

## **Diagnosis of disorders of sex development (DSDs): impact of molecular diagnostics on clinical practice**

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**Key Points:** Advances in molecular diagnosis contribute to the management of DSD, from genetic counselling to assisted reproductive technology, prenatal diagnosis and postnatal treatment.

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## **Abstract**

Disorders of sex development (DSD) consist of a diverse group of conditions that can sometimes be challenging to diagnose accurately using standard phenotypic and biochemical approaches. Obtaining a specific diagnosis can be important for identifying potentially life-threatening associated disorders, as well as providing information to guide parents in the most appropriate management for their child. Recent advances in molecular methodologies have helped to identify several novel causes of DSD, and have led to the adoption of molecular tests into clinical practice for diagnosis and genetic counselling. Occasionally, pre-implantation diagnosis in assisted reproductive technology, prenatal diagnosis of at-risk pregnancies and confirmatory testing of positive newborn screening results is performed. Among the genetic tests available, most use a candidate-gene approach, while new high-throughput DNA analysis could enable a genetic diagnosis to be made where the aetiology is unknown or differential diagnosis wide. Nonetheless, genetic tests have also been associated with several concerns; a diagnosis is not always possible even using new molecular approaches, resulting in anxiety for the parents, and incidental information obtained may be a concern. Careful selection of the genetic test indicated for each condition remains important for good clinical practice. The purpose of this brief review is to describe some advances in molecular biological techniques for diagnosing DSD.

## Introduction

Mammalian sex development is a complex process that depends on three general steps.

The first step is establishment of the chromosomal complement at fertilisation so that the typical male has XY chromosomes and the typical female XX chromosomes (sometimes referred to as “chromosomal sex”).

The second step is gonad determination (“sex determination”). This process involves development of the bipotential gonad into either a testis or ovary (“gonadal sex”). The presence of the *SRY* gene on the Y chromosome is usually sufficient to promote testis development, whereas an ovary will usually develop in the absence of *SRY* expression<sup>1</sup>.

The final step is differentiation of the internal and external genitalia (“sex differentiation”). This process occurs under the influence of hormones secreted by the gonad and results in the “phenotypic sex”. Early in gestation, all embryos have both paramesonephric ducts (Müllerian ducts) and mesonephric ducts (Wolffian ducts). If a testis develops, anti-Müllerian Hormone (AMH), a glycoprotein secreted from the Sertoli cells, acts on its receptor in the Müllerian ducts causing their regression. Testosterone secreted by the testicular Leydig cells acts on the androgen receptor in the Wolffian ducts inducing development of the epididymis, deferent ducts and seminal vesicles. Testosterone is further reduced to dihydrotestosterone (DHT), which acts on androgen receptors to cause androgenisation (masculinisation) of the external genitalia. Although, the female reproductive tract develops even in the absence of any gonad, advances in our knowledge have demonstrated that ovarian development and its maintenance are active processes<sup>1</sup>.

Mutations or alterations in the genes regulating gonad determination and sex differentiation can lead to disorders of sex development (DSD)<sup>2</sup>. Based on the Chicago Consensus, these can be broken down into three broad categories: sex chromosome DSD, 46,XY DSD and 46,XX DSD. The karyotype *per se* does not make a child “male” or “female” and ideally a specific diagnosis is reached for each individual. However, the ability to detect an X and Y chromosome rapidly using techniques such as fluorescent in-situ hybridisation (FISH) means that a broad diagnostic category can be reached in

around 48 hours and counselling and biochemical and genetic investigations focussed accordingly.

Sex chromosome DSD results from differences in sex chromosome number, and theoretically includes the sex chromosome aneuploidies such as Turner syndrome (45,X) and Klinefelter syndrome (47,XXY). Early presentation with atypical genitalia can occur in some children with 45,X/46,XY mosaicism or 46,XX/46,XY ovotesticular DSD.

46,XY DSD results from incomplete intrauterine virilisation and is characterised by ambiguous or “female” external genitalia with or without the presence of Müllerian structures. 46,XY DSD can result from impaired production of testosterone, decreased conversion of testosterone into DHT or from impairment of the peripheral action of these hormones<sup>3</sup>. At histological analysis, testicular tissue can be absent, partially or completely dysgenetic, or even appear normal depending on the underlying condition<sup>4, 5</sup>.

46,XX DSD results from fetal exposure to androgen excess and is characterised by ambiguous or “male” external genitalia. Most often this is due to congenital adrenal hyperplasia (CAH). These patients usually have ovaries and Müllerian structures, except for a rare subgroup who have isolated testicular tissue (46,XX testicular DSD) or both testicular and ovarian tissue present (46,XX ovotesticular DSD)<sup>6</sup>.

Although DSD can present at many different ages, one of the most common presentations is with ambiguous (or atypical) genitalia in the newborn period. Sex assignment may be a complex process and especially challenging for babies with a 46,XY karyotype and poorly developed genitalia and non-palpable gonads<sup>7</sup>. Meticulous assessment and counselling by a multidisciplinary team is essential in this process. Reaching a specific diagnosis can be important for assessing the risk of potentially associated harmful conditions (e.g. renal, adrenal disorders), and to some extent predicting long-term outcome such as gender identity, endocrine function, potential fertility and gonadal tumour risk. The rapid identification of a molecular cause may sometimes be very helpful in decision making<sup>8</sup>.

