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Towards Improved Genetic Diagnosis of Human Differences of Sex Development

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Abstract

Despite being collectively among the most frequent congenital developmental conditions worldwide, differences of sex development (DSD) lack recognition and research funding. As a result, what constitutes optimal management remains uncertain. Identification of the individual conditions under the DSD umbrella is challenging and molecular genetic diagnosis is frequently not achieved, which has psychosocial and health-related repercussions for patients and their families. New genomic approaches have the potential to resolve this impasse through better detection of protein-coding variants and ascertainment of under-recognized etiology, such as

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Author contributions

The authors contributed equally to all aspects of the article.

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mosaic, structural, non-coding, or epigenetic variants. Ultimately, it is hoped that better outcomes data, improved understanding of the molecular causes, and greater public awareness will bring an end to the stigma often associated with DSD.

INTRODUCTION

Conditions affecting the development of chromosomal, gonadal, or anatomic sex, termed DSD (see Box 1), encompass a large spectrum of overlapping phenotypes and an equally wide array of etiologies[1–3]. They may present in isolated form, as an index feature of well-defined syndromes (Figure 1), or as an incompletely penetrant trait of many complex conditions[3,4]. They can have an origin in various developmental processes including gonadal formation, hormone biosynthesis or responses in target tissues, or signaling along the hypothalamus-pituitary-gonad axis. Phenotypes range from minor malformations (such as undescended testes or hypertrophy of the clitoris) to abnormal gonadal development leading to ambiguity of the genital anatomy or discordance between sex chromosomes and anatomy. The conditions under the DSD umbrella are each individually rare and multiple genetic etiologies have been demonstrated, ranging from missense single nucleotide variants (SNVs) to whole chromosome aneuploidies (Table 1). At least 75 genes have been associated with one occurrence of DSD in humans, with variable strength of evidence[2]. In addition, anatomical phenotypes and hormonal profiles frequently overlap between the various conditions under the DSD umbrella, making an accurate DSD diagnosis one of the most difficult in medicine.

However, the 2005 Consensus Conference and recent efforts towards standardization of care, such as those spearheaded by the DSD-Translational Research Network (DSD-TRN[5]) in the US or the International DSD (I-DSD) registry (initiated in Europe[6]), have emphasized the need to reach an early diagnosis for optimal outcomes[7,8]. In particular, potential impaired fertility[9,10], gender identity uncertainty, and self-image issues all contribute to poor psychosocial and medical quality-of-life outcomes[8]. An accurate diagnosis is critical as risk of gonadal cancer[11–13], comorbidities (Fig. 1), and associated risks for variant-carrying family members vary greatly among conditions. It is critical to refer patients to a specialized multidisciplinary team as soon as DSD is suspected. At birth, it is urgent to initiate treatment to avoid life-threatening crises in the salt-wasting forms of Congenital Adrenal Hyperplasia (CAH), as well as to accurately distinguish between conditions with overlapping phenotypes but different outcomes to optimally decide on management and sex of rearing. Finally, benefits of having a diagnosis for individuals living with a rare disorder, even if no treatment is available, have been well documented[14,15].

Yet, despite the high number of genes reported since the discovery of the first sex-determining gene, *SRY*, 30 years ago[16–20], the molecular mechanisms underlying human sex development remain far from understood, and a majority of patients do not receive a diagnosis. Even with massively parallel sequencing technology (also known as next-generation sequencing, NGS), causative variants are reported in only 35%−45% of XY DSD in research series[21,22], with even lower diagnostic rates in the clinical setting[23]. An

inability to discover and interpret variants, especially beyond protein-coding regions, and poor delivery of care have been major limitations to diagnostic efficacy.

In this review, we discuss approaches and hurdles to diagnosis, challenges with the interpretation of variants, pre-clinical genomic technologies bringing promise of improvement, and how genetic information for individuals with a DSD is interpreted at a societal level. We refer to recent expert reviews for description and management of individual DSD conditions and known underlying molecular mechanisms[2,3,9,24–28].

GENETIC TESTS IN CLINICAL USE

The traditional approach to DSD diagnosis has been step-wise stratification starting with clinical phenotyping and karyotyping to orient subsequent endocrine analysis, followed by genetic testing (which is often limited to individual candidate genes) to resolve intractable cases. Many algorithms have been put forth over the years to try and streamline the diagnostic process[16,25,29,30], but progress is needed on the genetic diagnostic front. Apart from CAH, where hormonal markers orient the diagnosis, and chromosomal DSD (such as 45,X and 47,XXY and variants), clinically available genetic testing techniques fail to make a molecular diagnosis in more than half of cases. Diagnostic yield is particularly low for 46, XY DSD with gonadal dysgenesis.

Newborn screening for CAH

The vast majority (90–95%) of DSD in 46,XX individuals with genital atypia is associated with CAH due to *CYP21A2* deficiency (~1:15,000 (ref[31])). Because the life-threatening salt-wasting crises associated with this condition can be treated, CYP21A2 deficiency is now part of newborn screening panels in many parts of the world. However, a recent survey of protocols used across US states showed that criteria and technology are not standardized, resulting in poor test positive predictive value (PPV) **[G]**, ranging from 0.7% to 50%[32]. This test is purely biochemical, as genetic testing for this gene is complex and not adapted to a screen. Most cases of CAH are diagnosed by endocrine testing alone and, as a result, our understanding of the correlation between genotype and outcomes remains insufficient to adapt treatment throughout life to specific genotypes[33].

Sex chromosome assessment by karyotype

For newborns with atypical genitalia, a karyotype is still the test of reference (Table 2). However, interphase fluorescent *in situ* hybridization (FISH) for X and Y chromosomes and *SRY* is recommended as it is faster (24 hrs versus 1–2 weeks). About ~15% of DSD are thought to be chromosomal DSD, including Klinefelter syndrome, Turner syndrome variants with Y chromosome contribution (typically mosaic 45,X/46XY, with or without isodicentric Y **[G]**), segmental deletions of the X, or translocations of an *SRY*-containing Y fragment to the X[5,23]. With non-invasive prenatal screening (NIPS) using cell-free fetal DNA **[G]** to detect aneuploidies, many parents may now learn the sex chromosomes of their child around 10 weeks of gestation and DSD are increasingly ascertained this way[34–36]. Accuracy of this screen is lower for sex chromosomes than for Down syndrome (where sensitivity and specificity are close to 100%[37]). PPV of NIPS for sex chromosome aneuploidies has been

reported at 32%−57.6%[38–40], with very low PPVs of 18–21% for 45,X (Turner syndrome), and PPVs ranging from 39%[38] to 90%[40] for Klinefelter syndrome (47,XXY).

If NIPS suggests a normal sex chromosome complement, subsequent ultrasounds showing genitalia typical of the other sex (reported in 1/14,300 pregnancies[36]) may indicate a DSD. In a series, ascertainment errors were reported in ~1/3 of cases (tube mislabeling or incorrect identification of sex on ultrasound) and a DSD actually diagnosed at birth in 36%[36]. A vanishing twin (of the opposite sex) is a frequent cause of apparent discrepancy in the remaining 30%[35]. As NIPS is only a screen, follow up with confirmatory genetic testing (with karyotype or microarray) is needed and protocols have been proposed for this[35].

Chromosomal microarrays

The advent of chromosomal microarrays revealed small, cryptic copy number variants **[G]** (CNVs) as an overlooked, albeit rare, DSD etiology[41–43]. Sex chromosome aneuploidies, and deletions or duplications of not only coding but also regulatory regions of *SOX9, WNT4,* or *NR0B1* have been shown to cause DSD (genes, conditions, and research-based methods of detection are reviewed in[41]). Gene expression dosage threshold effects may also explain the incomplete penetrance observed in several conditions and pedigrees, which brings an element of extreme complexity to diagnosis. CNVs may also underlie the elusive etiology of developmental forms of DSD, such as cloacal malformations[44] or Mayer Rokitansky Küster Heuser (MRKH) syndrome[45,46].

