Molecular and cytogenetic diagnosis of disorders/differences of sex development: molecular update of genes controlling sexual differentiation

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Sex determination and differentiation is a unique overly complex biological process controlled by a concerted action of a large network of genes, growth factors, hormones, and environmental determinants. The precise dosage and accurate spatial and temporal expression of those genes are essential for the normal sexual development. Recently, the advent of high-throughput technologies has greatly revolutionized our understanding of genetic pathways controlling sex development. Nevertheless, the condition is still highly challenging, and a small subset of patients receive a definitive molecular diagnosis. Large-scale collaborative basic science projects have been establishment, together with international registries for setting up standardized guidelines and diagnostic approaches that depend on careful clinical evaluation, hormonal and imaging analyses, and cytogenetic and molecular diagnostic tools. This review discusses the recent updates and how application of recent technologies in a stepwise fashion can significantly enhance the disorders of sexual development diagnostic rates and potentiate the management outcomes. Establishing a precise genetic diagnosis greatly affects the patient and family quality of life and allows for personalized management, predicting future risk and expected interventions, and provides better family counseling.

Keywords:
CNV, DSD, long-read sequencing, next-generation sequencing, sex determination, tumor risk

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Introduction

Sex development is a unique biological process controlled by a large genetic network that acts in a concerted manner. The proper dosage and the accurate spatial and temporal expression of those genes are essential for completion of the final sexual phenotype (Hiort, 2013; Rey and Grinspon 2020). The term disorders of sexual development (DSD) include conditions with differences in the urogenital development, associated with an atypical chromosomal, gonadal, or phenotypic sex (Hughes *et al.*, 2006). The phenotypes are largely diverse, and obtaining an accurate diagnosis is always challenging. The observed phenotype variability could result from the wide range of causative genetic alterations that may be monogenic, digenic, oligogenic, or due to variants in noncoding region or even epigenetic modulations. Worldwide, there is a considerable lack of standardization of diagnostic approaches and genetic testing and the majority of patients with DSD do not receive an accurate diagnosis (Audi *et al.*, 2018). In recent decades, DSD had come to the focus of attention of scientists, clinicians, and psychiatrists, and recently, all the old stigmatizing terms have been omitted, even the term 'disorder' has become interchangeable with other terms such as 'difference or variations.' Over the past few years, the use of whole-exome sequencing (WES) has increased the diagnostic yield and successfully identified many novel mutations and new DSD genes (Parivesh *et al.*, 2019). The adoption of targeted sequencing strategy using candidate gene panels has proved to be a rapid economic and reliable choice (Hughes *et al.*, 2019). Whole-genome sequencing (WGS) is a more complex and laborious choice. Data output is huge and difficult to interpret in undiagnosed patients. On the contrary, the use of chromosomal microarray analysis (CMA) in patients with associated somatic anomalies was able to detect multiple novel copy number variants that could not be detected on the gene exon level (White *et al.*, 2011; Norling *et al.*, 2013). Although CMA is now used for clinical diagnosis, whole-exome and whole-genome methodologies are still under transition from basic research to routine clinical diagnostics (Guerrero-Fernández *et al.*, 2018). Nevertheless, result interpretations must be done with extreme caution and the detection of novel findings must be carefully validated with proper scientific steps

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for each patient with DSD with proper phenotype/genotype correlation (Parivesh *et al.*, 2019). Annex 1 shows the List of Abbreviations.

Genetic control of normal gonadal development

Genetic sex is determined after fertilization by establishing XY or XX chromosomal constitution, which derives the bipotential gonad to differentiate into a testis or an ovary. 'Sex determination' is designated to describe the gonadal differentiation during early fetal development. Once the gonad is developed, the products of the gonad determine the hormonal environment of the embryo promoting the differentiation of internal duct systems, and the formation of the external genitalia 'sex differentiation' (Suzuki *et al.*, 2002; Hiort, 2013) (Fig. 1).

Gonadal ridge development

During the fourth week after fertilization, the urogenital ridge develops and divides into a urinary and an adrenogonadal ridge, the latter forms the adrenal cortex and the gonads (Chassot *et al.*, 2012; Rey *et al.*, 2020). The expression of sufficient levels of *WT1* and *NR5A1* (*SF1)* genes is essential for early gonadal ridge differentiation (Hossain and Saunders, 2001). *SF1* is required for gonadal differentiation and steroidogenesis as well as for Leydig cell function, spermatogenesis, and development of ovarian follicle (Suntharalingham *et al.*, 2015; Bashamboo *et al.*, 2016). Other genes such as *EMX2* and *LHX9* are also required for the formation of gonadal ridge in humans (Wilhelm *et al.*, 2007; Yang *et al.*, 2018).

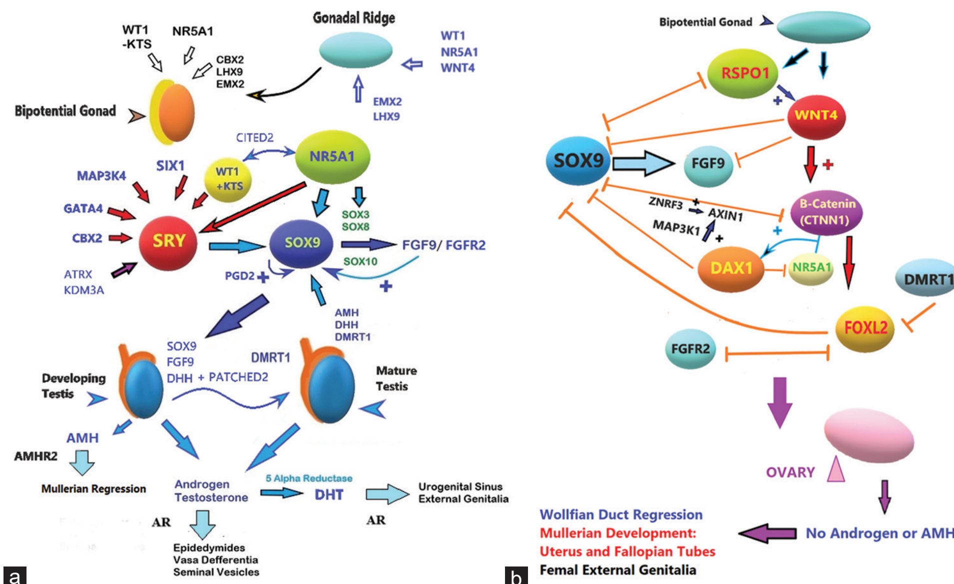
Primordial germ cells (PGCs)

At the fifth week, PGCs derived from pluripotent cells of the posterior proximal epiblast migrate to the gonadal ridges and colonize in the genital ridge. The bipotential PGCs start to express *DDX4* (a fertility factor), DAZL (could be crucial for spermatogenesis), and low levels of SYCP3 (a synaptonemal protein) (Hu *et al.*, 2015).