The purpose of this review is to analyse the contribution of and the advances in molecular biology techniques in the management of DSD.

## **Molecular approaches to the diagnosis of DSD**

Historically, candidate genes for DSD have been identified following analysis of deleted or duplicated chromosomal regions that segregate with DSD phenotypes (copy number variations)<sup>9</sup>, linkage analysis, from animal models, or when a biochemical profile suggests a block in a key enzyme or protein in a biochemical pathway. Following the identification of a potential DSD-causing gene, conventional Sanger sequencing has typically been used to analyse groups of patients depending on their phenotype and hormonal profiles. Nowadays, Sanger sequencing is still the main methodological tool used to investigate inherited mutations<sup>10</sup>. When there is clear biochemical evidence suggesting a block in a specific pathway, the chances of identifying a change in the relevant gene are generally high (e.g. *STAR*, *CYP17A1*, *POR*). However, where the potential cause of DSD is not clear or specifically indicated by the hormonal profile (e.g., testicular dysgenesis), a molecular diagnosis is only established in around 20% of cases using a candidate gene approach<sup>2</sup>.

In addition to candidate gene sequencing, specific copy number variations (CNV) can be analysed using techniques such as FISH, quantitative PCR and multiplex ligation-dependent probe amplification (MLPA). In a small number of cases, duplications or deletions of genes or specific exons can be identified. For example, in 46,XY gonadal dysgenesis a deletion of *SRY* is found in approximately 5% of cases and duplication of the region of Xp21 containing *NROB1* in a similar proportion<sup>11</sup>.

Recently, high-throughput molecular tools have allowed the simultaneous analysis of copy number variation or parallel sequencing of many genes with much greater efficiency<sup>12</sup>. Among these genetic tests, array-comparative genomic hybridization (array-CGH) and SNP genotyping array, whole-exome or -genome sequencing could enable a genetic diagnosis to be made where the differential diagnosis is wide, biochemical tests unclear or even in the absence of prior clinical suspicion<sup>13-15</sup>.

Array-CGH allows rapid screening to identify submicroscopic genomic imbalances resulting in the identification of known and potentially causative copy number variations (CNVs) on a genome wide scale. It is a powerful technique with high potential resolution. It is becoming a routine clinical diagnostic tool and is gradually replacing classical cytogenetic methods in some centres. Most of the clinically available aCGH platforms are designed to detect aneuploidies, well-characterized microdeletion/microduplications and subtelomeric or other unbalanced chromosomal rearrangements. In addition, aCGHs cover numerous CNVs of unclear significance scattered throughout the human genome, some of which may be relevant especially if

they contain candidate genes or occur *de novo*. However, this technology is not able to identify balanced chromosomal rearrangements such as translocations and inversions and some ploidies<sup>16</sup>, and low level mosaicism may not be detected (Table 1). Furthermore, validation of the data is necessary by other techniques such as FISH, Multiplex Ligation-dependent Probe Amplification (MLPA) or a quantitative copy number variant assay<sup>16</sup>. Nevertheless, customized array-CGH platforms are becoming a useful tool in genetic disorders and prenatal diagnostics and have been applied to investigate DSD patients with 46,XY gonadal dysgenesis and with unknown cause<sup>15,17</sup>.

More recently, next generation sequencing (NGS) technologies have revolutionized the identification of disease-causing genes. These sequencing methods employ massively parallel approaches to sequence multiple samples simultaneously<sup>18</sup>. NGS is predominantly a simple DNA variant test with the ability to detect single base-pair substitutions and small insertions and deletions involving a few nucleotides. If the patient's phenotype is more consistent with a chromosome abnormality, it is more appropriate to perform a karyotype and/or aCGH as an initial test, although newer algorithms are improving the detection of CNVs from high throughput sequencing approaches. In addition, if a specific diagnosis is suggested from biochemical or hormonal data then a more focussed approach is appropriate.

Some studies have claimed that NGS can identify variants with 99.9% sensitivity and specificity at a depth of 30-fold<sup>19</sup>. However, difficulties remain in terms of the quality and efficiency of capture methods and coverage, data transfer, storage and especially bioinformatic analysis. Strategies to address allelic variants of unknown significance should be developed. Multiple sources of error including sequencing errors, incorrect alignment (mismapping) and random sampling may occur in NGS, especially when read depth is low<sup>18</sup>. In general, NGS cannot identify variations larger than a few base pairs in size, such as indels (insertions and deletions), trinucleotide repeats and some copy number variations<sup>20</sup>. Confirmatory Sanger sequencing or MLPA/array-based tests are still necessary to confirm the results of NGS if the results are being used clinically, which increase the cost and time to reach the diagnosis<sup>21-23</sup>.

The three most common applications of NGS are in targeted arrays, whole exome sequencing (WES) and whole genome sequencing (WGS). Targeted arrays are designed to capture panels of known genes involved in conditions such as DSD, so that these genes can be sequenced at the same time and for relatively low cost. This approach may be useful when many different genes, some of which are quite large (e.g.

*MAP3K1*), can produce a common phenotype such as testicular dysgenesis. Sanger sequencing of these genes on a one-by-one basis would be laborious and costly in terms of manpower, and the development of targeted arrays linked to NGS might be a solution to screening for potentially disruptive changes (Table 1).

