---

**Article:**
Hughes, L.A., McKay Bounford, K., Webb, E. et al. (9 more authors) (2019) Next generation sequencing (NGS) to improve the diagnosis and management of patients with disorders of sex development (DSD). Endocrine Connections, 8 (2). pp. 100-110. ISSN 2049-3614

https://doi.org/10.1530/EC-18-0376

---

 Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

# Next Generation Sequencing (NGS) to improve the diagnosis and management of patients with Disorders of Sex Development (DSD).

Hughes, L.A[1]., McKay-Bounford, K[1]., Webb, E.A[2]., Dasani P[1]., Clokie, S[1]., Chandran, H, McCarthy, L[2]., Mohamed,Z[2]., Kirk, J.M.W[2]., Krone, N.P[2]., Allen, S[1]., Cole, T.R.P[1].

[1] West Midlands Regional Genetics Service, Birmingham Women's and Children's NHS Foundation Trust, Birmingham, B15 2TG, United Kingdom

[2] Department of Endocrinology & Diabetes, Birmingham Women's and Children's Hospital, Steelhouse Lane, Birmingham, B4 6NH, United Kingdom

Corresponding author and to whom requests for reprints should be addressed:
Dr Trevor Cole, Clinical Genetics Unit, West Midlands Regional Genetics Service, Birmingham Women's and Children's NHS Foundation Trust and Birmingham Health Partners, Mindelsohn Way, Birmingham, B15 2TG
Phone: 0121 335 8024 Fax: 0121 627 2618 Email: trevor.cole1@.nhs.net

Keywords: Next generation sequencing (NGS), disorders of sex development (DSD), gene, variant

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

## Abstract

Disorders of sex development (DSDs) are a diverse group of conditions where the chromosomal, gonadal or anatomical sex can be atypical. The highly heterogeneous nature of this group of conditions often makes determining a genetic diagnosis challenging. Prior to Next Generation Sequencing (NGS) technologies, genetic diagnostic tests were only available for a few of the many DSD associated genes, which consequently had to be tested sequentially. Genetic testing is key in establishing the diagnosis, allowing for personalised management of these patients. Pinpointing the molecular cause of a patient's DSD can significantly impact patient management by informing future

development needs, altering management strategies and identifying correct inheritance pattern when counselling family members.

We have developed a 30 gene NGS panel, designed to be used as a frontline test for all suspected cases of DSD (both 46,XX and 46,XY cases). We have confirmed a diagnosis in 25 of the 80 patients tested to date. Confirmed diagnoses were linked to mutations in *AMH, AMHR2, AR, HSD17B3, HSD3B2, MAMLD1, NR5A1, SRD5A2 & WT1* which have resulted in changes to patient management. The minimum diagnostic yield for patients with 46,XY DSD is 25/73. In 34/80 patients only benign or likely benign variants were identified, and in 21/80 patients only variants of uncertain significance, (VOUS) were identified, resulting in a diagnosis not being confirmed in these individuals. Our data supports previous studies, that an NGS panel approach is a clinically useful and cost effective frontline test for patients with DSDs.

## Introduction

Disorders of sex development (DSD) encompass a wide range of conditions with diverse clinical features, pathophysiology and clinical management[1–3]. The recently revised stratified DSD diagnostic pathway consists of clinical examination, biochemical investigations and karyotype determination[4,5]. Once a presumptive diagnosis has been made, targeted sequencing of candidate genes may then be performed at a later stage[5]. Whilst reaching the correct diagnosis impacts significantly on management decisions, determining the aetiology of genital ambiguity in patients with DSD on the basis of clinical and biochemical assessment remains challenging[6]. In DSD where no clear abnormality in the steroidogenesis pathway is present, the yield from genetic testing had historically remained low, and with single-gene sequencing was both costly and time consuming[4].

Improvements in gene sequencing technology in conjunction with rapidly falling costs have led to the use of targeted next-generation sequencing (NGS) assays. These enable multiple known disease causative genes to be sequenced in parallel alongside initial clinical assessment and biochemical investigations, potentially avoiding the need for additional expensive biochemical and radiological investigations[7]. Reaching a timely diagnosis is extremely important as it ends diagnostic uncertainty, avoids further unnecessary investigations, enables appropriate disease-specific counseling (including assessment of future fertility potential and malignancy risk) and implementation of personalised

 Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

medical plans in accordance with current disease specific consensus guidelines[8]. Additionally, in the long-term accurate early diagnosis will support the development of better designed outcome studies.

The use of targeted next generation sequencing panels for molecular diagnosis of DSD patients has already been reported successfully in several previous publications[9–12]. These publications show diagnostic yield and clinical utility in predominantly 46,XY DSD cohorts using panels of 64-219 genes. We present our data showing sequence analysis of a cohort of 80 DSD patients using a 30 gene panel.

## Materials & Methods

### Patients

Eighty patients with a DSD were referred to the West Midlands Regional Genetics Laboratory (WMRGL) at the Birmingham Women's and Children's NHS Foundation Trust for diagnostic DSD testing between March 2014 and March 2017, comprising of 73 patients with 46,XY DSD and 7 with 46,XX DSD. Referrals were from Clinical Genetics, Urology or Endocrinology specialists. Karyotyping and/or microarray results were typically available to confirm the patient's karyotype. Single gene testing may have also been performed in advance of the NGS screen but a pathogenic mutation had not been identified. Where DNA was available, cascade testing was performed on parental or sibling samples to confirm segregation or to confirm a diagnosis in the proband's similarly affected siblings. Data from cascade testing is not included here; all figures therefore represent only probands referred for diagnostic testing who underwent analysis via the NGS panel. Consent was obtained for clinical testing from all patients in this study. Patients undertaking routine clinical testing in this report are not identifiable. This report has been registered with the audit committee at the Birmingham Women's and Children' Hospital NHS Foundation trust (CARMS-30120).