Chromosomal microarrays are the reference method for detection of CNV, which can be identified even in mosaic form (if the CNV is present in more than 20–30% of cells). They often provide answers in syndromic cases of DSD and occasionally in isolated cases (for example 14% of cases in the DSD-TRN registry were diagnosed by chromosomal microarray [5]). Clinical laboratories typically report CNVs larger than 25–50 kb (Table 2), which is larger than some DSD genes, although the current high density of probes on the arrays could allow detection of much smaller CNVs. A research bioinformatics tool was designed specifically for detection and functional annotation of smaller (> 1 kb) CNVs in DSD[47]. In 52 cases it identified >300 CNVs overlapping 68 DSD genes. Validation of the pathogenicity of these variants awaits replication in other cohorts and *in vitro* validation experiments prior to clinical deployment of the tool.

Single gene testing

Single gene testing is recommended when CAH is suspected clinically, as some degree of phenotype-genotype correlation may exist to distinguish the salt-wasting, simple virilizing, and non-classical forms[48,49]. For this and other conditions, targeted Sanger sequencing is useful once a variant has been identified in a proband to ascertain the phase of compound heterozygous variants and inheritance or *de novo* status. Because of the phenotypic overlap between conditions and the large number of candidate genes, single candidate gene testing (whether by NGS or Sanger sequencing) is not typically efficient in other cases. Indeed, analysis of the two most frequently ordered single gene tests for 46,XY DSD (*AR* and *SRD5A2)* in the DSD-TRN Registry showed that negative results were returned for ~40% of *AR* and ~ 55% of *SRD5A2* tests[5].

Exome sequencing

To identify SNVs or small INDELs in the dozens of known DSD genes, the method of choice is now massively parallel sequencing using short (~150 bp) reads covering the protein-coding regions of genes (clinical exome sequencing methods and best practices were recently reviewed in[50]). It can be performed with enrichment (targeted capture of a panel of known causative genes) or without enrichment ('whole' exome capture, with reporting limited to selected genes). Expertly curated enriched panels (from 30 to 180 DSD genes) have shown great diagnostic rates[22,51–57], as well as time- and cost-efficiency[53] in a research setting, but those are not typically commercially available. Clinical whole exome sequencing (WES), interpreted by an expert board, resulted in 35% firm diagnoses in 46,XY DSD[21].

WES and panels for DSD testing have respective advantages (reviewed in[2,55,58]). While panels need to be re-engineered when a new causative gene is discovered, and patients retested, WES allows both easy reanalysis and gene discovery. However capture kits have evolved dramatically, and some DSD genes were poorly captured in older exome tests[5], and variants in un-captured regions would be missed even upon reanalysis. For WES, whether targeted (to known clinical genes, covering ~30Mb of the genome), whole exome (45Mb), or expanded (into adjacent non-coding regions, 60Mb) capture is performed could greatly influence the number of discoverable variants. Sequencing platforms and analysis pipelines also evolve rapidly, and it is increasingly difficult for patients and providers alike to understand what the results of a test mean, as the 'same' test a few years later may result in a different outcome.

Whole genome sequencing

Whole genome sequencing (WGS), while based on the same short-read sequencing (SRS) principle, avoids the capture limitation of exome or panels. WGS has been shown to expand diagnostic utility and improve clinical management in rare pediatric disorders [59,60]. WES misses about 650 true variants (3% of coding variants) per individual, which WGS is able to detect thanks to more even coverage of GC-rich and hard-to-capture exons[61]. Also, the proportion of false-positive SNVs is much higher for WES than for WGS (78%versus 17% (ref[61])). Thus, WGS alone should increase diagnostic yield in the protein-coding regions of known DSD genes. Early indications suggest that this may be the case, but supporting evidence has yet to be published.

In addition, WGS allows detection of variants outside of exons, for example in regulatory elements found in promoters, introns or enhancers, a known source of DSD variants. Currently, 89% of pathogenic variants reported in the ClinVar database are in coding sequences (99% if adjacent regions are included)[62]. However, the exome represents only 1–2% of the genome and many causal variants are expected to be missed using exome sequencing alone. In the USA, Children's National Hospital and Rady Institute have recently pioneered shifting their accredited pipeline to WGS as the primary technology for clinical genetic testing, but DSD panels are not yet available. With a cost still much higher than WES, and limited interpretability of variants in non-coding regions, the current appeal of WGS as a clinical test remains limited for providers in spite of the clear technical advantages.

IMPROVING DETECTION OF DSD VARIANTS

Current clinical technology detects mostly coding SNVs and large CNVs in the heterozygous or homozygous state. Detection yield should increase when methods become clinically available for detecting other types of variants and etiology, such as variants in mosaic states, oligogenic etiology, complex genomic variants and epigenetic variants—all known but under-ascertained causes of DSD. Emerging, pre-clinical approaches for improving detection of these classes of variants are discussed below.

Mosaicism

Chimerism **[G]** [63,64] and mosaicism **[G]** [65] have been reported as a DSD etiology and possibly the cause of missing diagnoses, phenotypic variability, and reduced penetrance. Turner syndrome variants with mosaic 45,X/46,XY typically present with mixed gonadal dysgenesis, with different tissue anatomy in the two gonads. Mosaic X-inactivation explained phenotypic variability in 46,XX individuals with a Y-to-X translocation[66]. A mosaic frameshift mutation in *SRY* in a man with testicular dysgenesis presented as a typical female phenotype in two 46,XY daughters, who inherited the mutation in a non-mosaic state[67]. Mosaicism for variants in other DSD genes may be under-recognized because of the lack of appropriate clinically available technology. In particular, better detection of mosaicism is expected to improve the diagnostic yield in ovotesticular DSD and other conditions resulting in asymmetric genital phenotypes, cases where the phenotype is at the mild end of the spectrum for a known condition; or in genes known for syndromic DSD when DSD condition presents in isolated form.

Next-generation sequencing was shown to be more sensitive than karyotype or microarray for detection of Y material in Turner syndrome variants[68], but standard variant calling pipelines are not adequate to detect low level mosaicism or chimerism. They assume a diploid genome, and the depth of coverage (~100x for WES; ~30X for WGS) is too low for robust mosaic variant calling. Targeted short-read sequencing applied to high read depth (1000x) should allow robust detection of these otherwise cryptic DSD etiologies[69].

Oligogenic etiology

Involvement of variants in multiple genes has been hypothesized to explain phenotypic variability in individuals sharing genetic etiology. For example, variants in *NR5A1*/SF-1 are associated with phenotypes ranging from isolated adrenal insufficiency, premature ovarian insufficiency, or ovotesticular DSD in 46,XX individuals, to undescended testes or infertility in typical males, and ambiguous genitalia or gonadal dysgenesis with female genitalia in 46,XY individuals. An oligogenic mode of inheritance, with variants in other genes explaining the variability in phenotype, has been proposed in this[70,71] and other forms of DSD[9,72,73]. However, current bioinformatics pipelines are not designed for efficient identification of oligogeny. Publications have started sharing lists of other variants found even in resolved cases[74] to help identify contribution of other genes to DSD phenotypic variability and pathogenicity[69].

Structural variation

Structural variants **[G]** (SVs) interrupting the coding regions of DSD genes or modifying the genomic architecture around regulatory regions can result in DSD. While microarrays reliably identify large gains or losses of genetic material, they are blind to balanced rearrangements, inversions, or translocations. The short-read sequencing methodology common to both WES and WGS does not accurately detect large or complex SVs[75]. Several technologies that can detect complex SVs have been developed, with complementary strengths, and their combined use will be necessary to recognize the full extent of the currently under-ascertained role of SVs in DSD. Below, we discuss optical genome mapping (OGM) and long-read sequencing (LRS), and the current barriers to their clinical use (for details about techniques and algorithms to identify SVs in SRS, LRS and OGM see[76,77]).

