**differences of sex development in the newborn: from clinical scenario to molecular diagnosis**

**Differences of sex development in the newborn: from clinical scenario to molecular diagnosis**

**Running title: Clinical and molecular features of DSDs**

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We searched PubMed for articles published in English between Jan 1, 2010, and March, 2021, using the MeSH terms “sexual development, “disorders of sex development” and “ambiguous genitalia”. Preference was given to practice guidelines, international consensus statements. Relevant papers from reference lists of the chosen papers where added.

**Abstract**

Differences/disorders of sex development (DSD) are defined as a group of congenital conditions in which the development of chromosomal, gonadal or anatomical sex is atypical. The incidence of DSD is 1:4500 births. The current classification divides DSDs into 3 categories according to chromosomal sex: 46,XX DSD, 46,XY DSD and sex chromosome DSD. DSD phenotypes can be concordant with the genotype (apparently normal external genitalia associated with gonadal dysgenesis), or can range from simply hypospadias to completely masculinised or feminised genitalia with a discordant karyotype. Numerous genes implicated in genital development have been reported. The search of genetic variants represents a central element of the extended investigation, as an improved knowledge of the genetic aetiology helps the immediate and long-term management of children with DSDs, in term of sex of rearing, hormone therapy, surgery, fertility and cancer risk. This review aims to assess the current role of molecular diagnosis in DSD management.

Keywords: atypical genitalia, differences of sex development, disorders of sex development, molecular diagnosis.

## Introduction

Disorders or differences[1] of sex development (DSD) are defined as a group of congenital conditions in which the development of chromosomal, gonadal or anatomical sex is atypical [2]. The term DSD was introduced at the Chicago Consensus Conference in 2005 [2]. The incidence of DSD is estimated to be 1 in 4500 births, but varies among different ethnic groups and seems to be higher in the southern African population [3]. Sex development occurs in 2 phases "sex determination" and "sexual differentiation" [4,5]. Female and male embryos show a bipotential morphologically identical gonadal primordium until gestational week 6, as both Wolfian and Mullerian ducts coexist [6]. "Sex determination" starts when the undifferentiated gonad is stimulated to differentiate into testicle or ovary according to sex chromosomes [7]. Sexual determination depends on multiple factors, such as genetics and environment [8]. "Sexual differentiation" is the process leading to phenotypic sex development through the action of gonadal hormones [4]. Testicular hormones, such as testosterone and insulin-like peptide 3 (INSL3) produced by Leydig cells, play an essential role in the development of male internal genitalia (epididymis, vas deferens, seminal vesicles), and testicular descent [9,10]. The conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase promotes the development of male external genitalia (penis, scrotum and prostate). The production of anti-Müllerian hormone (AMH) by Sertoli cells stimulates the regression of the Müllerian ducts. The lack of AMH secretion allows the development of Müllerian ducts into uterus, Fallopian tubes, and upper third of vagina in females, while the regression of Wolffian ducts is promoted by the chicken ovalbumin upstream transcription factor II (COUP-TFII). The absence of high levels of testosterone and DHT prevents the virilization of the external genitalia in the female foetus [11].

Normal sex development results from the action of activating and repressing factors in a precise space-time pattern [12]. Genetic mutation in any of these factors can result in a DSD.

DSD diagnosis and management are complex for clinicians and demand a multidisciplinary team approach [13]. The expert team should include a paediatric endocrinologist, a geneticist, a psychiatrist or psychologist, a radiologist expert in US, and a surgeon [12,14,15]. Several issues need to be assessed,

such as the possible development of an adrenal crisis or the assignment of the sex of rearing. Additionally, DSDs may be part of complex genetic syndromes characterized by multiple malformations [16].

Several diagnostic algorithms for DSD have been published [4,12,17–22].

The aim of this review is to gather the most recent evidences on DSDs in the newborn and to underline the role of molecular diagnosis in the management of the disease.

## Classification

The classification divides DSDs in 3 categories [2,3].

- 46,XX DSD that includes virilized females and XX sex reversal;
- 46,XY DSD that includes patients with abnormal testicular differentiation and XY sex reversal;
- sex chromosome DSD that includes Turner Syndrome, Klinefelter Syndrome, 45,X/46,XY gonadal dysgenesis, and ovotesticular disorders.

## Diagnosis

Diagnosis should be based on family and perinatal history, physical examination, biochemical, molecular and histological data (Figure 1 A, B and C). Molecular diagnosis is essential in the management of a child with DSD, as it provides information about gonadal function during foetal and postnatal life, potential future fertility and cancer risk. Furthermore, it may give the possibility to offer a genetic counselling to the parents.

