

CLINICAL REPORT

Whole genome sequencing identifies a cryptic *SOX9* regulatory element duplication underlying a case of 46,XX ovotesticular difference of sexual development

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Email: pedro.sanchez@cshs.org**Abstract**

Ovotesticular differences of sexual development (OT-DSD) are rare genetic variances defined by the coexistence of both testicular and ovarian tissues. Various molecular etiologies including *SRY* translocation or *SOX9* pathogenic variants with different modes of inheritance have been associated with 46,XX OT-DSD. Here we describe a child diagnosed with *SRY*-negative 46,XX OT-DSD after completing a series of complex clinical genetic analyses, including chromosomal microarray, DSD gene panel (sequencing and deletion/duplication analysis), whole exome sequencing, and whole genome sequencing. Of these, only whole genome sequencing reported a pathogenic duplication in a non-coding region that contains the RevSex regulatory element, which modifies *SOX9* expression and is associated with 46,XX OT-DSD and complete sex reversal. This is the first clinical RevSex duplication detected by clinical whole genome sequencing. We highlight the utility of whole genome sequencing in shortening the diagnostic odyssey and the importance of optimal counseling through a team-based multi-specialty approach for patients with DSDs.

KEYWORDSdifference of sexual development, OT-DSD, ovotesticular DSD, *SOX9* duplication, whole genome sequencing

1 | INTRODUCTION

Ovotesticular differences of sexual development (OT-DSD) are defined by the coexistence of both testicular and ovarian tissues. OT-DSD accounts for about 4% of all intersex variances and affects about 1 in 100,000 newborns (Nistal et al., 2015). The 46,XX karyotype is the most common subtype in the United States and around the world,

presenting in 70% of OT-DSD patients (Krob et al., 1994). A translocation of the Sex-determining Region Y (*SRY*) gene onto one of the X chromosomes is the leading cause found in 80% of individuals (Ono & Harley, 2013). The *SOX9* gene on chromosome 17 encodes a protein which functions downstream of *SRY* in typical male sexual development. Its pathogenic variants are also known to cause several forms of OT-DSD. The 46,XX OT-DSD has variable expressivity, ranging from a phenotypical male to one with ambiguous genitalia (sometimes referred to as androgynous or atypical) (Note for editorial, “androgynous” is a term used by a growing minority to replace “ambiguous,” as

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ambiguous suggests the morphology is “undefined” and patiently requires assignment and repair to become non-ambiguous—versus a choice or decision of the family or patient. We chose the descriptor, atypical or androgynous, because atypical genitals represent a defined natural variation that can present among those who are of intermediate sex or intersex [do not fit the male and female binary]). At birth, our proband was noted to have palpable gonads within both rugated labia but was otherwise healthy without concerns. With the advances in technology, an increasing number of prenatal and postnatal genetic analyses have been incorporated into family planning and genetic counseling. However, interpreting such results in the context of the mercurial nature of OT-DSD can be challenging. Here we present a child with a 46,XX OT-DSD duplication of the *SOX9* upstream regulatory sequence RevSex, identified by clinical whole genome sequencing. We highlight the numerous and complex genetic tests the patient underwent in a clinical setting, showcasing the potential benefits from early whole genome sequencing.

2 | CLINICAL REPORT

The child or proband was conceived naturally to a G2P2002 mother. It was an uneventful pregnancy and he was born full-term via an uncomplicated spontaneous vaginal delivery. In the pregnancy involving our proband, NIPT for common aneuploidy was completed but testing for fetal chromosomal sex was not pursued.

At the birth of our proband, he was noted to have atypical genitalia but was otherwise healthy without concerns. His phallus had a perineal meatus, severe ventral chordee, and dorsal hooded prepuce. The scrotum was bifid with both testes palpable within. Abdominal ultrasound showed no Mullerian or adnexal structures. Initial lab evaluation showed low total testosterone (113 ng/dl), low anti-mullerian hormone (19.47 ng/ml), and follicle-stimulating hormone (4.09 mIU/ml). These results fall within the typical range when ovarian tissue is present and suggest the diagnosis of an OT-DSD. Karyotyping and fluorescence in situ hybridization analysis revealed a 46,XX

karyotype with absent *SRY*. A multidisciplinary discussion of care involving endocrine, urology, and medical genetics was initiated. After consultation with family members, the parents decided to raise the child to support a boy gender and pursue treatment to increase phallus size with testosterone cypionate, 25 mg intramuscular monthly for 3 months, followed by a multi-staged reconstruction surgery of genitalia. During the first stage of reconstruction, gonadal biopsy was performed and identified the presence of both ovarian and testicular tissue, confirming 46,XX OT-DSD.

