The Use of Genetics for Reaching a Diagnosis in XY DSD

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Abstract

Reaching a firm diagnosis is vital for the long-term management of a patient with a difference or disorder of sex development (DSD). This is especially the case in XY DSD where the diagnostic yield is particularly low. Molecular genetic technology is playing an increasingly important role in the diagnostic process, and it is highly likely that it will be used more often at an earlier stage in the diagnostic process. In many cases of DSD, the clinical utility of molecular genetics is unequivocally clear, but in many other cases there is a need for careful exploration of the benefit of genetic diagnosis through long-term monitoring of these cases. Furthermore, the incorporation of molecular genetics into the diagnostic process requires a careful appreciation of the strengths and weaknesses of the evolving technology, and the interpretation of the results requires a clear understanding of the wide range of conditions that are associated with DSD.

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Introduction

Differences and disorders of sex development (DSD) is a collective term for a group of relatively rare congenital conditions that are associated with an alteration in chromosomal, gonadal, or anatomic sex [Hughes et al., 2006]. Atypical genitalia at birth are the most common manifestation of DSD occurring in approximately 1 in 300 births [Ahmed et al., 2004]. However, genitalia that are sufficiently atypical to require any investigations during early infancy are rarer with a birth prevalence of about 1 in 1,200 in term infants [Rodie et al., 2022]. In addition, three-quarters of cases of atypical genitalia that present with a hypospadias have a 46,XY karyotype and are raised as boys [Ahmed et al., 2004; Rodie et al., 2022]. A substantial proportion of these boys will be profoundly undermasculinised, and at least 20–25% may not have a satisfactory outcome [Al-Juraibah et al., 2019; Tack et al., 2020]. In the past, many infants with XY DSD were raised as girls, but as the pendulum shifts towards raising them as boys [Kolesinska et al., 2016], it is likely that these boys and young men will have more extensive health care needs. While there are many factors that determine long-term outcome, the underlying cause of the DSD will

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Table 1. Causes of XY DSD

Disorders of gonadal development
Gonadal agenesis
Gonadal dysgenesis, complete and partial forms
Ovotesticular DSD
Disorders of cholesterol synthesis
Smith-Lemli-Opitz syndrome
Disorders of androgen synthesis
Enzymatic defects in adrenal steroidogenesis
STAR deficiency
P450scc deficiency
3β-hydroxysteroid dehydrogenase II deficiency
17α-hydroxylase and 17,20 lyase deficiency
P450 oxidoreductase defect
Enzymatic defects in testicular steroidogenesis
P450 oxidoreductase defect
Isolated 17,20-lyase deficiency
Cytochrome b5 defect
17β-hydroxysteroid dehydrogenase III deficiency
Enzymatic defects in alternative pathway to DHT
17β-hydroxysteroid dehydrogenase deficiency due to *AKR1C2* and *AKR1C4* defects
Disorders of testosterone metabolism
5α-reductase type 2 deficiency
Disorders of androgen action
Androgen insensitivity syndrome, complete and partial forms
Persistence of müllerian ducts
Defect in AMH synthesis
Defect in AMH receptor
Disorders of Leydig cell function
LH deficiency (hypogonadotrophic hypogonadism)
Impaired Leydig cell differentiation (LHCGR defects), complete and partial forms
Other causes of XY DSD
Maternal intake of endocrine disruptors
Associated with impaired prenatal growth
Genetic malformation syndromes
Isolated or complex atypical genitalia of unknown cause (non-specific XY DSD)

play a major part in influencing long-term outcome. XY DSD can have multiple causes (Table 1), and a clear diagnosis may allow the adoption of a more individualized approach rather than a common management pathway that is simply based on the broad phenotype such as hypospadias. With the advances in laboratory-based diagnostic technology, a clear diagnosis is more likely than ever before, but this requires a systematic and a multidisciplinary approach, especially as the upscaling of genetic testing has introduced challenges in deciphering which variants are disease causing. The introduction of published guidelines for variant interpretation has sought to standardise variant interpretation with respect to disease and inheritance pattern but challenges still exist [Richards et al., 2015; Ellard et al., 2019]. In a survey performed about 5 years ago, expert centres that encountered an infant with XY DSD performed comprehensive genetic investigations to a variable extent [Kyriakou et al., 2016]. This may be due to the burden that a comprehensive approach places on local health care resources, but it is also possible that the clinical case for reaching a firm diagnosis, especially through detailed molecular genetic investigations, has not been made sufficiently strongly for boys with XY DSD. This review will explore the evidence that exists for the routine use of genetic investigations for some conditions that result in XY DSD.

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Diagnostic Technologies in Genetics

In the case of XY DSD, genetic testing has increasingly become part of standard clinical practice [Kyriakou et al., 2016; Audi et al., 2018; Ahmed et al., 2021], but there is a wide range of techniques that may be involved in the diagnostic process, each having a different investigative application and genetic resolution. Although standardized approaches exist based on the specific chromosomal sex, the diagnostic strategy may vary depending on the patient's characteristics and the likelihood of a genetic condition. The typical three-tier investigative approach to a newborn with suspected XY DSD includes the following successive steps: (1) defining the sex chromosome complement in combination with ultrasound to define internal genitalia and biochemical testing for life-threatening comorbidity; (2) an extensive biochemical evaluation of testosterone synthesis and action; and (3) specific genomic testing for causative variants. Although this order has been used in clinical practice for many years, it is increasingly becoming clear that genetic analysis is being performed at an earlier stage in the diagnostic process either in parallel with biochemical investigations or even prior to performing detailed endocrine investigations or irrespective of those results [Kyriakou et al., 2016; Alhomaidah et al., 2017; Nixon et al., 2017; Audi et al., 2018; Johnson et al., 2020; Ea et al., 2021]. Here, we discuss key genetic technologies that have been adapted for clinical diagnosis in DSD.