Medical history

A family tree should be always assessed in order to recognize a specific pattern of inheritance. For example, X-linked recessive mode of inheritance is suggestive of androgen insensitivity syndrome (AIS). In contrast, parental consanguinity, family history of salt-losing, unexplained infant deaths or DSDs may suggest one of the autosomal recessive disorders of steroidogenesis. Maternal history of infertility or miscarriage should be investigated. An androgen-secreting tumour or placental aromatase deficiency can lead to virilisation of a female embryo [23]. Finally, maternal exposure to drugs or toxins during early pregnancy may inhibit foetal virilization (i. e. progesterone in male embryos) [24].

Clinical presentation

DSDs may present with a genital phenotype concordant with the karyotype and dysgenetic gonads (i. e. 46,XX individual with normal external and internal female genitalia but dysgenetic ovaries). Nevertheless, more commonly DSD phenotypes result from defects of masculinization in 46,XY foetuses (ranging from simple hypospadias to female genitalia) or abnormal virilization of 46,XX foetuses (from simple hypertrophy of the clitoris to apparently male genitalia with "apparently undescended" testes) [12]. The presence of atypical genitalia is the major clinical sign in patients with 46,XY DSD and 46,XX DSD. However, mild forms of DSD may present with less severe clinical manifestations that challenge the decision to consider the patient affected by a DSD [12]. Other signs and symptoms may present later, such as delayed/absent puberty, or infertility in Klinefelter and Turner syndromes [3]. Wide phenotypic variation exists in patients with chromosomal mosaicism ranging from a phenotypically male with cryptorchidism and/or hypospadias, to a phenotypically female with gonadal dysgenesis. In the same patient a dysgenetic testis on one side may co-exist with an ovary or a streak gonad on the opposite side and differently represented Mullerian structures. Interestingly, 90–95% of patients with a 45,X/46,XY karyotype are phenotypically normal males [25,26] .

Congenital adrenal hyperplasia (CAH) due to 21 hydroxylase deficiency is the most common form of 46,XX DSD. Due to the high risk of life-threatening adrenal insufficiency in this condition, the first step in the evaluation of a newborn with atypical genitalia, is the evaluation of general condition, hydration, electrolyte balance, blood glucose and blood pressure.

The evaluation of the external genitalia

The evaluation of the external genitalia should be accurate in order to recognize any asymmetry or hyperpigmentation, the presence of a cloacal exstrophy, apparently male genitalia associated with bilateral undescended testes, micropenis and/or hypospadias, apparently female genitalia associated with enlarged clitoris, urogenital sinus, labioscrotal fusion ad/or palpable gonads [12,15].

Stretched dorsal length and diameter of the phallus/tubercle should be measured and the presence of chordee, as well as the location of the urethral meatus should be reported [2]. Penile length is measured from the base of the penis under the pubic symphysis to the tip of the glans with the penis gently stretched. If pubic fat tissue is abundant, depression is required so that the ruler touches the pubic symphysis to avoid a falsely reduced measurement. The average stretched penile length of a full term newborn baby is 3.5 (± 0.7) cm [27]. For the assessment of a preterm neonate, the typical penile length can be calculated using the formula –2.27 + (0.16 × weeks of gestation) [28]. Micropenis is defined as a stretched penile length 2.5 standard deviations below the mean for the age group. Micropenis not associated with cryptorchidism and/or hypospadias, is unusual in DSDs, while it may be suggestive of isolated gonadotropin-releasing hormone deficiency [29]. It is also important to evaluate the anogenital distance, as a biomarker of prenatal exposure to androgens [2,3]. A shorter anogenital distance occurs in patients with cryptorchidism and hypospadias due to abnormalities in foetal testicular development [30]. It is recommended that all patients with isolated perineal hypospadias or any form of familial hypospadias should be evaluated for DSDs [12,21,31].

Generally if gonads are palpable, they are more probably testes, or, occasionally, ovotestes, as ovaries tend to remain in the abdomino-pelvic position [32]. For this reason, apparently normal female

external genitalia with palpable gonads suggest a complete androgen insensitivity syndrome. At the opposite, the significant virilization of the external genitalia with bilateral non-palpable gonads may suggest an abnormally virilized female with CAH [3].

Labioscrotal hyperpigmentation is associated with excessive ACTH and proopiomelanocortin levels in patients with CAH [33]. Various degrees of labioscrotal fusion may be present with posterior fusion of the *labia majora*, partially fused hemiscrotum or completely fused scrotum. In addition, it is important to evaluate clitoris size. In newborn girls, the typical clitoral length ranges from 2.0 to 8.5 mm (mean 4.0 mm), the clitoral width from 2.0 to 6.0 mm (mean 3.32 mm), and the clitoral index (length x width) is 13.3 mm [34].

Prader's virilization stages [35] (figure 2) and the External Masculinisation Score [36] (figure 3) are the most used scoring systems, which have been used to objectively describe the degree of virilization in newborn females and the masculinization defects in newborn males, respectively [12,21,24]. In addition, the External Genitalia Score [37] (figure 4) has been recently proposed in both sexes, to evaluate the appearance of the genitalia more comprehensively across the phenotypic spectrum. Anogenital distances (AGDs) have been proposed as markers of androgenization in female and hypovirilization in male newborns, respectively [37,38] .

