The evolving role of whole-exome sequencing in the management of disorders of sex development

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RESEARCH

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Abstract

Objective: Disorders of sex development (DSD) are defined as congenital conditions in which the development of chromosomal, gonadal and anatomical sex is atypical. Despite wide laboratory and imaging investigations, the etiology of DSD is unknown in over 50% of patients.

Methods: We evaluated the etiology of DSD by whole-exome sequencing (WES) at a mean age of 10 years in nine patients for whom extensive evaluation, including hormonal, imaging and candidate gene approaches, had not identified an etiology.

Results: The eight 46,XY patients presented with micropenis, cryptorchidism and hypospadias at birth and the 46,XX patient presented with labia majora fusion. In seven patients (78%), pathogenic variants were identified for *RXFP2, HSD17B3, WT1, BMP4, POR, CHD7* and *SIN3A.* In two atients, no causative variants were found. Mutations in three genes were reported previously with different phenotypes: an 11-year-old boy with a novel *de novo* variant in *BMP4*; such variants are mainly associated with microphthalmia and in few cases with external genitalia anomalies in males, supporting the role of BMP4 in the development of male external genitalia; a 12-year-old boy with a known pathogenic variant in *RXFP2*, encoding insulin-like 3 hormone receptor, and previously reported in adult men with cryptorchidism; an 8-year-old boy with syndromic DSD had a *de novo* deletion in *SIN3A*.

Conclusions: Our findings of molecular etiologies for DSD in 78% of our patients indicate a major role for WES in early DSD diagnosis and management – and highlights the importance of rapid molecular diagnosis in early infancy for sex of rearing decisions.

Key Words

- disorders of sex development
- whole-exome sequencing
- bone morphogenetic protein 4
- insulin-like 3 hormone receptor

Endocrine Connections (2021) **10**, 620–629

Introduction

Disorders of sex development (DSD) are classified as a congenital discrepancy between external genitalia and gonadal and chromosomal sex (1). The prevalence of DSD, including hypospadias, is estimated at 5 out of 1000 newborns (2), with 75% of affected individuals having 46,XY karyotype (3). The current classification of DSD

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includes four categories 46,XY, 46,XX, sex chromosome DSD (1) and syndromic DSD. Syndromic DSD are conditions associated with congenital malformations in addition to the atypical genitalia. These may be due to monogenic defects, biochemical abnormalities of steroid synthesis, or microdeletions, duplications or unbalanced rearrangements (4).

The investigation of infants with ambiguous genitalia is challenging because determining the molecular etiology can, in some children, be crucial for reaching sex of rearing decisions. The etiology of 46,XY DSD is unknown in more than 50% of cases, despite extensive laboratory and imaging investigations, and the diagnosis is often deferred to the second decade of life (5). The evaluation of an infant with ambiguous genitalia includes clinical examination, karyotyping, and laboratory and imaging evaluations. Until the last decade, targeted gene sequencing was used to identify the genetic etiology of patients with DSD. However, in addition to being expensive and time-consuming, in many cases, this approach failed to identify the etiology. Therefore, it is currently recommended only when clinical and hormonal assessments point to a specific gene (5). Today, high-throughput sequencing (HTS) panels of genes involved in sex determination and differentiation are available. More than 60 genes have been described in association with DSD (6, 7). The recent availability of whole-exome sequencing (WES), mainly for research purposes, has led to improved accuracy of diagnosis of DSD patients, identifying causal variants in more than 50% of cases, as well as novel genes causing DSD (8). Here, we report a cohort of nine children with DSD, in which wide laboratory, imaging, and targeted hypothesis-driven sequencing investigations failed to identify the etiology of DSD. Use of WES in a research setting identified the genetic etiologies in seven (78%) of these patients.

Materials and methods

Patients

The cohort consisted of nine patients with atypical genitalia for whom extensive laboratory, imaging, and initial genetic assessments failed to identify the etiology of DSD. Excluded from the cohort were patients with ambiguous genitalia whose genetic etiology was identified by sequencing of candidate genes. All patients were followed in our clinic every 6 months.

Biochemical analysis

Hormonal levels were obtained at referral, during follow-up and at the last visit. At diagnosis, all patients were assessed for a baseline hormone profile, and LHRH stimulation test (100 µg LHRH, with blood sampling at baseline, 30, 60, and 90 min) and ACTH stimulation test (250 µg Synacthen, with blood sampling at baseline and 60 min) were performed. hCG stimulation test (100 IU/kg Pregnyl, with blood sampling at baseline and 72 h) was performed in patients with 46,XY DSD. Repeat LHRH stimulation test was performed at the time of the study. LH, FSH, testosterone, TSH, FT_4, and cortisol were measured by direct automated chemiluminescent IRMA using the ADVIA Centaur immunoassay system (Bayer Corporation, Tarrytown, NY). 17-OHP was measured by enzyme immunoassay (IBL International GmbH, Hamburg, Germany), and androstenedione was measured by chemiluminescent enzyme immunoassay (IMMULITE 2000, Siemens, Gwynedd, UK). Urinary glucocorticoid level was determined by gas chromatography–mass spectrometry (GCMS) to exclude adrenal enzyme deficiency.