Postnatal chromosomal sex can be studied by a number of methods. Preliminary sex chromosomes determination assay can be performed quickly using quantitative fluorescence PCR (QF-PCR). Microsatellite loci present on the sex chromosomes are amplified using fluorescently labelled primers, and subsequent electrophoresis separation of the labelled products by length allows the quantification of X and Y chromosomes [Mann et al., 2012] as well as detection of structural abnormalities, such as isodicentric X [Donaghue et al., 2003] or partial Y chromosome translocation on the X chromosome [Mansfield et al., 2015]. Although this test is highly accurate, the results require confirmation by karyotype analysis to exclude other chromosomal rearrangements. The conventional G-banded karyotyping is widely available as a gold standard method to identify chromosomal abnormalities larger than 5–10 Mb in size. The technique is based on precise microscopic visualisation of condensed metaphase chromosomes additionally stained by trypsin in combination with Giemsa or Leishman stain. The karyograms that are obtained can be interpreted by the chromosomal number, appearance, and banding patterns to identify aneuploid or mosaic karyotypes as well as relatively large chromosomal translocations, deletions, duplications, and inversions.

Whilst conventional karyotyping is more appropriate for routine clinical diagnosis, more speedy molecular cytogenetic techniques that do not require cell culturing can be employed for the detection of smaller submicroscopic copy number changes (CNVs). FISH technique uses specific fluorescently labelled DNA probes that hybridize to complementary target regions at any chromosomal site of interest. The common application of FISH in DSD is the identification of numerical and structural abnormalities of sex chromosomes, such as aneuploidies or isodicentric Y [Robinson et al., 1999]. Translocation of the *SRY* gene can also be tested by FISH in individuals with 46,XX testicular or ovotesticular DSD [Laino et al., 2014; Baetens et al., 2017]. However, rare locations of the translocated gene can also be uncovered [Peng et al., 2015]. Deletions and amplifications >50 kb can be identified for known chromosomal regions only since FISH probes are designed to be complementary to a known target DNA sequence which is subsequently indicated by the presence or absence of a fluorescent signal. The exclusion of the whole *SRY* gene deletion by FISH using a specific probe may not necessarily detect aberrations with partial gene loss [Jaillard et al., 2015]. Hence, the technique has also been widely employed as a confirmation tool for genomic changes identified previously by other methods such as chromosomal microarray [Brandt et al., 2013].

The resolution of genetic techniques drops further to the level of thousands of nucleotide pairs with chromosomal microarray technology (CMA) which allows the detection of CNVs that are <1 kb. Submicroscopic rearrangements as small as 0.3 kb have been identified using CMA in a cohort of patients with 46,XY DSD [White et al., 2013]. Two types of CMA are currently in use in clinical practice: microarray-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) arrays. Both are molecular cytogenetic methods whereby genome-wide screening for CNVs is performed by the hybridization of fragments of patient DNA with matching synthetic nucleotide probe sequences on the arrays which are fluorescently labelled. Array CGH includes the comparison of signals from differently labelled test and control DNA samples analysed on the same array chip, whereas SNP arrays measure the signal intensity as well as allele distribution first, comparing the results with a reference population frequency. In addition, the DNA probes can be synthesised in different lengths, allowing

the targeting of particular genomic regions that are functionally related to the regulation of sex development [Ylstra et al., 2006; Bashamboo et al., 2010b; Croft et al., 2018]. Unlike aCGH, the SNP array has wider diagnostic applications by detecting triploidy, mosaicism, and regions of homozygosity [Levy et al., 2018] which may overlap with genes that are known to cause an autosomal recessive DSD.

The prevalence of CNVs among individuals with DSD revealed by CMA varies considerably between studies, depending on the methodology employed, and ranges from 14 to 33% [Tannour-Louet et al., 2010; White et al., 2011; Norling et al., 2013; Délot et al., 2017; Audi et al., 2018]. The highest diagnostic rate was observed in those who had apparent dysmorphic features and developmental delay [Ledig et al., 2010; Baetens et al., 2014]. The diagnostic rate may also depend on the CNV detection limit [Amarillo et al., 2016]. In addition, novel genotype-phenotype associations can also be established. Deletions involving removal of the whole *DMRT1* gene were linked to a 46,XY complete or partial gonadal dysgenesis phenotype [Ledig et al., 2010; Tannour-Louet et al., 2010; Igarasha et al., 2013], whereas partial deletion of the gene may contribute to ovotesticular DSD [Ledig et al., 2012]. Although deleterious variants outside the coding region of the genome remain largely unexplored, some CNVs can cause inappropriate binding of the transcription factors to regulatory noncoding elements which may have negative implications on gene expression and result in DSD [Baetens et al., 2016; Croft et al., 2018]. Identification of CNVs in such regulatory regions led to the realisation that genes such as *GATA4* [White et al., 2011; Harrison et al., 2014] and *SOX9* [White et al., 2011; Kon et al., 2015] were involved in gonadal development.

While CMA identifies CNVs between 10 kb and 5 Mb in size and which may affect several genes, smaller genomic rearrangements, such as single nucleotide (SNV) or insertion-deletion variants (INDELs), can be captured by gene sequencing technology. The 2 approaches that are widely used include the traditional Sanger sequencing, approaching a single gene or small set of genes, and a massive parallel sequencing, also known as next-generation sequencing (NGS), a high-throughput screening technology (HTS) which can analyse multiple short DNA target sequences (<300 kb) simultaneously in one sample. Most of the sequencing methods are based on the principle of synthesis of new DNA strands [Sanger et al., 1977] where oligonucleotide primers are incorporated into a new DNA strand using a mixture of deoxynucleotide triphosphates and chain-terminating deoxynucleotide tri-phosphates for each nucleotide that is fluorescently labelled. The Sanger sequencing of the coding exonic and flanking intronic regions is usually a method of choice when one specific genetic condition is highly suspected. For example, *AR* and *SRD5A2* are the most requested genes to be sequenced using this approach in 46,XY DSD [Kyriakou et al., 2016], while other commonly requested gene lists include *HSD17B3* and others depending on which particular familial variant is suspected. However, many DSD conditions may be masked under an oligogenic basis of disease transmission, meaning that a simultaneous contribution of multiple gene variation may lead to a DSD phenotype [Camats et al., 2018; Fluck et al., 2019; Martinez de la Piscina et al., 2020]. More robust NGS may be chosen to discover novel variants in targeted gene panels (usually dozens of genes) or on a wider whole-exome (WES) and whole-genome (WGS) scale. Moreover, the target DNA can now be read in longer fragments (several kb) before the sequences are mapped together to unique positions in reference genomes using bioinformatics software. The long-read sequencing has allowed to fill in the gap in the reference data for the centromeric region of the Y chromosome [Jain et al., 2018] and has become a promising method for attempting to read full RNA sequences [Barseghyan et al., 2018].