Optical Genome Mapping detects variants 500 bp to Mbp in size.—OGM (supported by the Bionano Genomics platform) can identify large and/or complex SVs with high specificity and sensitivity[78–80]. OGM images megabase-size DNA molecules fluorescently labeled at specific sequence motifs throughout the genome. The resultant pattern of fluorescent tags within long DNA molecules is used for *de novo* assembly of each allele of the sample genome[81]. Because genomic architecture is preserved in the ultralong DNA molecules, comparison of the sample-specific maps with reference maps allows for identification of large deletions, insertions, inversions, translocations and complex rearrangements, including those not associated with copy number variation. The resolution limit of the method is dictated by both the spacing of the fluorescent tags along the genome and the pixel resolution of the camera capturing the signal. OGM can currently resolve SVs starting at about 500 bp in size, but this could evolve in the future with different labeling enzymes and improved sensors. The ability of OGM to identify clinically relevant SVs has been shown in cancer[82–84] and Duchenne muscular dystrophy[85]. The first clinical application of OGM was approved in 2019[86] and it is being adopted in place of Southern blotting to identify the 3.3 kb D4Z4 repeats in facioscapulohumeral muscular dystrophy[87], even in a low-level mosaic state.

Because OGM does not provide base-to-base information, a novel software was developed and integrated into the Genoox sequence analysis platform. It uses an advanced reference graph structure and short read WGS data from the same sample to refine breakpoint localization of OGM-based SV calls (from multiple kbp to ~200 bp). Integrated datasets enable more precise identification of breakpoints that might interrupt genes and facilitate interpretation of SVs. Identification of high-quality, clinically relevant SVs in OGM datasets is facilitated by *nanotatoR*[88], a newly developed annotation tool that provides a variety of filtration options, information on the identity of and distance to overlapping genes and parental zygosity, and enables integration with gene expression (RNA-Seq) data if available. A limitation is that the Bionano Genomics Database contains data for only ~230 healthy individuals. Although other databases, such as the Database of Structural Variants (DGV), have larger numbers (~55,000), most are aggregated from different studies and techniques, which makes it difficult to achieve a harmonized dataset wherein frequency values translate to true population frequency. In addition, comparison of SVs to reported variants is more complex than for SNVs, as SVs of different sizes that interrupt a gene in

the same region may have a similar effect but will be categorized as different. Just as the exponential expansion of public databases of control variants (such as gnomAD) and disease variants (such as ClinVar) has revolutionized interpretation of SRS data, interpretation of non-coding and structural variants will become more accurate as more samples are analyzed and databases expand.

Long-read sequencing excels at detection of SVs in the 50 bp-5 kbp range.—LRS, sometimes called third-generation sequencing, produces reads over 10 kb and up to megabases in length (compared to the typical 150 bp of clinical SRS), greatly facilitating mapping of sequences in areas of the genome difficult to sequence because of repetitive elements or pseudogenes. The role of LRS in medical genetics is emerging[89]. Two main technologies currently dominate: nanopore-based sequencing (developed by Oxford Nanopore Technologies) and single-molecule real-time (SMRT) sequencing (developed by Pacific Biosciences). Because long stretches of DNA are sequenced from a single molecule, LRS allows phasing of variants along a haplotype, variants to be resolved in low complexity regions, and detection of SVs missed by SRS[90]. In comparisons of SMRT, OGM and WGS, OGM shone at detecting SVs in the 1kb-1Mb range but only SMRT was able to detect ~90% of insertions and >75% of deletions in the 50 bp to 1 kbp range [75]. Bioinformatics tools to identify more complex SVs and annotate variants are still being developed (for methods to identify SVs in LRS and SRS, see[76,91]).

Nanopore sequencing recently enabled telomere-to-telomere assembly of the X chromosome[92], which may be useful for X-linked DSD (assembly of the Y chromosome is still to be tackled). Because of its high per-base error rate, LRS cannot currently be used to detect SNVs but should be very helpful to identify SVs too small to be accurately ascertained with OGM and CNVs smaller than the clinical threshold for microarrays. However, clinical applicability of LRS is still a few years away because of its current very high compute requirements and cost.

Epigenetic variation

Genital atypia is part of several imprinting disorders such as Prader Willi (OMIM 176270), Beckwith-Wiedemann (OMIM 130650), and IMAGe[93] (OMIM 614732) syndromes. Changes in methylation in the promoters of DSD genes and histone modifications have been shown to affect mammalian sex determination (reviewed in[2,94]) and at least one case has been reported of differential methylation upstream of *SRY* in a 46,XY woman compared to her father[95].

The diagnostic utility of genome-wide DNA methylation analysis has been recently demonstrated[96] including in syndromes with genital involvement (Cornelia de Lange, Kabuki, genitopatellar, ATRX, and CHARGE syndromes). Systematic analysis of epimutations **[G]** (single locus) and episignatures **[G]** (across multiple loci) is likely to both identify new DSD etiology and help resolve variants of unknown significance (VUS). In addition to existing technology using bisulfite conversion followed by SRS or SNP arrays, LRS via nanopore-based sequencing[97] or SMRT[98,99] technology and OGM[86] all hold the promise of detecting epigenetic and genetic variation on the same long

DNA molecule[100,101]. Improving integration of epigenetic data (DNA methylation, histone modifications, imprinted regions) into sequence and SV analysis algorithms will be critical to increase DSD diagnostic yield, as was shown for microarrays[47].

IMPROVING VALIDATION OF DSD VARIANTS

Typically, WES and WGS identify ~21,000 and ~3 million variants per genome respectively, with WGS variants largely found in non-coding regions, where interpretability is even lower than in coding sequence. Variant filtration using a pre-established list of genes relevant to the phenotype, population frequency, predicted protein-damaging effects, and previously reported occurrence in the same condition helps prioritize potentially pathogenic variants. However, the yield of likely pathogenic variants that can be confidently called is low because evidence of causation is very low for many of the dozens of DSD genes. Many genes have been published once, with limited *in vitro* or *in vivo* evidence of their deleteriousness. Existing guidelines (for example, ref 103 and the SVI General Recommendations for Using ACMG/AMP Criteria to incorporate results of validation studies into variant interpretation algorithms have focused on traditional, low throughput assays, which cannot keep pace with the amount of data produced by NGS. Multiplexed, high-throughput assays are necessary[102], but designing assays specific for DSD is complex as they need to be relevant to each gene and affected tissue type.

Improving reference database curation

The 2015 publication of the 28 ACMG/APA criteria[103] for classification of variants (from pathogenic to benign) was followed by the creation of tools to automate classification, such as Varsome[104], Franklin and Sherloc[105]. These, and clinical platforms, use the ClinVar and/or UniProt variant repositories to score the ‘reputable source’ criteria (PP5 and BP6[103]). However, a recent detailed analysis showed that annotation of DSD genes in ClinVar is grossly insufficient[2]. Many published pathogenic variants, supported by *in vitro* validation, have not yet been curated and included in the database. As a result, evidence of pathogenicity may be over- or under-estimated, even for well-known DSD genes (Table 1 and Supplementary Table 1). For data on more genes, types of variants, and associated references, see[2].) For example, ClinVar contained only 109 variants in the androgen receptor gene (*AR*)[2], while the AR mutation database (maintained until 2014) annotated >550 variants[106]. For Persistent Müllerian Duct Syndrome (PMDS), caused by variants in either the Anti-Müllerian Hormone (*AMH*) or its receptor (*AMHR2*), ClinVar contains 4 *AMH* and 5 *AMHR2* pathogenic variants. They were curated from historical publications and deposited by OMIM, but another 20+ variants from the largest published series[107,108] were not.