Genetic analyses

This study was approved by the Ethics Committee of Ha'Emek Medical Center and by the Genetics Committee of the Israeli Ministry of Health. Blood samples were collected after the parents signed the appropriate consent form. Genomic DNA was extracted from peripheral mononuclear cells using the Blood Amp Kit (Qiagen Inc.). Targeted gene-by-gene sequencing was performed either when a candidate gene was suspected based on the phenotype and hormonal results that indicated a specific etiology, or based on the availability of specific gene testing in the genetics laboratory. Sanger sequencing of the coding exons and untranslated regions was used to identify pathogenic variants in candidate genes *AR*, *NR5A1*, *SRD5A2*, *CHD7*, *LHR*, *WT1*, *GPR54* and *DHCR7*. The specific variant p.R80Q in the *HSD17B3* gene, to which most cases of 17βHSD deficiency are attributed in the Israeli-Arab population, was analyzed when clinical and hormonal findings suggested a deficiency of this enzyme (9).

Exon enrichment was performed using Agilent SureSelect Human All Exon V4. Paired-end sequencing was performed on the Illumina HiSeq2000 platform with an average sequencing coverage of 50× as described elsewhere (10, 11). Details of the exome sequencing procedures are listed in the supplementary materials (see section on

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supplementary materials given at the end of this article) and potentially pathogenic variants were verified by Sanger sequencing (Supplementary Data 1). The samples were analyzed as trios.

Results

Of the nine recruited patients, two were from consanguineous families (cases 2 and 5). Ultrasonography scan of the fetus during pregnancy identified a female phenotype in four out of seven patients, whereas karyotyping after birth revealed the 46,XY genotype in all of them. Median age at presentation was 21 days (range 7–455). All male genotype patients presented with severe atypical genitalia, including all or part of the following: cryptorchidism, hypospadias, small testicular volume, and bifid scrotum (Table 1). Only one patient presented with atypical female phenotype (case 5). Additional organ anomalies were found in six out of the nine patients (Table 1). Median age at last visit was 12.6 years, and pubertal stages at last visit are summarized in Table 1. ACTH stimulation test revealed normal cortisol and 17-OHP responses in all subjects apart from case 5, in whom elevated peak 17-OHP concomitant with low peak cortisol suggested the diagnosis of congenital adrenal hyperplasia (Table 2). LHRH stimulation tests performed at presentation revealed an elevated LH peak in seven patients and an elevated FSH peak in four patients. hCG stimulation test revealed a variable rise in testosterone response after 72 h, but the results were inconclusive for a specific etiology (Table 2). Urinary GCMS profile was normal in all patients except for case 5, in whom the ratio between the adrenal metabolites indicated a deficiency of oxidoreductase. Repeat LHRH stimulation tests were performed at the median age of 12 years (Table 2), revealing elevated peak LH in three patients and elevated peak FSH in four patients, consistent with the diagnosis of primary testicular failure. This wide hormonal investigation did not lead to a specific individual's etiology for DSD.

Eight patients had 46,XY karyotype, and only one had 46,XX karyotype. Targeted gene-by-gene approach and sequencing of specific candidate genes were negative for pathogenic variants in all patients. Patients underwent WES at the median age of 10 years, revealing three previously described pathogenic variants and four novel variants that constitute strong candidates for explaining the etiology of DSD (Table 3). In the other two patients, variants that could explain the phenotype were not observed. Case 1 had the previously reported variant c.664A>C, p.T222P in *LGR8*, also known as *RXFP2*, inherited from his mother (9). Case 2 had a novel homozygous autosomal recessive variant of *HSD17B3,* resulting in 17βHSD deficiency (c.673G>A, p.V225M), previously described by our group (10). Case 3 had a novel *de novo* splice-site variant of Wilms Tumor 1 gene (*WT1*), c.1433-3C>G. Case 4 had a novel *de novo* missense variant of *BMP4*, c.209G>T, p.R70L. Case 5 had the previously described homozygous variant of the cytochrome P450 oxidoreductase gene (*POR*) (11). Case 6, who had syndromic DSD, had a previously reported *de novo* autosomal dominant variant of *CHD7* causing Charge syndrome, c.1480C>T, p.R494T (12) and case 8, who also had syndromic DSD, carried a *de novo* deletion, c.2809_2810del, p.K937QfsTer2 of *SIN3A* (SIN3 transcription regulator family member A).

Detailed description of the patients

Case 1

The proband, born to unrelated healthy parents, was referred to our clinic at the age of 10 days for investigation of atypical genitalia. His karyotype was 46,XY. Hormonal analysis indicated an elevated LH peak following LHRH stimulation and normal basal and hCG-stimulated testosterone values. He underwent bilateral orchiopexy at the age of 17 months. Sequencing of four different candidate genes for pathogenic variants, and for the common pathogenic variant of *HSD17B3* in the Israeli-Arab population, p.R80Q, was negative. WES identified a missense variant of *RXFP2*, which is maternally inherited and has been previously described in association with testicular maldescent (12). At the age of 13 years, he had a pubertal stage of Tanner P3 with short penile length and testicular volume of 4 mL. Peak LH and FSH following LHRH stimulation were exaggerated, indicating primary testicular insufficiency

Case 2

The proband, female phenotype baby was referred due to palpable masses in both inguinal canals at the age of 3 months. Cleft soft palate was found in physical examination. Her karyotype was 46,XY. Laboratory evaluation revealed elevated peak LH following LHRH stimulation. A low basal testosterone: androstenedione ratio of 0.39 (normal range >0.8), suggested 17βHSD deficiency; however, sequencing of the common Israeli-Arab population variant p.R80Q, which was only available at that time, was negative for the variant. Sequencing of candidate genes, including *SRD5A2, LHR, AR* and *GPR54*,

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Table 1 Clinical characteristics of all patients.