### Gene selection

Thirty genes with a reported clinical association with a DSD were selected following discussion between the WMRGL and clinical specialists in Genetics and Endocrinology (table 1). Genes include those thought to be involved in 46,XY DSD and 46,XX DSD, and are tested as a single panel pipeline covering both of these groups of patients. The *CYP21A2* gene associated with 95% of cases of Congenital Adrenal Hyperplasia (CAH) is not included in this panel. This is because this patient group typically have a clinical diagnosis prior to genetic testing, and also the *CYP21A2* pseudogene makes accurate mapping of short reads to the functional gene very difficult.

**Sample preparation**

Genomic DNA was typically extracted from peripheral blood samples using Qiasymphony technology (Qiagen) following the manufacturer's instructions. The concentration of all genomic DNA samples were assessed using a Qubit (Life technologies) prior to sequencing.

**Next Generation Sequencing (NGS)**

Library preparation was initially performed by a customised TruSeq Custom Amplicon (TSCA, Illumina Inc, San Diego, California) 30 gene panel run on the MiSeq (Illumina Inc, San Diego). Exons were targeted with 25 base pairs of padding on either side, resulting in 431 amplicons of 425bp. Enrichment was performed on 250ng of genomic DNA, and sequencing using 250 base paired-end reads. TruSight One (TSO) technology was then used to capture exonic regions of 4,813 genes, sequencing 24 samples on a HiSeq 2500 (Illumina Inc, San Diego, California). The same 30 DSD genes were analysed as a virtual panel using TSO. A depth of coverage of 20x was considered sufficient for either approach, and a technical report was generated indicating the proportion of each gene covered to this level. All patients that were initially run by TSCA but where a diagnosis was not confirmed were subsequently retested using TSO. NGS was performed on probands only and analysis for copy number variation in these genes was not performed.

**Bioinformatic analysis**

Bioinformatic analysis was performed using an in-house pipeline where sequence reads were mapped to the human genome hg19 reference. Several programmes are incorporated in the pipeline; Trimmomatic (quality trimming of reads), BWA mem (alignment to hg19), Samblaster (duplicate marking), Abra (realigning), Platypus (variant calling), Annovar (variant annotation) PLINK (IBS calculation) and Picard (calculating hybridisation and mapping metrics). Custom python code and bedtools were used to calculate coverage and the Python module pandas to produce patient specific Excel files.

**Variant interpretation and reporting**

Variants were classified following the Association of Clinical Genetic Science (ACGS, www.acgs.uk.com) best practice guidelines, based on the American College of Medical Genetics and genomics recommendations[13]. This included utilisation of in-house frequency data, population frequency data (dbSNP, 1000 genomes & EXAC), in-silico tools including Polyphen, Align GVGD and splice tools (searched through the alamut interface), the Human Gene Mutation Database (HGMD Professional, Biobase Corporation), and evidence from peer-reviewed literature. The five classes are described in table 2. For suspected compound heterozygous mutations, parental samples were

 Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

requested to confirm that the mutations were on opposite alleles (in trans). Regions of interest were all exonic regions plus 30bp upstream and 10bp downstream of each exon. Intronic variants outside of these regions were considered as deep intronic variants (DIVs) and no further investigation was undertaken. In some cases, Sanger sequencing was performed to complete gene coverage to a depth of 20x. For example, where a single heterozygous mutation in a likely candidate gene associated with a recessive condition had been identified.

## Results

The DSD NGS panel provided a molecular diagnosis in 25 out of the 80 patients tested (table 3). A diagnosis was deemed as confirmed where variants of class 4 or class 5 were identified which were consistent with the inheritance pattern for that gene, and where disruption of the gene was in keeping with the patient's phenotype. On 4 occasions a class 3 variant was found in combination with a class 5 mutation (patients 2, 11, 15 & 20). Although technically class 3 variants are of uncertain significance, their presence in combination with a class 5 variant in these patients, when considered with the clinical information provided led us to believe that these findings were causally related to the clinical features. The overall diagnostic yield for this panel is therefore currently 25/80 for all samples and 25/73 for 46,XY DSD. This figure represents the likely minimum detection rate of this panel as some samples were received with an initial request for Sanger sequencing of a specific gene, and typically only included for panel testing if negative on Sanger sequencing.

Class 3 variants (listed in table 4) were typically missense mutations which had not been previously reported, and therefore no clinical information was available. These were included in the clinical report with a statement that a diagnosis had not been confirmed due to the uncertainty around the pathogenicity of such variants. Where only class 1 and/or 2 variants were identified, patient reports stated that no evidence of a pathogenic mutation had been identified. A summary of the findings can be seen in figure 1. Both previously reported and novel pathogenic mutations and variants were identified in *AMH, AMHR2, AR, DHCR7, HSD17B3, HSD3B2, LHCGR, MAMLD1, NR5A1, SRD5A2 and WT1* (table 5). Diagnosis due to mutations in the *AR* gene (7 patients) were the most commonly observed (table 3) followed by diagnosis due to HSD17B3 (5 patients) and SRD5A2 (4 patients).

## Discussion

DSDs, estimated to be present in 1.7% of live births[14] are a diagnostic challenge due to variable expressivity and pleiotrophy, clinical overlap of the different DSD, and their significant aetiological heterogeneity. Historically a genetic diagnosis was made in as few as 13% of cases [15]. We present data from 80 patients who underwent routine diagnostic testing for DSD using a 30 gene NGS panel. This diagnostic DSD panel was utilised irrespective of clinical and biochemical features, unless a specific single Sanger sequence request was made based on phenotypic assessment. The diagnostic yield of this DSD panel was shown to be 25/80 for all DSD's, higher for 46,XY DSD (25/73), and would have been higher in this cohort if all cases with a suspected diagnosis, (all subsequently confirmed on Sanger Sequencing) , had not been filtered out prior to implementation of the panel test. Pathogenic (or likely pathogenic) mutations in the *AMH*, *AMHR2*, *AR*, *HSD17B3*, *HSD3B2*, *MAMLD1*, *NR5A1*, *SRD5A2* and *WT1* genes were identified. Our detection rate and findings are similar to previous studies, summarised in Table 6. Dong *et al* demonstrated an increased detection rate of 9/13 in 46,XY DSD patients using a panel of 219 genes[10], however the study included small patient numbers and so may not be representative.