Genetic pathway of testicular differentiation

Sex-determining region on chromosome Y (*SRY)* gene was identified in 1990 and mapped to the Yp11.2 (Sinclair *et al.*, 1990). During the seventh week, a brief and transient *SRY* expression is triggered in the XY gonad to initiate a cascade of events, ultimately leading to testicular organogenesis. Testes become morphologically identifiable at 9–10 gestational weeks. *SRY* is activated by the + KTS splice variant of *WT1* (Hossain and Saunders, 2001), *SF1,* and a transcription factor *SP1* (Assumpção *et al.*, 2005; Buaas *et al.*, 2009). *WT1* gene interacts with *CITED2,* a transcription activator (Buaas *et al.*, 2009), to regulate the expression of *NR5A1(SF1)* gene and increase *SRY* levels. *GATA4* and *MAP3K4* genes are also implied in *SRY* activation (Miyamoto *et al.*, 2008; Warr *et al.*, 2012), whereas *KDM3A* and *ATRX* genes are involved in epigenetic regulation of *SRY* expression (Kuroki *et al.*, 2013; Tachibana, 2015). The precise timing and threshold level of *SRY*

Figure 1



(a) Most important genes involved in testicular determination and the male pathway. (b) Most important genes involved in ovarian determination and the female pathway.

expression are essential for upregulating the main target gene, *SOX9*, a transcription factor necessary for skeletal development and is a master regulator of testis determination (Hiramatsu *et al.*, 2009). *SRY* binds to multiple elements within gonad-specific enhancer in *SOX9* called 'TESCO' along with the steroidogenic factor-1 (*SF1*). Once *SOX9* has reached a specific expression level, it enhances *FGF9* expression, which results in a feed-forward loop maintaining high *SOX9* levels. Other members of the SOX family such as *SOX3*, *SOX8*, and *SOX10* also interact with *SF1* to maintain *SOX9* expression (Polanco *et al.*, 2010; Zhen-Yu and Wan-Xi, 2017). The repression of ovarian gene *FOXL2* by *SOX9* (Wilhelm *et al.*, 2009) and the increased *FGF9* threshold result in downregulation of *WNT4* expression, leading to progression of the male pathway and inhibition of the female pathway. In absence of *SRY* gene, *SOX9* is silenced and the female pathway proceeds (Grinspon and Rey, 2016). Primitive Sertoli cells start development and expression of *SOX9, DHH,* and *FGF9* (Yao *et al.*, 2002; Cory *et al.*, 2007; Werner *et al.*, 2015), which are involved in Leydig cell differentiation, in addition to growth factors, like NGFs and PDGFs (Basciani *et al.*, 2010; Barrionuevo *et al.*, 2012). Under the control of *SF1, MAMLD1* is expressed in fetal Sertoli and Leydig cells (Fukami *et al.*, 2008). Leydig cells also start to secrete the androgen testosterone, which further directs testicular morphogenesis as well as different aspects of male differentiation. Desert hedgehog (*DHH*) in association with its receptor, *PATCHED2*, triggers Leydig cell differentiation (Yao *et al.*, 2002; Barsoum *et al.*, 2009) and may be involved in Sertoli-peritubular cell interaction and deposition of basal lamina (Pierucci Alves *et al.*, 2001). *HHAT* gene induces post-translational modifications of *DHH*. Mutations in *HHAT* were found to cause complete 46, XY gonadal dysgenesis with a female phenotype (Callier *et al.*, 2014). *DAX1 (NR0B1)* gene that maps to the dosage-sensitive sex-reversal region on Xp21 is essential for normal testicular cord formation (Park *et al.*, 2008). Although *DAX1* expression persists in the developing ovary, it becomes downregulated before *SRY* expression peaks by about 10 days (Guo *et al.*, 1995; Hanley *et al.*, 2000). However, its continued expression in Sertoli cells may help spermatogenic cell development in response to steroids and pituitary hormones. Moreover, *DAX1* is essential for the development of a functioning hypothalamus-pituitary-gonadal axis. Duplication of *DAX1* antagonizes the synergistic action of *SF1* and *SRY* on *SOX9,* resulting in 46, XY DSD (Ludbrook *et al.*, 2012; García-Acero *et al.*, 2019). Finally, *ARX* is an X-linked gene identified in infants with lissencephaly and genital abnormalities and may lead to a block in Leydig cell differentiation (Kitamura *et al.*, 2002).

Genetic pathway of ovarian differentiation

In the absence of *SRY* in XX gonads, *WNT4* expression increases, leading to stabilization of β-catenin (*CTNNB1)* and silencing of *FGF9* and *SOX9* (Kim *et al.*, 2013; Zhen-Yu and Wan-Xi, 2017). *SF1* and β-catenin form a complex that upregulates the *DAX1* gene (Mizusaki *et al.*, 2003), which antagonizes *SF1* and thereby inhibits steroidogenic enzymes required for testosterone production. *WNT4* is also involved in the internal genital tract development. Similarly, *RSPO1* is expressed in the bipotential gonadal ridge and increases in the XX gonads in the absence of *SRY*. *RSPO1* stimulates the expression of *WNT4* and leads to increase of the cytoplasmic β-catenin (Tomizuka *et al.*, 2008). β-catenin also activates *FOXL2* transcription factor, and through antagonizing *WT1*, *FOXL2* further represses *SF1* expression (Biason-Lauber, 2012; Ungewitter and Yao, 2013; Takasawa *et al.*, 2014). The high levels of WNT4/β-catenin and *FOXL2* impede *FGF9* and *SOX9*, leading to ovarian pathway stabilization (Lin and Capel, 2015). *FOXL2* has been recently found to be required for maintenance of the granulosa cell lineage in ovaries (Gomes *et al.*, 2020). A novel function of *RSPO1* is the independent regulation of estrogen receptor expression (Geng *et al.*, 2020). Loss-of-function mutations in the human *RSPO1* gene were reported with ovotesticular DSD, probably owing to upregulation of *SOX9* (Tomaselli *et al.*, 2008; Grinspon and Rey, 2016). However, *FOXL2* mutations result in a range of phenotypes, including streak gonads and ovarian failure associated with blepharophimosis, ptosis, and epicanthus inversus (BPES) (De Baere *et al.*, 2001; Beysen *et al.*, 2009). Recently, COUP-TF2 (*NR2F2*) gene has been identified as a suppressor of *SOX9* gene in early gonadal embryogenesis; in addition, *CBX2* has also been found to have a role in ovarian function and in regulation of genes associated with ovarian maintenance, suggesting that *CBX2* is essential for gonad formation in both sexes (Bouazzi *et al.*, 2019). Fetal ovarian development begins at 50–55 days of embryonic life, and by 20 weeks, seven million germ cells are formed. Of them, only 400 ovulate in adults, whereas the majority undergo atresia. Annex 2 shows the most important Genes Involved in Gonadal Determination and differentiation.

