16q23.1-q23.2 | AD | *Wwox*−/−: impaired steroidogenesis, impaired theca and Leydig cell development, postnatal lethality, bone growth defects<br>*Wwox*$^{gt/gt}$: atrophic seminiferous tubules and reduced fertility, shorter lifespan, increased B-cell lymphoma's | Missense, CNV | GD, ambiguous genitalia | / |
| *ZFPM2* | 8q22.3 | AD | *Zfpm2*$^{-/-}$: failure of testicular cord formation | Missense, deletion, translocation | GD, ambiguous genitalia, | Congenital heart defects (atrial septum defects, ventricular septum defects, tetralogy of Fallot), diaphragmatic hernia |
| *ZNRF3* | 22q12.1 | ? | *Znrf3*$^{-/-}$: XY partial or complete gonadal sex reversal | Missense, splice site | Partial and complete GD | / |
| Genes involved in 46,XX DSD | | | | | | |
| *BRCA2* | 13q13.1 | ? | *Drosophila Dmbrca2*$^{-/-}$: underdeveloped ovaries with fewer ovarioles and atypical egg chambers | Nonsense, frameshift | Complete ovarian dysgenesis, primary amenorrhea, hypergonadotropic hypogonadism | Microcephaly, café-au-lait spots, acute myelocytic leukemia |
| *CYP21A2* | 6p21.33 | AR | / | Variable | Virilization of the external genitalia | Adrenal insufficiency |
| *ESR2* | 14q23.2-q23.3 | ? | See above | Missense | Ovarian dysgenesis, primary amenorrhea | / |
| *FSHR* | 2p16.3 | AR | *Fshr*$^{-/-}$: disordered estrous cycles, ovulatory defects, atrophic ovaries, and a thread-like uterus | Missense | Hypergonadotropic ovarian dysgenesis | Primary ovarian insufficiency<br>Male infertility |
| *LHX1* | 17q12 | AD | *Lhx*−/−: normal ovaries, no uterus, oviducts and the upper region of the vagina | Missense | Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome | / |

(*continued on next page*)

**Table 1** (*continued*)

| Gene | Locus | Inheritance | Mouse Phenotype | Human Phenotype | | |
|---|---|---|---|---|---|---|
| | | | | Mutation type | DSD related | Other |
| *NR2F2* | 15q26.2 | ? | *Nr2f2*$^{-/-}$: embryonically lethal because of angiogenesis and heart defects<br>*Nrf2*−/+: reduced fertility | Frameshift deletion | Ovarian dysgenesis, ovotesticular DSD, ambiguous genitalia | Congenital heart disease |
| *NR5A1* | 9q33 | AD | See above | Missense | 46,XX testicular DSD<br>46,XX ovotesticular DSD | POI |
| *NUP107* | 12q15 | AR | *Nup107*$^{-/-}$: | Missense | Ovarian dysgenesis, absence of spontaneous puberty | Nephrotic syndrome |
| *PSMC3IP* | 17q21.2 | AR | *Hop2*$^{-/-}$: deficient gametogenesis | In-frame deletion | Ovarian dysgenesis | / |
| *RSPO1* | 1p34.3 | AR | *Rspo1*$^{-/-}$: XX mice display testicular vascularization and steroidogenesis | Missense | XX testicular DSD | Palmoplantar hyperkeratosis |
| *SOX3* | Xq27.1 | X | *Sox3*$^{+}$: follicular atresia, ovulation of defective oocytes, and severely reduced fertility | CNV | XX testicular DSD | Intellectual disability, hypopituitarism |
| SOX9 | 17q24-q25 | AD | *Sox9* overexpression: testicular development | Regulatory CNV | XX testicular DSD | See above |
| SOX10 | 22q13.1 | AD | Transgenic expression results in XX sex reversal | Duplications | XX testicular DSD, male external genitalia with hypospadias | Prominent eyes, borderline hypertelorism, epicanthic folds, gray right eyebrow, depressed nasal bridge, apparently low set ears, and hypotonia |
| SRY | Yp11.3 | Y | *Sry* translocation: XX female-to-male sex reversal | Translocation | XX testicular/ovotesticular DSD | / |
| WNT4 | 1p36.12 | AD/AR | *Wnt4*$^{-/-}$: XX gonads show testicular vascularization and androgen production Reduced amount of oocytes | Missense | Müllerian aplasia and hyperandrogenism<br>Sex reversal | SERKAL syndrome: sex reversal and kidney, adrenal, and lung dysgenesis |
| WT1 | 11p13 | AD | See above | Frameshift deletion, nonsense | Streak gonads, atypical female genitalia, clitoromegaly, short and blind-ending vagina | Nephrotic syndrome |
| WWOX | 16q23.1-q23.2 | AD | See above | Missense | Ovarian dysgenesis | / |
| Genes involved in steroid hormone biosynthesis | | | | | | |
| *AKR1C genes* | 10p15 | AR, multigenic | / | Missense, splice site | Cryptorchidism and undervirilized external genitalia, sex reversal, defective androgen synthesis | / |