WES is generally more expensive, but the cost of this approach is falling and the clinical application is increasing in some settings<sup>18, 24-26</sup>. WGS is being used in some research settings but is currently more costly. Some of the major challenges with these approaches are the number of variants detected and incidental or “bystander information”, information that is discovered in non-relevant genes that may have health-related consequences. For example, WES typically generates 15,000-20,000 variants within the coding region while WGS typically generates 3-4 million variants that differ from the human reference sequence<sup>23</sup>. The vast majority of these variants are benign, and only 1-2% are associated with a potential phenotype. Several filters should be used for the selection of deleterious variants: variants with low allelic frequency (<1-5%) in a reference population and synonymous and noncoding variants are generally excluded, whereas, nonsense, frameshifts, indels and canonical splice site variants are more likely to be deleterious, especially if a review of the literature supports the variants’ pathogenicity. Indeed, incidental findings of potentially deleterious variants have been reported in normal populations (3.4% for European descent and 1.2% for African descent) so clear structures need to be in place to know what information an individual might want to be told and to have variants verified in a clinically approved test and with the support of genetic counselling<sup>27</sup>. As the cost of WES continues to drop, it can be envisaged that this could be a frontline test in some centres, with initial analysis of DSD-related genes in the first instance<sup>28</sup>.

Another challenge of DSD is that some causes occur *de novo* or even in dominantly inherited forms, and the inability of affected individuals to reproduce means that large pedigrees with affected and unaffected individuals are often not available. The use of family trios (parents and affected child) is very important to facilitate the variant filtering process<sup>23</sup>, especially for detecting *de novo* changes. The potential significance of findings in novel genes is strengthened when changes in the same new gene are found in multiple independent families or individuals with DSD and not in large ancestrally-matched-population control databases. International research collaborations may be needed to reach this goal<sup>29</sup>.

## **Molecular approach to prenatal diagnosis of DSD**

Prenatal detection of ambiguous genitalia or genotype-phenotype discordance has increased over time because of the widespread use of advanced technical imaging and molecular approaches to determine fetal genetic sex<sup>30</sup>. A prenatal karyotype/phenotype discrepancy has been reported to occur in as many as approximately 1:2,500 pregnancies<sup>31</sup>. Ambiguous genitalia should be suspected when typical male or female genitalia are not visualised on prenatal ultrasound performed by an experienced sonographer after 14 weeks of gestation (wg). Several approaches have been developed over the years to determine fetal karyotype, the most recognised is the karyotyping of cells cultured from the CVS or amniotic fluid at approximately 11-14 to 16 wg, respectively. However, both these procedures have small, but measurable rates of pregnancy loss, varying from 0.5 to 2%<sup>32,33</sup>. Amniocentesis can only be performed in the second trimester of gestation and, although CVS provides reliable results in the first trimester, its use before the 11<sup>th</sup> wg is associated with limb defects<sup>34</sup>.

The identification of circulating cell-free fetal DNA (cffDNA) in maternal blood in 1997 provided an important alternative approach for noninvasive prenatal diagnosis<sup>34, 35</sup> and nowadays, PCR-amplification of cffDNA is the gold standard method to define earlier fetal sex without any risk of miscarriage<sup>36</sup>.

The sensitivity and specificity of fetal sexing by cffDNA can be influenced by methodologies, the sample types and gestational age. Different Y chromosome markers (SRY, DYS14) have been used for Y chromosome detection proposes<sup>37, 38</sup>. However, two recent meta-analysis studies did not observe discernable differences between both markers, with the methodology used and gestational age at sample collection being the main factors influencing the diagnostic accuracy of sex determination using cffDNA<sup>36, 37</sup>.

There is an agreement that fetal sex can only be determined with an accuracy close to 100%, after the 8<sup>th</sup> wg<sup>34, 36, 37, 39</sup>. However, a great debate remains in the literature regarding the earliest gestational age associated with the higher sensitivity and specificity. In a recent meta-analysis, which included 10,587 tests, the sensitivity and specificity at 5<sup>th</sup> wg were 93% and 95%, respectively. To avoid false-negative results, before reporting a test as female (Y chromosome negative), all negative tests collected before the 8<sup>th</sup> wg should be repeated in the following weeks<sup>40</sup>. Recently cffDNA analysis using NGS has been reported for the diagnosis of some monogenic or X-linked genetic disorders but the use in clinical practice is still limited<sup>37, 41</sup>.

### **Molecular approach to Sex Chromosomal DSD (45,X/46,XY mosaicism)**

DSD due to chromosomal abnormalities can include Turner Syndrome (45,X), Klinefelter Syndrome (47,XXY) 45,X/46,XY mosaicism (also known as mixed gonadal dysgenesis, MGD), and 46,XX/46,XY chimerism. Despite the fact that most children with a 45/46,XY karyotype have normal male phenotype at birth<sup>42</sup>, the phenotype can be highly variable and it is still one of the more prevalent conditions to present with atypical genitalia postnatally. The most common feature in this group of children is asymmetric development of gonads often with a dysgenetic testis in one side and a streak gonad in the other<sup>43, 44</sup>. The karyotype is 45,X/46,XY or its variants with a 45,X cell line and one or more lineages with a normal or structural abnormal Y chromosome. The 45,X/46,XY mosaic is also associated with short stature, cardiovascular and renal anomalies among other features, but different phenotypes may be determined by the presence of Y deletions, ring chromosomes and isochromosomes. Surveillance for Turner Syndrome associated features is recommended.