Separating out analysis of 46,XY DSD from those with 46,XX DSD results in an improved 46,XY yield to 25/73 but highlights the 0/7 diagnostic yield of individuals with a 46,XX DSD. Difficulty in confirming a molecular diagnosis in those with an 46,XX DSD has also been seen in other studies[11,12]. Sample numbers for those with 46,XX DSD are very small in this study and therefore are unlikely to be representative of the true diagnostic capability of the panel for these patients. In addition, other causes of 46,XX DSD such as translocation of SRY to the X chromosome, duplications of *SOX9* or CAH due to CYP21A2 deficiency are not detectable by this method. Patients would typically have had karyotype and/or microarray prior to testing on the panel and would have been tested separately for CYP21A2 deficiency if CAH was suspected. Increased sample numbers and incorporation of more 46,XX DSD associated genes as they are identified may allow a more accurate estimate of the panel's usefulness for those with 46,XX DSD. It will also be important to include new 46,XX DSD genes that are likely to be identified in current international exome/genome sequencing projects such as the 100,000 genome project in England.[16]

Novel mutations and variants in several genes were identified where functional studies were not available. Variants were considered likely to be causative if they were observed in trans (on opposite chromosome alleles) with a known pathogenic mutation, in a disease gene showing autosomal

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

recessive inheritance (4 patients in our cohort). In some cases, segregation studies confirming the bi-allelic nature of the findings also supported a likely pathogenic role. This information has expanded our knowledge of likely diagnostic DSD variants for future investigation of DSD patients. Novel VOUS in the absence of a confirmed diagnosis were also seen. Whilst their significance currently remains uncertain, wider data sharing through publication of studies such as this is crucial to further our understanding of such variants. The large number of VOUS in this cohort is predominantly due to limited clinical information related to some DSD related genes, for example only VOUS were detected in the *CBX2* gene. Clinicians will have to manage any patient confusion or anxiety within the current uncertainty until more data is available. When designing future NGS DSD panels it will remain important to recognise that increasing the number of genes, especially those with limited data, will generate greater numbers of VOUS with increased cost and complexity of analysis. This should be balanced against the potential for gene discovery.

Segregation studies have been helpful in determination of pathogenicity in several families, as illustrated by patient 23, who had an Androgen Receptor (*AR)* variant initially reported as a VOUS. The patient's older brother, previously shown to have a 47,XXY karyotype and a "milder" phenotype was subsequently shown to have the same *AR* variant. Given his additional X chromosome, without skewed X inactivation a milder phenotype would be expected. This increased our confidence in calling the variant a "likely pathogenic" mutation and highlights the importance of reviewing interpretation of variants when new information becomes available.

NGS technology has also allowed the identification of mosaic mutations in the *AR* gene, which may have gone undetected by other methods. Identification of an accurate number of reads enhances our knowledge of the level of mosaicism present. It should be noted, however, that the results represent the mutation load in peripheral blood (70% patient 8 and 30% patient 24) and not necessarily other relevant tissues. Of the mutations identified, both had been previously reported in non-mosaic form in the literature[17,18]. Mosaic mutations have however been reported in the *AR* gene[19,20] and taken together with the clinical features, allowed us to conclude that these mutations in mosaic form were likely to be contributing to the phenotype in these patients. Importantly for patient management, *AR* mutations in mosaic form are believed to pose a risk of virilisation in patients due to the presence of wild type androgen receptor[19]. Distinguishing mosaic from non-mosaic forms therefore can have significant consequences for patient management and genetic recurrence risk.

Identifying the correct genetic diagnosis modifies the patient management and impacts on the accuracy of information and choices available to family members. The former is clearly illustrated in case 21 where mutations in two different DSD related genes were present in the same family; *HSD17B3* and *AR.* The family were requesting gonadectomy pre-adolescence in a 46,XY DSD female, on the basis that her cousins who were 46,XY DSD females, due to a homozygous *HSD17B3* mutations, had virilised and been managed with gonadectomy in another centre. Identification of a previously unidentified mutation in *AR* in this family meant 3 girls with 46,XY changed their subsequent management. In 4 cases the diagnosis in affected siblings has been confirmed (listed in table 3 patients 7, 17, 21 & 23) including cases enabling early prenatal (patient 20) or neonatal (patient 17) diagnosis, and thus implementation of appropriate management from birth. This highlights the importance of identifying the molecular diagnosis not only for the proband but also for the wider family.

The panel also identified pathogenic mutations which were thought to be co-incidental and not to be related to the initial clinical presentation in the proband. In patient 18 with Congenital Adrenal Hyperplasia (CAH) due to HSD3B2 deficiency, the patient was also shown to be a carrier for the common splice mutation c.964-1G>C in the *DHCR7* gene which is linked to Smith Lemli Opitz syndrome (SLOS). Whilst such incidental findings can be challenging for patient counselling, the information provided may also be of great significance. This will be especially true when previously undetected autosomal recessive mutations are uncovered in highly consanguineous families, which are common within many DSD cohorts.

Where a clinician has a strong suspicion of the involvement of a specific gene, Sanger sequencing may be more cost effective, especially where the number of amplicons is relatively small. For example pathogenic mutations in the *AMH*, *AMHR2*, *AR* and *SRD5A2* gene were identified in this way. When the original clinical diagnosis is incorrect however, the potential cost savings rapidly disappear if sequential Sanger sequencing is required, and therefore any benefit is highly dependent on the clinical expertise and the specificity of the additional non-genetic investigations. There may also be atypical presentations not yet recognised for mutations in some genes, and therefore a wider panel approach has the potential to address this.