Sex differentiation

Fetal Leydig cells could be identified by the start of the eighth week (Codesal *et al.*, 1990) and soon begin to produce testosterone (by 63 days), which results in stabilization of the Wolffian ducts and differentiation of the vas deference, epididymis, and seminal vesicles and masculinization of external genitalia (Wilhelm

et al., 2007). On the contrary, anti-Müllerian hormone (*AMH*), produced by fetal Sertoli cells, acts through its specific type II receptor (*AMHR2*) to preclude Müllerian differentiation through local diffusion, resulting in their regression. Insulin-like growth factor 3 (*INSL3*), produced by Leydig cells, is responsible for the transabdominal testicular descent, whereas movement through the inguinal canal is mediated by androgens (Ahmed *et al.*, 2013). The differentiation of fetal Leydig cells depends initially on PDGFs, secreted by Sertoli cell (Ivell *et al.*, 2013), whereas further differentiation and proliferation depends on hCG, secreted from the placenta, in first and second trimesters, and then on the effect of fetal LH on the LH/CG receptor (Qiaoand Han, 2019). 5α-reductase enzyme converts testosterone to dihydrotestosterone (DHT), which promotes virilization of the external genitalia (Auchus, 2004). In XX embryo, Mullerian and external genital development occurs independent of ovarian differentiation owing to absence of testosterone and AMH. Müllerian ducts form the uterus, fallopian tubes, and upper third of the vagina with regression of Wolffian ducts. *NR2F2* is also responsible for eliminating the Wolffian duct in female embryos, independently of the androgen absence (Zhao *et al.*, 2017).

Classification of DSD

DSD could result from genetic defects in the pathways responsible for gonadal determination or owing to defects in hormone production or sensitivity. The International Consensus Conference, organized by the Lawson Wilkins Pediatric Endocrine Society (LWPES) and the European Society for Pediatric Endocrinology (ESPE), 2006, had adopted a simplified classification and terminology 'disorders of sex development' or 'DSD' in place of previously used stigmatizing descriptions (Hughes *et al.*, 2006).

Diagnostic approaches to patients with DSD

Standardization of clinical assessment of DSD individuals has been recommended by the European Cooperation in Science and Technology (COST) Action 'DSDnet'. The Consensus Statement provided recommendations depending on lifelong evidence-based experience for DSD care and data collection across ages (Audi *et al.*, 2018; Parivesh *et al.*, 2019). Large-scale collaborative basic science projects (as the EuroDSD project) have been established, together with international registries, such as the I-CAH Registry and I-DSD Registry and the DSD Translational Research Network for setting up standardized guidelines and DSD diagnostic approaches (Guerrero-Fernández *et al.*, 2018). The recommended guidelines included the following:

Primary clinical evaluation

Informed consent

Patients or parents should be thoroughly informed before conducting any diagnostic and/or therapeutic procedure. Whenever possible, it is recommended to postpone the decision of performing irreversible procedures, such as genital surgery till the patient is old enough to participate actively in the decision.

History taking and physical examination

(1) Personal history of parental consanguinity, prenatal exposure to androgens or androgen antagonist, maternal virilization during pregnancy, and other complaints.
(2) Family history of genital ambiguity, hypospadias, salt-losing crisis or infant deaths, infertility, amenorrhea, or premature menopause.
(3) Physical examination of the external genitalia with virilization grading using the Quigley scale (Quigley *et al.*, 1995). Pubertal assessment using the Tanner classification (Tanner *et al.*, 1969) and anthropometric measurements should be also performed. Examination of other body system is important for ruling out any associated somatic abnormalities.
(4) Sex identity and sex role evaluation by masculinity/femininity scale is important for proper sex assignment (Dessouky, 2001; Ercan *et al.*, 2013).

First-line diagnostic tests

Karyotype analysis of peripheral blood lymphocytes is essential as a first-line diagnostic evaluation to initially diagnose the patient into one of the three main DSD categories.

Hormonal evaluation

(1) Neonatal screening for 21-hydroxylase deficiency congenital adrenal hyperplasia (CAH) by measuring 17-hydroxyprogesterone (Speiser *et al.*, 2018).
(2) For 46, XX DSD, hormonal assay of serum testosterone and its precursors, ACTH and urine cortisol level for the diagnosis of pituitary-adrenal function.
(3) For 46, XY DSD, measurement of basal and post-HCG serum testosterone to assess Leydig cell function and its precursors and DHT is

indicated for classification of defects of hormonal biosynthesis and measurement of AMH and inhibin B to assess Sertoli cell function.
(4) Serum FSH and LH in cases of gonadal dysgenesis (Mazen *et al.*, 2008).
(5) Measuring the ratio of the urine concentrations of precursor metabolites and the steroid products of the suspected enzymatic defect could be more sensitive than blood levels.

Abdominal ultrasonography

It is indicated for detection of the type and position of gonads as well as uterus and vagina (Steven *et al.*, 2012).

Second-line diagnostic tests

Imaging and laparoscopic approach

Genitogram or cystoscopy/vaginoscopy is recommended for assessment of the genitourinary system. Laparoscopic examination with gonadal biopsy taking is essential for diagnosis of ovotesticular DSD (OT DSD) and for assessment of the type of gonadal tissue and early detection of gonadoblastoma and for prophylactic gonadectomy (Liu *et al.*, 2014; Faure Conter *et al.*, 2019; Rangarajan *et al.*, 2021).

Cytogenetic and molecular approach

A stepwise cytogenetic and molecular approach should follow the primary clinical, hormonal, and imaging evaluation. This will be discussed in detail in the next section.

Chromatographic mass spectrometry

Currently, biochemical workup is strongly recommended, as it can detect the underlying pathology and the required hormonal treatments, even if genetic diagnosis was not established. Chromatographic mass spectrometry is recommended for the precise measurement of steroid hormone (Oliveira *et al.*, 2020). Although this method is not yet widely available, standardization between laboratories has been started.

Psychological support

After the primary medical evaluation and information about the condition, continued psychosocial support is often required, especially in the process of diagnosis and decision making. Communication within a patient's social network might also help in providing support, acceptance, and social advices (Pasterski *et al.*, 2014).

Genetic counseling should discuss management options and helps decision making, taking into consideration to assess Leydig cell function expected morbidities, tumor formation, and future fertility. It should also address recurrence risk and prenatal diagnosis options (Helmy and Mohamed, 2014).

Cytogenetic and molecular diagnosis of DSD

Conventional cytogenetic analysis

Approximately 75% of patients with DSD have a 46, XY karyotype, whereas 46, XX karyotype is detected in ~10–15% (Juniarto *et al.*, 2016) [Table 1]. The remainder has numerical or structural anomalies of the sex chromosomes. The major advantage of karyotyping is that it provides the opportunity to study the whole genome in one set and can detect the balanced chromosomal rearrangements, which is not available in other techniques including copy number variation (CNV) methods or wide genome molecular techniques; in addition, it can precisely detect mosaicism. Nevertheless, the karyotype resolution is limited to about 5 Mb (Yatsenko and Witchel, 2017).