Detection of an abnormal Y chromosome in routine analysis depends on the type and the size of the missing Y segment. Classical cytogenetic results can be supplemented with FISH techniques to ensure a better characterisation of chromosomal abnormalities. FISH can be useful in identifying the nature and the origin of unknown markers and chromosomal rearrangements, which may have important implications in sex determination and in the development of gonadal tumours<sup>45</sup>.

Array CGH (aCGH) represents an alternative approach to sex chromosomal complement analysis<sup>46</sup>. In a comparative study between SNP array analysis and classical karyotyping in patients with Turner Syndrome, it has been demonstrated that SNP array analysis can have advantages over karyotyping for the identification of cryptic Y chromosome material<sup>47</sup>. However, karyotyping retains an important advantage over arrays for the identification of complex mosaicisms, including translocations and rare X chromosome structural variants. SNP genotyping is unable to detect fully balanced X-autosome translocations, and it is currently unclear whether low level mosaicism will be missed<sup>46, 47</sup>.

Mutational analysis by NGS may also be relevant in DSD patients with sex chromosome mosaicism<sup>48</sup>.

### **Molecular approach to 46,XY DSD**

46,XY DSD can present with a large phenotypic spectrum of features. Where biochemical data or the presence of associated features point to a specific single gene cause, traditional approaches to sequencing are usually informative. However, in many cases of testicular dysgenesis or the broad group of “partial androgen insensitivity”, it is much more difficult to reach a specific diagnosis using routine techniques. New molecular tools may help in the investigation of these patients (Figure 1). Occasionally, specific genetic investigations have been performed in fetal DNA to investigate a known molecular defect in families with a previously affected child<sup>49-52</sup>. Defining the molecular basis of some forms of 46,XY DSD can have important clinical implications (Table 2).

#### ***46,XY caused by abnormal gonadal determination***

Abnormalities in the expression of genes involved in the process of gonadal determination can disrupt testicular development. Complete and partial forms of 46,XY gonadal dysgenesis result from complete or partial failure of this process, respectively. Individuals with 46,XY complete gonadal dysgenesis (CGD) usually present in adolescence with absent puberty, typical female internal and external genitalia, and streak gonads. In contrast, partial testicular dysgenesis usually presents at birth with ambiguous genitalia; variable internal and external genitalia are seen<sup>53</sup>. *SRY* and *NR5A1* (encoding SF-1) mutations are the most frequent molecular causes of isolated 46,XY GD and recently variations in *MAP3K1* have also been identified<sup>54</sup>. Gonadal dysgenesis may be isolated or may be part of a syndrome. When additional features are found, a candidate-gene approach is generally successful (for example, Denys-Drash Syndrome and *WT1*; heart defects and *GATA4*; or campomelic dysplasia and *SOX9*).

Human sex development is sensitive to gene dosage effects and duplication and deletion events contribute to anomalies in gonadal development. Abnormal copy number variation or expression of several genes involved in testis determination have been identified in patients with 46,XY gonadal dysgenesis<sup>11</sup>. Commercial genetic tests to identify gene copy number variation, for *SRY*, *SOX9*, *NR0B1/DAX1*, *DMRT1*, *NR5A1/SF1*, *WNT4*, and *WT1*, and sequencing for each candidate gene are clinically available. Investigation of gene dosage imbalances using MLPA analysis has provided a good complement to DNA sequencing in patients with 46,XY gonadal dysgenesis, enabling simultaneous screening for deletions and duplications of several genes<sup>11, 15, 55</sup>.

The evaluation of the entire genome by aCGH to identify submicroscopic genomic imbalances on a genome wide scale, or whole-exome or -genome sequencing may allow molecular diagnosis of known genes and novel candidate genes for 46,XY gonadal dysgenesis<sup>13-15</sup>.

For example, studies using array-CGH technique resulted in the identification of known or potentially causative aberrations in some patients with dysgenetic DSD<sup>15, 17, 56</sup>. In one such report, Affymetrix Genome-Wide Human SNP Arrays 6.0 were used to analyse copy number variation in 23 individuals with gonadal dysgenesis without a genetic aetiology. Three copy number changes potentially caused gonadal dysgenesis were found: a large duplication on the X chromosome that included *NROB1*, a rearrangement that appears to affect the *SOX9* region and a small deletion immediately downstream of *GATA4* suggesting that rearrangements of non-coding sequences disturbing gene regulation may account for a proportion of DSD cases<sup>17</sup>.

In another study, a customized 1 M array-CGH platform with whole-genome coverage and probe enrichment targeting 78 genes involved in sex development was used to analyze 9 patients with 46,XY gonadal DSD. Three novel candidate regions for 46,XY GD were identified in two patients: an interstitial duplication of the *SUPT3H* gene and a deletion of *C2ORF80*. A large duplication highlighting *PIP5K1B*, *PRKACG* and *FAM189A2* were also detected but functional analyzes of these genes are not available<sup>15</sup>.