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

Despite the diagnostic rate of 25/73 for 46,XY DSD, the failure to achieve a diagnosis in 55 patients confirms the need for further development. Of note, the panel cannot currently detect copy number changes (CNVs), and so further development should include detection of CNVs that are below the resolution of the current chromosomal microarray assay. The 30 genes in our panel were selected due to published evidence of their involvement in DSD, but new gene discoveries in studies such as the 100,000 genome project[16] should enhance the diagnostic utility, especially for 46,XX DSD. Eggers et al demonstrated a 118/278 diagnostic rate for 46,XY DSD patients by including 64 DSD genes[11], and Dong et al reported a 6/13 rate by including 219 genes[10], thus diagnostic capability may be improved by increasing the gene number. These benefits may be marginal however as only one likely pathogenic mutation in the Dong series was in the additional 179 genes not included in this 30 gene panel. Comparison of these studies could indicate that a greater impact on the detection rate may be due to patient selection. It will be important, therefore, that further candidate gene inclusion is critically evaluated as the addition of genes without clear clinical utility will likely result in increasing cost and numbers of VOUS without necessarily increasing diagnostic capability. The future of this investigative pathway may well be transformed by implementation of whole exome or whole genome sequencing, but any benefits of diagnostic detection will have to be weighed against increased cost and clinical complexities resulting from VOUS and co-incidental findings. It therefore remains important to optimise such NGS panels for DSD so that a valid comparison can be made in future.

This data demonstrates clear advantages of an NGS panel approach for highly heterogenous conditions such as DSD. Despite the limitations of the panel including incomplete coverage and inability to detect copy number changes, the results presented here demonstrate that an NGS based panel approach is a useful frontline tool for diagnosing DSDs. In addition to a diagnostic yield of at least 25/80 we have shown examples of cases where the information provided from the panel has identified diagnoses in complex families with the potential for multiple aetiologies, cases where panel findings have significantly impacted management and treatment decisions, and examples of novel variants being identified, thus expanding our current knowledge. As more and more patients are tested, the information provided by such panels will continue to grow and improve our understanding of these complex conditions and hopefully improve the diagnostic capability of such tests. Despite its limitations, the clinical benefit of this approach is clearly demonstrated for DSD patients allowing for timely accurate diagnoses, more informed management strategies and improved counselling for patients and their families.

## Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

## Funding

This work did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

## References

1. Biason-Lauber, A. Control of sex development. *Best Pr. Res Clin Endocrinol Metab* **24,** 163–186 (2010).

2. Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O. & Fujii-Kuriyama, Y. Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. *Proc Natl Acad Sci U S A* **83,** 2841–2845 (1986).

3. Arboleda, V. A., Sandberg, D. E. & Vilain, E. DSDs: genetics, underlying pathologies and psychosexual differentiation. *Nat. Rev. Endocrinol.* **10,** 603–15 (2014).

4. Ahmed, S. F., Achermann, J. C., Arlt, W., Balen, A. H., Conway, G., Edwards, Z. L., Elford, S., Hughes, I. A., Izatt, L., Krone, N. *et al.* UK guidance on the initial evaluation of an infant or an adolescent with a suspected disorder of sex development. *Clin. Endocrinol. (Oxf).* **75,** 12–26 (2011).

5. Lee, P. A., Nordenström, A., Houk, C. P., Ahmed, S. F., Auchus, R., Baratz, A., Baratz Dalke, K., Liao, L.-M., Lin-Su, K., Looijenga, L. H. J. *et al.* Global Disorders of Sex Development Update since 2006: Perceptions, Approach and Care. *Horm. Res. Paediatr.* **85,** 158–80 (2016).

6. Lee, Y. S., Kirk, J. M., Stanhope, R. G., Johnston, D. I., Harland, S., Auchus, R. J., Andersson, S. & Hughes, I. A.. Phenotypic variability in 17beta-hydroxysteroid dehydrogenase-3 deficiency and diagnostic pitfalls. *Clin Endocrinol* **67,** 20–28 (2007).

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019


7. Ahmed, S. F., Bashamboo, A., Lucas-Herald, A. & McElreavey, K. Understanding the genetic aetiology in patients with XY DSD. *Br. Med. Bull.* **106,** 67–89 (2013).

8. Lee, P. A., Houk, C. P., Ahmed, S. F., Hughes, I. A. & Endocrinology, I. C. C. on I. organized by the L. W. P. E. S. and the E. S. for P. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics* **118,** e488-500 (2006).

9. Baxter, R. M., Arboleda, V. A., Lee, H., Barseghyan, H., Adam, M. P., Fechner, P. Y., Bargman, R., Keegan, C., Travers, S., Schelley, S. *et al*. Exome sequencing for the diagnosis of 46,XY disorders of sex development. *J. Clin. Endocrinol. Metab.* **100,** E333-44 (2015).

10. Dong, Y., Yi, Y., Yao, H., Yang, Z., Hu, H., Liu, J., Gao, C., Zhang, M., Zhou, L., Asan, Yi, X. & Liang, Z. Targeted next-generation sequencing identification of mutations in patients with disorders of sex development. *BMC Med. Genet.* **17,** 23 (2016).

11. Eggers, S., Sadedin, S., van den Bergen, J. A., Robevska, G., Ohnesorg, T., Hewitt, J., Lambeth, L., Bouty, A., Knarston, I. M., Tan, T. Y. *et al.* Disorders of sex development: insights from targeted gene sequencing of a large international patient cohort. *Genome Biol.* **17,** 243 (2016).

12. Kim, J. H., Kang, E., Heo, S. H., Kim, G.-H., Jang, J.-H., Cho, E.-H., Lee, B. H., Yoo, H.-W. & Choi, J.-H. Diagnostic yield of targeted gene panel sequencing to identify the genetic etiology of disorders of sex development. *Mol. Cell. Endocrinol.* **444,** 19–25 (2017).

13. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E. *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* **17,** 405–423 (2015).