Furthermore, phenotypic description associated with the deposited variant is often absent or sparse. For syndromes where the genital phenotype is incompletely penetrant or for genes where different types of mutations cause different conditions, it is critical to know the phenotypes associated with reported variants. For example, *AR* variants can cause X-linked spinobulbar atrophy (not a DSD) or androgen insensitivity syndrome (AIS). One of the largest depositors into ClinVar (Invitae) does not specify which of the conditions is linked

to variants and many other depositors do not indicate a phenotype at all. As a result, out of the ~400 *AR* variants in Clinvar, 190 provide a phenotypic description that includes DSD but only ~100 seem to be specifically linked to an actual DSD case.

Another problem is that the sex chromosome complement associated with the reported variant typically is not provided. Finding a variant associated with DSD in an XX individual may not be relevant to explaining an XY individual's condition, as some genes only affect genital phenotype (either in syndromic or isolated forms) in an XX or XY genetic background[3]. For example, *AMHR2* variants cause PMDS in men but not in XX women, who do not express AMH during fetal development. Reference databases for control, non-pathogenic variants (such as gnomAD) have started systematically reporting XX or XY status, and this needs to be adopted in databases aggregating disease variants as well. While the Leiden Open Variation Database reports zygosity and sex chromosome complement, when known, it is not routinely used by clinical algorithms. For these databases to realize their full potential, genotype-phenotype correlation, when known, needs to be curated, with an indication of known inheritance models. New, unpublished variants should be associated with a phenotypic description (for example. Did the individual have the condition for which this gene is known? In syndromic conditions, which of the incompletely penetrant features were present?). This will require a massive effort by expert curators. Unfortunately, unlike other conditions, there is no current working group for curation of DSD variants funded by the ClinGen consortium. For example, the only annotation currently available in ClinGen for *SRY* is "sufficient evidence for haploinsufficiency", without specification that this is true only in XY, and without mention of DSD caused by abnormal presence of *SRY* in XX individuals or by SNVs in *SRY* in XY individuals. A PanelApp effort sponsored by Genomics England is underway to crowd-source evaluation of existing clinical panels, including for DSD. For any such effort, specific functional protein regions and variants will need to be curated in addition to gene names, and the curated database integrated into analytic pipelines. We have started a special interest group on the Franklin platform to curate DSD variants, which will be open to interested contributors worldwide and would have the advantage of being directly integrated into the sequence analysis platform.

Validation of VUS

The emergence of databases reporting population frequency has helped prioritize variants tremendously, but new approaches need to be developed and brought to the clinical testing realm to enable classification of the thousands of variants uncovered by next-generation genome sequencing and mapping.

Integration with transcriptome analysis.—RNA-seq profiles can be utilized for both assessing functional consequences of VUSs and molecular phenotyping of a disease state. RNA-seq data can be interrogated directly if a variant is expected to lead to changes in splicing, allelic skewing, RNA instability, or expression levels. Alternatively, RNA-Seq datasets from multiple DSD samples can be grouped based on expression profile and those groups investigated to search for common causative variants. To facilitate these analyses, platforms integrating RNA Seq profiles with WGS and microarray data need to be made user-friendly and publicly available for wider use. However, the utility of transcriptome-

based VUS validation is limited by the availability of a relevant tissue on which to perform RNA-seq. Only about half of known DSD genes are expressed in blood cells, and a slightly higher percentage in skin fibroblasts[109]. For many DSD, whose origin is in the developing embryo, the relevant tissue is not available. Even a close proxy, such as adult genital or gonad tissue, is rarely available.

Interpretation of non-coding variants.—Pathogenic non-coding variants in known DSD genes have been reported (reviewed in[9,110]) and causative SVs have been identified outside of coding regions, in promoters and enhancers[111]. For example, duplication of an enhancer upstream of the *AR* gene was shown to increase expression[76] and a recurrent variant in the promoter region resulted in aberrant translation and complete AIS[112]. A 3-bp deletion in the promoter of *SRY* that partially deletes a binding site for the SP1 transcription factor resulted in 46,XY complete gonadal dysgenesis. Other examples include variants in regulatory regions for *SOX3*, *DMRT1*, or *NR0B1*/DAX-1. The best studied example is the amazingly complex *SOX9* regulatory region[110]. Variants affecting non-coding regions of *SOX9* lead to at least 4 different conditions, including 46,XX testicular DSD and long-bone disorder campomelic dysplasia, which is associated with DSD in 75% of 46,XY individuals. Non-conservation between mouse and human genomes complicates the study of DSD variants in animal models.

Non-coding variants are currently under-ascertained (only a few hundred regulatory variants have been classified as pathogenic in human disease[113–115]), and validation of such variants is difficult. Integration of regulatory region annotation into WGS, OGM, or LRS analysis pipelines is still being developed. High-throughput tests of chromatin accessibility, configuration and binding site occupancy need to be applied to DSD datasets and tissues to prioritize variants that may affect functional regions of the genome, such as enhancers, relevant transcription factor binding sites, or regions of open chromatin[116,117,118]. Focusing variant searches to the few percent of the genome with open chromatin and potential regulatory function vastly reduces the logistical complexity of WGS interpretation. High-throughput cis-regulatory element sequencing (CRE-seq) reporter assays to test the impact of regulatory variants on gene expression[119] should also be useful to classify VUS identified in DSD datasets.

DSD-specific cellular models.—For many DSD genes, such as *SOX9*, non-coding variants cannot be tested in animal models because of lack of evolutionary sequence conservation. Cellular models could be powerful tools to evaluate VUS and candidate genes affecting the molecular pathways present in the developing gonad[120]. The pluripotent human testicular embryonal carcinoma NT2/D1 cell line[121–125] expresses the regulatory pathways downstream of *SRY* and *SOX9* and responds to *SOX9* perturbation similarly to what is observed during early sex determination. *SOX9* is key to maintaining the integrity of Sertoli cells[126,127] and controls a vast number of biological processes in gonadal development[128]. Over- or under-expression of *SOX9* occurs in many XX and XY DSD. NT2/D1is therefore a powerful screening tool to detect patterns of gene network perturbation caused by DSD variants.

Attempts to reprogram skin fibroblasts into Sertoli cells, either via iPS cells or by direct transformation, have also emerged [129,130]. These would allow the generation of case-specific cell models for high-throughput testing of the downstream effects of VUSs on molecular pathways and cellular phenotype (cell adhesion, division, response to hormones, etc.).

IMPROVING CLINICAL PRACTICE

The aspirational goals set by the International Rare Diseases Research Consortium (IRDiRC) include a call for reaching an accurate diagnosis within one year of an individual coming to medical attention. Yet many individuals with DSD undergo years of endocrine testing, imaging, and diagnostic surgeries without reaching a diagnosis. Hormone assays may not reliably distinguish between conditions (Box 2), and practice varies considerably[131]. A European effort is under way to standardize laboratory assessment in DSD[131,132]. But beyond endocrine testing, the consensus statements of 2006 and 2016[1,7] emphasized the need for interdisciplinary care, which needs to include expert competence for ordering appropriate genetic tests and interpreting their results. DSD-TRN registry data showed that 97% of undiagnosed patients at centers in the network had not exhausted clinically available genetic tests[5]. Causes are likely to include reluctance by insurance companies or health authorities to authorize testing, limited access to testing, and discomfort toward genetic testing, but may also reflect clinician habits or the lack of a genetics provider on the team altogether[133,134].

Prioritizing genetic tests

Prioritizing genetics as part of an integrated approach with parallel anatomical and biochemical assessment prior to interdisciplinary decision-making is recommended[5,26,135] and should be considered standard of care. Cost-effectiveness of early genetic testing has been reported for DSD and other conditions[22,51,54,56,136] (Box 2). Results of WES and gene panels are now available in a few weeks, on par with some endocrine tests, allowing genetic testing to become a front-line approach that can be ordered early in the diagnostic process to orient and focus expensive, and possibly invasive, phenotypic exploration and discovery of comorbidities. The feasibility and usefulness of stat exomes (results within 2 days) for diagnosis of critically ill newborns (including syndromic DSD, such as CHARGE, Kabuki, or Noonan syndromes) has been demonstrated and implemented in various settings[137–141]. For DSD gene panels, most facilities offer 2–3 week turnarounds, which would be sufficient once life-threatening adrenal insufficiency has been ruled out.