Many studies of whole exome sequencing in patients with 46,XY due to gonadal dysgenesis are currently underway but, to date, published results are limited. In a pilot study of 10 DSD patients using exome sequencing, a molecular cause was confirmed in five individuals with a previous known genetic diagnosis and potentially in 2/5 patients in whom no molecular diagnosis could be obtained by current diagnostic approach<sup>57</sup>. Another report used exome sequencing to describe the first missense mutations in the coding sequence of *FOG2* associated with 46,XY DSD in humans<sup>58</sup>. The first loss-of-function mutation in hedgehog acyl-transferase (*HHAT*) was also found in a child with testicular dysgenesis and chondrodysplasia, due to disrupted hedgehog palmitoylation and signalling<sup>59</sup>. Exome sequencing has also identified disruption of known genes, such as *NR5A1/SF-1*<sup>60</sup>. In a recent report, the underlying genetic etiology of 46,XY DSD patients without a previous genetic diagnosis was investigated by exome sequencing followed by analysis of a list of all known human DSD-associated genes. The

identification of the genetic diagnosis was possible in more than a third of cases<sup>28</sup>. It is likely that further reports of novel causes of DSD will emerge in the near future.

#### ***46,XY DSD caused by abnormal production of testosterone***

Most cases of 46,XY DSD resulting from enzymatic defects in adrenal and/or gonadal steroid synthesis have a hormonal profile in basal and/or stimulated conditions (after ACTH or hCG stimulation tests) that indicates the causative defect of the disorder (e.g., 3- $\beta$  hydroxysteroid dehydrogenase deficiency type 2, 17 $\alpha$ -hydroxylase deficiency or 17- $\beta$  hydroxysteroid dehydrogenase deficiency type 3) (Figure 1). The hormonal diagnosis is based on the accumulation of steroid precursors above the enzymatic defect and, frequently, the molecular defect can be identified by Sanger sequencing<sup>61</sup>. In two children with atypical genitalia due to 17 $\beta$ -HSD3 enzyme deficiency and normal D4/T ratio after hCG stimulation, Sanger sequencing disclosed mutations in the *HSD17B3* gene confirming the diagnosis of 17 $\beta$ -HSD3 deficiency. Therefore, molecular genetic analysis provides more accurate diagnosis of 17 $\beta$ -HSD3 deficiency than serum D4/T ratio<sup>62</sup>

#### ***46,XY DSD caused by androgen insensitivity***

Androgen insensitivity syndrome is the most frequently recognized 46,XY DSD and has a X-linked pattern of inheritance. Girls with complete androgen insensitivity syndrome (CAIS) present with a typical phenotype (breast development, primary amenorrhea and scarce pubic hair) and hormonal profile during and after puberty. Before puberty, there is an overlap between the CAIS phenotype and 46,XY DSD caused by mutations in the *LHCGR*, *NR5A1*, *CYP17A1* or *HSD17B3* genes<sup>63</sup>. The identification of defects in these genes by sequencing may establish the diagnosis avoiding the need for an hCG stimulation test. In contrast, the phenotype of partial androgen insensitivity syndrome (PAIS) is quite variable, and the clinical presentation depends on the degree of responsiveness of the external genitalia to androgens. Ambiguous genitalia with microphallus, severe hypospadias, bifid scrotum and palpable gonads, is the most frequent phenotype of PAIS<sup>64</sup>. The large phenotype spectrum of PAIS patients can cause misdiagnosis with several 46,XY DSDs due to defects in androgen production<sup>65</sup>. PAIS diagnosis is unequivocally established by the identification of a molecular defect in the AR gene.

AR mutations are identified in almost all CAIS patients and in many patients with PAIS<sup>66, 67</sup>. In our experience, Sanger sequencing identifies AR mutations in 89% and 77% of families with CAIS and PAIS, respectively. These patients were at postpubertal age and had typical AIS phenotype (gynecomastia, high LH and testosterone basal levels, affected brothers or X-linked inherited pattern). Other studies have suggested the prevalence of AR mutations in individuals with a “PAIS”-like phenotype is considerably lower<sup>68</sup>.

The knowledge of the AR defect may not indicate the potential androgen responsiveness in puberty because the phenotype depends on the residual mutant AR function that may be influenced by different factors, such as co-regulatory proteins<sup>69</sup>. A NGS approach may allow the opportunity to identify genetic modifiers, although these are difficult studies to perform with certainty, unless large kindred are available<sup>21</sup>.

#### ***46,XY DSD caused by 5 $\alpha$ -reductase type 2 (5 $\alpha$ -RD2) deficiency***

The biochemical diagnosis of 46,XY DSD caused by 5 $\alpha$ -reductase type 2 (5 $\alpha$ -RD2) deficiency is based on an elevated serum testosterone (T)/dihydrotestosterone (DHT) ratio. To determine the T/DHT ratio correctly, DHT should be measured using a very specific assay, as the commercial immunoassays show high cross-reactivity with testosterone. The analytical performance of DHT assays has improved with LCMS-based technology but this methodology is not available in most laboratories<sup>70</sup>. To analyse this ratio, T should be in the post-pubertal range. Therefore, in pre-pubertal patients, it is necessary to increase testosterone levels using hCG stimulation or exogenous testosterone enanthate injection<sup>71, 72</sup>. However, especially in newborns, a normal T/DHT ratio does not exclude 5 $\alpha$ -RD2 because the transcription of the isoenzyme 5 $\alpha$ -RD1, normally not detected at this age, may occur<sup>73, 74</sup>. Considering the prevalence of normal T/DHT ratios in affected patients, mutational analysis of the *SRD5A2* gene is indicated as the first approach especially in neonates<sup>75, 76</sup>. This is clinically important as the diagnosis of a *SRD5A2* gene mutation in neonates with ambiguous genitalia may influence the choice of sex-of-rearing based on the significant association of 5 $\alpha$ -RD2 deficiency with a change to male social sex. Moreover, many adults with 5 $\alpha$ -RD2 deficiency reared as male report a statistically significant better quality of life than adults reared as females<sup>77, 78</sup>. In addition, male patients may possibly have children after intrauterine insemination<sup>79</sup>.