Associated anthropometric and/or facial abnormalities, mid-line defects, major malformations of heart, kidneys and brain, anomalies of limbs, fingers and toes may provide important information to orient genetic tests, since approximately 25% of DSD cases are part of a complex genetic syndrome [8,12,16].

Laboratory

Perinatal history and physical examination should guide laboratory investigations [21]. **First line tests** include serum electrolytes, 17-hydroxyprogesterone (17-OHP), testosterone, gonadotrophins (LH, FSH), AMH, plasma renin activity, androstenedione, DHT and urine analysis [3,21]. Hormone and electrolyte levels change during the first weeks of life. Gonadotrophins, androgens and precursors may be detectable just after birth (24-36 hrs) and between the 2nd and the 6th month of life (during the so-called "minipuberty") [10], so it is important to consider an early neonatal collection of blood and urine as well as further samples collected at a later stage during minipuberty.

Infants with salt-losing forms of CAH usually show electrolyte abnormalities after the second week of life and serum 17-OHP is usually unreliable during the first 36 hours of life [24]. Where newborn screening [39] for CAH is not available, blood and urine sample for steroid profile should be collected before starting replacement therapy. Plasma or serum steroids should be measured by liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) or immunoassays after organic solvent extraction. LC-MS/MS allows the analysis of multiple analytes from a single sample while maintaining analytical specificity [40]. Urinary steroid profile (USP) analysis by gas chromatography mass spectrometry (GC-MS) provides qualitative and quantitative data on the excretion of steroid metabolites. The results of these initial investigations shall often dictate the second line tests [21].

Sex chromosome analysis should be performed as early as possible, preferably by a rapid quantitative fluorescent polymerase chain reaction or fluorescent in-situ hybridization (FISH) analysis including probes for SRY, followed by complete karyotyping including hidden mosaicisms. This test defines the category of DSD [2].

**Second-line investigations** are often needed in the diagnostic workup of DSDs [21]. The human chorionic gonadotrophin (hCG) stimulation test [8,13] is useful to assess testicular function after minipuberty (when the hypothalamic-pituitary-gonadal axis is physiologically suppressed) and to

detect the presence of defects in testosterone biosynthesis [24]. In combination with the USP, the results of the hCG test can be informative to direct the diagnosis of 46,XY DSDs [24]. ACTH stimulation test may be required when a rare defect of adrenal and/or gonadal steroidogenesis is suspected [13].

Imaging

Aims of imaging studies are to evaluate not only gonads, pelvic, perineal and inguinal regions, but also kidneys and adrenal glands, due to the well-known association between DSDs, renal and adrenal defects [12]. Ultrasound (US) is the first-choice method to study inguinal gonads, the presence of ovaries and uterus, and abnormalities of the adrenal glands and kidneys. US has the strength of being radiation free, but the weakness of being operator-dependent. US is sometimes inappropriate to study abdominal testis or streak gonads [12], because dysgenetic gonads may be too small to be accurately visualized at US, but this does not indicate their absence [3,8]. Magnetic resonance imaging (MRI) is used to study Mullerian structures, but it is not completely reliable for the assessment of intra-abdominal testes or streak gonads [12]. If US and MRI are inconclusive, a laparoscopy, should be performed [24]. Laparoscopy facilitates the inspection of intraperitoneal structures in order to better evaluate gonads, genital ducts, and suspension ligaments [12,18], and helps in the decision to remove, relocate or to take a biopsy of dysgenetic gonads [12].

**From clinical scenario to genetics**

The gonadal primordium is morphologically identical in both sexes and its development is regulated by the action of multiple genes, which often participate in the formation of other organs [3,6]. This process concerns a complex regulatory network involving activation and repression pathways.

- GATA binding protein 4 (*GATA4*), encodes for a transcription factor essential for the initial development of many organs, including the heart. It represents the gene that probably initiates the formation of the gonadal crest;
- empty spiracles homeobox2 (*EMX2*), is involved in the formation of the central nervous system and the entire uro-genital system;
- Wilms' tumor 1 (*WT1*) gene is essential in the early stages of nephrogenesis and actively intervenes in the development of the testis, as well as the gonadal primordium;
- LIM homeobox factor 9 (*LHX9*) is initially active in the gonadal and adrenal primordia of both sexes and is later expressed in a dimorphic manner during the formation of male and female gonads;
- *NR5A1* gene, encoding for steroidogenic factor 1 (SF1), activates a cascade of genetic events, including upregulation of SOX9 in Sertoli cells leading to tubular cord formation and testicular organogenesis [41].
- chromobox homolog2 (*CBX2*) is a gene related to the development of the gonadal primordium, but which is also expressed in the later stages of gonadal differentiation [6].

Male sex determination is mainly governed by The Sex Determining Region on the Y chromosome (*SRY*) gene [3,8].