14. Blackless, M., Charuvastra, A., Derryck, A., Fausto-Sterling, A., Lauzanne, K. & Lee, E. How sexually dimorphic are we? Review and synthesis. *Am. J. Hum. Biol.* **12,** 151–166 (2000).

15. Arboleda, V. A., Lee, H., Sánchez, F. J., Délot, E. C., Sandberg, D. E., Grody, W. W., Nelson, S. F. & Vilain, E. Targeted massively parallel sequencing provides comprehensive genetic diagnosis for patients with disorders of sex development. *Clin. Genet.* **83,** 35–43 (2013).

16. Siva, N. UK gears up to decode 100,000 genomes from NHS patients. *Lancet* **385,** 103–104

(2015).

17. Galani, A., Sofocleous, C., Karahaliou, F., Papathanasiou, A., Kitsiou-Tzeli, S. & Kalpini-Mavrou, A. Sex-reversed phenotype in association with two novel mutations c.2494delA and c.T3004C in the ligand-binding domain of the androgen receptor gene. *Fertil. Steril.* **90,** 2008.e1-2008.e4 (2008).

18. Marcelli, M., Tilley, W. D., Wilson, C. M., Wilson, J. D., Griffin, J. E. & McPhaul, M. J. A single nucleotide substitution introduces a premature termination codon into the androgen receptor gene of a patient with receptor-negative androgen resistance. *J. Clin. Invest.* **85,** 1522–8 (1990).

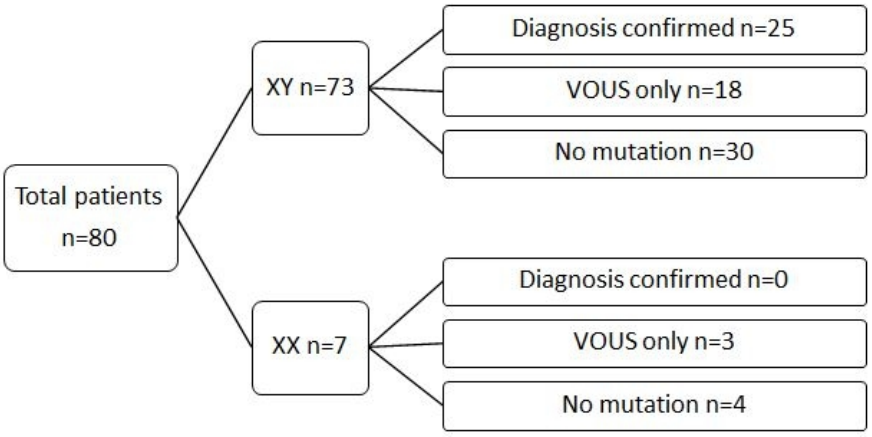
19. Holterhus, P. M., Wiebel, J., Sinnecker, G. H., Brüggenwirth, H. T., Sippell, W. G., Brinkmann, A. O., Kruse, K. & Hiort, O. Clinical and molecular spectrum of somatic mosaicism in androgen insensitivity syndrome. *Pediatr. Res.* **46,** 684–90 (1999).

20. Köhler, B., Lumbroso, S., Leger, J., Audran, F., Grau, E. S., Kurtz, F., Pinto, G., Salerno, M., Semitcheva, T., Czernichow, P. *et al*. Androgen Insensitivity Syndrome: Somatic Mosaicism of the Androgen Receptor in Seven Families and Consequences for Sex Assignment and Genetic Counseling. *J. Clin. Endocrinol. Metab.* **90,** 106–111 (2005).

**Figure 1: Summary of findings of DSD panel.**

Results of the panel are separated by karyotype (XX or XY) and by result. 'Diagnosis confirmed' indicates patients where a pathogenic variant was detected compatible with the patient's phenotype. 'VOUS (Variant Of Uncertain Significance) only' indicates solely Class 3 variants were detected and therefore a diagnosis could not be confirmed. 'No mutation' indicates only Class 1 (polymorphisms) or Class 2 variants (unlikely to be pathogenic) were detected.

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019




184x126mm (96 x 96 DPI)

| 46,XY DSD | Gene Name | Location |
|---|---|---|
| Disorders of testicular development | ARX | Xp22.13 |
| | ATRX | Xq13.3 |
| | CBX2 | 17q25 |
| | DHH | 12q13.1 |
| | DMRT1 | 9p24.3 |
| | MAMLD1 | Xq28 |
| | NR0B1 | Xp21.3 |
| | NR5A1 | 9q33 |
| | SOX9 | 17q24-q25 |
| | SRY | Yp11.3 |
| | TSPYL1 | 6q22-23 |
| | WNT4 | 1p35 |
| | WT1 | 11p13 |
| Disorders of Hormone synthesis or action | AMH | 19p13.3-p13.2 |
| | AMHR2 | 12q13 |
| | AR | Xq11-q12 |
| | CYB5A | 18q23 |
| | CYP11A1 | 15q23-24 |
| | CYP17A1 | 10q24.3 |
| | DHCR7 | 11q12-q13 |
| | HSD3B2 | 1p13.1 |
| | HSD17B3 | 9q22 |
| | LHCGR | 2p21 |
| | POR | 7q11.2 |
| | SRD5A2 | 2p23 |
| | StAR | 8p11.2 |

**Table 1a**

| 46,XX DSD | Gene Name | Location |
|---|---|---|
| Disorders of ovarian development | RSPO1 | 1p34.3 |
| | SOX9 | 17q24 |
| | SRY | Yp11.3 |
| | WNT4 | 1p35 |
| Androgen Excess | CYP11B1 | 8q21-q22 |
| | CYP19A1 | 15q21 |
| | HSD3B2 | 1p13 |
| | NR3C1 | 5q31 |
| | POR | 7q11.2 |

**Table 1b**

**Table 1: Genes included in the DSD panel**

A summary of all genes and their chromosomal location which are included in the panel for both 46,XY DSD (table 1a) and 46,XX DSD (table 1b). CAG repeat in *AR* not analysed to avoid incidental diagnosis of Spinal bulbar muscular atrophy.