### ***46,XY DSD of unknown aetiology***

Despite advances in our knowledge of the genetic basis of sex development a significant number of people with 46,XY DSD do not have an aetiologic diagnosis. In these patients, chromosomal abnormalities are not identified by classical cytogenetic techniques and the hormonal profile is not suggestive of the causative disorder. In this group of patients without a suspected target gene, next generation sequencing techniques may allow the identification of novel or unsuspected known genes associated with DSDs, especially if there are multiple affected family members. Studying trios will be important, especially for identifying *de novo* changes, and multicentric and international collaborative approaches may identify multiple variants in the same gene in independent families, which will be useful especially if supported by gene/protein expression data or animal models. However, proving causation in some cases may be challenging, especially if there are strong modifier effects from other genetic/oligogenic or epigenetic influences, or from environmental effects. Defining monogenic disorders may become increasingly more difficult and, as we enter a grey area, we may find a subset of conditions where there are strong genetic associations, weak genetic associations, or where no apparent genetic association (at least on the exome level) exists.

### **Molecular approaches to 46,XX DSD**

#### ***CAH due to 21 hydroxylase deficiency***

The diagnosis of 21-hydroxylase deficiency is essentially based on measurements of adrenal steroids, and markedly increased serum 17OH-progesterone levels in a baby with ambiguous genitalia define the diagnosis of classical form. However, molecular methodologies can be useful in specific conditions, such as in male newborns with normal genitalia and moderately increased 17OH-progesterone levels, to allow differential diagnosis of classical and non-classical forms of CAH, with therapeutic implications<sup>80, 81, 82</sup>.

One of the most recognized uses of molecular biology is for the diagnosis and treatment of at-risk pregnancies. This therapy has been used in tertiary centres for over 30 years and has been proven effective in reducing genital virilisation of affected female fetuses with CAH<sup>83, 84, 85</sup>. In brief, this treatment involves administration of dexamethasone (DEX), in order to suppress the adrenal androgen secretion. The success

of treatment depends on its onset, ideally no later than the 7 wg, before the period of genital sensitivity to androgen action, and is maintained until the end of pregnancy in affected fetuses<sup>84</sup>.

In an attempt to avoid DEX exposure of all male fetuses and in unaffected female fetuses, new approaches for prenatal diagnosis have been developed. Despite the descriptions that fetal sex determination using cffDNA have the highest sensitivity and specificity at 8 wg, recently a representative series with 258 at-risk pregnancies for CAH described a sensitivity of 96% for identification of Y-chromosomal material at the 4 wg<sup>86</sup>.

More recently, attempts to determine earlier *CYP21A2* genotype in cffDNA, to minimize the time of DEX exposure in female fetuses, have been proposed.<sup>87</sup> NGS of cffDNA in maternal plasma, at 5.4 wg, allowed the diagnosis of affected and unaffected female fetuses by linkage analysis of SNPs flanking the *CYP21A2*<sup>87</sup>.

Despite these advances, prenatal DEX therapy cannot be considered a standard care, and some adverse effects have been observed. Prenatally treated children have birth weight within the normal range, but reduced by approximately 0.4-0.6 kg, and the long term significance of this finding remains of concern<sup>88</sup>. In another study, unaffected children treated prenatally with DEX had poorer verbal working memory, rated lower on self-perception of scholastic competence and had increased self-rated social anxiety<sup>89</sup>. Until the long term risks can be defined, prenatal DEX therapy should be performed only in tertiary centers that can follow all exposed children for long periods<sup>90</sup>. Fully informed consent from parents is needed.

### ***Other CAH Diagnoses***

Besides 21OH deficiency, other adrenal enzymatic defects can also present with moderately increased neonatal 17OHP levels and occasionally these patients are picked up in 21OHD neonatal screening programs, such as 3 $\beta$ -hydroxysteroid dehydrogenase deficiency type 2 (3 $\beta$ HSD2) and P450 oxidoreductase deficiency (POR)<sup>91-94</sup>. Molecular analysis can be useful as a confirmatory test in these patients<sup>91-94</sup>. Indeed, in POR deficiency, both sexes can present with ambiguous genitalia. Additionally, POR deficiency can be associated with Antley-Bixler syndrome; however, these clinical manifestations may be not evident at birth or may even be absent<sup>95</sup>.

### ***46,XX testicular DSD and ovotesticular DSD***

Patients with 46,XX testicular DSD and male genitalia often have a translocated *SRY* gene, but the genetic basis of other forms of 46,XX testicular DSD (with ambiguous genitalia) or ovotesticular DSD is less well established<sup>96-98</sup>.