QF-PCR

It is a noninvasive prenatal diagnosis (NIPD) that can identify Y-specific markers. It is indicated for urgent

Table 1 Main DSD categories

Sex chromosome DSD	Turner's syndrome: 45, X and variants, including mosaicism
	Klinefelter syndrome: 47, XXY and variants
	45, X/46, XY: mixed gonadal dysgenesis and ovotesticular DSD
	46, XX/46, XY chimerism; ovotesticular DSD
	Structural sex chromosomal abnormalities
46, XX DSD	Gonadal dysgenesis: Female gonadal dysgenesis Testicular DSD Ovotesticular DSD
	Androgen excess: Fetal 21 hydroxylase deficiency Fetal 11 hydroxylase deficiency Fetoplacental aromatase deficiency Maternal luteoma Exogenous androgen excess
	Others: MURCS, MRKH syndrome
DSD 46, XY	Gonadal dysgenesis: Complete gonadal dysgenesis Partial gonadal dysgenesis Gonadal regression Ovotesticular DSDs
	Disorders in androgen synthesis or action: 5 alpha reductase deficiency Androgen insensitivity syndrome AMH and AMH receptor defects
DSD associated with other somatic anomalies	For example, syndromes of multiple X or other X linked or autosomal gene abnormalities as ATRX syndrome, Campomelic dysplasia and Frasier syndrome, respectively

AMH, anti Müllerian hormone; DSD, disorders of sexual development

genetic diagnosis if DSD is suspected prenatally based on family history or if fetal genital ultrasound is discordant with the genetic sex. QF-PCR could be conducted on cell-free fetal DNA in maternal peripheral blood from the sixth week if parents consider pregnancy termination (if legislation permits) (Atef *et al.*, 2011; Zargari *et al.*, 2011).

Fluorescence in-situ hybridization (FISH) technique

FISH is a major diagnostic tool in the workup of patients with DSD. It depends on the use of fluorescently labeled specific DNA sequence probes and hybridization with the patient DNA in a chromosomal preparation. The resolution of the FISH technique is about 100 kb–1 Mb, which is markedly higher than conventional karyotyping. Different types of probes are commercially available, which has greatly increased the accuracy of detection of chromosomal abnormalities. FISH can use cells in any stage of the cell cycle as well as archived tissue (Kamel *et al.*, 2015). Moreover, FISH is the best to precisely detect mosaicism through facilitating the study of a huge number of nuclei using the fluorescent enumeration X and Y centromere signals (Maciel-Guerra *et al.*, 2012).

Sanger sequencing

Sanger sequencing is a traditional method to study coding regions (exons), flanking regions, and sometimes promoter regions of a candidate gene. It is a targeted approach for mutational analysis of known DSD genes. It is always performed for diagnosing monogenic causes of DSD (Soliman *et al.*, 2015; Hassan *et al.*, 2016; Jiang *et al.*, 2020). The diagnostic yield of individual candidate gene sequencing is generally high if biochemical analysis suggests an abnormality of a specific pathway, but if the condition is not clear as in testicular dysgenesis, the molecular diagnosis is about 20% (Hughes *et al.*, 2006). The most commonly studied DSD genes by the Sanger sequencing method include *SRD5A2*, *HSD17B3*, *AR*, *CYP21A2*, *CYP11B1*, *SF1*, and *WT1* (Hiort *et al.*, 2014). Sanger sequencing method is limited by being laborious and can only analyze one gene at the time. However, it is still widely used in laboratories and is useful for segregation of a specific gene variant in a family and for validation of high-throughput sequencing methods (HTS) such as DSD panels, WES, and WGS.

CNV analysis

Multiplex ligation-dependent probe amplification (MLPA)

It is a multiplex PCR method that can precisely identify CNVs in specific DSD-associated loci with detection of breakpoint site (Schouten *et al.*, 2002). Multiple studies detected variable CNVs in DSD genes using the MLPA analysis including *DAX1* duplication (García-Acero *et al.*, 2020), partial *NR5A1* gene deletion (Barbaro *et al.*, 2011; Nagy *et al.*, 2019), and *CYP17A1* deletion in CAH (Turkkahraman *et al.*, 2015). Moreover, a heterozygous duplication of entire *SOX9* coding region and ~483 kb upstream was reported in OT DSD using the MLPA technique (López-Hernández *et al.*, 2018).

Quantitative real-time PCR

A real-time (RT) PCR, also known as quantitative PCR (qPCR), and reverse transcription PCR (RT-PCR) is a PCR-based technique that is able to amplify a target DNA sequence with quantification of the concentration (in real time). It depends on sequence-specific probes that are labeled with a fluorescent reporter and is frequently used in evaluating DNA copy number, SNP detection, and allelic discrimination and also mRNA expression, when preceded by reverse transcription PCR. The main applications are confirmation of MLPA and microarray gene expression data (Segundo-Val and Sanz-Lozano, 2016; López-Hernández *et al.*, 2018). RT-PCR is a precise method for measuring DNA concentration in multiple samples at a time, with provision of immediate results. A disadvantage is that fluorescence-detecting thermocyclers are expensive; thus, it is especially useful in large commercial laboratories (Bustin *et al.*, 2009).

Chromosomal microarray techniques (CMA): aCGH and SNP array

It is a molecular cytogenetic technique that allows the detection of whole genome copy number changes below the threshold of a standard karyotyping (<5 Mb). The gene content of imbalances can be thus established and the OMIM morbid genes could be blotted against the patient's phenotype (genotype/phenotype correlation). In some genetic diagnostic centers, CMA has replaced the traditional karyotype, whereas in others, it is used as a second-line tool for DSD associated with malformations or other system involvement. The basic principles and techniques of SNP array are similar to the array CGH, but it favors it by detecting single nucleotide polymorphism (SNP) all over the genome. SNP array enables genotyping in addition to the standard CNVs data; thus, its main advantage is that it can determine both CNVs and LOH (loss of heterozygosity) and can detect mosaicism (Keren, 2014).