No.	Origin	Consanguinity	Prenatal ultrasound phenotype	Age[a] (days)	Phenotype[a]	Karyotype	Age[b] (years)	Phenotype[b]			Others
								PH	Penile length (cm)	TV (mL)	
1	Arab-Muslim	No	Female	10	Bilateral UDT, micropenis, hypospadias G4	46,XY	13.5	P3	5.5	4	ADHD, learning difficulties
2	Arab-Muslim	Yes	Female	90	Labioscrotal folds, clitoromegaly, single orifice	46,XY	14.3	P3	7	05-Jun	Cleft soft palate
3	Arab-Muslim	No	NA	7	Bilateral UDT, micropenis, hypospadias G4	46,XY	13.1	P3	5	Rt. 2 Lt. NP	None
4	Arab-Muslim	No	Female	12	Rt. UDT, hypospadias G4, bifid scrotum	46,XY	12.6	P3	3.5	4	Subaortic membrane, ASD, recurrent UTI, mild sensorineural hearing impairment
5	Arab-Muslim	Yes	Female	30	Fusion of labia majora, small vaginal–urethral orifice	46,XX	11.8	P1		B1	None
6	Druze	No	Male (micropenis)	25	NP testis, micropenis, hypospadias G4	46,XY	8.4	P1	3.3	NP	Autism, mental retardation, heart anomalies, deafness, dysmorphism
7	Arab-Muslim	No	Female	14	Hypoplastic bifid scrotum, micropenis & chorde, small TV	46,XY	12.1	P2	2.5	5	None
8	Jewish-Morocco	No	Male	455	Bilateral UDT, micropenis	46,XY	8.1	P1	2.5	1	ADHD, autism, mental retardation, convulsive disorder, hydrocephalus, SOD, ASD GHD
9	Jewish-Morocco	No	Male	21	Bilateral UDT, micropenis, hypospadias G4	46,XY	13.9	P3	3	5	Learning and behavioral difficulties
	Median	2 (22%)		21 (7-455)			12.6 (8.4-14.3)				

[a]At diagnosis; [b]At last visit.
ADHD, attention deficit hyperactivity disorder; ASD, atrial septal defect; G4, grade 4; GHD, GH deficiency; NA, not available; NP, nonpalpable; PH, pubic hair (Tanner stage); SOD, septo-optic dysplasia; TV, testicular volume; UDT, undescended testis; UTI, urinary tract infection.

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was negative for pathogenic variants. WES performed at the age of 8 years revealed a novel missense variant of the *HSD17B3* gene previously reported by us (13). The parents were heterozygous for the identified mutation. At the age of 14.3 years, he had a pubertal stage of Tanner P3, G3, and elevated basal LH and FSH.

Case 3

The patient was reviewed at our institute at the age of 7 days for assessment of atypical genitalia. He had normal kidneys and absence of Mullerian duct remnant on ultrasonographic imaging. His karyotype was 46,XY. A candidate gene approach excluded pathogenic variants in *SRD5A2*, *AR* and *NR5A1*. WES performed at the age of 11 years identified a novel *de novo* splice-site variant of the *WT1* gene (c.1433-3C>G). At the age of 12.3 years, he had pubertal stage Tanner P3, G1, and elevated basal and peak LH and FSH. Annually repeated ultrasonographic imaging demonstrated normal kidneys with no abnormal findings.

Case 4

The proband was first seen in our clinic at the age of 12 days due to atypical genitalia. He was born to unrelated parents after *in vitro* fertilization twin pregnancy. Prenatal ultrasound demonstrated a female fetus. In addition, he had a subaortic membrane and atrial septal defect. No other anomalies were found. His karyotype was 46,XY. A hormonal evaluation indicated normal gonadotropin and testosterone levels. Sequencing of the *SRD5A2, AR*, and *WT1* genes did not reveal pathogenic variants. WES revealed a *de novo* missense variant of *BMP4* predicted as pathogenic. This variant is absent from all public single-nucleotide polymorphism databases. At the age of 12.6 years, he had pubertal stage Tanner P3, penile length of 3.5 cm and testicular volume of 4 mL.

Case 5

The proband was born to first-cousin parents and first seen in our clinic at the age of 30 days with the fusion of the labia majora and small vaginal–urethral orifice. Her karyotype was 46,XX. Results of the ACTH stimulation test suggested congenital adrenal hyperplasia due to 21-hydroxylase deficiency. However, sequencing of *CYP21A* did not detect a pathogenic variant. GCMS was consistent with POR deficiency, but the sequencing of the *POR* gene revealed no pathological variant. At the age of 11 years, using WES, a homozygous missense variant of *POR*, previously described in an Israeli-Bedouin family, was identified (14). The parents were heterozygous for the identified mutation. At the age of 11.8 years, she had no signs of puberty with low peak LH response to LHRH stimulation test and estrogen supplementation therapy was initiated.