The diagnostic yield of WES is not that different to NGS-based targeted gene panel analysis [Baxter et al., 2015; Délot et al., 2017; Xu et al., 2019], while a thorough selection algorithm based on stepwise genetic testing may increase the diagnostic yield up to two-thirds [Xu et al., 2019; Jacobson et al., 2020; Mazen et al., 2021]. On the other hand, WES allowed the discovery of novel DSD candidate genes, such as *FANCA* [Mazen et al., 2021], *FOG2/ZFPM* [Bashamboo et al., 2014], *HHAT* [Callier et al., 2014; Mazen et al., 2021], *SOX8* [Portnoi et al., 2018], *NR2F2* [Bashamboo et al., 2018], *ZNRF3* [Harris et al., 2018], *EP300, KDM6A,* and *CDT1* [Gazdagh et al., 2016]. Others may be discovered by overlapping WES results with animal model experiments [Barseghayn et al., 2018]. The moderate diagnostic success of WES in DSD patients may be explained by limitations of the technique itself (Table 2) or by the increasing belief that other genetic mechanisms exist within the subset of non-coding DNA that may be involved in the regulation of sex development [Atlas et al., 2021]. The viability of the latter is supported by discoveries in the field of androgen insensitivity syndrome (AIS). Using different strategies, various genetic alterations immediately upstream of the *AR* gene were identified in patients with AIS proven not to be due to a variant in the coding region of *AR* [Lower et al., 2004;

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Table 2. Advantages, disadvantages, and the applicability of molecular genetic techniques in the field of DSD diagnostics

Range of techniques	Genomic resolution	Applicability in DSD	Advantages	Limitations
QF-PCR	Variable and depends on coverage of informative markers	Early detection of sex chromosome aneuploidies based on PCR amplification of sex chromosomes	Short turnaround time of approximately 1 working day; small amount of sample required (>1 mL of blood); unlike in karyotyping, can be performed using inviable foetal cells as no cell culture is required for PCR; accurate and cost effective	Changes outside the target sequences of the chromosomal markers cannot be captured;balanced rearrangements, including partial monosomy, and low-level mosaicism cannot be found even within the target region
G-banded karyotype	Larger than 5–10 Mb in size	Conventional cytogenetic technique to produce a visible representation of a chromosome complement by condensing and G-banding (Giemsa staining) of chromosomes pre-treated with trypsin. The alternation pattern of light and dark bands allows to detect microscopic numerical and structural changes including aneuploidy, translocations, deletions, and duplications.	Widely available single assay baseline genomic screen for numerical and structural chromosomal abnormalities; able to detect most sex chromosome mosaicism	The turnaround time for results is 7–10 days as cell culture is required; requires a same-day specimen with fresh viable cells; technique is limited by resolution of 5 Mb and should be combined with other methods to detect low levels of mosaicism
FISH	Usually lies between 50 and 500 kb	Molecular cytogenic technique that uses fluorescent probes to identify either the position of genes, some structural variations (translocations and insertions), CNVs associated with specific genes (amplifications and deletions), or to span entire chromosomes in complex rearrangements (spectral karyotyping and multi-colour FISH)	Relatively inexpensive compared to other molecular genetic techniques; rapid turnaround time of 24–48 h	Resolution limitation; detects only known chromosomal imbalances determined by the specific probe
Chromosomal microarrays: aCGH and SNP array	10 kb, assuming sufficient SNP coverage	Genome-wide screening for CNV at a kilobase level resolution by the hybridization of fragments of patient DNA with matching synthetic nucleotide probe sequences on the arrays labelled with fluorochromes. Known as the first-line approach in case of multiple congenital malformations and unexplained developmental or intellectual disorders. Considered part of first line genetic testing in DSD	Whole genome screening; exon coverage may allow detection of intragenic CNVs including those affecting a single exon; SNP array has wider diagnostic potential compared to aCGH as it can detect polyploidy, chimerism, mosaicism, and regions of homozygosity	aCGH cannot detect balanced chromosomal rearrangements and may not detect mosaicism unless combined with conventional cytogenetic techniques; some CNVs identified by microarray are challenging to interpret in relation to the degree of pathogenicity; guidance seeks to standardise the interpretation and reporting of CNVs
Single gene sequencing (Sanger sequencing)	1 bp	The method is based on in vitro DNA replication methodology when the sequences of fragmented DNA are sequentially identified from fluorescent signals in order to capture genomic variants at the nucleotide level. Can be used for familial variant testing or smaller studies, e.g., to validate the single gene variants identified through HTS assays or research studies	Rapid turnaround time from 3 to 14 days; reduced chance of incidental findings; may be used to ensure sequencing coverage of the targeted region, e.g., complete sequencing in regions not covered by HTS	Time-consuming; low discovery power; sequence read length is limited by 700–900 bp

Table 2 (continued)

Range of techniques	Genomic resolution	Applicability in DSD	Advantages	Limitations
HTS	1 bp	NGS of targeted gene panels, spanning the protein coding regions of common DSD genes, provides a molecular diagnosis for about 50% of patients. Genomic sequences of these genes can be determined for multiple samples simultaneously and quantitatively compared to reference data	More time efficient and cost effective than single gene approach; greater diagnostic yield	Read length limit (usually less than 200 bp); initial gene panel development is time consuming, labour intensive, and has limited future flexibility for the addition of new DSD-related genes; data output requires expert processing and analysis that depend on the availability of infrastructure and bioinformatic expertise; causative variants outside the targeted regions will not be identified
		Unlike targeted gene panel approach, WES can identify novel candidate DSD genes within the protein-coding regions of genome	Time efficient and cost effective; strong discovery power; opportunity to use WES to target a large DSD gene panel to aid interpretation of results and to easily modify targeted panels as novel DSD related genes are identified	Sequencing depth may be insufficient for use in clinical settings; may not detect structural variation, aneuploidy, and low-level mosaicism; may not detect low-frequency variants due to the higher sequencing error rates or poor exome coverage
		Whole genome sequencing (WGS) provides a comprehensive picture of the entire genome to detect SNV, CNV, INDELs, and larger structural variants such as ploidy changes and balanced translocations or inversions	WGS displays the broadest diagnostic perspectives due to entire genome coverage; provides more consistent coverage of gene sequences throughout the genome, including the non-coding regions	Expensive method requiring extensive data storage space and bioinformatic expertise; use in clinical diagnostics is currently limited; interpretation of genomic variation in non-coding regions remains challenging