A major difficulty is choosing the list of candidate genes when interpreting WES data or designing an enriched panel. In published research studies, the number of genes included in targeted panels ranged from 30 to 180[22,52–54,56]. The lack of consensus is also evidenced by the extremely diverse panels offered by companies for clinical testing. In the US, several facilities use NGS to support their panels: some offer true panels, with capture enriched for a chosen list of genes; and some offer *in silico* panels with exome-wide capture and interpretation limited to the panel genes. Enrichment technology is proprietary and not disclosed and the actual number of bases covered or depth of coverage for individual genes is not described. The number of genes varies even for similar test descriptions.

The ‘abnormal genitalia’ panels offered by Blueprint Genetics and the University of Chicago have 62 and 72 genes respectively. The ambiguously named ‘Disorder of male sex development’ (is it appropriate for males or for XY individuals?) panel from Invitae has 8 genes, the ‘neonatal 46,XY DSD panel’ from GeneDx has 19 genes, and the ‘46,XY DSD/complete gonadal dysgenesis’ panel from the University of Chicago has 26 genes. Average turnaround time ranges from 2 to 6 weeks and quoted price varies from $250 to $4,000. For facilities offering WES it is unclear what genes would be included in the analysis or if competency exists to specifically interpret DSD variants. In the Netherlands, Radboud-UMC offers a DSD/Primary adrenal deficiency WES-based test using a 143-gene panel with a 3–4 month turnaround for 900 euros in or a 15 working day turnaround for 4000 euros. Variability in practice is likely to be as great in other countries, but information is typically not made publicly available. Thus, establishing standardized testing protocols should be a priority of the field to improve test uptake and diagnostic yields.

Using diagnoses to improve care

A critical aspect of patient-centered care is diagnostic accuracy, which provides the means to avoid inappropriate treatments, adjust interventions to the specific condition, and improve outcomes[142]. For example, many individuals receive a working diagnosis of partial androgen insensitivity (PAIS), whereby an impaired tissue response to testosterone during development results in atypical external genitalia in 46,XY individuals. However, in only a minority of suspected *de novo* PAIS cases is a mutation actually found in the Androgen Receptor (*AR*) gene[106,143,144]. Those misdiagnosed may carry mutations in *SRD5A2*, *NR5A1*/SF-1, or *LHCGR* that can partially mimic PAIS. It is therefore critical to obtain a molecular diagnosis prior to deciding on a course of treatment, as response to androgen therapy could be very different depending on the gene mutated.

A precise genetic diagnosis is also needed to predict associated conditions. For example, *WT1* heterozygous mutations can present as isolated DSD in XY individuals, but patients are at risk of tumors and kidney failure. Different types of variants in *WT1* result in Denys-Drash syndrome (SNVs in exons 8/9) and Frasier syndrome (splice variants, reviewed in[145]). Wilms’ tumor is almost always associated with Denys-Drash, whereas risk of gonadoblastoma (but not Wilms’ tumor) is high in Frasier syndrome but kidney disease may be less severe [146]. Other congenital malformations, in particular cardiovascular and skeletal abnormalities, are frequently associated with DSD and must be ascertained and monitored[3,23,147].

Finally, it is important to determine the mode of inheritance not only for genetic counseling but also for carrier risk assessment. For example, many *NR5A1*/SF-1 mutations are inherited from seemingly unaffected parents[148,149] who are at risk of early ovarian or testicular failure, information important to their family and life planning.

Need for large registries

Because each individual DSD condition is rare, and best practices often unclear, large registries are needed to capture longitudinal clinical data, in association with molecular variants when known. These registries need to include presentations and natural history, as

well as the effects of hormonal or surgical interventions (or non-intervention) on gender identity, sexual function, psychosocial outcomes and quality of life.

The DSD-TRN has launched an effort to standardize practice and improve the standard of care at its member sites. It uses newly-designed, DSD-specific standardized forms to support all aspects of care (endocrine, genetic, anatomical, and psychosocial)[5,8]. The DSD-TRN registry collects this standardized information longitudinally to allow for investigation into long-term outcomes. The I-DSD registry[6] has also recently started to collect longitudinal data in a standardized fashion[150]. DSD-Life , a large European effort to recruit participants to measure psychosocial adaptation, psychosexual health and mental health, reported that participation varied by condition[151]. It is critical that clinicians and families of all ethnic and socio-economic backgrounds are invited to actively participate in these registries so that an accurate, global picture of lives with DSD can be captured and serve all equitably.

CONCLUSIONS

DSD is a hyper-specialized subspecialty of pediatric endocrinology and urology, but awareness is growing, fueled by highly successful advocacy efforts which place front and center the issues faced by individuals with a DSD. Yet, the global impact of DSD remains under-recognized, and individuals with DSD continue to face stigma and societal attempts at discrimination (Box 3).

Estimates of DSD incidence vary widely, depending on the conditions included; even expert providers disagree on what should be included, and the conditions referred to specialized centers vary widely[133]. When mild malformations are included, estimates can reach 1.7% live births[152]; isolated hypospadias alone has been calculated to affect about 1 in 125 boys[153]. For severe conditions under the DSD umbrella, recent studies cite aggregated incidences between 0.2% and 0.5%[26,110], making DSD one of the most frequent birth defects. An incidence of 0.5% for DSD equates to ~39 million affected families worldwide, which is similar to the number of people living with HIV/AIDS (according to HIV.gov) and 3–4 times more than the number of people with Parkinson's disease (according to the Parkinson's Foundation). As a comparison, arguably the best known rare genetic disease, cystic fibrosis, affects an estimated 70–100,000 people globally (according to the Cystic Fibrosis Foundation, Cystic Fibrosis Worldwide and ref. [154].

While a multidisciplinary approach to care is becoming the norm, it requires important resources and competencies not currently available in many settings. Major fundraising initiatives for medical research and infrastructure development will be necessary to increase the global awareness of DSD, support systematic large-scale variant validation studies and design of condition-specific bioinformatic analysis tools, and deploy evidence-based best practices in all aspects of clinical practice to benefit the DSD community at large, improve outcomes, and reduce stigma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLOSSARY

Positive predictive value (PPV) [G]:
The PPV of a clinical test is the ratio of the number of individuals confirmed to have the condition being tested for to those who tested positive with the test, irrespective of disease status. It predicts the likelihood of someone who tests positive of having the condition.

Isodicentric Y [G]:
Abnormal Y chromosome resulting in two centromeres and two identical arms (Yp or Yq). Breakpoints vary, but individuals with 2 short (Yp) arms may have two copies of the testis-determining gene *SRY* (located in Yp11.2), while those with 2 long arms typically do not carry *SRY*.

Cell-free fetal DNA [G]:
Fragments of DNA of fetal origin circulating in the maternal blood during pregnancy, which can be tested to screen for aneuploidies such as trisomy 21 or to ascertain the sex chromosome complement of the fetus.

Copy number variants [G]:
Variants, typically larger than 50 bp, that result in increased or decreased ploidy, such as a deletion on an autosome resulting in a single copy of the region instead of two. Copy number variants are a type of structural variant.

Chimerism [G]:
a condition whereby two different genomes are found in a single individual, usually as a result of fusion of two zygotes during a twin pregnancy. DSD can arise when the two genomes have a different sex chromosome complement (XX and XY).

Mosaicism [G]:
a condition whereby two different genomes are found in a single individual, typically resulting from a post fertilization mutation that is found only in the daughter cells of a subset of embryonic cells and thereby results in different phenotypic expression in different tissues. A frequent type of mosaicism associated with DSD is Turner syndrome variants with 45,X/46,XY mosaic karyotypes.