Case 6

The 46,XY proband was born to unrelated parents and was referred to us at the age of 25 days due to atypical genitalia. In addition, he had dysmorphic facial features, severe hypotonia, right-sided aortic arch and conductive hearing impairment. Brain MRI demonstrated hypoplastic pons with mild fourth-ventricle dilatation. His clinical characteristics suggested syndromic DSD. LHRH and ACTH stimulation tests were within the normal range, but he had low peak testosterone values following hCG stimulation. The candidate gene approach revealed no pathogenic variants in *DHCR7*, *NR5A1* or *WT1*. WES performed at 3.5 years of age revealed a previously described *de novo* and heterozygous missense variant (c.1480C>T, p.R494T) of the *CHD7* gene (15). The proband was later diagnosed with severe mental retardation and autism. At the age of 8 years, he had a small penile length and nonpalpable testes.

Case 8

The 46,XY proband was referred to our clinic due to micropenis at the age of 1.1 years. His parents were unrelated. In addition, he had mental retardation, hydrocephalous, attention deficit hyperactivity disorder (ADHD), convulsive disorder, cardiac anomalies, short stature with growth hormone deficiency, and autism. No other anomalies were found. WES performed at the age of 12.75 years identified *de novo* pathogenic deletion of 2 nucleotides in *SIN3A*, c.2809_2810del (p.K937QfsTer2). At the age of 8 years, he had severe micropenis and a testicular volume of 1 mL.

Discussion

Using WES, we identified pathogenic variants that explained the phenotype of DSD in 78% of our cohort. Patients underwent WES at a mean age of 10 years, following lack of success with traditional diagnostic strategies (16), including wide hormonal assessments, imaging, and targeted gene sequencing, in finding the etiology of DSD. These traditional approaches have been found to identify the etiology of DSD in only 20% of cases, and a specific

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Table 2 Hormonal results of all patients.

No.	Age[a] (days)	LHRH test[b]				hCG test		Age[c] (years)	LHRH test[c]				T (ng/mL)	GCMS	Other tests
		LH (mIU/L)	LH peak (mIU/L)	FSH (mIU/L)	FSH peak mIU/L	T (ng/mL)	T[d] (ng/mL)		LH (mIU/L)	LH peak (mIU/L)	FSH (mIU/L)	FSH peak (mIU/L)			
1	14	**2.8**	**46**	<0.4	3.9	2.59	4.65	13	**2.8**	**29.4**	**8.5**	**21.4**	2.87	N	
2	90	**7.2**	**52**	0.57	1.46	0.7	2.13	14.9	**14.3**	ND	**43.2**	ND	3.5	N	T:A = 0.39
3	7	2.0	**13.6**	**5.1**	**19.7**	1.1	10.8	12.3	**6.4**	**66**	**74**	**160**	0.6	N	
4	224	<0.5	**5.3**	1.1	5.3	0.24	3.35	12.6	2.5	ND	**5.66**	ND	1.58	N	
5	30	ND	ND	ND	ND	ND	ND	11.8	<0.07	1.5	**3.5**	**14.3**	<0.24	Compatible with P450 oxidoreductase deficiency	
6	25	<0.5	**6.5**	1.4	**20.4**	<0.1	1.9	8.4	<0.07	ND	0.8	ND	0.14	N	
7	14	**19.7**	**86**	**8.7**	**19.8**	1.76	3.2	12.7	0.85	ND	**3.06**	ND	0.5	N	
8	455	<0.07	ND	1.35	ND	<0.1	ND	8.7	<0.07	ND	1.5	ND	<0.07	ND	
9	21	2.3	**39.6**	**5.99**	**33.1**	1.25	1.69	12.0	1.5	**27.6**	**11.2**	**28.4**	1.2	N	
Normal ranges[b]		<0.3–2.5	1.3–3.8	<0.5–2.2	2.6–6.3	<0.03	3 times basal		<0.3–2.5	1.3–3.8	<0.5–2.2	2.6–6.3	2.3–8.65[e]		

Bold faced numbers represent values above the normal ranges.
[a]At diagnosis; [b]Normal range for pre-pubertal male; [c]At last visit; [d]After 72 h; [e]Normal range for adult male.
N, normal; ND, not determined; T, testosterone; T:A, testosterone:androstenedione ratio.

diagnosis is often deferred to the second decade of life (6, 17, 18, 19). Recently, HTS panels have been used for the diagnosis of DSD (6, 9, 20, 21). An international study including 326 patients with DSD identified its etiologies in 43% of them using a HTS panel of 64 known genes (9). The use of the HTS panel reduced costs, enabled an earlier specific diagnosis and facilitated clinical management. However, these panels cover only genes that are known to be involved in sex development and determination. In contrast, WES theoretically sequences all genes in the human genome and as new genes causing DSD are discovered, the patient datasets can be reanalyzed for pathogenic variants that were not previously recognized. Since WES generates a wealth of genetic data, it has been recommended that the molecular results, together with the clinical and hormonal findings, be interpreted by a multidisciplinary team that includes clinicians and medical geneticists (5). Here, using WES, we identified seven causative variants (four novel and three previously reported) that explained the etiology of DSD.