Subsequent genetic analysis consisted of a Single Nucleotide Polymorphism (SNP) Chromosomal Microarray followed by targeted next-generation sequencing and deletion/duplication panel analysis of the coding regions of multiple genes of sexual development (*ANOS1*, *AR*, *ATRX*, *CHD7*, *DHCR7*, *DHH*, *FGFR1*, *HESX1*, *MAP3K1*, *NROB1*, *NR5A1*, *SOX9*, *SRD5A2*, *SRY*, and *WT1*). Clinical reports for both did not reveal a genetic cause for disorder. Clinical exome trio sequencing was then pursued and did not identify any clinically significant variants. After extensive discussion with the family, whole genome sequencing (40X coverage) was performed. A 281 kilobase (kb) duplication of chromosome 17q24.3 was identified in the proband (Figure 1) and was located 411 to 692 kb upstream of the *SOX9* gene (chr17:69,424,545-69,705,568; NCBI build hg19). Genome sequencing at 40X coverage allowed for nucleotide-level resolution of breakpoints. Analysis of sequencing reads at both ends of the variant demonstrated that the duplication was in tandem with a head-to-tail orientation (Figure 2). Although no genes were present within this region, the duplication involved a known regulatory element named RevSex that modulates *SOX9* expression during development. Similar duplications encompassing the RevSex region have been previously reported in 46,XX OT-DSD individuals (Figure 3) (Benko et al., 2011; Cox et al., 2011; Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, Van Den Bergen, & Sreenivasan, 2018; Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, van den Bergen, Sreenivasan, Knarston, et al., 2018; Croft, Ohnesorg, & Sinclair, 2018; Hyon et al., 2015; Kim et al., 2015; Ohnesorg et al., 2017; Tan et al., 2020; Xiao et al., 2013a; Xiao et al., 2013b). A re-analysis of the

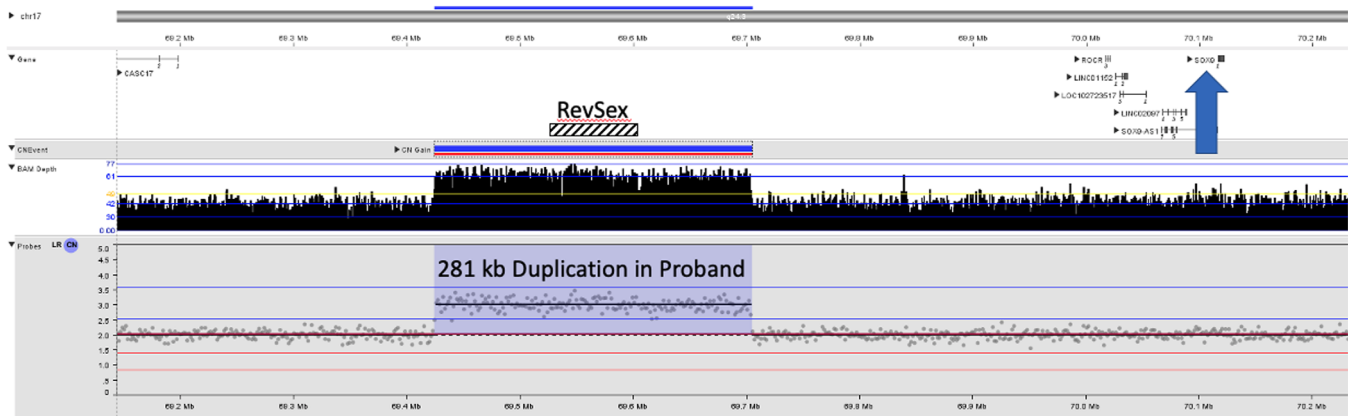


FIGURE 1 A 281 kilobase (kb) pathogenic copy number gain was detected at chromosome band 17q24.3, located 411 to 692 kb upstream of the *SOX9* gene (arrow). The duplication encompasses the ~78 kb RevSex region located approximately 517–595 kb upstream of *SOX9* (striped bar)