Clinical utility: it has been recently recognized that recurrent, clinically significant regions of CNV may be found in ~13–20% of individuals of syndromic and nonsyndromic DSD, with a higher diagnostic yield in the syndromic type. (Tannour-Louet *et al.*, 2010;

White *et al.*, 2011; Igarashi *et al.*, 2013; Baetens *et al.*, 2014). In addition, novel rearrangements including known DSD genes and other genomic areas were found in many patients with syndromic DSD (Le Caignec *et al.*, 2007; Schlaubitz *et al.*, 2007; van Silfhout *et al.*, 2009). A relatively common deletion in chromosome 9p24.3, encompassing *DMRT1* gene, was reported in 46, XY DSD cases with gonadal dysgenesis and female phenotype, with a varying severity according to the deletion size (Tannour-Louet *et al.*, 2010; Ledig *et al.*, 2012; Igarashi *et al.*, 2013). 9q33.3, encompassing variable sequence lengths of *SF1* gene, is another deletion region reported in 46, XY patients with female or ambiguous external genitalia and testicular dysgenesis or ovotestis. Duplications of Xp21.1 region encompassing *DAX1* were also detected in 46, XY DSD cases with a female phenotype and gonadal dysgenesis (White *et al.*, 2011; Ledig *et al.*, 2012).

Rearrangements of noncoding sequences disturbing gene regulation may account for a significant proportion of DSD (white *et al.*, 2011). Copy number losses involving the upstream regions of *SOX9*, *GATA4*, and *DAX1* genes have been reported in patients with isolated 46, XY gonadal dysgenesis (Benko *et al.*, 2011; White *et al.*, 2011; Kim *et al.*, 2015). In addition, Kim *et al.* (2015) identified duplications upstream of *SOX9* gene in three families with an isolated 46, XX DSD. A noncoding regulatory region located in a gene desert 517-595 kb upstream of the *SOX9* promoter has been proposed to contain a gonad-specific *SOX9* transcriptional enhancer (Benko *et al.*, 2011). Recently, a novel gonadal regulatory element upstream of *SOX9*, enhancer 13 (Enh13), was found to be essential for initiation of testicular differentiation in mouse (Gonen *et al.*, 2018).

CMA could also detect CNVs encompassing novel genes or chromosomal locations for DSD. A commonly deleted region, spanning *MAMLD1* gene, has been identified in individuals with 46, XY DSD associated with myotubular myopathy (Bartsch *et al.*, 1999). Subsequently, further studies have confirmed that mutation of *MAMLD1* may contribute to a small subset of 46, XY DSD (Tsai *et al.*, 2005; Fukami *et al.*, 2006). The role of *SOX3* was identified by Sutton *et al.* (2011) who screened 16 SRY-negative 46, XX male patients for CNV and identified rearrangements encompassing or in close proximity to the *SOX3* gene in three patients: two of them had duplications encompassing *SOX3*, and the third patient had a single microdeletion immediately upstream of *SOX3*, suggesting that altered regulation is the cause of the phenotype. Sutton *et al.* (2011) concluded that *SOX3* has a role in the pathway of sexual development and that *SOX3* and SRY are functionally interchangeable in sex determination. In a large cohort study of 116 patients with idiopathic DSD, chromosomal rearrangements were detected in 21.5%, which encompassed known DSD genes (*SRY* or *DMRT1)* as well as novel ones such as *FGFR2*, *KANK1*, *ADCY2*, and *ZEB2* (Tannour-Louet *et al.*, 2010). Using a customized 1 M array CGH platform, Norling *et al.* (2013) have identified five novel candidate genes for XY gonadal dysgenesis in two out of nine patients (22%), including interstitial duplication of the *SUPT3H* gene and a deletion of *C2ORF80* in two DSD siblings and a large 9q21.11 duplication encompassing *PIP5K1B*, *PRKACG*, and *FAM189A2.* All the five genes were found to be expressed in testicular tissues, and one is shown to cause gonadal DSD in mice. Many candidate chromosomal rearrangements with a potential role in DSD-related phenotypes have been reviewed by Sreenivasan *et al.*, 2018. They included deletions of 9p23-24 encompassing *DMRT1* and *KANK1* in XY gonadal dysgenesis, deletions at 12p13 and 16p11.2 in hypospadias, deletions in 1p36.3, 9p24.3, and 19q12-13.1 in ambiguous genitalia, and deletions in 10p14 and Xq28 in cryptorchidism. Copy number gains at Xq28 encompassing the *VAMP7* gene have been reported in newborns with congenital genitourinary tract masculinization disorders (Tannour-Louet *et al.*, 2010, 2014; Chávez-López *et al.*, 2020).

Custom-designed, target gene-specific array is a recent powerful tool for high-resolution detection of genomic CNVs, through customized array designs. The possibility of increasing the probe density loaded on chips allowed detection of genomic changes at a single-exon level, with more precise breakpoint determination (Piluso *et al.*, 2011; White *et al.*, 2011; Norling *et al.*, 2013). They have been recently used to study the potential pathogenicity of small CNVs as well as copy-neutral regions of homozygosity in DSD genes (Amarillo *et al.*, 2016). Using this method, the pathogenicity evidence of recent DSD genes such as *WWOX* was strengthened and genetic imprinting was described as a novel recurrent pathogenic mechanism in DSD. Other regions were suggested to be related to epigenetic mechanism and RNA expression during gonadal development (Parivesh *et al.*, 2019).

Gene expression analysis and methylation studies: Recently, there have been reports of changes in the methylation patterns of the key sex determination genes in animal DSD. An example is persistent hypermethylation of Sry gene with a resultant downregulation in mRNA and protein expression that was experimentally documented as the cause of ovotestis and female phenotype in Sry-positive XY dogs (Jeong *et al.*, 2016). Methylation studies of the promoter regions of Sox9, Sox3, and Wnt4

genes in XX DSD dogs with ovotestis and testis phenotypes revealed hypomethylation in Sox9 and hypermethylation in Wnt4 and in Sox3 to the levels similar to XY dogs (Salamon *et al.*, 2017). On the contrary, recent studies in humans using genome-wide transcriptome analysis and DNA methylation profiling revealed that dosage-sensitive sex-chromosome genes regulate co-expression networks of sex chromosome dosage-sensitive autosomal genes with diverse domains of critical cellular functions and specific immune activation pathways are upregulated by supernumerary X chromosomes (Raznahan *et al.*, 2018; Skakkebæk *et al.*, 2018). Methylation studies can help understand the phenotypic variability in cases of X chromosome aneuploidy and X- autosomal translocations.

High-throughput screening techniques (HTS)

Individual gene sequencing is being widely replaced by HTS strategies. Next-generation sequencing (NGS), deep sequencing, or massively parallel sequencing are alternative terms that describe a high-throughput DNA sequencing technology that depends on sequencing of millions of DNA fragments in parallel. Each of the genome bases (three billion) is sequenced multiple times to provide the high depth required for delivery of accurate data. Many NGS platforms have been developed in recent years providing an easier technology with higher depth and a lower cost. NGS could be used to sequence the entire genome (whole genome) or all of the 22 000 coding genes (whole exome) or a panel of candidate genes and genomic regions of interest (targeted NGS-based panels) (Guerrero-Fernández *et al.*, 2018; Parivesh *et al.*, 2019; Mazen *et al.*, 2021).