Structural variants [G]:
Structural variants include copy number variants, insertions, translocations or inversions. They can be balanced (when the rearrangement does not result in loss or gain of genomic material) or unbalanced.

Epimutation [G]:
A heritable variant that modifies gene expression through gain or loss of DNA methylation or other modification of chromatin without affecting the underlying DNA sequence.

Episignature [G]:
A unique pattern of epigenetic variation (typically DNA methylation) occurring at multiple nucleotide locations throughout the genome. Episignatures have shown potential diagnostic value in syndromic conditions where no underlying genetic etiology is found.

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Box 1.

DSD nomenclature

Patient-centered and multidisciplinary care, promoted by the Consensus Statement[1], has forced clinical teams to reflect further on the nomenclature for the conditions they care for. The term ‘Disorders of Sex Development’ was coined at the 2005 Chicago Consensus Conference as an answer to several historical issues[1]. One was the inherently confusing meaning of the word ‘Intersex’, which has a strong significance for identity in addition to a medical meaning. ‘Intersex’ suggested that, from a clinical standpoint, there is a requirement for ambiguous genitalia to ‘qualify’ for this diagnosis. Conditions with no external ambiguity, such as complete androgen insensitivity syndrome (CAIS), would illogically be excluded from the Intersex category, while congenital adrenal hyperplasia (CAH), which is often accompanied with ambiguous external genitalia in XX individuals, would be considered by some advocacy groups, such as the CARES Foundation, as a ‘disorder affecting the adrenal glands’ without acknowledgement of the word ‘Intersex.’ It was also felt that a clear medical slant to naming was needed to ensure that all the tools of evidence-based medicine would be available to systematically evaluate the effects of clinical management on outcomes. Finally, it was proposed that the term should be as precise as possible and should reflect the genetic etiology when available.

The Global DSD Update[7], a follow-up to the Chicago Consensus Conference, summarized the positive aspects of the DSD nomenclature, including facilitated access to health care and insurance, an umbrella term helping generate a comprehensive and integrated model of care, and distinction from other conditions such as gender dysphoria. It also warned against the negative aspects: stigma associated with the word ‘Disorder’, the perceived implication that ‘sex’ implies sexual behavior, and the possible risk of increasing participant refusal in research under the DSD heading.

Now that the medical and health insurance community are aware of the complexities of DSD, the role of the word ‘disorder’ as a catalyst is waning, and it is time to retire it. The word ‘Difference’ has emerged as a trending alternative, and is increasingly being used in publications over the past few years. ‘Difference’ does not carry the potential stigma of ‘Disorder’, has been used by advocacy organizations and the National Health Service in the UK, and allows the recognizable acronym DSD to be retained, which is widely used throughout the medical literature.

Box 2:

A case for early comprehensive NGS testing in DSD diagnosis

Here, we outline the diagnostic process and timeline (see figure) for an individual taken from the DSD-TRN registry.

Family history.

Ultrasound during the sixth pregnancy of second cousins identified female genitalia in the fetus while Non-Invasive Prenatal Screening (NIPS) suggested the presence of a Y chromosome. An older brother had been born with ambiguous genitalia, but no cause had been established. An ultrasound at birth showed testes in the labioscrotal folds.

Endocrine testing.

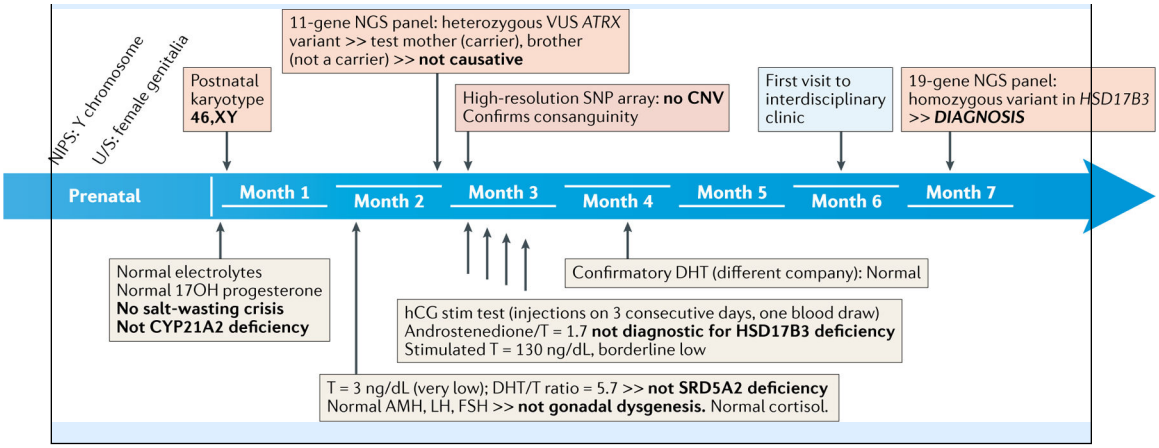
While 21-hydroxylase deficiency does not cause genital atypia in XY individuals, measurement of electrolytes and 17-OH progesterone was performed on day 2 and showed normal levels. A second blood draw at about five weeks of age showed normal gonadotropins, cortisol, Anti-Müllerian hormone, very low testosterone (T), and a dihydrotestosterone (DHT)-to-testosterone ratio not diagnostic for SRD5A2 deficiency. A confirmatory DHT test was normal. The child was subjected to a human chorionic gonadotropin (hCG) stimulation test in pursuit of a HSD17B3 deficiency diagnosis. This rare recessive condition results in ambiguous genitalia in XY individuals owing to impaired testosterone synthesis. Stimulated testosterone was borderline low, but the test was not diagnostic.

Genetic testing.

The 46,XY karyotype was confirmed at birth. A microarray identified regions of homozygosity, as expected in this consanguineous family, but no copy number variants. An 11-gene NGS panel reported a variant of uncertain significance in *ATRX;* testing of the mother and affected brother ruled out this etiology. Finally, a firm diagnosis was made at age $7^{1/2}$ months when another NGS panel (of 19 genes) identified a homozygous variant in *HSD17B3*.

Conclusion.

A large candidate gene panel or whole exome sequencing, ordered as an emergency test at birth, would have reassured the family within two weeks, as this condition has no known comorbidities. It would also have been very cost-effective, avoiding 7 blood draws from 3 individuals, 7 endocrine tests, an hCG stimulation, an inadequate NGS panel, and a microarray. Understanding the genetic diagnosis will orient management. Prenatal testing can be offered for future pregnancies. Early treatment may result in more typical male anatomy in adulthood. Sisters of prepubertal age should be tested: while XX individuals are asymptomatic, some XY individuals are born with typical female anatomy but develop signs of virilization and hirsutism at puberty, leading to a change to male gender in about 50% of cases. Alternatively, removal of the (infertile) gonads prior to puberty prevents the phenotypic changes (for complexities of management and gender outcomes in this condition, see[156–159]).



Box 3:

Societal Challenges: DSD, genetic diagnosis, and sports

Athletic authorities have attempted to prevent women athletes with a DSD from competing in women's events for years, often using genetic data as justification. In 1968, genetic screening was imposed for all women athletes, initially as a sex chromatin test and later as a molecular genetic test. It is estimated that women were banned from events at almost every Olympic games between 1968 and 1984, while others likely 'self-disqualified'[160]. Women who were removed had no proven advantage (such as women with complete androgen insensitivity), yet this genetically-based policy would potentially let 46,XX men compete. Under pressure from sports leaders and geneticists (such as Arne Ljungvist and Albert de la Chapelle, respectively) the history of discriminatory rulings targeting women with a Y chromosome concluded in 1999, with the declaration that the presence of a Y chromosome no longer precluded eligibility for women's competitions (see[161] for a detailed account).