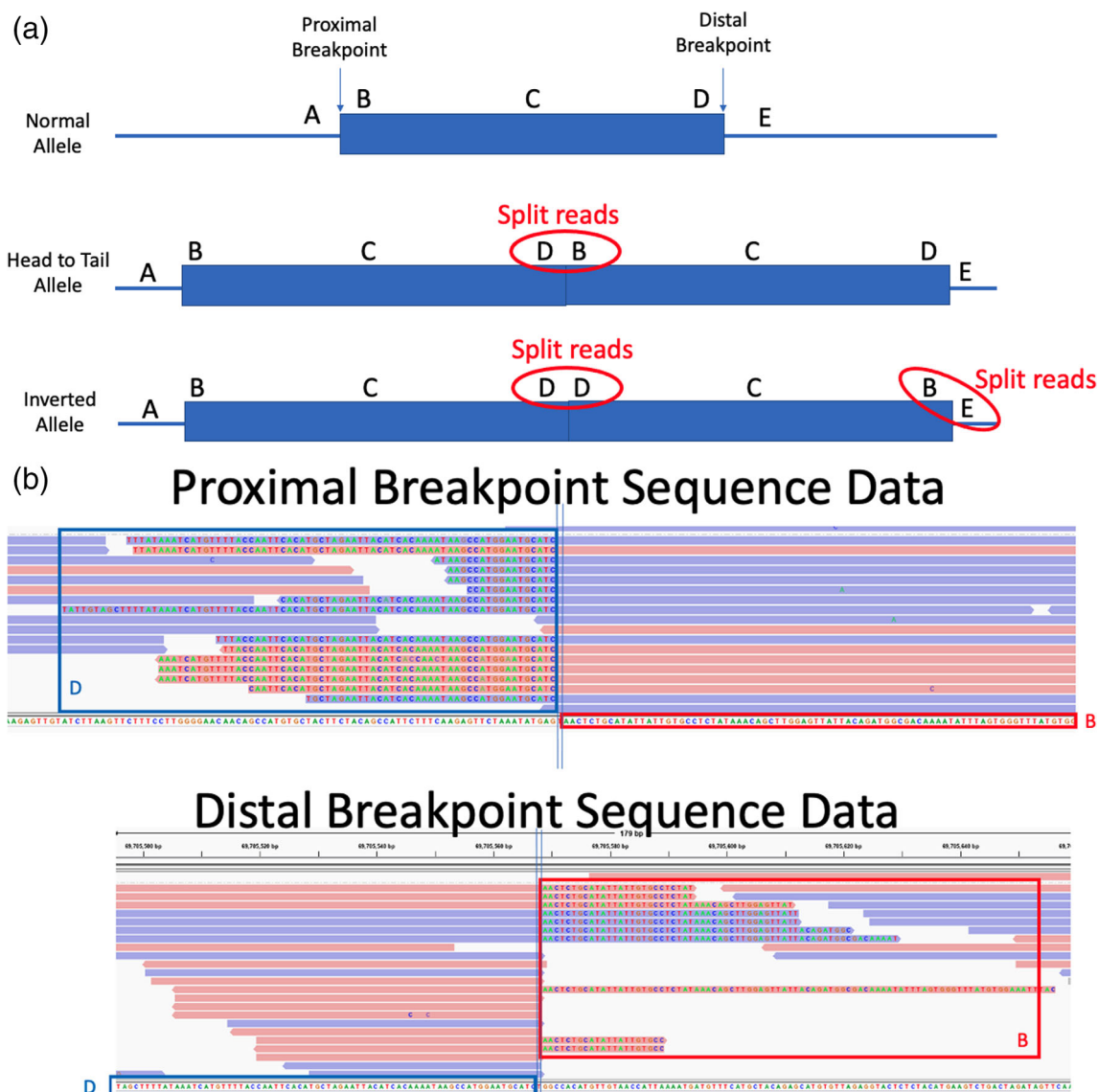


FIGURE 2 (a) Schematic of a normal allele and corresponding duplications (blue box) located in tandem and oriented either head-to-tail or inverted. Letters A-E represent NGS reads at locations within the duplication or at the breakpoints. Split reads containing sequence from D and B regions would indicate a tandem duplication-oriented head-to-tail. Split reads containing sequence from B and E regions or inverted D regions would indicate a tandem-inverted allele. (b) Representative sequencing data identifying D-B split reads from at the proximal breakpoint (~chr17:69,424,458–69,424,648) and distal breakpoint (~chr17:69,705,484–69,705,624) of the RevSex duplication. At both proximal and distal breakpoints, split reads corresponding to the D region (blue box) and B region (red box) are detected

proband's original chromosomal SNP microarray data was requested. The commercial laboratory confirmed the presence of the duplication that was initially filtered out of the analysis due to the size threshold and absence of coding genes; a new amended clinical report was subsequently issued. Of note, all genetic/genomic tests were completed on a clinical basis in laboratories within the United States that were CAP certified and CLIA approved.