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

| Variant class | Variant description | Confirmation by Sanger | Reported |
|---|---|---|---|
| **1** | Polymorphism | No | No |
| **2** | Unlikely to be pathogenic | No | Yes |
| **3** | Variant of uncertain significance (VOUS) | Yes | Yes |
| **4** | Likely to be pathogenic | Yes | Yes |
| **5** | Clearly pathogenic | Yes | Yes |

**Table 2: Classification and follow up of variants.** All variants determined to be class 3-5 were confirmed using Sanger sequencing and all were included in the clinical reports. Variants considered to be unlikely to be pathogenic (class 2) were not confirmed by Sanger sequencing and were recorded for information only in the clinical report. Class 1 variants were not reported.

| Patient | Sex | Reason for referral | Gene | Allele 1 | Allele 2 | Results reported and follow up |
|---|---|---|---|---|---|---|
| **1** | XY M | ?PAIS | AR | c.2402C>T p.(Thr801Ile) | | Confirmed diagnosis of PAIS. |
| **2** | XY M | ?PMDS | AMH | c.283C>T p.(Arg95*) | c.905G>A p.(Arg302Gln) | Consistent with diagnosis of PMDS. |
| **3** | XY F | ?46,XY DSD | NR5A1 | c.1171A>T p.(Lys391*) | Normal | Consistent with a diagnosis of a 46,XY DSD. **Novel mutation**. |
| **4** | XY$^{p}$ F | ?46,XY DSD | HSD17B3 | c.614T>A p.(Val205Glu) | c.645A>T p.(Glu215Asp) | Supports diagnosis of 46,XY DSD due to HSD17B3 deficiency. |
| **5** | XY$^{p}$ F | ?46,XY DSD | HSD17B3 | c.194C>T p.(Ser65Leu) | c.729_735del7 p.(Ile244Argfs*11) | Supports diagnosis of 46,XY DSD due to HSD17B3 deficiency. |
| **6** | XY$^{p}$ M | ?46,XY DSD | SRD5A2 | c.698+1G>T | c.698+1G>T | Consistent with diagnosis of 46,XY due to SRD5A2 deficiency. |
| **7** | XY$^{p}$ M | X-linked hypospadias | MAMLD1 | c.1366C>T p.(Arg456*) | | Consistent with MAMLD1 associated hypospadias. Confirmed in 2 affected brothers and mother (carrier). Carrier of PMDS (c.35T>G p.(Val12Gly) in AMH). |
| **8** | XY$^{p}$ M | ?46,XY DSD | AR | c.2391G>A p.(Trp797*) | | Mosaic (70% of reads). Likely causally related to clinical features. |
| **9** | XY F | ?XY DSD | NR5A1 | c.69 C>A p.(Tyr23*) | Normal | Consistent with diagnosis of 46,XY DSD. **Novel mutation**. |
| **10** | XY F | ?46,XY DSD | HSD17B3 | c.695C>T p.(Ser232Leu) | c.695C>T p.(Ser232Leu) | Confirms diagnosis 46,XY DSD due to HSD17B3 deficiency. |
| **11** | XY$^{p}$ M | ?PMDS | AMHR2 | c.813_817delGCTCT, p.(Leu272Trpfs*24) | c.931G>A, p.(Gly311Ser) | Consistent with features of PMDS. **Novel mutation and novel variant.** |
| **12** | XY F | ?46,XY DSD | SRD5A2 | c.737G>A, p.(Arg246Gln) | c.737G>A, p.(Arg246Gln) | Consistent with diagnosis of SRD5A2 deficiency. |
| **13** | XY$^{p}$ M | Penoscrotal hypospadias | SRD5A2 | c.586G>A, p.(Gly196Ser) | c.586G>A, p.(Gly196Ser) | Consistent with diagnosis of SRD5A2 deficiency assuming XY. |
| **14** | XY M | Gynaecomastia, Hypospadias, micropenis | AR | c.2057T>C p.(Val686Ala) | | Consistent with clinical features. Confirmed inherited from mother. |
| **15** | XY M | Ambiguous genitalia | HSD17B3 | c.277+4A>T | c.133C>T p.(Arg45Trp) | Consistent with clinical features. c.13C>T p.(Arg45Trp) is **novel variant.** |
| **16** | XY F | ?AIS | AR | c.2343G>A p.(Met 781Ile) | | Consistent with diagnosis of AIS. |
| **17** | XY M | ?PMDS | AMHR2 | c.289C>T p.(Arg97*) | c.289C>T p.(Arg97*) | Confirms diagnosis of PMDS. Both parents carriers. Also had another child affected child who was homozygous for the same mutation (detected in neonatal period). |
| **18** | XY M | ?XY DSD | HSD3B2 | c.518T>G p.(Leu173Arg) | c.518T>G p.(Leu173Arg) | Confirms diagnosis of CAH due to HSD3B2 deficiency. Both parents are carriers. Patient also a carrier of the c.964-1G>C splice mutation in DHCR7. |
| **19** | XY$^{p}$ F | ?46, XY DSD | HSD17B3 | c.277+4A>T | c.645A>T p.(Glu215Asp) | Confirmed diagnosis of 46, XY DSD due to 17-Beta Hydroxysteroid dehydrogenase deficiency. Each parent carries 1 mutation. |
| **20** | XY M | Undervirilised male | SRD5A2 | c.307C>T p.(Arg103*) | c.107A>G, p.(His36Arg) | Consistent with clinical features. Parental samples confirmed compound heterozygous. Follow up biochemical testing confirmed SRD5A2 deficiency. **Novel variant**. |
| **21** | XY F | ?XY DSD | AR | c.2407dupC p.(Gln803Profs*27) | | Confirms diagnosis of AIS. Two affected siblings also have mutation. **Novel mutation.** Also heterozygous for HSD17B3 familial mutation. **c**.803G>A p.(Cys268Tyr). |
| **22** | XY M | Ambiguous genitalia | WT1 | c.1087A>T p.(Arg363*) | Normal | May be contributing to features. Confirmed de-novo. Tumour screening initiated. |
| **23** | XY M | Severe hypospadias | AR | c.2384T>A p.(Phe795Tyr) | | Initially reported as VOUS. Once identified in affected (milder) brother who's karyotype was 47,XXY more confident that linked to features. **Novel variant.** |
| **24** | XY M | Severe hypospadias and penile transposition | AR | c.2645T>C p.(Leu882Pro) | | Mosaic (30% of reads). Likely causally related to phenotype. |
| **25** | XY M | ?PMDS | AMH | c.649C>T p.(Gln217*) | c.649C>T p.(Gln217*) | Consistent with diagnosis of PMDS. **Novel mutation**. |