| | | | | | | |
|---|---|---|---|---|---|---|
| *CYB5A* | 18q22.3 | AR | / | Splice site | Ambiguous genitalia | Methemoglobinemia |
| *CYP11A1* | 15q24.1 | AR | *Cyp11a1*$^{-/-}$: steroid hormone biosynthesis defects | Variable | Ambiguous to female external genitalia<br>Virilization in XX individuals | Adrenal insufficiency, salt wasting, hyponatraemia, hyperkalaemia, hypovolemia, acidosis, death in infancy |
| *CYP17A1* | 10q24.32 | AR | / | Variable | Ambiguous to female external genitalia in XY individuals, hypoplastic internal genitalia, cryptorchidism<br>Virilization in XX individuals | Decreased glucocorticoids, increased mineralocorticoids<br>High blood pressure, hypokalaemia |
| *CYP21A1* | 6p21.33 | AR | / | Variable | Virilization in XX individuals | Adrenal insufficiency |
| *HSD3B2* | 1p12 | AR | / | Variable | Undervirilization in XY individuals<br>Virilization in XX individuals | Possible salt wasting |
| *HSD17B3* | 9q22.32 | AR | / | Variable | Female-like or ambiguous genitalia with cryptorchidism | / |
| *POR* | | AR | *Cpr*$^{-/-}$: abnormal vasculogenesis and hematopoiesis, defects in brain and limb development | Variable | Undervirilization in XY individuals<br>Virilization in XX individuals | Antley-Bixler syndrome |
| *SRD5A2* | 2p23.1 | AR | / | Variable | Ambiguous genitalia, micropenis and prostate hypoplasia | / |
| *STAR* | 8p11.23 | AR | Star$^{-/-}$: female external genitalia in both sexes, adrenocortical insufficiency | Variable | Female external genitalia in XY individuals, cryptorchidism, underdeveloped internal genitalia | Adrenal insufficiency, salt wasting, hyponatraemia, hyperkalaemia, hypovolemia, acidosis, death in infancy |

AD = autosomal dominant, AR = autosomal recessive, DSD = disorders of sex development, GD = complete gonadal dysgenesis, CNV = copy number variant, GOF = gain of function, LOF = loss of function, POI = primary ovarian insufficiency, PT = phenotype, X = X-linked.

## Old genes, new functions - novel genes, new pathways

Genome wide sequencing technologies have facilitated the discovery of novel disease genes and new functional tools such as CRISPR/Cas9 gene editing enable to study the functional consequences of genetic variants more efficiently. As a consequence, novel DSD genes, and new roles for known DSD genes are increasingly reported and major progress has been made in understanding testicular development in 46,XX individuals.

### *Unraveling the molecular genetic background of 46,XX ovotesticular DSD*

#### *NR5A1 and WT1*

Almost simultaneously, three independent groups reported on the identification of the *NR5A1* Arg92Trp variant in nine individuals with 46,XX (ovo)testicular DSD [55–57]. Remarkably this variant affects the same codon as the previously identified Arg92Gln variant, which had been found in a patient with 46,XY DSD and adrenal insufficiency [70]. Furthermore, the Arg92Trp variant was also found in 46,XY undervirilized individuals and the Arg92Gln variant was identified in a 46,XX DSD case [56]. Very recently, a third 46,XX DSD associated variant was identified, Ala260Val [58].

Interestingly, these variants disrupt both the testicular and ovarian pathways. During ovarian development, NR5A1 and β-catenin form a complex that upregulates the *NR0B1* gene, which is involved in *SOX9* repression in female embryos. It has been hypothesized that the mutations resulting in 46,XX ovotesticular DSD impede the action of this NR5A1/β-catenin complex and thus undo the NR0B1-mediated repression of *SOX9*. *In vitro* assays in which the complex and a *NR0B1* promoter construct were co-transfected supported this hypothesis [58].