A *de novo* missense variant, c.209G>T, p.R70L, in the *BMP4* gene was identified in case 4, an 11.3-year-old 46,XY male who presented at the age of 12 days with atypical external genitalia. BMP4 is a member of a large cytokine family related to the transforming growth factor beta proteins. BMP4 heterozygous loss-of-function variants (MIM 112262) have been described in association with autosomal dominant microphthalmia with brain and digital anomalies (MCOPS6), a syndrome that is characterized by ocular, digital and brain anomalies, cleft lip and palate, and renal malformations (22, 23). In mice, Bmp4 is expressed in both the mesenchyme and the urethral epithelium and it is essential for outgrowth of the genital tubercle (24). Mice lacking *Bmp4* show hypoplasia of the genital tubercle together with reduced expression of other outgrowth factors, including *Wnt5a, Hoxd13* and *p63* (25). The development of male external genitalia consists of two phases. The first is development of the genital tubercle, which is regulated by BMP proteins, including BMP4, and the second is from 8 weeks onward, when the gonads have differentiated into testes in 46,XY individuals and the hormone-dependent phase begins, when testosterone causes elongation of the genital tubercle and the urethral groove terminates (24). Consistent with observations in mice lacking *Bmp4*, variants in the human *BMP4* gene have been reported in a few cases associated with hypospadias (6, 26). Eight missense variants were identified in Chinese patients with hypospadias by direct sequence analysis of *BMP4* and *BMP7* (26). Furthermore, HTS was performed in 70

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Table 3 Molecular findings in all patients.

No	Karyotype	EMS (37)	Targeted gene approach	Age[a]	WES findings					Non-pathogenic variants
			All negative		Gene (Transcript ID)	DNA	Protein	Inheritance	Type of mutation (ACMG classification)	
1	46,XY	5	*DHCR7, SRD5A2, AR, NR5A1, GPR54,* R80Q mutation of *HSD17B3*	12.8	*RXFP2 (*ENST00000307765.5)	c.664A>C	p.T222P	AD	Missense – previously described maternally inherited (likely pathogenic)	
2	46,XY	6	*SRD5A2, LHR, AR,* R80Q mutation of *HSD17B3*	8.0	*HSD17B3* (ENST00000375263.3*)*	c.673G>A	p.V225M	AR	Missense – novel (pathogenic)	
3	46,XY	5.5	*SRD5A2, AR, NR5A1*	11.0	*WT1(ENST00000332351.3)*	c.1433-3C>G		AD	Splice – novel *de novo* (pathogenic)	*PROKR2* (c.809G>A, p. R270H) *FANCC* (c.77C>T, p.S26F)
4	46,XY	4	*SRD5A2, AR, WT1*	11.3	*BMP4(ENST00000245451.4)*	c.809G>T	p.R70L	AD	Missense – novel *de novo (*pathogenic)	
5	46,XX	_	*CYP21A, POR*	11.0	*POR (ENST00000461988.1)*	c.1615G>A	p.G539R	AR	Missense – previously described (pathogenic)	
6	46,XY	5	*NR5A1, WT1, DHCR7*	3.5	*CHD7 (ENST00000423902.2)*	c.1480C>T	p.R494T	AD	Nonsense – previously described *de novo* (pathogenic)	
7	46,XY	8	*SRD5A2, NR5A1, AR*	11.0		No pathological variants				
8	46,XY	8.5	*SRD5A2, AR, LHR*	12.75	*SIN3A (ENST00000394947.3)*	c.2809_2810del	p.K937QfsTer2	AD	Del-novel *de novo* (pathogenic)	*TOE1* (rs145913038) *WDR60* (c.2257+1G>A) *IGSF1* (c.3551G>A)
9	46,XY	6	ND	8.0		VUS				*CDON* (c.746C>T) *WDR81* (c.2894C>T), p.P865L) *PTCH1* (c.2485G>A), p.V829M) *POMT1* (c.221G>T, p.D741Y)
	Mean (range)			10 (3.5-12.8)						

[a]At the time that WES was performed.

AD, autosomal dominant; AR, autosomal recessive; ND, not done; EMS, external masculinization score; VUS, variants of unknown significance (37).

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patients with variable DSD phenotypes revealed three heterozygous missense variants of BMP4 that were predicted to be damaging (20). Two cases had an additional *SRD5A2* variant on one allele, suggesting dysgenic or polygenic inheritance. Interestingly, variants in *BMP4* have been reported in association with a combined pituitary hormone deficiency, suggesting that BMP4 participates in an early stage of pituitary development by inducing the formation of Rathke's pouch (27, 28, 29). Other than a subaortic membrane, atrial septal defect, severe hypospadias, and cryptorchidism, the boy had no other anomalies. This case highlights the role of BMP4 in external genital development.

Case 1 had a previously described heterozygous T222P variant in the *RXFP2* gene. Insulin-like 3 hormone and its receptor *Rxfp2* have been shown to play an important role in testicular descent in mice, with mice lacking *Rxfp2* having an abdominal testis (30). The T222P variant has been reported in adult males with cryptorchidism attributed to reduced activity of the RXFP2 protein caused by poor membrane expression of the mutant receptor (12). However, other authors have called into question the role of RXFP2 variants in XY DSD, since no association between T222P or *RXFP2* variant and male cryptorchidism has been reported (31, 32).