Mutations in the genes encoding for the proteins necessary for testosterone biosynthesis are associated with **46,XY DSD** [3] (Table 1) [SF1 (*NR5A1*), LH receptor (*LHR*), steroidogenic acute regulatory peptide (*StAR*), cholesterol desmolase (*CYP11A1*), 17α-hydroxylase/17,20-lyase (*CYP17A1*), 3β-hydroxysteroid dehydrogenase type 2 (*HSD3B2*) [42], 17β-hydroxysteroid

dehydrogenase type 3 (*HSD17B3*), 3α-hydroxysteroid dehydrogenase (*AKR1C2/4*), P450-oxidoreductase (*POR*), and 5α-reductase type 2 (*SRD5A2*) [43]]. Other genes associated with **46,XY DSD** (Table 1) include *CBX2*, *DHH*, *DMRT1*, *DMRT2*, *MAP3K1*, and *SOX8*. Loss of function *SOX9* mutations are typically associated with gonadal dysgenesis and campomelic dysplasia., mutations in *GATA4* may also be associated with congenital heart disease in addition to testicular anomalies, *WT1* mutations may present with various phenotypes including Denys-Drash, Meacham, Frasier and WAGR syndromes. In the absence of SRY, female-specific pathways involving genes such as *RSPO1*, *WNT4*, or *NR0B1* are set on, promoting the development of the ovaries [43–46]. Homozygous recessive missense mutation of *WNT4* gene are associated with a syndrome characterized by female to male sex reversal associated with renal, adrenal, and lung dysgenesis [47]. Genes associated with ovarian dysgenesis include *LHX8*, *MCM8*, *MCM9*, *NOBOX*, and *FSHR* [3] (Table 1).

CAH due to mutations in the 21-hydroxylase (*CYP21A2*) gene, is the most common form of **46,XX DSD**. Other genes, which are involved in steroidogenesis and may cause DSDs, are *CYP11B1*, *HSD3B2* [44], *POR*, and *CYP19A1* [48] (Table 1). The classical presentation of CAH due to 21-hydroxylase deficiency may varies ranging from clitoromegaly to perineal hypospadias with chordee to complete fusion of labiourethral and labioscrotal folds such that the female infant appears as a male with bilateral undescended testes (Prader stage 5) [3]. Male infants usually present with normal male sexual development (except for male infants with *HSD3B2* and *POR* mutations that may present with undervirilization), hyperpigmentation of the scrotum may be evident. Failure to thrive, poor feeding, lethargy, dehydration, hypotension, hypoglycemia, hyponatremia, hyperkalemia and metabolic acidosis (due to adrenal insufficiency with gluco and mineralocorticoid deficiency) usually appears during the 2$^{nd}$ or 3$^{rd}$ week of life.

Patients with **46,XX sex reversal** can be classified into two major groups depending on the presence of the SRY. Ovotesticular disorder is defined by the presence of both ovarian follicles and seminiferous tubules in the same patient (ovarian, testicular, ovotesticular, and dysgenetic tissue).

The specific phenotype depends on relative gene expression patterns and the function of the gonads is particularly related to hormone secretion. Duplications involving the SOX9 locus or potential SOX9 regulatory elements have been associated with XX testicular and XX ovotesticular DSD [45]. Others genes associated with ovotesticular DSD include *NR5A1, SOX3, SOX10, WNT4, and RSPO1* [46].

The Xp21.2 region of the X chromosome, is a locus with high genetics complexity. This region contains the receptor subfamily 0, group B, member 1 (*NR0B1*) gene that encodes the nuclear receptor protein DAX1 involved in the development and differentiation of the hypothalamus-pituitary-gonadal and adrenal axes. Duplications of this region are associated with **46,XY sex reversal**[3], deletion or mutations are associated with adrenal hypoplasia *congenita* in a 46,XY individual [49], while microdeletion are associated with **46,XX sex reversal** [50].

Persistent Mùllerian Duct Syndrome (PMDS) is a rare autosomal recessive disorder characterized by the persistence of Mùllerian structures in males, associated with normal phallic development and normal testicular function, albeit most men are infertile [51]. Most case of PMDS are due to *AMH* or AMH receptor (*AMHR2*) mutations.

**Sex chromosome DSD** include Turner Syndrome, Klinefelter Syndrome, and chromosomal mosaicisms 45,X/46,XY and 46,XX/46,XY with gonadal dysgenesis. These conditions account for about 15% of DSDs [5].

Turner syndrome is characterized by aneuploidy (45,X karyotype) or structural rearrangements of the X chromosomes. Structural rearrangements include isochromosome Xq, partial deletions, and ring X chromosome. Incidence is 1 in 2500 live-born females. Patients with Turner syndrome may be diagnosed in the neonatal period because they present low birth weight, short neck, and lymphedema of hands and feet. Other typical presentations include short stature and absent puberty. Characteristic features include epicanthal folds, down slanting palpebral fissures, low set ears or dysplastic external ears, neck pterygium, large mouth with downturned corners, micrognathia, nail

dysplasia, left-sided cardiac anomalies (coarctation of the aorta, left heart hypoplasia), and horseshoe kidneys. In general, patients with Turner syndrome do not present with atypical genitalia.