However, a common molecular origin for these conditions is suggested based on families in which patients with these two phenotypes are identified<sup>99</sup>. To date, genetic aetiologies of ovotesticular DSD include translocation of *SRY* in a small number of these patients, inactivating mutations in single genes (such as *RSPO1*, with palmoplantar hyperkeratosis and predisposition to squamous cell skin tumours) and disruption of regulatory regions resulting in altered expression of genes involved in testis determination (such as *SOX9* and *SOX3*)<sup>100-102</sup>.

Prenatal diagnosis of 46,XX testicular DSD has been reported in isolated cases, in which a discrepancy between the karyotype and genitalia was observed during screening for chromosomal aberrations<sup>96, 103</sup>. FISH analysis of amniotic fluid cells could be useful to identify *SRY*, as well as PCR amplification of the *SRY* sequence in fetal DNA samples<sup>103</sup>.

Although ovotestes most commonly occur with a 46,XX karyotype, they can occur with 46,XX/46,XY chimerism or more rarely in an individual with a 46,XY karyotype with disruption of testis-determining genes. For example, results of high-resolution array-comparative genomic hybridization (CGH) reported a female patient with 46,XY ovotesticular DSD with testicular tissue on one side and an ovary harboring germ cells on the other, who had a deletion of ~35 kb affecting exons 3 and 4 of the *DMRT1* gene. This report suggests that *DMRT1* haploinsufficiency is sufficient to cause 46,XY gonadal dysgenesis and 46,XY ovotesticular DSD<sup>104</sup>.

## **Genetic counselling**

Genetic counselling is the process by which parents or relatives at risk of DSD are informed of the nature and consequences of the disorder, and the probability of developing it or and of passing it on to their offspring. The optimal time to determine the genetic risk and discuss prenatal testing is before pregnancy<sup>105</sup>. Family members at risk of an autosomal recessive or X-linked form of 46,XY DSD can be tested after the identification of a specific molecular defect in an affected family member. Prenatal genetic testing can show that the fetus is unaffected by the disorder or give parents the opportunity to prepare themselves for the birth of a child who has a genetic condition<sup>106</sup>.

Nonetheless, genetic tests are also associated with several concerns; parents experience stress and anxiety regarding the results, and a specific molecular diagnosis is not always possible, especially in 46,XY DSD. Parents carrying mutations may feel guilty about their child's condition and psychological support is an important component of genetic counselling.

Additionally, genetic tests are generally expensive and can cause discrimination and stigmatisation. For these reasons, genetic testing should be requested after an extensive discussion about how the result may be useful for patients, their relatives or for management decisions<sup>29, 107</sup>. A geneticist with experience of DSD is an invaluable member of the DSD team.

### **Conclusion**

Recent methodological advances in the diagnosis of monogenic diseases have not only elucidated the molecular aetiology of patients with DSD, but also yielded valuable information on biological processes of sex development. Although many of these techniques are not yet available for routine diagnosis, in the near future they will likely contribute to the diagnosis of DSD in many patients quickly and inexpensively. However, careful selection of the genetic test indicated for each condition is still important for good clinical practice. Multidisciplinary approaches including psychological support are essential to prevent unnecessary stress during this challenging process.

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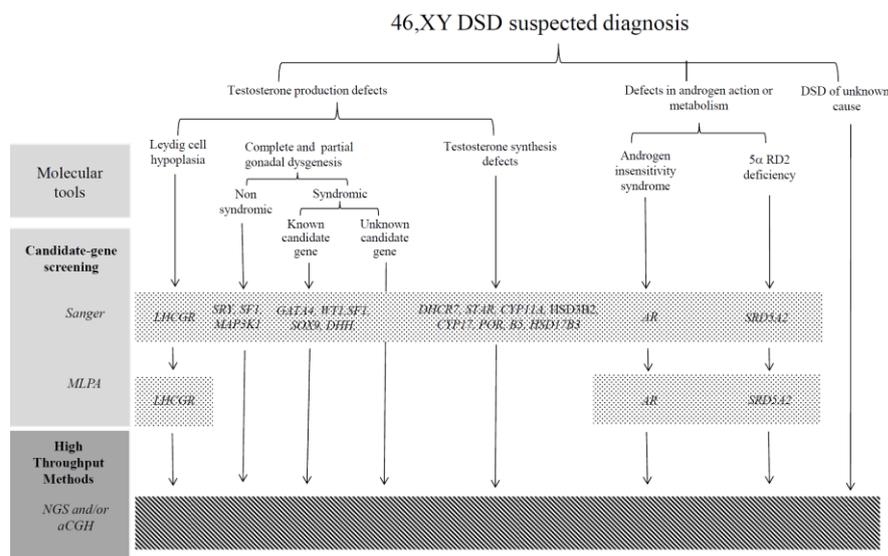
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## Figure Legends

**Figure 1-**Molecular approach for genetic diagnosis of individuals with 46,XY disorders of sex development (DSDs). 46,XY DSD results from incomplete intrauterine virilisation due to defects in testosterone production, androgen action or metabolism, or due to undetermined causes. This chart indicates a potential sequence of the molecular approaches to define the genetic aetiology of 46,XY DSD. The arrows indicate the steps of investigation based on previously described molecular defects associated with 46,XY DSD. In testosterone production defects, and androgen action or metabolism defects, the candidate-gene approach is usually the first step. Human sex development is sensitive to gene dosage effects and duplication and deletions events may be ruled out in gonadal dysgenesis, as well as obvious single gene conditions due to associated syndromes. Considering the low frequency of diagnosis throughout a candidate gene approach, aCGH and NGS should be the first step of molecular investigation in isolated gonadal dysgenesis as well as in undetermined DSD, unless associated features are present. Targeted array based approaches, and whole-exome or -genome sequencing may enable molecular diagnosis to be reached involving known genes and novel candidate genes for 46,XY.