Targeted NGS-based panels: a panel of candidate genes and genomic regions of interest with a strong coverage is increasingly considered as a rapid and less time consuming front-line tool for diagnosing patients with DSD. Targeted exome sequencing has been proved to be efficient and overweighs Sanger sequencing in the diagnostic yield and can diagnose complex patients with multiple etiologies (Hughes *et al.*, 2019). However, this approach has the limitation of incomplete genome coverage. Variable results were obtained from different gene panels, ranging from 28 to 45% diagnostic yield, depending on the chosen genes and the patient number and selection criteria (Eggers *et al.*, 2016; Fan *et al.*, 2017; Kim *et al.*, 2017; Ozen *et al.*, 2017; Hughes *et al.*, 2019). As more panels are selected and more patients are studied, the data provided will continue to grow with more informed management strategies. The ability of NGS to detect CNVs depends on depth of coverage designed to work with WGS or WES data. In addition, SNP sequencing backbone, when combined with a gene mutation panel, allows for simultaneous detection of gene mutations and genome-wide CNVs (Shen *et al.*, 2019).

Whole Exome Sequencing: WES strategies are applied to the whole-genome coding regions and have the benefit of the ability to identify novel DSD genes. The use of WES as a first-line approach would result in a much higher amount of data necessitating thorough bioinformatics analysis with lower gene coverage that might lead to missing of pathological variants. It detects multiple gene variants of unknown significance (VUS), which should be carefully analyzed, complemented with other genetic and hormonal diagnosis. Parental samples should be also requested to confirm that the mutations are de novo variants or inherited from heterozygous parents, if homozygous. It was strongly recommended by COST Action that all obtained molecular DSD data, in addition to clinical and biochemical data, should be prospectively collected in international databases such as I-DSD Registry (*http://www.i-dsd.org*) to improve the future diagnostic algorithm of patients with DSD. During the recent years, WES has been progressively improving with decreasing of prices, and more data are published with more improvement of bioinformatic tools. This makes WES one of the most important and valuable methods in understanding genes that control variable DSD pathologies and complex phenotypes. WES has led to discovery of novel DSD genes such as *FOG2/ZFPM2*, *HHAT*, *FGFR1*, *NR2F2*, *ZNRF3*, *SOX8*, and *DHX37* (Bashamboo *et al.*, 2014; Callier *et al.*, 2014; Mazen *et al.*, 2016; Bashamboo *et al.*, 2018; Harris *et al.*, 2018; Portnoi *et al.*, 2018, McElreavey *et al.*, 2020, respectively). It has also detected novel variants in known DSD genes associated with novel phenotypes such as for *NR5A1* (Baetens *et al.*, 2017; Bashamboo *et al.*, 2017; Swartz *et al.*, 2017) and confirmed the pathogenicity of variants in previously suspected genes (Werner *et al.*, 2015; Ben Hadj Hmida *et al.*, 2016; Baetens *et al.*, 2017). Moreover, a subset monogenic disorders suspected by clinical, hormonal, and imaging studies may not reveal any disease-causing mutations in the exons of the corresponding gene. A new model for abnormal AR-5~UTR translation that mediates repression of the normal ORF translational protein was described by Hornig and colleagues in two unrelated patients with complete androgen insensitivity syndrome The authors highlighted the importance of 5~UTR mutations affecting uORFs for the pathogenesis of monogenic disorders in general (Hornig *et al.*, 2016 a, b).

Whole Genome Sequencing: as many of the DSD-related genes are transcriptional regulators such as *SRY*, *SOX9*, *NR5A1*, and *FOXL2* genes, it could be

hypothesized that a subset of undiagnosed patients with complex forms of DSD may result from variants affecting noncoding regulatory elements (Baetens *et al.*, 2017). In addition, pathogenic variants in control regions can lead to the same phenotypes that result from mutations in a gene-coding region. WGS will be able to detect CNVs in noncoding regulatory regions with a higher diagnostic yield. Nevertheless, with taking into consideration that the exome constitutes slightly over 1% of the genome, WES is thus much more cost-effective than the entire genome. Furthermore, the data output from WGS is huge and need a sophisticated bioinformatic analysis. Approximately three to four million variants different from the human reference genome are obtained from WGS compared with 15 000–20 000 variants within exons. Most of these variants are nonpathogenic, and only 1–2% are potentially associated with a phenotype (Kolesinska *et al.*, 2018). Multiple filters should be applied for the selection of deleterious variants including nonsense mutations, frameshift mutations, insertions and deletions, and canonical splice site variants. It is very recommended to use family trios (parents and affected child) to facilitate the process of variant filtering. The pathogenic relevance of novel genes is strengthened when changes in the same gene are detected in other individuals with DSD and in independent families and not in control databases of ancestrally matched populations (Parivesh *et al.*, 2019; Roca *et al.*, 2019; Shen *et al.*, 2019).

Long-read sequencing (LRS): although NGS technology has reduced the sequencing cost significantly, the short-read amplification has a bias of the inability to identify variants of larger sequences (Chaisson *et al.*, 2015; Hehir-Kwa *et al.*, 2018; Lavrichenko *et al.*, 2021). Recently, the advent of third-generation LRS such as single-molecule real-time (SMRT) sequencing (Pacific Biosciences, PacBio) (California, United States) and nanopore sequencing (Oxford Nanopore Technologies, ONT, Oxford, United Kingdom) has focused on overcoming challenges faced by NGS. It has also the advantage of transcriptome analysis by sequencing full-lengths of cDNA molecules, thus enabling detection of regions encompassing splice junction abnormalities, multiple-mapping loci, and repeat-regions all over the coding and the noncoding transcriptome (Kuo *et al.*, 2017). LRS has been successfully used in many neurodegenerative diseases resulting from microsatellite repeats expansion (Liu *et al.*, 2017; Ebbert *et al.*, 2018). Compared with Illumina SRS, LRS could effectively recognize details of breakpoints in novel chromothripsis rearrangements and detect the parental origin and structural abnormalities with precise delineation of novel variants resulting from retrotransposon insertions in two patients with congenital abnormalities (CretuStancu *et al.*, 2017; Vollger *et al.*, 2019). Nevertheless, application of LRS in clinical diagnosis is still in its early steps. It has strong limitations owing to the computing time and bioinformatic resources. PacBio technology is highly accurate, but time consuming, whereas. Nanopore sequencing is fast but lacks base-calling accuracy. They are being developed to overcome these limitations before widespread application (Volden *et al.*, 2018).