Klinefelter syndrome is characterized by a 47,XXY karyotype. The incidence is approximately 1 in 500 males. Affected boys have normal external genital development. They may present with tall stature, small testes, delayed puberty, infertility, and gynecomastia. Boys with Klinefelter syndrome often manifest dyslexia, behavioral difficulties, and defects in executive function. Autism spectrum disorders are more common than the general population [3].

Individuals born with chromosomal mosaicisms, previously known as mixed gonadal dysgenesis (MGD), have a wide range of anatomical findings. Clinical presentation at birth can be various (from a male newborn to a newborn with atypical genitalia and Turnerian features), as well as the karyotype (e.g. 45,X/46,XY or 46,XX/46,XY and others) and gonadal characteristics (e.g. streak gonads or normal ovaries/testes).

## Molecular genetic diagnosis in DSD

Establishing a specific molecular diagnosis is helpful in the clinical management of patients with DSDs and in the accurate counseling for the family [21]. The molecular diagnosis may inform about genotype-phenotype correlation and provides additional prognostic information regarding gender identity, fertility, and cancer risk. The molecular diagnosis should be oriented by karyotype, clinical phenotype, hormonal profile, family history (consanguinity, DSDs in other family members).

So far, the list of genes known for being involved in human 46,XY and 46,XX DSD (including gonadal dysgenesis, primary and secondary gonadal insufficiency, steroidogenesis disorders, androgen resistance, isolated urogenital anomalies and syndromic conditions associated with atypical genitalia) amounts approximately to 62 genes for 46,XY DSDs and 61 genes for 46, XX DSDs [17] (Table 1).

Karyotype analysis is essential in the initial classification of any DSD into one of three categories (Table 1 and Figure 1).

### Karyotype and fluorescence in-situ hybridisation (FISH)

Karyotype is an image of chromosomes that assesses their number and structure. Classic karyotyping identifies aneuploidies and the chromosomal sex of an individual, but its resolution is limited to the detection of very large (>10 Mb) chromosomal rearrangements [52]. When a DSD is suspected during pregnancy (based on family history or foetal genitalia appearance at US) the first approach is to determine the foetal genetic sex through the karyotype or the carrier status of a genetic condition already known in the family. Invasive prenatal diagnosis can be made through chorionic villus biopsy (from 9 to 11 weeks of gestation) or amniotic fluid cells analysis (from 15 to 20 weeks). Nowadays, a non-invasive prenatal diagnosis is the preferred approach (NIPD), because it is not associated with risk of termination of pregnancy, and can be performed from the $6^{th}$-$7^{th}$ week of gestation. It consists in the identification of Y chromosome-specific markers in maternal peripheral blood cell-free foetal DNA [53]. In the event that an abnormal karyotype is identified, the diagnosis of *sex chromosome DSD* can be made, and no further genetic analysis is usually required.

FISH or PCR with specific markers for X and Y chromosomes (X and Y centromeres and SRY) can be used to assess for sex chromosome mosaicism and SRY presence [3].

**Chromosomal Microarray Analysis and Multiplex Ligation-dependent Probe Amplification**

Chromosomal Microarray Analysis (CMA), through Comparative Genomic Hybridization (CGH) and Single Nucleotide Polymorphism (SNP) array, compared with classical karyotyping techniques, achieves much higher precision in the identification of genetic material gain or loss (duplication or deletion) but may be less effective at detecting sex chromosome mosaicism [53]. A CGH array measures the variation in DNA probes in a sample compared to control samples, and the results are reported as copy number variants (CNV) [54]. CNVs may explain a significant number of undiagnosed cases of DSDs [52].

SNP arrays detect changes in copy number evaluating changes in intensity of a particular SNP bound to a fluorescent probe suggesting a duplication or deletion of the analyzed region. SNP arrays can also detect loss of heterozygosity [52].

Microarray manufacturers have combined the two technologies together to be able to both identify gain or loss of genetic material and regions of heterozygosity and homozygosity [54].

In some centers, these tests have replaced the traditional karyotype whereas CMA is often used as a second-line investigation in the study of DSD associated with multiple malformations. CNV may be present in one-fifth of DSD patients, more commonly in syndromic forms. Several genes regulating sex development have been shown to exert a dose-dependent effect. Duplications of FGF9, SOX3 or SOX9 have been reported in 46,XX DSD and duplications of DAX1 and WNT4 in 46,XY DSD. Deletions of ATRX, DMRT1, EMX2 or WT1 have been reported in 46,XY DSD [52,53] (Table 1).