QF-PCR, quantitative fluorescent PCR; FISH, fluorescence in situ hybridization; aCGH, comparative genomic hybridization; SNP array, single nucleotide polymorphism array; HTS, high-throughput screening; NGS, next-generation sequencing; WES, whole-exome sequencing; WGS, whole-genome sequencing; SNV, single nucleotide polymorphism; CNV, copy number variant; INDEL, insertion-deletion

Hornig et al., 2016; Batista et al., 2019]. Further, studies involving *AR* variant-negative individuals with AIS revealed a deficiency in androgen-responsive protein (apolipoprotein D) indicating functional AIS [Hornig et al., 2016]. An extended investigation where epigenetic regulation of *AR* was assessed at both transcription and translation levels resulted in molecular diagnosis of AIS type II due to the production of aberrant AR promoter transcripts [Hornig et al., 2018]. In another study, the existence of an androgen-responsive transcriptome was also hypothesised due to the identification of several non-coding RNAs that displayed different expression levels before and after hCG stimulation in boys with a DSD phenotype [Rodie et al., 2017]. Given the capability of WGS to iden-

tify CNV, SNVs, and INDELs throughout the genome, it is possible to reach a better understanding of DSD mechanisms although the complexity of variants would render interpretation difficult [Bocher et al., 2020]. Other novel genome-wide techniques are being developed and require validation for utilisation in research and clinical settings. Optical genome mapping (OGM) is designed to identify megabase-level complex structural variations that may occur in high repeat regions and, therefore, are difficult to be detected by short-read methods. Using fluorescently labelled sequence motifs, attached to each of the DNA strands at different density, and imaging instrument, the molecules are assembled to a reference genome with a high level of precision [Dremsek et al., 2021]. Epi-

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genetic modifications of histones and particularly genomic rearrangements involving CpG islands may result in aberrant methylation and subsequently transcriptional activation or repression [Jeziorska et al., 2017]. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) and single-cell ChIP-seq techniques are used to identify the way histone modifications contribute to a specific phenotype [Nakato et al., 2021]. In addition to *AR* studies in humans, DNA methylation of CpG sites in promoter regions of *SRY* [Jeong et al., 2016], *SOX3, SOX9,* and *WNT4* [Salamon et al., 2017] was studied in animals suggesting the importance of epigenetic regulation in DSD pathogenesis.

WT1 Disorders

WT1, a zinc finger transcription factor, is located on chromosome 11p13, and contains 10 exons, which encode a proline/glutamine rich transcriptional-regulation region and the 4 zinc fingers of the DNA-binding domain [Gessler et al., 1992]. Alternative splicing as well as alternative translation start sites and RNA editing give rise to more than 30 *WT1* isoforms [Scharnhorst et al., 2001]. The relative ratios of *WT1* isoforms, as well as the alternative splice site in intron 9, which allows the omission or inclusion of 3 amino acids (lysine-threonine-serine [KTS]), regulate specific urogenital differentiation processes [Scharnhorst et al., 2001]. Abnormalities of *WT1* are associated with a large spectrum of disorders including Wilms' tumour (WT), glomerulopathy, congenital anomalies of the kidney and urinary tract, disorders of gonadal development, and gonadoblastoma [Dong et al., 2015]. These abnormalities can be categorised into distinct *WT1* associated syndromes. WAGR syndrome includes WT, aniridia, genitourinary malformations, mental retardation, and focal and segmental glomerular sclerosis (FSGS). WAGR syndrome results from a heterozygous contiguous gene deletion in the 11p13 region, leading to haploinsufficiency of several genes, including *WT1* and *PAX6* [Rose et al., 1990]. Denys-Drash syndrome (DDS) comprises steroid-resistant nephrotic syndrome with progression to end-stage kidney disease (ESKD) and a high risk of WT. The 46,XY DSD that arises in these syndromes is due to a disorder of gonadal development, mainly partial gonadal dysgenesis [Denys et al., 1967; Drash et al.,1970]. Germline *WT1* variants predominantly located in exon 8 or 9, coding for zinc fingers 2 or 3, alter the binding of the WT1 protein to DNA and are frequently identified in DDS patients [Tsuji et al.,

2021]. Frasier syndrome (FS) encompasses progressive glomerulopathy, 46,XY DSD associated with anomalies in gonadal development, as complete or partial gonadal dysgenesis, with a high risk of gonadoblastoma [Frasier et al., 1964]. This phenotype results from pathogenic variants predominantly located in the second splice donor site in intron 9 and which leads to an imbalance in the WT1(+KTS)/WT1(−KTS) protein ratio [Barbaux et al., 1997; Klamt et al., 1998; Wagner et al., 2003]. Meacham syndrome is a rare malformation syndrome characterized by congenital diaphragmatic abnormalities, cardiac malformations, and genitalia abnormalities [Meacham et al., 1991; Masswinkel-Mooij et al., 1992; Killeen et al., 2002; Suri et al., 2007]. Complete or partial gonadal dysgenesis has also been reported in 46,XY individuals with Meacham syndrome. Missense pathogenic *WT1* variants have been identified in some cases [Killeen et al., 2002; Suri et al., 2007]. In addition to the classical syndromes, many other clinical presentations have been described in patients with heterozygous variants in *WT1*, ranging from patients with nephrotic syndrome [Lipska et al., 2014; Ahn et al., 2017], women with secondary and primary amenorrhea without renal disorders [Wang et al., 2015], to patients with 46,XX testicular and ovarian-testicular DSD [Gomes et al., 2019].