Optical genome mapping: it depends on using optical imaging of fluorescently labeled megabase-size DNA molecules immobilized in a specialized nanochannel arrays (Hastie *et al.*, 2017). Long DNA molecules throughout the genome are labeled at specific sequence motifs, using restriction enzymes (Barseghyan *et al.*, 2018). The fluorescent tag patterns of each sample allele are then assembled with scaffold length values of above 60 Mbps (~10.5 Mbps for Nanopore) (Jain *et al.*, 2018). A map of the sample-specific pattern is compared with a reference in silico-digested maps that enables identification of large and complex rearrangements. Optical mapping has been successfully used in identification of potentially pathogenic structural variants in population-specific genome assemblies (Seo *et al.*, 2016; Shi *et al.*, 2016) and in muscle-wasting disorders such as Duchenne muscular dystrophies (Barseghyan *et al.*, 2017) with 100% diagnostic concordance rates (Barseghyan *et al.*, 2017). The main advantage of optical mapping over CMA is the higher resolution and the ability to identify balanced rearrangements. Although it is not yet applied in clinical diagnosis, Barseghyan *et al.* (2018) are working on employment of positional cloning for DSD diagnosis. It was also recently recommended that the integration of WGS with these technologies will tremendously improve precision and identification rates and increase the optical mapping's breakpoint uncertainty to less than 140 bp (instead of 3–5 kb) (Chaisson *et al.*, 2019), which could enable survey of all types of genomic variations in the near future.

Conclusion

The primary care of patients with DSD requires a multidisciplinary team of subspecialists, who work in close collaboration with a strong peer support network. This team should include an expert clinical geneticist who will direct the required genetic workups. Specialized pediatric and older patients' services should be available around the world, rolled out by a professional team who would address any health-related issues, answer questions about sex and future fertility, expected risk or health problems in later stages of life, as well as provide

genetic counseling. The genetic approach to a patient with DSD should be a complementary one, starting with karyotyping, followed by a stepwise sequencing of molecular analyses that include a high-throughput technology or CMA in patients with associated somatic abnormalities.

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Annex 1 List of abbreviations

Abbreviation	Full name
(5′-UTR)	5′ untranslated region
aCGH	Array comparative genomic hybridization
ACTH	Adrenocorticotropic hormone
AMH	Anti-Müllerian hormone
AMHR2	Anti-Muellerian hormone type-2 receptor
ATRX	Alpha-thalassemia mental retardation syndrome
CAH	Congenital adrenal hyperplasia
CMA	Chromosomal microarray analysis
CNV	Copy number variation
DHEA	Dehydroepiandrosterone
FISH	Fluorescence in-situ hybridization
FSH	Follicle-stimulating hormone
GSK3β	Cyclin-dependent kinase-5 and glycogen synthase kinase-3β
LH	Luteinizing hormone
LOH	Loss of heterozygosity
MLPA	Multiplex ligation-dependent probe amplification
MRKH	Mayer-Rokitansky-Küster-Hauser syndrome
MURCS	Mullerian-renal cervicothoracic-somite association
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
Q-F PCR	Quantitative fluorescence PCR
RT-PCR	Real-time PCR
SNP Array	Single nucleotide polymorphism array
TESCO	Testis-specific enhancer of So×9 core
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
β-hCG	Beta-human chorionic gonadotropin

Annex 2 Genes involved in gonadal differentiation

Gene	OMIM#	Chromosomal location	Expression	Function	Associated phenotype	Inheritance	References
WT1 (Wilms' tumor-associated gene 1)	607102	11p13	Urogenital ridge derivatives	Encodes a zinc finger DNA binding protein with transcriptional and post-transcriptional regulating capacity. WT1 is required for normal formation of the genitourinary system and mesothelial tissues	Denys-Drash syndrome	AD	Hossain and Saunders (2001); Wagner *et al*. (2002)
					Frasier syndrome		
					Wilms' tumor, type 1		
CITED2 (CBP/ p300-interacting transactivator, with glu/asp-rich c-terminal domain, 2)	602937	6q24.1	Widespread	WT1 cofactor, regulating SF1 expression in the adreno-gonadal primordium	Atrial septal defect 8	AD	Buaas *et al*. (2009)
					Ventricular septal defect 2		
NR5A1 (nuclear receptor subfamily 5, group a, member 1)	184757	9q33.3	Gonadal ridges, adrenal gland primordia, hypothalamus, and pituitary	Stabilization of intermediate mesoderm, and transcriptional regulation of several genes (StAR, steroid hydroxylases, aromatase, AMH, DAX1, and many other)	46, XX sex reversal	AD	Suntharalingham *et al*. (2015); Bashamboo et al. (2016)
					46XY sex reversal 3		
					Adrenocortical insufficiency		
					Premature ovarian failure7		
					Spermatogenic failure 8		
SRY (Sex-Determining Region Y)	480000	Yp11.2	genital ridge	Encodes a transcription factor that is a member of the high mobility group (HMG) box family of DNA binding proteins. It binds to SOX9 male sex determination to initiate of the male pathway	46XX sex reversal 1	XLD	Sinclair *et al*. (1990)
					46XY sex reversal 1	YL	
SOX9 (SRY-BOX 9) (a member of SOX 'SRY - related HMG box' gene family)	608160	17q24.3	Widespread	Transcription factor that plays a key role in chondrocytes differentiation and skeletal development and testis determination. With SF1, it regulates transcription of the AMH gene	Acampomelic campomelic dysplasia	AD	Hiramatsu *et al*. (2009)
					Campomelic dysplasia		
					Campomelic dysplasia with autosomal sex reversal		

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Annex 2 Contd...

Gene	OMIM#	Chromosomal location	Expression	Function	Associated phenotype	Inheritance	References
SOX3 (SRY-BOX 3)	313430	Xq27.1	Widespread	SOX3 is closely related to SOX1 and SOX2, all belong to the SOXB1 subfamily and are expressed throughout the developing central nervous system (CNS). So×3 induced testis differentiation mice by upregulating expression of So×9 via a mechanism similar to Sry	46, XX Sex Reversal 3		Sutton *et al*. (2011)
GATA4 (GATA-binding protein 4)	600576	8p23.1	Widespread	GATA-binding proteins are a group of transcription factors that control gene expression and differentiation. Regulation of coelomic epithelium thickening	Testicular anomalies with or without congenital heart disease	AD	Miyamoto *et al*. (2008)
					Atrial septal defect 2, Atrioventricular septal defect 4, Tetralogy of Fallot, Ventricular septal defect 1		
ZFPM2 (FOG2) (zinc finger protein, multitype 2) or	603693	8q23.1	Widespread	Thought to modulate the transcriptional activity of Gata4, a regulator of early testis development	46XY sex reversal 9	AD	Bashamboo *et al*. (2014)
FGF9 (Fibroblast growth factor 9)	600921	13q12.11	Gonads and several other tissues	Upregulation of SOX9 and downregulation of WNT4	Tetralogy of Fallot		Harris *et al*. (2019)
FGFR2 (FGF receptor 2)	176943	10q26.13	Gonads and several other tissues	Upregulation of SOX9 and downregulation of WNT4	Antley-Bixler syndrome		Siggers *et al*. (2014)
					Apert syndrome		
					Pfeiffer syndrome		
					Crouzon syndrome		
DMRT1 (Doublesex-related and mab3-related transcription factor-1)	602424	9p24.3	Gonads and several other tissues	encodes a male-specific transcriptional regulator with a conserved zinc finger-like DNA binding domain, the (DM domain), a key factor in sex determination and differentiation Antagonizes FOXL2	46XY sex reversal 4 (46, XY gonadal dysgenesis, partial or complete, with 9p24.3 deletion)		Brunner *et al*. (2001); Ledig *et al*. (2012)
MAP3K4 (mitogen-activated protein kinase kinase kinase 4)	602425	6q26	Widespread	Haploinsufficiency of Map3k4 disrupted early expression of Sry in mice leading to XY sex reversal			Warr *et al*. (2014)
CBX2 (Chromobox homolog gene 2; or M33 mouse homolog of)	602770	17q25.3	Widespread	Regulation of homeotic genes. Have a role in genital ridges development Represses WNT4 signaling	46XY sex reversal 5		Bouazzi *et al*. (2019)
NR2F2 (Nuclear receptor subfamily 2, group f, member 2; COUP-TFII)	107773	15q26.2	Widespread	The encoded protein is a ligand inducible transcription factor that is involved in the regulation of many genes	46, XX sex reversal 5 Congenital heart defects	AD	Bashamboo *et al*. (2018)