CNV arrays have been essential for regulatory region analysis, which is usually missed by focusing on the coding regions of a gene. The use of CGH arrays has been especially useful when examining variants related to SOX9 and its regulation, by discovering micro-duplications, whole gene duplication, and deletions related to campomelic dysplasia and DSD [55,56]. SOX9 multiple large CNVs, such as duplication and deletion, have been identified far upstream the coding region in

patients with 46,XX and 46,XY DSD, helping to define the minimal overlap regulatory region upstream SOX9, known as RevSex [57]. CGH technologies have also identified many putative regulatory regions in genes such as: SOX3, NR0B1, and GATA4 [58]. Using CMA both gain (duplication) or loss (deletion) of genetic material can be found. However, the interpretation of the result may be problematic. The results may provide a diagnosis when a specific pathogenic variant known to be associated with DSD is identified, but novel variants of uncertain clinical significance (VUS) may be also found [59].

Many candidate genes and chromosomal loci with potential roles in sex determination and DSD are awaiting further validation. These include deletions in the 9p23-24 chromosomal region encompassing DMRT1 and KANK1 in cases of XY gonadal dysgenesis [60–63], deletions at 12p13 and 16p11.2 in cases of hypospadias, deletions at 10p14 and Xq28 in cryptorchidism, deletions at 1p36.3, 9p24.3 and 19q12-13.1 in cases of atypical genitalia [64], copy number gains at Xq28 encompassing the VAMP7 gene in newborns with congenital genitourinary tract masculinization disorders [65].

CMA typically reports only CNV above a 25–50 kb cutoff, leaving a diagnostic gap for smaller CNV. Previous studies in DSD cohorts have identified smaller CNV, but the interpretation remains a challenge. High-resolution CNV maps (with CNV as small as 50 bp) are increasingly becoming available and are poised to bridge the gap with the smaller insertions or deletions of bases (INDELs) that are detectable using short-read sequencing. High-resolution CNV maps have been recently used to investigate the potential consequences of small CNV as well as genomic copy-neutral regions of homozygosity (ROH) in DSD genes [66]. Although CNVs seem to play a crucial role in DSDs, functional analyses are still needed to determine whether they are responsible for clinical phenotype [52]. Multiple ligation probe amplification (MLPA) is a more targeted PCR-based method technique that can detect up to 40 genomic loci copy numbers in a single reaction. MLPA is frequently utilized to confirm CNV array results, determine the mode of inheritance, and screen patient cohorts for known CNVs, which in turn are then fine-mapped using CNV arrays. MLPA can

also be used to detect CNV of single exons in genes of interest. However, due to the cost of the required probes, MLPA is not considered as the method of choice to determine the exact boundaries of genomic rearrangements[52]

**Gene-testing panels, Whole Exome Sequencing and Whole Genome Sequencing**

Although CMA is an effective diagnostic technique for detecting chromosomal abnormalities with higher precision than traditional karyotype, it is ineffective in detecting small genetic variations. Serial gene sequencing (by Sanger sequencing) was the historical method of choice, where the selection of candidate genes was oriented by clinical phenotype, imaging and endocrine tests [54].

Last generation gene panels are now preferred and typically based on targeted next-generation sequencing (NGS) through either targeted or full exome capture followed by limited interpretation. Additional Sanger sequencing may be performed to fill in genes with missing or insufficient read-depth coverage.

Huge improvements in genetic sequencing technology combined with a significant reduction in costs have provided a stimulus to change the clinical approach, as NGS, Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS) have become available in clinical practice. Diagnostic DNA laboratories are moving from single-gene sequencing (sequential analysis) to NGS assays (parallel testing), designed to sequence multiple DSD genes on a targeted panel or the whole-exome with predetermined filters that target DSD genes. A targeted panel is advantageous as it yields high-quality coverage of the genes of interest, whilst minimizing the risk of incidental findings [21].

The above cited panels are not standardized. Many commercial providers offer clinical genetic testing of DSD and many institutions have started developing their in-house tests.

Because such targeted sequencing provides high read depth for a set of genes, it allows the detection of non-reference alleles present at very low frequencies. Such panels could therefore be useful to detect mosaics that have been hypothesized to underlie the variable phenotypic expression

of DSDs. These panels need frequent updates when novel gene-disease associations are discovered [54].

WES and WGS are powerful tests that help to obtain a molecular diagnosis in patients with a suspected underlying genetic etiology, but with clinical features that overlap different conditions.

WES allow to identify and sequence only exons and the immediately adjacent intronic canonical splice sites, covering about 45 million base pairs (or ~1.5% of the genome) [5]. WES covers approximately 95% of the protein coding regions of the genome, which currently host 80-90% of known pathogenic variants [67]. The sequencing method is similar to those used in genetic panels, having the exon capture followed by NGS and validation of low-quality mutations via Sanger sequencing. The main advantage of WES is the ability to provide the sequence information of all known protein-coding regions of the genome, which are analyzed simultaneously. WES is used for searching mutations in known genes involved in sexual development, and has the potential for finding new DSD genes. Furthermore, since all exons are sequenced, the data can be easily re-analyzed as new gene associations are reported, a practice that has been shown to increase the diagnostic rate [54]. Overall, WES technology is very powerful; in many cases the use of WES instead of a targeted panel is reasonable, for example when a complex genetic syndrome or two different conditions are suspected in the same patient [59]. Recently, the use of WES for DSD research has focused on DSD familial cases and has also been used to validate the existence of potential genetic modifiers, which could sensitize an individual to develop a DSD [58]. Limitations in the diagnostic efficacy of WES include incomplete gene coverage, platform variability, variance interpretation difficulties, and ethical issues related to incidental findings [59].