However, this progress was undermined in 2018 when the International Association of Athletic Federation (IAAF, now World Athletics) introduced a new set of rules that lowered the testosterone limit to 5 nmol/L for eligibility to compete in the women's category of a small number of events, namely distance running from 400m to the mile[162]. No clear rationale was provided for the rule change but it affected only those events in which Caster Semenya competed, a multiple women's 800m champion whose biological sex had become a source of speculation. The controversy became an international outcry when, on May 1, 2019, the Court of Arbitration of Sports (CAS) sided with the IAAF, preventing Ms. Semenya and other women with naturally high testosterone levels from competing with other women unless they modified their natural biology.

The ruling is worrying for a number of scientific reasons. Only athletes with a DSD who carry a Y chromosome are targeted: a woman with an XX karyotype and a condition resulting in a testosterone level above 5 nmol/L (which has been reported, albeit rarely[163,164]) will remain eligible. (For a recent history of testosterone regulations in sports, refer to[165]). To take into account DSD mutations that cause complete or partial androgen sensitivity, only women athletes who are "sufficiently androgen-sensitive that their elevated testosterone levels have a material androgenising effect"[162] are affected by the ruling. However, there is currently no reliable diagnostic test to measure androgen sensitivity of muscles and no tests are performed on the athletes, which highlights the limitations of current genetic tests and their potential discriminatory consequences: although they can identify a genetic variant, physiological effects are not always clear. Finally, the new regulations are based on the unproven claim in the CAS Executive summary that "female athletes with 46 XY DSD enjoy a significant performance advantage over other female athletes without such DSD". The departure by CAS from the expectation that guidelines should rely on evidence is disturbing. CAS had suspended similar guidelines in 2016, arguing that the IAAF could not demonstrate that testosterone accounts for the entirety of the 10%−12% difference between male and female performance and requesting the IAAF to produce additional data to justify the

guidelines. The 2019 reversal of the 2016 ruling occurred without the production of such evidence.

We are now back to the pre-1999 position, whereby chromosomes define who is a man and who is a woman – a position that excluded countless athletes with 46, XY DSD conditions who were born, raised and self-identified continuously as women. With an increasing number of countries (including top Olympic countries such as Germany and Australia) allowing for non-binary gender identification on legal documents, the regulations are disconnected from societal trends and from the principles of the Olympic Charter: “Every individual must have the possibility of practicing sport without discrimination of any kind and in the Olympic spirit”[166]. The long journey of athletes with a DSD, and the discriminatory rulings they face, should inspire us to consider the complexity of interpreting genetic tests and cherish our most treasured value in science: evidence.

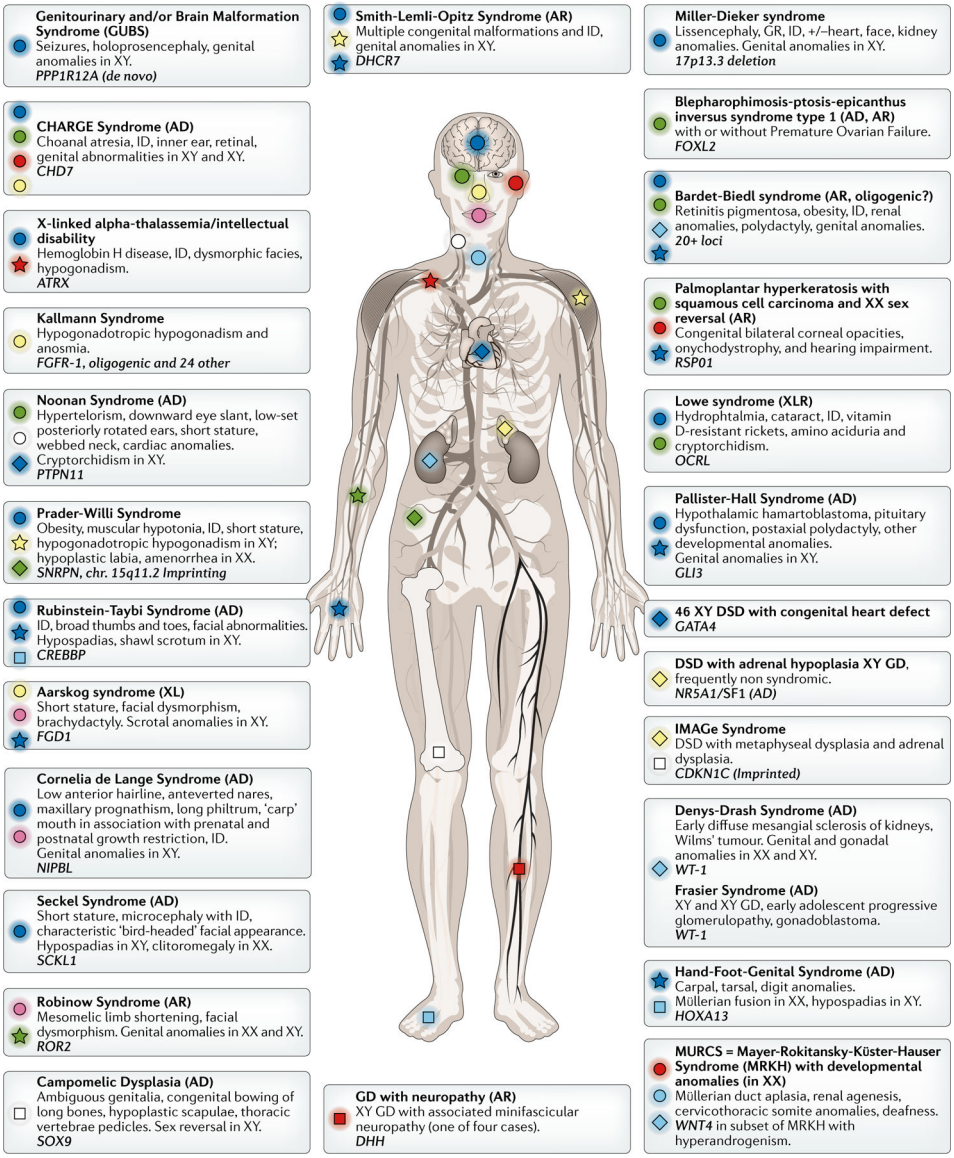


Figure 1: Disruption of processes in many different organs can result in isolated and syndromic forms of DSD.

A selection of conditions is represented. The main organs affected (each represented by a unique combination of colour and symbol), mode of inheritance and the main genetic etiology are shown. Although not recommended nomenclature by the 2006 Consensus Statement1, the term 'sex reversal' is used when it is part of the OMIM description. AD: autosomal dominant; AR: autosomal recessive; XL(R): X-linked (recessive), GD: gonadal dysgenesis; GR: growth retardation; ID: intellectual disability.

Table 1:
Major genetic etiology of 46,XX, 46, XY, and sex chromosome DSD

Additional rare syndromic forms of DSD are shown in Fig. 1.