WT1 abnormalities may also be present in non-syndromic patients [Ferrari et al., 2022]. Usually, WT1 renal disease is manifested by a progressive glomerulopathy associated with younger age at onset (infancy or childhood), but a slow progression of the renal disease, and its manifestations in patients not so young may also occur. Persistent proteinuria, which does not respond to standard steroid therapy and whose degree becomes progressively worse over time, is the common presentation of glomerulopathy. The disease can often progress to renal failure and renal transplant [Lipska et al., 2014; Ahn et al., 2017]. The renal pathology itself includes diffuse mesangial sclerosis in children with DDS and focal and segmental glomerular sclerosis in patients with FS syndrome [Frasier et al., 1964; Melo et al., 2002]. In addition, isolated steroid-resistant nephrotic syndrome may also result from WT1 variants, predominantly from exonic point variants [Ahn et al., 2017]. *WT1* disorders may also just solely present with a DSD phenotype [Kohler et al., 2011; Patel et al., 2013] and may develop renal disease later. It is observed that among the 46,XY carriers of pathogenic WT1 variants, the gonadoblastoma risk in patients with FS is much higher than in patients with DDS, although a careful evaluation of the gonadal tissue should be carried out in both conditions [Kohler et al., 2011; Eoz-

enou et al., 2020]. Pathogenic *WT1* variants have also been identified in 46,XX patients with *SRY*-negative testicular and ovotesticular phenotypes, but renal disease has rarely been described in these conditions [Melo et al., 2002; Achermann et al., 1999; Gomes et al., 2019; Eozenou et al., 2020]. The comparison of the phenotype and genotype of patients with *WT1* variants suggests that although the variable phenotypic expression is part of the spectrum of a single condition, a positive genotype-phenotype correlation regarding the degree of gonadal dysgenesis, gonadoblastoma, nephropathy, and WT can be found [Patel et al., 2013]. *WT1* molecular analysis is indicated in newborns with atypical external genitalia and in patients with a diagnosis of 46,XY partial or complete gonadal dysgenesis. Early identification of 46,XY DSD patients with high risk for developing nephrotic syndrome and ESKD is very important to provide advice regarding appropriate treatment strategies. On the other hand, in patients with a female phenotype and glomerulopathy, the early diagnosis of 46,XY gonadal dysgenesis will allow the correct hormone replacement to pubertal induction and maintenance of the female sex characteristics as a preventive surgical approach relatively to gonadal tumours. Furthermore, the identification of *WT1* variants could help to predict the genotype-specific risk of malignancy development. WT is frequently associated with *WT1* missense variants (exons 8 and 9; DDS) and deletion of the 11p13 region (WAGR syndrome), whereas risk of gonadoblastoma is higher in patients with *WT1* variants in the intron 9 splice donor site (FS). This knowledge allows for better targeted care with closer monitoring of the organs in question. Determining the mode of inheritance of a *WT1* variant is important for carrier risk assessment and also for genetic counselling. Thus, the diagnosis of heterozygous variants in *WT1* can be used to ensure early detection of associated conditions to guide early and optimal treatment and to provide genetic counselling.

Genetic mechanisms in WT1 disorders are highly complex, and several molecular and cytogenetic approaches, such as Sanger sequencing for intragenic *WT1* mutations screening, multiplex ligation-dependent probe amplification (MLPA) and/or FISH for analysis of small interstitial 11p13 microdeletions, and conventional or high-resolution karyotyping for other microscopic 11p13 rearrangements have been performed. Currently, high-resolution array-CGH has proven to be a more sensitive and cost-effective approach for CNVs analysis allowing for a determination of CNV boundaries and mapping of breakpoints. NGS methods have significantly improved the ability to identify molecular diagnoses underlying

pathogenic variants, including the *WT1* variants. So, individual *WT1* sequencing has been replaced by panels of DSD candidate genes and WES/WGS.

NR5A1/SF-1 Deficiency

After the first description of a 46,XY DSD patient with the p.Gly35Glu *NR5A1* gene variant [Woo et al., 2015], the spectrum of phenotypes associated with *NR5A1* variants has dramatically expanded. This initial patient bearing the heterozygous p.Gly35Glu variant presented with adrenal failure and gonadal dysgenesis with persistent müllerian derivatives [Correa et al., 2004]. The expected phenotype associated with *NR5A1* variants was changed when a heterozygous 8-bp microdeletion was found in a 46,XY DSD patient who presented with clitoromegaly, absence of uterus and gonads, and normal adrenal function [Lin et al., 2007]. After this first report, several cohorts of individuals with 46,XY DSD have shown that adrenal insufficiency is a rare finding in patients with *NR5A1* defects [Kohler et al., 2009; Guran et al., 2016; Sudhakar et al., 2019; Kalinchenko et al., 2020; Na et al., 2020]. Reported heterozygous *NR5A1* variants support the model that partial NR5A1 dysfunction can result in a variable impairment of Leydig cell function and androgen biosynthesis, leading to predominantly abnormal gonadal phenotypes which can range from complete testicular dysgenesis with müllerian structures through mild clitoromegaly or atypical genitalia without müllerian derivatives to proximal hypospadias associated with undescended testis [Philibert et al., 2007], micropenis with absent gonads [Schlaubitz et al., 2007], or even ovotestis in a 46,XY girl [Suntharalingham et al., 2015]. Currently, *NR5A1* variants represent one of the most frequent defects associated with 46,XY gonadal dysgenesis, accounting for up to 20% of cases [Pedace et al., 2014]. More than 80 different *NR5A1* variants, distributed across the full length of the protein, have been described and the majority are nonsynonymous heterozygous variants [Achermann et al., 2002; Pedace et al., 2014; Tantawy et al., 2014; Suntharalingham et al., 2015; Woo et al., 2015; Domenice et al., 2016; Fabbri et al., 2016; Na et al., 2020; Mönig et al., 2021] with the exception of 3 mild variants described in a homozygous state [Soardi et al., 2010; Warman et al., 2011; Na et al., 2020]. These findings reinforce the concept that *NR5A1* dosage is critical to normal gonadal development. However, a clear correlation between the location of a gene variant, its in vitro functional performance, and the associated phenotype is not observed.

Indeed, family members bearing the same *NR5A1* variant may present with variable phenotypes [Bashamboo et al., 2010a]. The reviewed data of 72 46,XY DSD patients with *NR5A1* variants reported in the literature, for whom information on presence or absence of müllerian derivatives was available, suggested that müllerian derivatives are present in about a quarter of the cases described above. Male infertility has been also related to the presence of *NR5A1* defects [Ferlin et al., 2015]. Patients with moderate/severe oligospermia or azoospermia and *NR5A1* variants may have normal testosterone and normal low or low inhibin B levels, but they are at a potential risk of deterioration of testicular hormonal secretion with age and may need counselling regarding preservation of sperm and regular monitoring of endocrine function. Considering the wide phenotypic variability and prognostic implications as well as the high frequency of *NR5A1* variants in dysgenetic 46,XY DSD, the molecular study of *NR5A1* is indicated in patients with those phenotypes.