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

**Table 3: Patient details with a confirmed molecular diagnosis**

Details of mutations and variants found in patient reported with a confirmed molecular diagnosis. Details of the karyotypic and phenotypic sex are in the second column with M and F representing phenotypic sex. P indicates presumed karyotype (reports not seen) from SRY sequence reads. Pathogenic mutations linked to the diagnoses listed in “gene column”. Allele 1 and 2 describe the mutations in the different alleles, a black box indicates an absent second X allele in XY individuals. Green = Normal (wildtype), Yellow = Class 3 variant (Variant Of Uncertain Significance (VOUS)), Orange = Class 4 variant (Likely pathogenic), Red = Class 5 variant (Clearly pathogenic). AIS = Androgen Insensitivity Syndrome, CAH = Congenital Adrenal Hyperplasia, PAIS = Partial Androgen Insensitivity Syndrome, PMDS = Persistent Mullerian Duct Syndrome.

| Patient | Sex | Reason for referral | Gene | Allele 1 | Allele 2 | Results reported and follow up |
|---|---|---|---|---|---|---|
| 26 | XY ? | N/A | POR | c.948-30G>A | Normal | A molecular diagnosis has not been confirmed |
| 27 | XY$^{p}$ M | 1$^{o}$ gonadal failure, short stature | WT1 | c.11C>G, p.(Pro4Arg) | Normal | A molecular diagnosis has not been confirmed |
| 28 | XY M | Mullerian resistant disorder | HSD17B3 | c.133C>T p.(Arg45Trp) | Normal | A molecular diagnosis has not been confirmed |
| 29 | XY M | ?46,XY DSD | RSPO1 | c.658C>T p.(Arg220Trp) | Normal | A molecular diagnosis has not been confirmed |
| 30 | XY M | Hypogonadism | HSD3B2 | c.809T>C p.(Ile270Thr) | Normal | A molecular diagnosis has not been confirmed |
| 31 | XY F | Facial dysmorphism, gastric motility issues, undescended testes, adrenal insufficiency | CBX2 | c.1411C>G p.(Pro471Ala) | Normal | A molecular diagnosis has not been confirmed. #Variant found in alternative transcript. |
| | | | CBX2# | c.616C>T p.(Gln206*) | Normal | |
| 32 | XX M | Hypospadias | NR5A1 | c.275G>A p.(Arg92Gln) | Normal | A molecular diagnosis has not been confirmed |
| 33 | XY F | ? Gonadal dysgenesis | CYP11A1 | c.1250T>G p.(Val417Gly) | Normal | A molecular diagnosis has not been confirmed |
| 34 | XY F | ? CYP11A1 imbalance | CYP11A1 | c.989C>T p.(Thr330Met) | Normal | A molecular diagnosis has not been confirmed |
| | | | MAMLDI | c.2009C>T p.(Thr670Ile) | | |
| 35 | XY F | Primary ovarian failure | WT1 | c.1493A>G p.(Glu498Gly) | Normal | A molecular diagnosis has not been confirmed |
| 36 | XY M | Severe penoscrotal hypospadias. | CBX2 | c.1416C>G p.(Asp472Glu) | Normal | A molecular diagnosis has not been confirmed |
| | | | HSD3B2 | c.500C>T p.(Ala167Val) | c.500C>T p.(Ala167Val) | |
| 37 | XY F | Tall stature, uterus present, no obvious ovaries | CBX2 | c.1411C>G p.Pro471Ala | Normal | A molecular diagnosis has not been confirmed |
| | | | AMH | c.53C>T p.(Ala18Val) | Normal | |
| | | | AMH | c.1556C>T p.(Ala519Val) | Normal | |
| 38 | XX F | Premature ovarian failure | CYP11B1 | c.1451T>A p.(Val484Asp) | Normal | A molecular diagnosis has not been confirmed |
| 39 | XY F | Clitoromegaly, no vaginal opening | AR | c.1174C>T p.(Pro392Ser) | | Pathogenicity of variant uncertain due to conflicting evidence |
| 40 | XY M | Penoscrotal hypospadias, micropenis & undescended testes | CBX2 | c.785G>A p.(Arg262Gln) | Normal | A molecular diagnosis has not been confirmed |
| 41 | XY M | Hypospadias and penoscrotal transposition | CYP11A1 | c.940G>A p.(Glu314Lys) | Normal | A molecular diagnosis has not been confirmed |
| | | | HSD17B3 | c.133C>T p.(Arg45Trp) | c.133C>T p.(Arg45Trp) | |
| 42 | XY M | ?46,XY DSD | LHCGR | c.828delC p.(Ser277Alafs*32) | Normal | A molecular diagnosis has not been confirmed. Variant likely to be pathogenic but absence of second mutation leads to uncertain significance. |
| | | | CBX2 | c.785G>A p.(Arg262Gln) | Normal | |
| 43 | XY M | Ambiguous genitalia | NR5A1 | c.146G>A p.(Cys49Tyr) | Normal | A molecular diagnosis has not been confirmed. De novo variant. |
| 44 | XY$^{p}$ F | ?46,XY DSD | NR5A1 | c.1019C>T p.(Ala340Val) | Normal | A molecular diagnosis has not been confirmed. Maternally inherited. |
| 45 | XY M | Ambiguous genitalia | HSD17B3 | c.202-22G>A | c.202-22G>A | A molecular diagnosis has not been confirmed. Recommend biochemical testing. |
| 46 | XX F | Ambiguous genitalia, complete labial fusion | LHCGR | c.458+3A>G | Normal | A molecular diagnosis has not been confirmed |
| | | | NR5A1 | c.486C>T p.(=) | Normal | |