3 | DISCUSSION

In 46,XX OT-DSD, multiple genes have been shown as contributors with distinct mechanisms leading to a broad spectrum of clinical

presentation. SRY translocation from Y to X chromosome and aberrations of downstream SOX9 are the most common causes (Ono & Harley, 2013). Emerging data also showed pathogenic variants of key genes involved in physiological sexual development such as NR5A, WNT4, SOX3, SOX5, RSP01, or WT1 can also lead to 46,XX OT-DSD via either promoting testiculogenesis or inhibiting oogenesis (Domenice et al., 2016; Jurayyan & Nasir, 2011; Tan et al., 2020).

Most relevant to our patient is the SOX9 protein. Activated downstream of SRY, SOX9 is a key player in the testiculogenesis pathway. Understanding the regulation of SOX9 expression is still a work in progress. TESCO, also known as the core sequence of Testis Specific Enhancer of SOX9, was the first SOX9 enhancer identified in the literature, but its deletion was not adequate to cause intersex variance

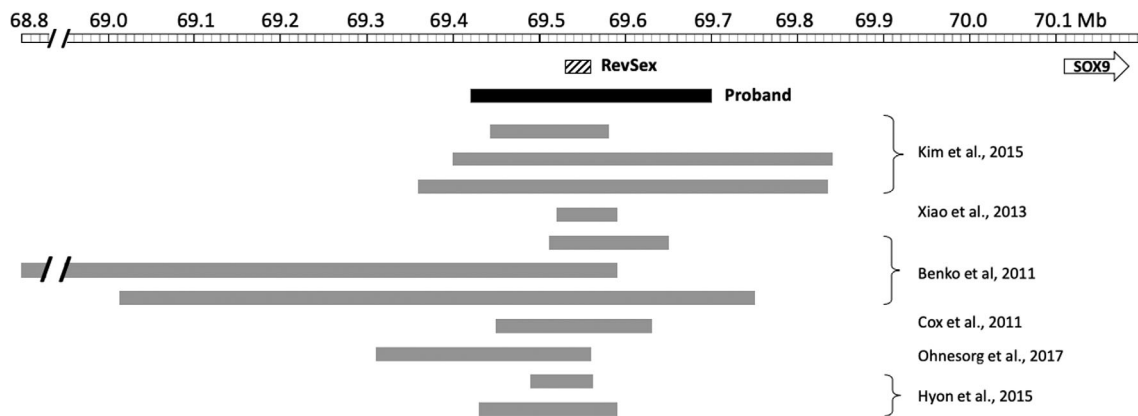


FIGURE 3 Schematic of the proband's duplication (black bar) in relation to previously published duplications (gray bars) involving the RevSex regulatory element (cross-hatched), upstream of the *SOX9* gene (arrow), in patients with 46,XX DSD

(Croft, Ohnesorg, & Sinclair, 2018; Gonen et al., 2017). In the past decade, more transcription regulators of *SOX9* have been identified. *Enh13* deletion and CNVs of *XYSR* were shown to cause XY sex reversal due to reduced *SOX9* expression (Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, van den Bergen, Sreenivasan, Knarston, et al., 2018; Gonen et al., 2018). An additional gene named *PPP2R3C* encodes the B gamma regulatory subunit of *PP2A*, which plays a crucial role in the phosphorylation-activation of *SOX9*. *PPP2R3C* functions as a downstream regulator whose deletion can also lead to XY sex reversal (Guran et al., 2019). Another *SOX9* enhancer named *eALDI* was discovered recently but its deletion has not been associated with any form of DSD yet (Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, van den Bergen, Sreenivasan, Knarston, et al., 2018). Additionally, CNVs of a *SOX9* enhancer named *RevSex* has been shown to result in XX sex reversal similar to our patient. The first case report of 46,XX OT-DSD caused by duplication of *RevSex* reported that family members of their proband, a brother and paternal uncle, were all karyotypically 46,XX but phenotypically male and infertile. The father who carried the same variant was unaffected (Cox et al., 2011). This could be explained by the current model that 46,XY males already express higher levels of *SOX9* during gonadogenesis, and further amplification of *SOX9* expression does not deter from a pattern of typical male sexual development (Benko et al., 2011). In the last decade, multiple unrelated individuals with 46,XX OT-DSD caused by CNVs of *RevSex* have been reported with the spectrum of typical and atypical genitalia (Benko et al., 2011; Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, van den Bergen, Sreenivasan, Knarston, et al., 2018; Kim et al., 2015). In our patient, the *RevSex* duplication resulted in androgynous genitalia, further contributing to the spectrum of variable presentation of this rare mutation. This phenomenon suggests that other genetic or non-genetic factors may contribute to the spatiotemporal expression of *SOX9* during development, leading to variable clinical outcomes.