17β-Hydroxysteroid Dehydrogenase Type 3 (17β-HSD3) Deficiency

17β-Hydroxysteroid dehydrogenase type 3 (17β-HSD3) deficiency is an autosomal recessive form of 46,XY DSD that consists of a defect in the conversion of androstenedione into testosterone and estrone into oestradiol [Rosler et al., 1983]. Despite the fact that this deficiency confers a spectrum of the 46,XY DSD phenotype, the most frequent presentation is a 46,XY newborn with an undervirilization of the external genitalia with a blind vaginal pouch, whose testes are often located in the inguinal canal or in a bifid scrotum and without müllerian derivatives [Andersson et al., 1996; Moghrabi et al., 1998; Mendonca et al., 2000; Lee et al., 2007]. Considering the female-like external genitalia, many individuals are assigned female at birth [Andersson et al., 1996; Inacio et al., 2011]. However, marked virilization is observed in puberty in individuals that did not undergo gonadectomy. In addition, social sex change from female to male is observed in around 39–64% of the individuals in adulthood [Inacio et al., 2011]. Clinically, the phenotype that is associated with 17β-HSD3 deficiency is very similar to partial AIS and 5α-reductase type 2 deficiency. In many cases, 17β-HSD3 deficiency can be identified by the increased concentrations of androstenedione and reduced levels of testosterone and subsequently confirmed by the genetic analysis of the *HSD17B3* gene [Boehmer et al., 1999; Mendonca et al., 2017]. A T/A ratio below 0.8 has been proposed as a

reliable cut-off for 17β-HSD3 deficiency for all age groups [Arnhold et al., 1988; Boehmer et al., 1999; Ahmed et al., 2000b]. In prepubertal individuals, a human chorionic gonadotropin (hCG) test may be required as an abnormal T/A ratio may only become biochemically evident following this stimulus [Kulle et al., 2017; Mendonca et al., 2017]. It is recommended that steroids with lower concentration such as androstenedione should be measured by HPLC with tandem mass spectrometry or immunoassays with a preceding extraction step [Khattab et al., 2015]. Despite this, commercial assays, especially automated immunoassays, are used by several biochemistry laboratories due to their low cost, simplicity, and fast turn-around times. Even when androstenedione is measured as recommended, the T/A ratio may not be sensitive enough in identifying genetically confirmed cases [Lee et al., 2007; Batista et al., 2018]. Lastly, an altered T/A ratio is not specific to this deficiency and may also be found in other DSD conditions such as gonadal dysgenesis [Ahmed et al., 2000b]. A clear diagnosis of 17β-HSD3 deficiency is crucial for the decision of sex assignment, considering the high rates of reassignment in those raised as female. In addition, the potential for spontaneous virilisation in those who do not have a gonadectomy can also be predicted when the diagnosis is clear. The biochemical diagnosis of 17β-HSD3 deficiency can also be hampered in those who have had a gonadectomy. Thus, there are several reasons why a molecular genetic diagnosis may have greater utility than a biochemical approach and it explains the rationale for the increased preference on a molecular genetic rather than a biochemical diagnosis in this condition [Kyriakou et al., 2016].

Androgen Insensitivity

Androgen insensitivity due to a resistance to androgen action leads to AIS, which has phenotypically consisted of complete (CAIS) and partial (PAIS) forms. PAIS is usually identified immediately after birth due to the presence of atypical genitalia. For CAIS, other than familial cases, the diagnosis is usually reached later in life, either at childhood, due to the presence of inguinal hernias, or at pubertal age, due to primary amenorrhea [Gulia et al., 2018; Melo et al., 2003]. Although AIS may be associated with elevated basal serum testosterone levels associated with high serum LH levels [Ahmed et al., 1999; Gulia et al., 2018], typically the biochemical findings are unremarkable. In fact, in some cases, the biochemical findings may overlap with those in a child with impaired gonadal

function [Ahmed et al., 1999]. In post-pubertal patients, the oestradiol levels are normal or slightly elevated for a male individual due to testosterone aromatization [Ahmed et al., 1999]. However, this hormonal pattern is only seen at mini-puberty or after puberty since the gonadotrophin axis is not activated during childhood. Genetic analyses reveal defects in both functional domains, DNA-binding and steroid-binding, of the coding region of *AR* as a cause of this condition [McPahul et al., 1993; Sultan et al., 1993; Ahmed et al., 2000a; Melo et al., 2003] that results in reduced androgen-binding activity. The *AR* locus is positioned between Xq13 and Xp11 [Migeon et al., 1981] and, therefore, the majority of variants are maternally inherited whilst about 30% are de novo. Although the presence of inactivating variants in *AR* may be evident in over 80% of girls and women with CAIS, *AR* variants in PAIS are much rarer occurring in less than 20% of cases of XY DSD [Ahmed et al., 2000a]. It is possible that in some cases, these variants may exist beyond the *AR*-coding region [Hornig and Holterhus, 2021], and this raises the need to explore more effective methods of selecting cases that may display androgen insensitivity. In the past, this has involved assessment of AR binding in genital skin fibroblasts [Evans et al., 1984], measurement of circulating androgen responsive proteins in response to androgen stimulation [Sinnecker et al., 1997; Bertelloni et al., 1997], or even clinical assessment of the genitalia in response to androgen stimulation [Stancampiano et al., 2022]. However, it may be possible to use other methods such as measurement of apolipoprotein D in genital skin fibroblasts [Hornig et al., 2016] or an androgen responsive transcriptome within circulating polymorphonuclear blood cells before and after androgen exposure [Rodie et al., 2017]. Although there are differences in the AR residual function among the AIS phenotypes, no difference has been observed in the hormonal levels across AIS phenotypes [Ahmed et al., 1999; Arnhol et al., 2011]. Long-term follow-up studies of young men identified in infancy show that those who had a genetically confirmed diagnosis of PAIS compared to those who were diagnosed clinically and did not have a variant in *AR* were more likely to have gynaecomastia and mastectomy, several genitoplasty-related procedures for hypospadias, and were more likely to have a trial of different forms of testosterone implying more challenging management of hypogonadism [Lucas-Herald et al., 2016]. Thus, identification of an *AR* variant can allow more tailored management as well as preparing the patient and the clinical service for a more challenging period. Given the low relative tumour risk in PAIS, a confirmed diagnosis also allows more tailored counselling regarding gonadectomy with the adoption of a wait and see approach [Tack et al., 2018].