Exome sequencing identifies on average 21000 variants in each case with respect to the reference human genome. For rare diseases such as DSDs, variants with global alternative allelic frequency greater than 1% are excluded from the downstream analysis as population polymorphisms and therefore probably benign. For this reason, sequencing the patient's exome as part of a trio, with phenotyped parents allows the identification of inherited or *de novo* variants, and is more effective

in identifying pathogenetic variants. This reduces dramatically the number of variants that need to be evaluated manually, reducing the time for the diagnosis [59]. Trio exome sequencing has been reported to increase the diagnostic yields from 22-23% to 31-33% [68,69], in comparison with proband-only sequencing. Finally, the analysis of the trio can clarify how the same inherited mutation can have different phenotypic expression.

Identified variants are typically filtered via utilization of disease-specific gene lists containing previously published genes associated with the disease. The variants are then classified into pathogenic (previously reported in humans as the recognized cause of the disorder) or likely pathogenic (previously unreported in a known human DSD gene and expected to cause the disorder) following the American College of Medical Genetics and Genomics (ACMG) guidelines. The ability of exome sequencing to provide an accurate genetic diagnosis heavily relies on the existence of current and accurate gene/phenotype data sets, such as those curated by OMIM (omim.org) or Orphanet (orpha.net), or the repositories of human variants such as ClinVar (ncbi.nlm.nih.gov/clinvar) or HGMD (hgmd.cf.ac.uk). *In silico* filtering of variants using data sets reduces analysis time and can help standardize the process [54].

An ethical conundrum has been linked to the capacity of exome sequencing to identify variants in genes beyond those known to be associated with the condition for which the patient is studied: what to do about incidental findings, such as those variants that may represent a life-threatening condition and may be medically manageable, but not directly related to DSD? Different strategies have been adopted by different regulatory institutions. The European Society of Human genetics recommends the use of *in silico* panels (complete exome capture, but analysis restricted to a set of relevant genes to be sought and reported to the patient in the clinical setting) to decrease the chance of inadvertently finding variants unrelated to the condition [70].

WGS has the capability to identify single-nucleotide variants and small INDELs with high precision not only in the exons, but also throughout the entire genome, in regions that may be involved in gene regulation. Because it involves untargeted capture, WGS has been shown to provide more

uniform reads depth across the genome and sequencing quality parameters than exome sequencing. It is therefore more reliable than exome sequencing even for variants in the protein-coding regions of the genome. Additionally, WGS has the potential to identify genetic aberrations larger than 1 kbp in size defined as structural variants (SV). SV are an important category of DNA variations observed in the human genome[54]. It has recently been shown that over 90% of genetic variants associated with complex multifactorial human conditions lie not within coding sequences of genes but in noncoding regions [71]. In genome-wide screens of DSD patients, duplications or deletions have been detected in the regulatory regions of known or suspected DSD genes, rather than in coding regions [52]. This is not surprising, given their role in the space-time 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 [72].

Current guidelines for genetic testing in DSD diagnostic process recommend a targeted approach when the clinical phenotype and the biochemical results are suggestive for specific genes, otherwise a massively parallel panel approach [29].

**Future perspectives in molecular diagnosis of DSD**

Many SVs are caused by non-allelic homologous recombination events between duplicated DNA sequences as large as 300 kbp in size. The repetitive nature of these regions makes them all but invisible to short-read sequencing platforms [73,74] or traditional cytogenetics methods such as CMAs, which can elucidate gain or loss of genetic material, but fail to detect the order/orientation of the SV and are virtually blind for detection of balanced events such as inversions and translocations. Recently, **optical genome mapping** has been proposed as the "go to" method for the identification of large/complex structural variants due to its high specificity and sensitivity [75]. Optical mapping offers key advantages as higher resolution and possible balanced event detection [5].

Another promising approach to diagnosing DSDs is analyzing the entire transcriptome (eg, from gonadal biopsy or differentiated induced pluripotent stem cells) and exploring for variations in RNA and DNA [76].

Finally, epigenetic variation may also cause DSD. For example, epigenetic changes have been shown to affect SRY regulation [77] and mutations in ATRX affect DNA methylation [78]. Probably, methyloma analysis should be explored in future diagnostic evaluations [12].