Previous reported patients with a *RevSex* duplication were detected by comparative genomic hybridization microarray (Croft, Ohnesorg, Hewitt, Bowles, Quinn, Tan, Corbin, Pelosi, van den Bergen, Sreenivasan, Knarston, et al., 2018; Kim et al., 2015; Tan

et al., 2020; Xiao et al., 2013a). Here we report the first patient with a *RevSex* duplication detected via whole genome sequencing. Because the duplication was located within non-coding regions, phenotype-driven genome sequencing was the key assay for detection of the pathogenic variant underlying our patients atypical phenotype. In next-generation sequencing (NGS), gene panels and exome sequencing assays, only the coding regions of the genome are analyzed; pathologic variants within non-coding regions of the genome are missed. Although microarrays are capable of detecting copy number variants (CNVs), if CNVs are smaller than the laboratory's reporting threshold or do not contain clinically relevant genes, they may be filtered out during analysis, as happened in our patient. In contrast, genome sequencing at 40X coverage was able to detect the duplication with nucleotide-level breakpoint resolution. At this coverage, the location and orientation of the duplication was also determined, which cannot be performed via microarrays.

In the last few years, CNV analysis based on NGS has been increasingly utilized in clinical testing through genome, exome, or gene panel sequencing (Kerkhof et al., 2017; Riggs et al., 2020; Schenkel et al., 2016; Yu et al., 2021). Deep-coverage genome sequencing may be more comprehensive than a combined chromosomal microarray and exome sequencing strategy, as it allows for concurrent detection of CNVs and sequence variants in both coding and non-coding regions within the same assay. Future reanalysis of genome data to investigate newly discovered genes and regulatory elements is also possible without need for resampling. In this capacity for a patient such as ours, genome sequencing may reduce the time to reach a definitive diagnosis, as well as potentially decrease the financial cost and/or emotional stress associated with the diagnostic odyssey for patients with complex genetic variances that underpin the sensitive topic of sex development. Having the diagnostic tools at our disposal to determine the root cause of an intersex variance could pave the way toward understanding that sex development is a dynamic process that results in the birth of children who do not always fit the typical male and female paradigm.

Despite its accuracy, noninvasive prenatal testing (NIPT) is of limited utility in the clinical decision-making of 46,XX OT-DSD. In NIPT,

cell-free fetal DNA in maternal serum is analyzed to detect fetal chromosomal aberrancies while fetal ultrasound is utilized to look for anatomical abnormalities. The reported sensitivity and specificity in determining fetal genetic sex are both close to 100%, while lower values were reported when NIPT was used to detect aneuploidies (Wright et al., 2012). Ultrasonographic determination in the second trimester is also reported to have an accuracy of around 100% after 12 weeks of gestation (Colmant et al., 2013). Although incongruence between findings on NIPT and prenatal ultrasound may clue clinicians into someone with a potential DSD, it showed limited use in affecting clinical decision prenatally when the underlying genetic cause is unclear. Even when a DSD is suspected based on NIPT findings, medical or surgical managements can only be pursued after birth.