In case 8, with syndromic DSD, a *de novo* pathogenic deletion in *SIN3A*, c.2809_2810del (p.K937QfsTer2), was identified. Heterozygous variants in the *SIN3A* gene (MIM 607776) are associated with Witteveen–Kolk syndrome and are characterized by neurological disorders, including developmental delay, microcephaly, intellectual disability, and autism spectrum disorders (33). Other variable features include characteristic facial dysmorphia (broad forehead, long face, downslanting palpebral fissures, depressed nasal bridge, large fleshy ears, long and smooth philtrum, small mouth, and pointed chin), short stature, microcephaly, joint hypermotility, and small hands and feet. Male genital abnormalities were reported in four cases (34), and genetic inactivation of *Sin3A* in the germline of XY mice leads to sterility resulting from early apoptotic death and a Sertoli-cell only phenotype (35). Case 8 carried a *de novo*, heterozygous loss-of-function variant and had features typical of Witteveen–Kolk syndrome, including intellectual disability, hydrocephalus, ADHD, convulsive disorder, cardiac anomalies, short stature with growth hormone deficiency, and autism. This variant is predicted to result in a truncated protein that will be recognized by the nonsense-mediated decay surveillance complexes and degraded.

Case 3 had a novel *de novo* splice-site variant of *WT1*. Mutations of the *WT1* gene (OMIM 607102) are associated with Denys–Drash syndrome, presenting with renal failure and high risk for Wilms tumor, and Frasier syndrome exhibiting nephrotic syndrome with a high risk for gonadoblastoma. In our case, primary testicular failure was observed in the patient at the age of 12 years, but with no renal anomalies.

Case 5 had a mutation that had been previously reported in an Israeli-Bedouin family (14). This *POR* mutation (OMIM 124015) has been reported in association with Antley–Bixler syndrome displaying genital atypia, disordered steroidogenesis and skeletal anomalies. In our case, the patient presented with labia majora fusion and primary adrenal insufficiency in infancy. At the age of 12 years, she had no signs of puberty, indicating the absence of gonadal steroid secretion.

Case 6 had a *de novo* heterozygous missense variant of the *CHD7* gene (OMIM 608892), exhibiting syndromic DSD with severe mental retardation and autism. *CHD7* has been reported as one of the genes causing syndromic DSD (15).

The sex of rearing decision is crucial for an individual's future, and takes into account many factors, including cultural background, future fertility, degree of virilization, potential adult sexual function, surgical intervention, life-long replacement therapy, and future malignancies. However, the main parameter to be considered is the likely gender identity in adulthood, which is strongly dependent on the specific DSD etiology (5, 36). In case 2, a female infant presented to our clinic at the age of 90 days with bilateral palpable masses. Genetic evaluation revealed a 46,XY karyotype. Hormonal evaluation and the candidate gene approach did not identify the etiology. It was only at the age of 8 years that WES identified a homozygous missense mutation of the *HSD17B3* gene. Knowing the etiology at infancy might have led to a different decision regarding sex of rearing in this case, highlighting the importance of WES in early molecular diagnosis of DSD and its important implications for the sex of rearing decision. Although WES identified the etiology of DSD in 78% of the cohort, 22% of the patients still remained with no diagnosis. Future directions to improve the accuracy of DSD diagnosis, might include using whole-genome sequencing, and improving bioinformatics methods by using available, rapid functional assays to prove causality of the identified variants (6). Moreover, repeat genetic testing is warranted because new genes are being discovered all the time.

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Conclusions

Our findings of molecular etiologies for DSD in 78% of our patients indicate a major role for WES in early DSD diagnosis and management, and highlight the importance of rapid molecular diagnosis in early infancy for sex of rearing decisions.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-21-0019.

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

Acknowledgements

The authors thank all of the patients and their parents for their participation in this study, and Camille Vainstein for professional English editing.

References

1 Houk CP & Lee PA. Consensus statement on terminology and management: disorders of sex development. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation* 2008 **2** 172–180. (https://doi.org/10.1159/000152032)

2 Ahmed SF, Dobbie R, Finlayson AR, Gilbert J, Youngson G, Chalmers J & Stone D. Prevalence of hypospadias and other genital anomalies among singleton births, 1988–1997, in Scotland. *Archives of Disease in Childhood: Fetal and Neonatal Edition* 2004 **89** F149–F151. (https://doi.org/10.1136/adc.2002.024034)

3 Rodie M, McGowan R, Mayo A, Midgley P, Driver CP, Kinney M, Young D & Ahmed SF. Factors that influence the decision to perform a karyotype in suspected disorders of sex development: lessons from the Scottish genital anomaly network register. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation* 2011 **5** 103–108. (https://doi.org/10.1159/000326815)

4 Hiort O & Gillessen-Kaesbach G. Disorders of sex development in developmental syndromes. In *Endocrine Involvement in Developmental Syndromes*, vol **14**, ch 12, pp 174–180. Eds Cappa M, Maghnie M, Loche S & Bottazzo GF. Basel: Karger 2009. (https://doi.org/10.1159/000207486)

5 Audi L, Ahmed SF, Krone N, Cools M, McElreavey K, Holterhus PM, Greenfield A, Bashamboo A, Hiort O, Wudy SA, *et al.* The EU COST Action. Approaches to molecular genetic diagnosis in the management of differences/disorders of sex development (DSD): position paper of EU COST Action BM 1303 'DSDnet'. *European Journal of Endocrinology* 2018 **179** R197–R206. (https://doi.org/10.1530/EJE-18-0256)

6 Croft B, Ayers K, Sinclair A & Ohnesorg T. Review disorders of sex development: the evolving role of genomics in diagnosis and gene discovery. *Birth Defects Research Part C: Embryo Today: Reviews* 2016 **108** 337–350. (https://doi.org/10.1002/bdrc.21148)