DSD	Inheritance[1]	Typical DSD genital phenotype	Genetic cause(s)[2]	Available tests	ClinVar limitations	Comments
46XX, DSD (~35 % of cases)						
Disorders of androgen excess: CAH	AR	Clitoromegaly, urogenital sinus, labioscrotal fusion. Normal ovaries.	*CYP21A2* in >90%	Newborn screening Gene panel for common variants Seq	Variant zygosity, sex chromosome complement, and genital phenotype are not indicated for these recessive conditions with sex-limited expression. Unclear which variants are linked to DSD.	Genetic diagnosis rarely pursued. Endocrine test: level of 17OHP
			CYP11B1	Seq		-
			CYP19A1	Seq		P450 aromatase deficiency
Testicular or ovotesticular DSD	*De novo*	Male or ambiguous genitalia. No uterus.	*SRY* translocation	FISH CMA Karyotype	Four pathogenic variants marked as copy number gain of Y including SRY. Whether patient is XX is not indicated.	Diagnosis of (ovo)testicular DSD requires histological examination of gonad.
			SOX9 CNV	CMA	Five variants curated from original papers.	
			SOX3 CNV	CMA	No *SOX3* CNV in database. Six reported variants linked to a different condition.	
			R92W variant in *NR5A1*	Seq	Shown as “conflicting interpretations of pathogenicity” despite strong published evidence for pathogenicity.	
			WT1	Seq	None of 637 variants indicate 46,XX DSD	
Premature ovarian insufficiency (POI) *	AR, AD, XL	Primary or secondary amenorrhea in phenotypic females.	*FSHR, BMP 15, FMR1, NR5A1* and 20 other genes	Seq	Variable. See examples in Supp. Table 1	Each gene explains a few cases. Etiology overlaps with POF and ODG.
MRKH	Not known	Aplasia of Mllerian structures	*Not known*	—	—	Candidate loci only. Not clinically tested.
46XY, DSD (~ 50% of cases)						
Disorders of gonadal development	Y-linked	Pure, partial, or mixed gonadal dysgenesis. Uterus or urogenital sinus present. Female or ambiguous genitalia.	*SRY* SNV	Seq CMA	Strong evidence. 28 likely/pathog enic variants, three VUS, none benign.	Disorders of sex determination
	AD		*NR5A1 SNV*		14 variants (36%) deposited by Invitae indicate Oligosynaptic infertility (OMIM #258150), a condition linked to a different gene.	
	AD		*SOX9 CNV, SNV*		Three variants curated from original papers. eight associated with	

DSD	Inheritance[1]	Typical DSD genital phenotype	Genetic cause(s)[2]	Available tests	ClinVar limitations	Comments
					campomelic dysplasia and sex reversal, but karyotype is not indicated. Only 17/160 variants associated with sex reversal. Most others do not indicate phenotype.	
	XL		*NROB1* dup		13 variants deposited by Invitae do not distinguish between phenotypes of loss of function variants (causing a different condition, AHC) and duplications.	
	AD		*MAP3K1 SNV*		Majority (21/30) of variants are reported as benign. Rapidly increasing numbers of benign, but not pathogenic, variants reported over time.	
	De novo		*9p24* deletion		n/a	
	AR		*CBX2 SNV*		Three variants including the two alleles of the original patient[155]; zygosity is not indicated for third variant.	
	AR		*DHH SNV*		35 variants, mostly VUS, with zygosity not indicated for this recessive condition.	
	AD		*WT1* SNV		637 variants. None of the entries specify with which of the several WT1-linked conditions they are associated.	
	AD		*DHX37 SNV*		Five, but not all, of the original published variants are in the database.	
Disorders of androgen action (AIS)	XL (~2/3), *de novo* (~1/3)	*Complete AIS (CAIS)*: Female external genitalia, functional often inguinal testes, blind vagina, no uterus. *Partial AIS (PAIS)*: Hypospadias, micropenis, gynecomastia.	*AR SNV, deletion*	Deletion by NGS or CMA (CAIS only) Seq	Only a fraction of known variants in the database. Most entries do not specify if variant is found in DSD condition.	The PAIS term should be reserved for cases in which a variant is found in the *AR* gene.
Disorders of androgen synthesis	AR	Hypospadias, micropenis/ clitoromegaly, urogenital sinus, labia or bifid scrotum. No uterus.	*SRD5A2*	Seq	Variant zygosity, sex chromosome complement, and genital phenotype are not indicated for these recessive conditions with sex-limited expression. Unclear which variants are Linked to DSD.	5α reductase deficiency
			CYP17A1			CAH
			HSD3B2			CAH
			StAR			Lipoid CAH
			HSD17B3			See Box 2

DSD	Inheritance[1]	Typical DSD genital phenotype	Genetic cause(s)[2]	Available tests	ClinVar limitations	Comments
			POR			Cytochrome P450 oxidoreductase deficiency
Persistent Müllerian Duct Syndrome	AR	Müllerian and Wolffian ducts coexist; inguinal hernias or undescended testes.	*AMHR2*, *AMH* SNV Indel	Seq	Historical variants are curated. For *AMH*, they are the only pathogenic variants in the database. For *AMHR2*, two other pathogenic variants are reported, but zygosity is not indicated. 13/14 variants from original published series are not in database.	-
Sex chromosome DSD (~15% of cases)						
Klinefelter syndrome	*De novo*	Gynecomastia, small testes, Leydig cell hyperplasia.	47,XXY	Karyotype	-	-
Turner syndrome variants	*De novo*	Various degrees of virilization. Mixed gonadal dysgenesis.	45,X/45,XY mosaics	Karyotype	-	-
Chimerism or Mosaicism	*De novo*	Ovotesticular DSD	46,XX/46,XY	Karyotype	-	-

[1]The most frequent mode inheritance is indicated; note that 'AD' may also include new mutations.

[2]Only the main genes involved are included. Where 'SNV' is indicated, most conditions can also be caused by small indels or splice variants, which are also detectable by sequencing.

[*]POI includes premature ovarian failure (POF) and hypergonadotropic ovarian failure (ODG) conditions. Those cases for which causative genes are known are named ODG1–8 and POF1–16 in the Online Mendelian Inheritance in Man (OMIM, omim.org) classification.

AR: autosomal recessive, AD: autosomal dominant, XL: X-linked, Seq: sequencing, FISH: fluorescence *in situ* hybridization, dup: duplication, CMA: chromosome microarray, CAH: congenital adrenal hyperplasia, 17OHP: 17-hydroxyprogesterone, CNV: copy number variant, MRKH: Mayer Rokitansky Küster Heuser syndrome, AHC: adrenal hypoplasia congenita, AIS: androgen insensitivity syndrome, VUS: variant of uncertain significance.

Table 2:

Clinical and preclinical tests for DSD.

Technique	Time taken[1]	Variants detected					Resolution	Diagnostic yield
		A/M	CNV	cSNV	ncSNV	SV		
Clinically available methods								
Karyotype	1–2 weeks	✓	✗	✗	✗	✓	>5 Mb	15% (mostly mosaics)
Interphase FISH (X, Y and *SRY* markers)	<3 days	✓	✗	✗	✗	✗	N/A	Rapid sex determination
Microarray	2–3 weeks	✓	✓	✗	✗	✗	>50 kb	15%
Single gene test or gene panel[2]	Up to 6 weeks	✗	✗	✓	(✓)	✗	SNV: 1 nt Indels: <50 bp	Panel-dependent
Exome sequencing	Up to 12 weeks (<1 week possible if urgent)	✗	✗	✓	✗	✗	SNV: 1 nt Indels: <50 bp	30–45%
Whole genome sequencing	Up to 16 weeks	✗	✓ (requires validation)	✓	✓	✓ (requires validation)	SNV: 1 nt	At least 30–45%
Pre-clinical methods								
Optical genome mapping[3]	Up to 12 weeks	✓	✓	✗	✗	✓	SV: >500 bp	Not known
Long-read sequencing[4]	Not known	✗	✓	✗ (not yet)	✗ (not yet)	✓	SV: 50 bp – 5 kb?	Not known

[1]Availability of test and time to results is highly dependent on location. Time estimates do not include time to obtain insurance pre-authorization to perform a test.

[2]The nature of the single nucleotide variants (SNVs) detected will depend on sequencing primer design (for single gene tests) or the variants included in the gene panel.

[3]Optical Genome Mapping is currently clinically approved in the USA only for FSHD (facioscapulohumeral muscular dystrophy), and has yet to be validated specifically in DSD. OGM is not designed for base-pair resolution and therefore cannot detect SNVs.

[4]Long-range sequencing is not in current clinical use. Although it does not yet have base-pair accuracy (the current error rate is ~5% at each nucleotide position), improved algorithms and detection methods should allow reliable detection of SNVs in the future. A/M: aneuplodies and/or mosaics, SV: structural variants, CNV: copy number variants, cSNV: coding SNV. ncSNV: non-coding SNV, N/A: not applicable.