The core of postnatal care of those with 46,XX OT-DSD centers on gender assignment and corresponding therapies. The gender identity of an individual is psychosocially influenced by self-perception and can be different from the biological sex assigned at birth (Westbrook & Schilt, 2014). Given the controversial data and diverse phenotypic variations of 46,XX DSD, a one-size-fits-all solution does not exist (Lee et al., 2006; Lee et al., 2016). Current recommendation by the Global Consortium of DSD emphasize the need for an individualized and multidisciplinary team-based approach of care with parent involvement in decision making (Lee et al., 2016). Gender assignment of individuals with DSD is typically conformed to the genital appearance of a typical male or female. Around 50% of patients with 46,XX OT-DSD were assigned to a gender that was perceived to be more closely aligned with the their sex assigned at birth, including our patient, and underwent reconstructive procedures (Krob et al., 1994). Long-term data on outcomes are limited in the literature, but several advocacy groups argue for delaying reconstructive surgeries that are solely done for cosmetic purposes until the individual born with the variance is of an age to participate in the decision-making process where they may decide not to undergo reconstructive surgery to create typical genitalia for themselves (Crawford et al., 2009; Wiersma, 2011). More research is warranted to better understand the best management and follow-up care for patients with 46, XX OT-DSD, especially in light of the fact that complications can potentially lead to irreversible impairments to the individual's reproductive functions (DE Mouriquand et al., 2016).

In the case where a boy/man gender is pursued, the reconstructive surgery typically focuses on repairing the hypospadias and are usually multistage. Additional revisions due to complications such as erectile dysfunction, urethral strictures, urethral cutaneous fistulas, recurrent chordee, meatal stenosis, or cosmetic concerns, are often needed (Renaux-Petel et al., 2019). Internal gonads and structures that are atypical for the assigned sex are generally removed. The risk of gonadal tumor in intersex patients varies across studies and subtypes. Cools et al. reported the overall prevalence of germ cell tumors in various patient series with DSDs to be estimated at 12% (97 of 817) (Cools et al., 2006; Looijenga et al., 2019; Morin et al., 2020; Slowikowska-Hilczler et al., 2015). Therefore, gender-typical gonads are usually left in place and monitored. Androgen therapy is often recommended for treatment of genitalia that presents with a smaller-

than-average phallus in order to conform with typical male genitals. (Lee et al., 2006) Cases of gender dysphoria in either direction of gender reassignment have been described. A variety of factors such as internal distress toward the condition itself makes quantifying the psychosocial effects of medical or surgical management particularly challenging (Lee et al., 2016). Therefore, care should be tailored to the unique biopsychosocial profile of each individual patient. Employing a multidisciplinary approach involving multiple specialties while encouraging active participation in treatment planning with parents will hopefully help our patient and others with similar variances to optimize their care.

In conclusion, we report a patient with 46,XX OT-DSD caused by the duplication of RevSex, a regulatory element of SOX9. This is the first clinical report of a patient with 46,XX OT-DSD due to RevSex duplication identified by clinical whole genome sequencing. This technology not only identified the duplication, but also confirmed the location and directionality of the duplicated sequence. Due to the ability of clinical genome sequencing to detect a wide range of sequence and copy number variants in coding and non-coding regions, genome sequencing should be considered as an alternative to exome sequencing or microarray for patients with DSDs to arrive at a quicker diagnosis. With a growing level of support and interest in incorporating molecular testing into clinical practice, we encourage the expansion of efforts to increase and improve medical education and genetic/genomic literacy (Sanchez-Lara et al., 2021). This should include not only strengths and limitations of tests but also an awareness of the potential level of inter- and intralaboratory variability when it comes to sample and data quality control, analytic pipelines, as well as laboratory-specific interpretation and reporting practices. We strongly recommend that clinicians carefully choose their preferred laboratory testing partners and provide them with immediate feedback when discordant results are discovered. Although our own practice is now incorporating rapid clinical genome sequencing to shorten the diagnostic odyssey in select patients, we continue to recommend an individualized approach using all clinical testing modalities and information available. An ongoing multidisciplinary approach involving the patient, the parents and experts from multiple specialties is ideal to customize counseling, gender assignment, and treatment strategies for the patient.

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CONFLICT OF INTEREST

Andrea Behlmann is a clinical laboratory director employed by PerkinElmer Genomics. The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

The contributions of each of the authors is as listed: Conceptualization Bahareh M. Schweiger, Andrew Freedman and Pedro A.

Sanchez-Lara; Patient recruitment and clinical assessment, Pedro A. Sanchez-Lara, Katheryn Grand, Bahareh M. Schweiger, Andrea Behlmann and Andrew Freedman; Writing—Original Draft Preparation, Zhiyu Qian; Writing—Review and Editing, Zhiyu Qian, Pedro A. Sanchez-Lara, Katheryn Grand, Maria C. Nieto, Andrea Behlmann, Bahareh M. Schweiger, Andrew Freedman. Supervision, Pedro A. Sanchez-Lara. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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