5α-Reductase Type 2 Deficiency

Elevated serum testosterone-to-DHT ratio (T/DHT) is often described as the classical hallmark of 5α-reductase type 2 deficiency. Typically, testosterone levels are normal to moderately high, and DHT levels are low to undetectable [Mendonca et al., 2016]. Steroidal laboratory analysis is most often performed by direct immunoassays on automated platforms, impacting analytical specificity. This specificity is improved by liquid chromatography linked with tandem mass spectrometry (LC-MS/MS), but it is not available everywhere [Kyriakou et al., 2016]. Regardless of the steroidal lab analysis technique, the levels of both hormones (T and DHT) are detectable at diagnostic levels only during the physiologic testosterone surge that occurs between birth and age 1–3 months (mini-puberty). As an alternative, the human chorionic gonadotropin (hCG) stimulation can be performed in prepubertal patients. While normal male individuals have T/DHT ratios between 8 and 16, in patients with 5α-reductase-2 deficiency, the T/DHT ratio ranges from 35 to 84 [Hochberg et al., 1996; Bertelloni et al., 2007, 2016; Maimoun et al., 2010; Chan et al., 2013; Mendonca et al., 2016]. Although this raised ratio has a high level of specificity for 5α-reductase-2 deficiency, the ratio is not sensitive enough as several cases have been reported with a lower ratio [Maimoun et al., 2010; Lucas-Herald, 2015]. Urinary steroid profile analysis by gas chromatography-mass spectrometry (GC-MS) can also provide qualitative and quantitative data on the excretion of steroid metabolites. The ratio of 5α- and 5β-reduced urinary metabolites can also lead to a diagnosis of 5α-reductase type 2 deficiency [Chan et al., 2013]. The advantage of these urinary metabolites is that they can also be measured and assessed in cases that have had a gonadectomy. However, these urinary metabolites are not reliable before 3 months of age as diagnostic pairs of 5β-to-5α-reduced metabolites are not detectable until then. Mutation analysis of the 5α-reductase type 2 gene (*SRD5A2*) is a relatively easy method for the diagnosis of 5α-reductase type 2 deficiency. *SRD5A2* comprises 5 exons, and most variants are located at exons 1 (33% of all variants) and 4 (19%) [Batista et al., 2020]. By direct gene sequencing, most suspected 5a-reductase type 2 deficiency cases showed allelic variants in *SRD5A2* either in a homozygous (70%) or com-

pound heterozygous (30%) state. The positions 196, 227, 235, and 246 are hotspots of the *SRD5A2* defects. Collectively, they make up 25% of all reported variants in the *SRD5A2* gene reported as causative of 5α-reductase type 2 deficiency [Batista et al., 2020]. In the beginning, the 5α-reductase-2 deficiency was reported in clusters worldwide, but it has been reported in several countries nowadays [Batista et al., 2020]. Given that most patients identify themselves as males, regardless of the sex of rearing and the degree of external genitalia virilization [Loch Batista et al., 2019], excluding 5a-reductase-2 deficiency is imperative in the infant with 46,XY DSD where a female sex of rearing is being contemplated. Although a combination of laboratory and genetic studies would be ideal, the molecular genetic diagnosis is increasingly being preferred for reaching a diagnosis of 5α-reductase type 2 deficiency [Kyriakou et al., 2016].

Congenital Hypogonadotrophic Hypogonadism

Congenital hypogonadotrophic hypogonadism (CHH) is a group of rare conditions caused by gonadotrophin deficiency. The group can be broadly divided into 2 sub-groups; there is one group that is due to a defect of GnRH neuron development and migration and is associated with anosmia/hyposmia. The other sub-group is due to defects in GnRH secretion or function and results in normosmic CHH. In addition to olfactory defects, patients with CHH may have a wide range of associated clinical features, sometimes as part of a recognised syndrome such as Kallmann syndrome or CHARGE syndrome. Although the diagnosis of CHH is typically reached during the second or third decades of life when clinical and biochemical profiles are typically compatible with the classic CHH phenotype, in young children, CHH can present with micropenis and/or undescended testes. More recently, cases of CHH have also been described with other forms of atypical genitalia including hypospadias [Eggers et al., 2016; Wang et al., 2017]. The clinical diagnosis of CHH, especially when there are no associated features, is challenging in childhood given that the gonadotrophin axis is quiescent in childhood and the most common cause of delayed puberty during adolescence is constitutional delay of growth and puberty (CDGP). The mini-puberty period in early infancy is an important window of opportunity for the early biochemical diagnosis of CHH but requires multiple sampling [Swee et al., 2019]. Biochemical parameters including basal LH, FSH, sex steroids, inhibin B, anti-müllerian hormone concentration,

and GnRH-stimulated LH can be helpful for pointing towards a diagnosis of CHH but have insufficient specificity for discriminating CHH from CDGP [Coutant et al., 2010; Harrington and Palmert, 2012; Palmert et al., 2012; Abitbol et al., 2016; Mosbah et al., 2020]. Since *KAL1*, subsequently renamed as *ANOS1*, was discovered in 1991 [Franco et al., 1991], genetic molecular testing of CHH has assumed an important role. With the introduction of high throughput sequencing technologies, to date, there have been reports of variants in more than 30 genes that cause CHH [Maione et al., 2018], and the overall genetic diagnostic yield of CHH has risen to 50% [Cangiano et al., 2016; Quaynor et al., 2016]. Furthermore, oligogenicity has been identified in around 15% of CHH cases as an explanation for the phenotypic heterogeneity [Cangiano et al., 2016]. Reaching a genetic diagnosis has also allowed the clinician to search for other associated extra-gonadal features, for example, heart or hearing defects in cases with CHD7 variants or renal defects in cases with *ANOS1* variants [Quinton et al., 2001]. A confirmed diagnosis of CHH at an early stage can also allow an informed discussion on the management of pubertal development. In addition, genetic screening also gives important data for early patient identification and subsequently receiving early treatment. For example, in male infants with micropenis and undescended testes, CHH genetic screening could confirm the definite diagnosis in the early age and raise alert for clinician for provide early treatment that could reach the better outcome [Bouvattier et al., 2011].