Abbreviations to be included under Figure 1

aCGH: array comparative genomic hybridisation; NGS: next generation sequencing



**Table 1: Molecular techniques applied to identify genetic causes of DSD**

Molecular Technique		Application	Time to get data without analysis (day)	Availability	Approximate costs	Limitations
aCGH, SNP array CGH		CNVs, Mosaicisms, uniparental disomy, LOH	Up to 4	Clinical & Research	£250 (N.B Standard clinical FISH for SRY/XCEN probes £150; standard clinical G-banded karyotype £200)	Unable to detect balanced translocations, inversions without loss of genomic material and polyploidies (triploidy and tetraploidy)
Sanger Sequencing		SNVs, small indels, microsatellite analysis	Up to 2 (most clinical tests longer)	Clinical & Research	£150-£600 (clinical testing depending on the size of the gene, laboratory and turn around time; targeted sequencing of known changes in a family less expensive)	Does not detect large deletions or insertions and can only analyze specific and small regions at a time, labor intensive
qPCR		CNVs, SNPs, gene expression	1.5	Clinical & Research	£150	Analyze specific and small regions at a time
MLPA		CNVs, SNPs methylation defects	Up to 3	Clinical & Research	Variable depending on panel	Analyze specific and small regions at a time
Next Generation Sequencing	Targeted Custom Capture Array	SNVs, CNVs	Up to 4	Clinical & Research	£800 (DSD clinical panel, including validation of positive findings); £800 (multigene clinical panel, including most DSD-related genes, including validation of positive findings); £130 (research panel of 150 genes, not including labour costs for processing or analysis, or clinical validation)	High cost, labor intensive, bioinformatic support needed, may not detect moderate insertions and deletions, “bystander” information about other genes may be obtained
	Exome	SNVs, CNVs,	Up to 16	Research	£500-£900 (depending on sequencing depth and cost of bioinformatic analysis)	
	Whole Genome	SNVs, CNVs,	Up to 21	Research	Research	

Note: approximate costs are given in pounds sterling (January 2015). Prices vary greatly depending on the type of test, laboratory/country and turn around time. Clinical testing is generally more expensive and some clinical high throughput analysis needs validation of key findings before test results can be reported.

CGH: array comparative genomic hybridization; CNV: copy number variation; LOH: loss of heterozygosity; MLPA: Multiplex Ligation-dependent Probe Amplification; qPCR: quantitative PCR SNPs: single nucleotide polymorphisms; SNVs: single nucleotide variants.

**Table 2: Selected examples where a genetic/chromosomal diagnosis can influence clinical management**

Condition (Gene)	Challenge	Benefit of genetic diagnosis
45,X/46,XY mosaicism	Variable phenotype; associated features; significant risk of germ cell neoplasia	Screening for cardiac and renal anomalies and Turner Syndrome surveillance; growth monitoring; gonadal biopsies on account of tumor risk
Chromosomal deletion (e.g 11p13/WAGR, 10q, 9p)	Associated features including another organ systems; potential learning difficulties	Focused screening of other systems depending on the condition (e.g. eyes, renal, cardiac); close monitoring by child development team and early support and intervention if developmental delay occurs; counselling of family and contact with appropriate support groups and multidisciplinary services for children with complex needs
Denys-Drash Spectrum ( <i>WT1</i> )	Risk of Wilms' tumor or nephropathy; significant risk of germ cell neoplasia	Defines individuals who need regular screening for Wilms' tumor or who might develop a renal phenotype later; higher risk of germ cell tumors, so gonadectomy or careful monitoring is required
Steroidogenic factor-1 ( <i>NR5A1</i> )	Variable phenotype; potential primary ovarian insufficiency (POI) or progressive infertility in family members; potential associated adrenal disease	Monitoring adrenal function; identifying female relatives who are at risk of developing POI and who could benefit from counseling and monitoring of ovarian function; identification of male relatives who may have infertility or a progressive endocrinopathy
17 $\alpha$ -hydroxylase deficiency ( <i>CYP17A1</i> )	Associated hypokalaemia, hypertension	Allows treatment and prevention of biochemical and blood pressure effects
5 $\alpha$ -reductase 2 deficiency ( <i>SRD5A2</i> )	Often associated with social sex change in puberty, potentially better QoL as male than female in adulthood; possible fertility in males	Male sex assignment avoids social sex change and potentially better QoL
21-hydroxylase deficiency ( <i>CYP21A2</i> )	Risk of salt wasting in children with 21-hydroxylase deficiency identified in neonatal screening	Allows genetic counselling and withdrawn of mineralocorticoid and salt replacement in patients with SV form

WAGR, Wilms tumour-Aniridia-Genitourinary-Retardation; QoL, quality of life; SV, simple virilising