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Annex 2 Contd...

Gene	OMIM#	Chromosomal location	Expression	Function	Associated phenotype	Inheritance	References
DHH (Desert hedgehog signaling molecule)	605423	12q13.12	Sertoli cells (testis), Schwann cells (peripheral nerves)	The hedgehog gene family encodes signaling molecules that play an important role in regulating morphogenesis	46XY sex reversal 7		Pieruccin*et al*. (2001)
					46XY gonadal dysgenesis with minifascicular neuropathy		
HHAT (hedgehog acyltransferase)	605743	1q32.2	Widespread	It encodes an enzyme that acts within the secretory pathway to catalyze amino-terminal palmitoylation of 'hedgehog'			Callier *et al*. (2014)
DHX37 (DEAH-BOX HELICASE 37)	617362	12q24.31	in testes, epididymis, Leydig cell cytoplasm and in germ cells at different stages of maturation.in	It encodes a DEAH-box proteins, implicated in a number of cellular processes Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division	46, XY sex reversal 11	AD	McElreavey *et al*. (2020)
MAMLD1 (Mastermind-like domain containing 1)	300120	Xq28	specific expression in fetal Sertoli and Leydig cells around the critical period for sex development	It encodes a mastermind-like domain containing protein. This protein may function as a transcriptional co-activator. high levels of expression in skeletal muscle	Hypospadias 2, X-linked X-linked myotubular myopathy		Fukami *et al*. (2008)
ATRX (Alpha-thalassemia/ mental retardation syndrome, Helicase 2, X-Linked)	300032	Xq21.1	Widespread	Nucleotide excision repair and initiation of transcription	Alpha-thalassemia/ mental retardation syndrome Mental retardation-hypotonic facies syndrome, X-linked		Gibbons and Higgs (2000)
KDM3A (Lysine-Specific Demethylase 3A)	611512	2p11.2	Testis, ovary, kidney, lung, heart, brain, liver, skeletal muscle, pancreas, and spleen	Demethylases histone H3 (epigenetic regulation by modification of chromatin conformation)	–		Kuroki *et al*. (2013)
VAMP7 (Vesicle-Associated Membrane Protein 7)	300053	Xq28		It encodes a transmembrane protein localizes to late endosomes and lysosomes and is involved in the fusion of transport vesicles to their target membranes. Alternate splicing results in multiple transcript	duplication and overexpression plays a role in the human masculinization disorders of the urogenital tract leading to cryptorchidism, hypospadias	XLR	Tannour-Louet *et al*. (2014); Chávez-López *et al*. (2020)
DAX1 (NR0B1) (Nuclear receptor subfamily 0, group B, member 1).	300473	Xp21.2	Gonads, pituitary, adrenals	Antagonizes SRY, SOX9. Essential for normal testicular and ovarian development and for proper formation of the adult adrenal gland	46XY sex reversal 2, dosage-sensitive		Guo *et al*. (1995); García-Acero *et al*. (2019)

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Annex 2 Contd...

Gene	OMIM#	Chromosomal location	Expression	Function	Associated phenotype	Inheritance	References
					Adrenal hypoplasia, congenital		
AXIN1 axis inhibitor 1	603816	16p13.3		It has both positive and negative regulatory roles in Wnt-beta-catenin signaling.in absence of Wnt signaling, it promotes polyubiquitination and degradation of cytoplasmic beta-catenin			Lui *et al.* (2011)
WNT4 (wingless-type mmtv integration site family, member 4)	603490	1p36.12	Widespread	Wnt signaling stabilizes β-catenin, thus antagonizes the β-catenin destruction complex. Wnt4 upregulates Da×1	female sex reversal and dysgenesis of kidneys, adrenals, and lungs (SERKAL syndrome)	AR	Kim *et al.* (2013)
					Mullerian aplasia and hyperandrogenism	AD	
CTNNB1 (catenin, beta-1)	116806	3p22.1	Widespread	An adherens junction protein. critical for the establishment and maintenance of epithelial layers; Upregulates WNT4, FST and FOXL2			Kim *et al.* (2013)
MAP3K1 (Mitogen-activated protein kinase kinase kinase 1)	600982	5q11.2	Widespread	MAP3K1 is a mitogen-activated protein kinase. It directly activates MAPK8 and MAP2K4. Generates anti-apoptotic signaling as a full-length protein, but it induces apoptosis following cleavage by caspases	46XY sex reversal 6	AD	Mazen *et al.* (2016)
AMH (Anti-Mullerian hormone)	600957	19p13.3	Enriched expression in testicular tissue	Results in regression of Mullerian ducts	Persistent Mullerian duct syndrome, type I	AR	Mazen *et al.* (2017)
AMHR2 (Anti-Mullerian hormone receptor, type II)	600956	12q13.13		It binds the ligand on their own but require the presence of a type I receptor for signal transduction	Persistent Mullerian duct syndrome, type II		Mazen *et al.* (2017)
RSPO1 (R-SPONDIN 1)	609595	1p34.3		RSPO1 is produced by fibroblasts and regulates keratinocyte proliferation and differentiation. It synergize with WNT4 in XX gonads	Palmoplantar hyperkeratosis and true hermaphroditism		Biason-Lauber (2012)
					Palmoplantar hyperkeratosis with squamous cell carcinoma of skin and sex reversal		
FOXL2 (forkhead transcription factor FOXL2)	605597	3q22.3	specifically expressed in eyelids and in fetal and adult ovarian follicular cells	is the earliest known marker of ovarian differentiation in mammals and may play a role in ovarian somatic cell differentiation and in further follicle development and/or maintenance			Ungewtter and Yao (2013)

Genes involved in gonadal determination and differentiation.