**Table 4a: Patients with Variants of Uncertain Significance (VOUS) where a diagnosis was not confirmed**

Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

| Patient | Sex | Reason for referral | Gene | Allele 1 | Allele 2 | Results reported and follow up |
|---|---|---|---|---|---|---|
| 3 | XY F | ?46,XY DSD | ATRX | c.2595C>G p.(His865Gln) | | ATRX & AMH variants found in in addition to NR5A1 class 4 mutation (table 3). |
| | | | AMH | c.-2C>T | Normal | |
| 4 | XY F | ? 46,XY DSD | MAMLD1 | c.2744A>C p.(Asp915Ala) | | MAMLD1 variant found in addition to HSD17B3 mutations (table 3) |
| 7 | XY M | X-linkedhypospadias | AMH | c.35T>G p.(Val12Gly) | Normal | AMH & CBX2 variants found in addition to MAMLD1 class 4 mutation (table 3) |
| | | | AMH | c.-74C>G | Normal | |
| | | | CBX2 | c.565G>A p.(Ala189Thr) | Normal | |
| 19 | XY F | ?46,XY DSD | MAMLD1 | c.728G>A p.(Cys243Tyr | Normal | MAMLD1 variant found in addition to HSD17B3 mutations (table 3) |
| 22 | XY M | Ambiguous genitalia | ATRX | c.546A>G p.(=) | Normal | ATRX variant found in addition to WT1 mutation (table 3) |

**Table 4b: Patients with variants of Uncertain Significance where a diagnosis has been confirmed.**

**Table 4: Details of Variants of Uncertain Significance (VOUS) identified.**

Details of all VOUS found in this study. Table 4a indicates patients where only VOUS were found i.e. no pathogenic or likely pathogenic variants were identified. As such a diagnosis could not be confirmed in these patients. Table 4b indicates patients where VOUS were found in addition to the pathogenic/likely pathogenic mutations which were believed to be causative of the patients phenotype. Details of the karyotypic and phenotypic sex are in the second column with M and F representing phenotypic sex. P indicates presumed karyotype (reports not seen) from SRY sequence reads. Allele 1 and 2 describe the variants in the different alleles, a black box indicates an absent second X allele in XY individuals. Green = Normal (wildtype), Yellow = Class 3 variant (Variant Of Uncertain Significance (VOUS)), Red = Class 5 variant (Clearly pathogenic).

**Table 5: Summary of the frequency of mutations and variants of uncertain significance found for each gene.**

The total number of pathogenic mutations (class 4 and 5 variants) and Variants of Uncertain Significance (VOUS, class 3) variants identified in the patients tested. Each mutated allele is given a score of 1 therefore a patient homozygous for a pathogenic mutation (score=2) would be equally represented in the table as a patient who is compound heterozygous for 2 pathogenic mutations.

| Gene | Pathogenic (Class 4 or 5) | VOUS Class (3) | Gene | Pathogenic (Class 4 or 5) | VOUS |
|---|---|---|---|---|---|
| AMH | 3 | 3 | HSD3B2 | 2 | 3 |
| AMHR2 | 3 | 1 | LHCGR | 1 | 1 |
| AR | 7 | 1 | MAMLD1 | 1 | 1 |
| ARX | 0 | 0 | NR0B1 | 0 | 0 |
| ATRX | 0 | 0 | NR3C1 | 0 | 0 |
| CBX2 | 0 | 6 | NR5A1 | 2 | 4 |
| CYB5A | 0 | 0 | POR | 0 | 1 |
| CYP11A1 | 0 | 3 | RSPO1 | 0 | 1 |
| CYP11B1 | 0 | 1 | SOX9 | 0 | 0 |
| CYP17A1 | 0 | 0 | SRD5A2 | 7 | 1 |
| CYP19A1 | 0 | 0 | SRY | 0 | 0 |
| DHCR7 | 1 | 0 | STAR | 0 | 0 |
| DHH | 0 | 0 | TSPYL1 | 0 | 0 |
| DMRT1 | 0 | 0 | WNT4 | 0 | 0 |
| HSD17B3 | 9 | 6 | WT1 | 1 | 2 |

 Accepted Manuscript published as EC-18-0376.R1. Accepted for publication: 16-Jan-2019

| Paper | Number of genes in panel | Number of patients tested and karyotypic sex | Diagnostic Yield Overall | Diagnostic Yield 46,XY | Diagnostic Yield 46,XX |
|---|---|---|---|---|---|
| **Baxter, 2015 (ref 9)** | 64 | 40 XY | - | 14/40 | - |
| **Dong, 2016 (ref 10)** | 219 | 13 XY, 8 XX | 8/21* | 6/13 | 2/8 |
| **Eggers, 2016 (ref 11)** | 64 | 278 XY, 48 XX | 126/326 | 118/278 | 8/48 |
| **Kim, 2017 (ref 12)** | 67 | 37 XY, 7 XX | 13/44 | 13/37 | 0/7 |
| **Hughes, 2018** | 30 | 73 XY, 7 XX | 25/80 | 25/73 | 0/7 |

**Table 6: Summary of previous studies using NGS analysis for DSDs**

*Dong et al report their detection rate of 46,XY DSD as 9/13 however 3 of these patients had Variants of Uncertain Significance (VOUS) only and therefore using the same parameters as in this study a diagnosis would not be confirmed. Numbers modified above to allow more accurate comparison with this study.