Challenges in the Use of Genetics for Reaching a Clinical Diagnosis

As illustrated through the aforementioned examples, genetic testing is now established as essential in confirming the diagnosis of DSD and typically involves the application of a range of techniques which are chosen depending on the clinical presentation and results of first line investigations including chromosomal analysis. Establishing a genetic diagnosis is not only important for the management of the individual with DSD but also for the parents and potentially wider family, allowing the opportunity for discussion about future reproductive risk and genetic counselling [Ahmed et al., 2021]. However, the heterogeneous nature of many DSDs has meant that the success in identifying a genetic cause in XY DSD remains relatively low [Ahmed et al., 2021]. In XY DSD, where there are no biochemical abnormalities of gonadal function, in over half of individuals the molecular diagnosis

remains elusive [Parivesh et al., 2019]. For this reason, in recent years, diagnostic genetic laboratories are moving away from the use of targeted single analysis, adopting HTS techniques, sequencing multiple DSD-related genes simultaneously on a targeted gene panel or through whole genome or exome sequencing (WGES) with bespoke filters applied to achieve adequate coverage of DSD-related genes. However, it is important that the relative advantages and disadvantages of different techniques used in the genetic diagnosis of DSD are recognised (Table 2).

Adopting a targeted panel approach of multiple DSD-related genes is more time efficient and cost effective than a single gene approach providing a greater diagnostic yield [Dong et al., 2016; Eggers et al., 2016]. However, panel development is time consuming, labour intensive, and has limited future flexibility for the addition of new DSD-related genes in comparison to alternative HTS techniques such as WGES. The advantage of WGES is the potential to identify new DSD-related genes in the research setting as well as the relative flexibility to modify diagnostic testing pipelines to accommodate these findings. In comparison to WES, WGS has more consistent coverage of gene sequences throughout the genome, including the non-coding regions. Whilst interpretation of genomic variation in non-coding regions remains challenging and, at present, out of the scope of diagnostic laboratories, it is anticipated that the ENCODE Encyclopedia will improve the understanding of molecular pathways, transcriptional regulatory mechanisms, and their impact on human diseases [ENCODE Project Consortium et al., 2020]. Previously the application of WGS was limited by cost and lengthy turnaround time; technological advance as well as the introduction of bioinformatic expertise has demonstrated the potential for rapid turnover of results in diagnostic laboratories [Mestek-Lamia et al., 2018]. It is likely that through future improvement in HTS techniques, bioinformatics, and algorithm design it will be possible to identify structural variation, CNV, and low-level mosaicism [Anjum et al., 2015; King et al., 2017] but the role of WGS requires careful evaluation in the field of DSD [Délot and Vilain, 2021].

As the application of newer genomic technologies has become more widely available, it has also led to challenges in the interpretation of complex genomic findings, especially the interpretation of variants of uncertain significance (VUS). The American College of Medical Genetics and Genomics (ACMG) guidelines have been adopted by most diagnostic laboratories and provide a framework for the interpretation of gene variants [Richards et al., 2015]. Despite the use of ACMG, the clinical significance of many variants remains uncertain causing diagnostic uncertainty, which is challenging for clinical teams, patient,s and their families [vanBever et al., 2020]. Lack of consensus about the application of ACMG guidelines as well as acknowledgement of its limitations for certain conditions has led to disease-specific reporting frameworks and recommendations for future revision [Berwouts et al., 2012; Amendola et al., 2016; Tavtigian et al., 2018; Walsh et al., 2019]. A decade ago, a survey performed in Europe of 910 human molecular genetic testing laboratories led to a response from 291 (32%) from 29 countries [Berwouts et al., 2012]. A quarter of these laboratories reported that they were accredited or certified, a fifth reported that they did not participate in any external quality assessment, and 28% did not use reference materials. However, all respondents expressed a preference to work in an accredited laboratory. Quality assurance is very well established in healthcare diagnostic laboratories [Ahmed-Nejad et al., 2021] in many countries, and guidelines have also been issued for laboratories involved in NGS [Matthijs et al., 2016]; for instance, in the UK, medical laboratories are accredited by UKAS (The United Kingdom Accreditation Service) to the ISO 15189:2012 standard. Healthcare scientists interpreting and reporting results in these laboratories are required to be registered with the Health and Care Professions Council (HCPC). In the UK, diagnostic genetic testing for DSD in England is now included in the National Genomic Test Directory (https://www.england.nhs.uk/publication/national-genomic-test-directories/) and in both England and Scotland it is also provided through a network of NHS genomic laboratory centres. Diagnostic interpretation of the genetic findings requires a very careful and methodical approach and, to deliver a high-quality service, centres that provide a diagnostic genetic service for DSD should have detailed phenotypic information in addition to the genetic findings. In the genomic era, it is likely that there will be an increase in genetic findings which will require greater investment in resources to ensure careful clinical phenotyping, adequate provision of bioinformatic infrastructure, and specialist multidisciplinary teams to aid the interpretation and reporting of complex results. Some centres have developed a diagnostic board consisting of the clinical geneticist, the molecular geneticist, the clinical biochemist, and the paediatric endocrinologist [Alhomaidah et al. et al., 2017]. This diagnostic board has the capacity to review its own activities and remain up-to-date with continuing advances in this field. Close involvement of the clinical genetics service through this board can also ensure that the MDT covers all aspects of genetic counselling. Con-

firming pathogenicity by exclusive use of the existing in silico tools is challenging and at the same time performing functional studies in all cases of novel variants is also challenging and expensive. Whilst links with research laboratories that may already be involved in some functional research is desirable, it is unrealistic to expect that this will always be the case; thus, novel high through-put methods are required for exploring pathogenicity [Aref-Eshgi et al., 2020].

Conclusion

In conclusion, diagnostic genetics is a cornerstone in the management of DSD. It is likely that molecular genetics will be used at a much earlier stage in the diagnostic process, and although in many cases the findings as well as the clinical utility of the tests are unequivocally clear, in some other clinical situations, the benefits are less clear and need careful exploration through long-term moni-

toring of these cases. Furthermore, not only does the incorporation of molecular genetics into the diagnostic process require a careful appreciation of the strengths and weaknesses of the evolving technology, the interpretation of the results requires a clear understanding of the wide range of conditions that are associated with DSD.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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