Another gene recently implicated in the pathogenesis of 46,XX ovotesticular DSD is *WT1*, formerly associated with 46,XY DSD. A *de novo* frameshift variant was identified in a girl with 46,XX testicular DSD, c.1453_1456del, p.(Arg485Glyfs*14). Structural protein remodeling suggests an increased activation of target genes, however these assumptions remain to be experimentally validated [59]. Previously, a *WT1* variant had also been described in a 46,XX girl with focal segmental glomerulosclerosis and streak gonads, adding additional arguments for the involvement of *WT1* mutations in XX gonadal development [71].

#### *BRCA2*

Very recently, the *BRCA2* gene, well known as breast and ovarian cancer predisposition gene, has also been involved in gonadal (ovarian) development and/or maintenance. Two compound heterozygous truncating variants were found in two sisters with XX ovarian dysgenesis, microcephaly and a history of leukemia in one of them. These variants resulted in reduced *BRCA2* levels and impaired DNA damage repair. It was hypothesized that the loss of DNA damage repair capacity induced specific somatic mutations affecting the rapidly dividing cells in the ovaries. However, the fact that both sisters have ovarian dysgenesis is less in favor of this somatic mutation hypothesis. Furthermore, *Dmbrca2−/−* - the fly orthologue of *BRCA2* - *Drosophilae* are sterile and both sexes are characterized by gonadal dysgenesis, suggesting that the ovarian dysgenesis is a direct consequence of *BRCA2* loss [72].

#### *NR2F2*

Bashamboo et al. reported on the identification of two novel, near-identical frameshift variants in *NR2F2* in three unrelated 46,XX children with a syndromic phenotype, including ovarian dysgenesis or (ovo)testicular development, congenital heart disease and BPES in two of them. Protein expression analysis in first-term human embryonic gonads showed that NR2F2 is present in the stromal cell population, and expression is mutually exclusive with FOXL2 [73]. Further functional validation should uncover the specific pathways in which NR2F2 acts and how it contributes to human sex development.

*ESR2*

Mutations in the *ESR2* gene, encoding the estrogen receptor β, have recently been associated with both 46,XY and 46,XX DSD. Our group identified a homozygous in-frame deletion in a 46,XY female, who in addition had anal atresia and eye abnormalities and two missense variants in women with isolated 46,XY DSD. Increased transcriptional activity for the deletion and one of the missense variants was shown, however the exact pathogenetic mechanism remains enigmatic [74]. Moreover, a heterozygous *ESR2* missense variant was found in a 46,XX girl with absent ovarian function, potentially implicating proper ESR2 signaling in both testicular and ovarian development and/or maintenance [75].

*New insights in the pathogenesis of 46,XY DSD*

*SOX8*

Portnoi et al. reported on the identification of two *SOX8* encompassing structural variants (SVs) and one missense variant in three unrelated 46,XY DSD girls. *In vitro* functional validation revealed that the mutant SOX8 protein cannot synergize with NR5A1 to promote target gene expression. Furthermore, *SOX8* variation was significantly higher in a cohort of individuals with male infertility or POI, suggesting a common etiology between these reproductive phenotypes [41].

*ZNRF3*

*Zinc and ring finger 3* (*ZNRF3*) has been put forward as a new important player in the mutual antagonism between the male and female pathways. ZNRF3 appears to antagonize WNT signaling, forms a direct target of RSPO1-mediated inhibition of testicular development and is necessary for murine sex determination. On the other hand, XY *Znrf3*$^{-/-}$ mice exhibit gonadal sex reversal and the observed phenotypes are associated with ectopic Wnt/β-catenin activity and reduced *Sox9* expression. These results confirm the potential of *Znrf3* to initiate testis development in mice. In humans, three *ZNRF3* variants have been found in girls with 46,XY complete gonadal dysgenesis. Functional validation in human cell lines and zebrafish embryos showed that two of these variants disrupt *ZNRF3* activity [76].

*Non-coding variation: the dark side of our genome*

Besides the importance of coding variation in the pathogenesis of DSD conditions, there is emerging evidence for the importance of regulatory regions in DSD pathogenesis. This is not surprising, given their role in the spatiotemporal expression of many genes implicated in sex development. A very recent example in humans is 46,XX or 46,XY DSD, caused by duplication or deletion of core enhancers upstream of *SOX9* [77]. A more exhaustive review on the involvement of the non-coding genome in DSD pathogenesis can be found in [78] and references therein.

## Molecular genetic diagnosis of DSD in a clinical setting

Following these technological advancements, the diagnostic approach for identifying the underlying molecular defect in individuals with a DSD condition has shifted from a sequential gene-by-gene approach to massively parallel sequencing of gene panels, targeted exome or genome sequencing (WES/WGS). The most adequate molecular genetic strategy will be individually determined based on initial karyotyping, the clinical phenotype, hormonal profiles, the familial history and the familial context (e.g. presence of consanguinity) [79,80].