7 Dong Y, Yi Y, Yao H, Yang Z, Hu H, Liu J, Gao C, Zhang M, Zhou L, Asan, *et al.* Targeted next-generation sequencing identification of mutations in patients with disorders of sex development. *BMC Medical Genetics* 2016 **17** article no. 23. (https://doi.org/10.1186/s12881-016-0286-2)

8 Eggers S, Sadedin S, van den Bergen JA, Robevska G, Ohnesorg T, Hewitt J, Lambeth L, Bouty A, Knarston IM, Tan TY, *et al.* Disorders of sex development: insights from targeted gene sequencing of a large international patient cohort. *Genome Biology* 2016 **17** article no. 243. (https://doi.org/10.1186/s13059-016-1105-y)

9 Boehmer AL, Brinkmann AO, Sandkuijl LA, Halley DJ, Niermeijer MF, Andersson S, de Jong FH, Kayserili H, de Vroede MA, Otten BJ, *et al.* 17β-Hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and de novo mutations. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 4713–4721. (https://doi.org/10.1210/jcem.84.12.6174)

10 Murphy MW, Lee JK, Rojo S, Gearhart MD, Kurahashi K, Banerjee S, Loeuille GA, Bashamboo A, McElreavey K, Zarkower D, *et al.* An ancient protein–DNA interaction underlying metazoan sex determination. *Nature Structural and Molecular Biology* 2015 **22** 442–451. (https://doi.org/10.1038/nsmb.3032)

11 Richards CS, Bale S, Bellissimo DB, Das S, Grody WW, Hegde MR, Lyon E, Ward BE & Molecular Subcommittee of the ACMG Laboratory Quality Assurance Committee. ACMG recommendations for standards for interpretation and reporting of sequence variations: revisions 2007. *Genetics in Medicine* 2007 **10** 294–300. (https://doi.org/10.1097/GIM.0b013e31816b5cae)

12 Bogatcheva NV, Ferlin A, Feng S, Truong A, Gianesello L, Foresta C & Agoulnik AI. T222P mutation of the insulin-like 3 hormone receptor LGR8 is associated with testicular maldescent and hinders receptor expression on the cell surface membrane. *American Journal of Physiology – Endocrinology and Metabolism* 2007 **292** E138–E144. (https://doi.org/10.1152/ajpendo.00228.2006)

13 Bertalan R, Admoni O, Bashamboo A, Tenenbaum-Rakover Y & McElreavey K. A novel *HSD17B3* gene mutation in a 46,XY female-phenotype newborn identified by whole-exome sequencing. *Clinical Endocrinology* 2017 **87** 407–408. (https://doi.org/10.1111/cen.13396)

14 Hershkovitz E, Parvari R, Wudy SA, Hartmann MF, Gomes LG, Loewental N & Miller WL. Homozygous mutation G539R in the gene for P450 oxidoreductase in a family previously diagnosed as having 17,20-lyase deficiency. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 3584–3588. (https://doi.org/10.1210/jc.2008-0051)

15 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, McPherson JD, Gibbs RA, White LD, Hefner M, *et al.* Spectrum of CHD7 mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *American Journal of Human Genetics* 2006 **78** 303–314. (https://doi.org/10.1086/500273)

16 Baetens D, Mladenov W, Delle Chiaie B, Menten B, Desloovere A, Iotova V, Callewaert B, Van Laecke E, Hoebeke P, De Baere E, *et al.* Extensive clinical, hormonal and genetic screening in a large consecutive series of 46,XY neonates and infants with atypical sexual development. *Orphanet Journal of Rare Diseases* 2014 **9** 209. (https://doi.org/10.1186/s13023-014-0209-2)

17 Lambert SM, Vilain EJ & Kolon TF. A practical approach to ambiguous genitalia in the newborn period. *Urologic Clinics of North America* 2010 **37** 195–205. (https://doi.org/10.1016/j.ucl.2010.03.014)

18 Ostrer H. Disorders of sex development (DSDs): an update. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 1503–1509. (https://doi.org/10.1210/jc.2013-3690)

19 García-Acero M, Moreno O, Suárez F & Rojas A. Disorders of sexual development: current status and progress in the diagnostic approach. *Current Urology* 2020 **13** 169–178. (https://doi.org/10.1159/000499274)

20 Wang H, Zhang L, Wang N, Zhu H, Han B, Sun F, Yao H, Zhang Q, Zhu W, Cheng T, *et al*. Next-generation sequencing reveals genetic landscape in 46, XY disorders of sexual development patients with variable phenotypes. *Human Genetics* 2018 **137** 265–277. (https://doi.org/10.1007/s00439-018-1879-y)

21 Xu Y, Wang Y, Li N, Yao R, Li G, Li J, Ding Y, Chen Y, Huang X, Chen Y, *et al.* New insights from unbiased panel and whole-exome sequencing in a large Chinese cohort with disorders of sex development. *European Journal of Endocrinology* 2019 **181** 311–323. (https://doi.org/10.1530/EJE-19-0111)

22 Bakrania P, Efthymiou M, Klein JC, Salt A, Bunyan DJ, Wyatt A, Ponting CP, Martin A, Williams S, Lindley V, *et al.* Mutations in BMP4 cause eye, brain, and digit developmental anomalies: overlap between the BMP4 and hedgehog signaling pathways. *American Journal of Human Genetics* 2008 **82** 304–319. (https://doi.org/10.1016/j.ajhg.2007.09.023)