**Management**

The uncertainty about the child's sex of rearing in case of atypical genitalia or the presence of a disagreement between the genital phenotype of the newborn and the prenatal karyotyping is a stressful situation for parents and clinicians [8,24] Correct gender assignment, as well the long-term management of DSDs, require a multidisciplinary expert team, involving a paediatric endocrinologist, a geneticist, a psychiatrist or psychologist, and a surgeon. The decision about the sex of rearing, and the eventual corrective surgery, should be made on the basis of a thorough investigation and parents should be involved in the decision-making process.

Many progresses have been made in the process of sex of rearing assignment on the basis of several factors, not only genital phenotype and surgical options. These factors include molecular diagnosis, surgical options, need for life-long replacement therapy, potential fertility, family opinion and cultural issues. For example considerations regarding sex assignment in a 46,XX patient with ovotesticular disorder should consider the presence of sufficient ovarian tissue with follicles to allow for pregnancy [3]. More than 90% of 46,XX CAH patients and all 46,XY patients with complete androgen resistance, which are assigned to female sex in infancy, have a female gender identity as adults [3]. Brain hormonal imprinting (due to testosterone) and its impact in later behaviour, has been considered more and more important in the decision-making process of sex of

rearing [8]. It is important to note that sex does not indicate gender; sex refers to the biology of the internal and external genital structures that is traditionally considered to be a binary categorization. Gender identity is the self-defined experience of one's gender [3] and depends by both psychological and social factors [8].

Pharmacological treatment depends on the choice of the sex of rearing and the underlying diagnosis.

Multidisciplinary care begins at diagnosis and continues across the lifespan of the individual [14]. Parents should be always informed and appropriate information adjusted to age [14] should be also provided to older-children and adolescents with DSDs.

Other several issues should be addressed such as the psychosocial wellbeing, sexual satisfaction and the risk of gender dysphoria, fertility options and risk of tumour development [5,24] .

The various forms of DSD have a variable risk of developing malignancies [8], specifically gonadal germ-cell tumours, seminomas and nonseminomas [14,79] .The risk of developing a tumour is much higher in patients with gonadal dysgenesis, especially forms that arise from early gonadal differentiation defects, such as mutations in *SRY* and *WT1*, than in patients with ovotesticular DSD or steroidogenesis disorders and retained gonads [14]. The presence of a Y chromosome in a dysgenetic gonad is associated with a high risk of neoplastic transformation into gonadoblastoma. For this reason, gonadectomy has been suggested, early or later in life, in 46,XY and 45,X/46, XY DSD that present retained gonads [3,14]. However, true malignancy risk is difficult to ascertain as many different phenotypes are included under the definition of ovotesticular DSD. Therefore over the past decade, new treatment algorithms for the management of MGD have emerged, focusing primarily on gonadal management [80]. The decision-making process leading to gonadectomy should always involve the expert team, if possible, it should be individualized on the basis of the specific molecular etiology [3,81].

## Conclusion

DSD is a group of genetically heterogeneous diseases that need thoughtful evaluation by a multidisciplinary team. Molecular technologies can help to clarify the aetiology and facilitate the diagnosis of DSDs. An early diagnosis, so as an early and correct sex assignment, are necessary in order to adequately take care of these patients and their families and ensure them the best possible quality of life, including the possibility of fertility and cancer prevention when needed.

## Authors' Contributions

AI and MDP collected published data and drafted the manuscript; AB, FB, DC and GR contributed to the design of the study and critically revised the manuscript, LDS and CB conceived the study, participated in its design and coordination and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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**Captions to figures**

**Figure 1. (Ref 12, 22)**
A. Suggested practical diagnostic approach to infant with atypical genitalia and/or DSD suspect.
B. Suggested practical diagnostic approach to infant with 46, XX DSD.
C. Suggested practical diagnostic approach to infant with 46, XY DSD.

**Figure 2. Different degrees of virilization according to the Prader's stages of virilisation (Ref 35).**
Prader stage 0 corresponds to females with normal external genitalia.
Prader stage I is characterized by a slightly enlarged clitoris.
Prader stage II distinguishes a mild degree of virilization.
Prader stage III-V is recognized as ambiguous genitalia.
Prader VI stage indicates a normal male presentation with typical external genitalia and normal testes in the scrotum.

**Figure 3. The external masculinization score (EMS)**
EMS provides an objective score of the extent of masculinization of the external genitalia. Each individual feature of the genitalia (phallus size, labioscrotal fusion, site of the gonads and location of urethral meatus) can be individually scored to provide a score out of 12. L/S, labioscrotal; Ing, inguinal; Abs, abdominal or absent on examination (ref 36).

**Fig 4.The External Genitalia Score (EGS)**
EGS can be applied in both typical male and female babies and in babies who have variations in their genital characteristics. EGS describe phenotypic features at 5 anatomical landmarks of the genitalia: degree of labioscrotal fusion, length of the genital tubercle, position of the urethral meatus, and location of the right and left gonad. The final score is the sum of points allocated to feature 1-5; GT, genital tubercle (ref 37).