Today panel sequencing, in which a set of known DSD genes is sequenced and for which robust coverage is guaranteed, is the first-tier approach. Different panels have been published, all of them reporting a diagnostic yield between 20.5 and 45%. An overview of the genes listed in these panels is given in [80–85]. Apart from single nucleotide variation (SNV) analysis, copy number analysis is

important to complete molecular diagnostics. Several algorithms for copy number assessment on MPS data are available. Given the decrease in the per-base sequencing costs, WES- or WGS-based panel testing will soon become routine practice. Systematic variant classification following the American College of Medical Genetics (ACMG) guidelines enables interpretation of variants, which is crucial for individual clinical management [86]. It is expected that WGS-based analyses will combine both SNV and SV assessment, both in coding and non-coding regions, thus representing a one-test-for-all approach. Apart from the data-analysis and challenging variant interpretation, costs for storage should also be considered [79,80]. If testing of known genes in a clinical context does not reveal (likely) pathogenic variants underlying disease, available WES and WGS data can be opened in a research context after consent of the patients. Current guidelines for genetic testing in DSD diagnostics recommend a targeted approach when the clinical phenotype and the biochemical results are suggestive for specific genes, and a massively parallel panel approach if this is not the case [79].

## Conclusion & future perspectives

Our understanding of the genetics and molecular mechanisms behind human sex development and DSD has significantly increased over the past decade, greatly facilitated by advances in MPS and novel tools for functional studies. This progress imposes new challenges to the field as recent findings emphasize that variation in one gene can result in different DSD phenotypes. This suggests that DSD conditions, and biological sex in general, cannot be seen as strictly separated entities but should be perceived as a spectrum. A possible explanation for this intrafamilial phenotypic variability is the influence of modifier genes and oligogenic inheritance, as was recently suggested for *NR5A1*-associated phenotypes. Werner et al. reported on the modifying role of *TBX2* variants on 46,XY DSD caused by a *NR5A1* mutation [87]. Camats et al. searched for modifying factors by performing WES on a cohort of 46,XY DSD cases with heterozygous *NR5A1* mutations. They analyzed a set of DSD genes and *NR5A1*-associated genes and found 19 potentially deleterious variants in 18 genes, which might contribute to the uniqueness of each of the investigated *NR5A1*-associated phenotypes [88]. This oligogenic, multiple-hit theory can probably be extrapolated to other DSD genes with phenotypic variability. At the same time, it poses an additional difficulty for the interpretation of genomic investigations in DSD. The inclusion of non-coding genomic data complicates interpretation even further. Tackling these challenges will provide new breakthroughs in the molecular underpinnings of human sex development and DSD conditions in the following years.

**Practice points**

- The diagnosis of DSD conditions is based on the interplay of three important pillars: physical examination, biochemical analysis and genetic tests, which are performed in parallel. The diagnostic work-up and care for a child with a DSD should be managed by a multidisciplinary team of experts, in which each member has specific know-how on (at least) one of the aspects of these conditions. Team members involved in the initial diagnostic phase include a (pediatric) endocrinologist, a (pediatric) urologist, a (child) psychologist and a clinical geneticist/clinical molecular geneticist.
- The approach for identifying the underlying genetic defect in individuals with a DSD condition is shifting from a gene-oriented approach in specific diagnoses to gene panel testing, based on massively parallel sequencing. Today, gene panel testing, in which known DSD genes are sequenced in parallel and for which robust coverage is guaranteed, is the first-tier approach, except in cases where a clear monogenic cause is suspected (e.g. 21-hydroxylase deficiency, androgen insensitivity).

**Research agenda**

- The search for molecular mechanisms involved in DSD has mainly focused on the coding part of the genome so far. Expanding the search window towards the non-coding genome will result in new mechanistic pathogenetic insight.
- Recent insight into the pathogenesis of 46,XX DSD has highlighted the fact that genes may have previously unanticipated roles in both XX and XY DSD, as well as in gonadal maintenance (e.g. *NR5A1* mutations can cause 46,XY DSD, 46,XX DSD and primary ovarian insufficiency). In that respect, DSD conditions could be considered as a spectrum rather than separate entities. These findings might also apply to other established DSD genes.
- Today DSD research is often limited in the available tools for *in vivo* validation of findings in relevant tissues such as human embryonic gonadal material, as access to such tissues is limited. Generation of human cellular model systems might overcome this limitation.

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