23 Reis LM, Tyler RC, Schilter KF, Abdul-Rahman O, Innis JW, Kozel BA, Schneider AS, Bardakjian TM, Lose EJ, Martin DM, *et al.* BMP4 loss-of-function mutations in developmental eye disorders including SHORT syndrome. *Human Genetics* 2011 **130** 495–504. (https://doi.org/10.1007/s00439-011-0968-y)

24 Suzuki K, Bachiller D, Chen YP, Kamikawa M, Ogi H, Haraguchi R, Ogino Y, Minami Y, Mishina Y, Ahn K, *et al*. Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. *Development* 2003 **130** 6209–6220. (https://doi.org/10.1242/dev.00846)

25 Kajioka D, Suzuki K, Nakada S, Matsushita S, Miyagawa S, Takeo T, Nakagata N & Yamada G. Bmp4 is an essential growth factor for the initiation of genital tubercle (GT) outgrowth. *Congenital Anomalies* 2020 **60** 15–21. (https://doi.org/10.1111/cga.12326)

26 Chen T, Li Q, Xu J, Ding K, Wang Y, Wang W, Li S & Shen Y. Mutation screening of *BMP4*, *BMP7*, *HOXA4* and *HOXB6* genes in Chinese patients with hypospadias. *European Journal of Human Genetics* 2007 **15** 23–28. (https://doi.org/10.1038/sj.ejhg.5201722)

27 Castinetti F, Reynaud R, Saveanu A, Jullien N, Quentien MH, Rochette C, Barlier A, Enjalbert A & Brue T. Mechanisms in endocrinology: an update in the genetic aetiologies of combined pituitary hormone deficiency. *European Journal of Endocrinology* 2016 **174** R239–R247. (https://doi.org/10.1530/EJE-15-1095)

28 Breitfeld J, Martens S, Klammt J, Schlicke M, Pfäffle R, Krause K, Weidle K, Schleinitz D, Stumvoll M, Führer D, *et al.* Genetic analyses of bone morphogenetic protein 2, 4 and 7 in congenital combined pituitary hormone deficiency. *BMC Endocrine Disorders* 2013 **13** article no. 56. (https://doi.org/10.1186/1472-6823-13-56)

29 Rodríguez-Contreras FJ, Marbán-Calzón M, Vallespín E, Del Pozo Á, Solís-López M, Lobato-Vidal N, Fernández-Elvira M, Del Valle Rex-Romero M, Heath KE, González-Casado I, *et al.* Loss of function *BMP4* mutation supports the implication of the BMP/TGF-β pathway in the etiology of combined pituitary hormone deficiency. *American Journal of Medical Genetics: Part A* 2019 **179** 1591–1597. (https://doi.org/10.1002/ajmg.a.61201)

30 Feng S, Ferlin A, Truong A, Bathgate R, Wade JD, Corbett S, Han S, Tannour-Louet M, Lamb DJ, Foresta C, *et al.* INSL3/RXFP2 signaling in testicular descent. *Annals of the New York Academy of Sciences* 2009 **1160** 197–204. (https://doi.org/10.1111/j.1749-6632.2009.03841.x)

31 Houate BEI, Rouba H, Imken L, Sibai H, Chafik A, Boulouiz R, Chadli E, Hassar M, McElreavey K & Barakat A. No association between T222P/LGR8 mutation and cryptorchidism in the Moroccan population. *Hormone Research* 2008 **70** 236–239. (https://doi.org/10.1159/000151596)

32 Nuti F, Marinari E, Erdei E, El-Hamshari M, Echavarria MG, Ars E, Balercia G, Merksz M, Giachini C, Shaeer KZ, *et al.* The leucine-rich repeat-containing G protein-coupled receptor 8 gene T222P mutation does not cause cryptorchidism. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 1072–1076. (https://doi.org/10.1210/jc.2007-1993)

33 van Dongen LCM, Wingbermühle E, Dingemans AJM, Bos-Roubos AG, Vermeulen K, Pop-Purceleanu M, Kleefstra T & Egger JIM. Behavior and cognitive functioning in Witteveen–Kolk syndrome. *American Journal of Medical Genetics: Part A* 2020 **182** 2384–2390. (https://doi.org/10.1002/ajmg.a.61775)

34 Cushman LJ, Torres-Martinez W, Cherry AM, Manning MA, Abdul-Rahman O, Anderson CE, Punnett HH, Thurston VC, Sweeney D & Vance GH. A report of three patients with an interstitial deletion of chromosome 15q24. *American Journal of Medical Genetics: Part A* 2005 **137** 65–71. (https://doi.org/10.1002/ajmg.a.30836)

35 Pellegrino J, Castrillon DH & Davis G. Chromatin associated Sin3A is essential for male germ cell lineage in the mouse. *Developmental Biology* 2012 **369** 349–355. (https://doi.org/10.1016/j.ydbio.2012.07.006)

36 Alhomaidah D, McGowan R & Ahmed SF. The current state of diagnostic genetics for conditions affecting sex development. *Clinical Genetics* 2017 **91** 157–162. (https://doi.org/10.1111/cge.12912)

37 Ahmed SF, Khwaja O & Hughes IA. The role of a clinical score in the assessment of ambiguous genitalia. *BJU International* 2000 **85** 120–124. (https://doi.org/10.1046/j.1464-410x.2000.00354.x)

Received in final form 9 May 2021
Accepted 18 May 2021
Accepted Manuscript published online 19 May 2021