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Clinical and Genetic Evaluation of Undervirilized Boys With Bifid Scrotum

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Thesis Introduction:

Bifid scrotum and hypospadias suggest undervirilization, yet boys presenting with these findings often do not receive a genetic diagnosis. Some of these individuals fall on the spectrum toward genital ambiguity, which often has an identifiable genetic etiology. In some cases, identifying an underlying genetic diagnosis can help optimize clinical care. The objectives of this work include characterizing current practice for genetic evaluation for patients with bifid scrotum, identifying approaches with a good diagnostic yield, as well as using whole-exome sequencing to study ten undervirilized 46,XY subjects with bifid scrotum.

These studies demonstrate that the majority of individuals with bifid scrotum do not receive a genetic diagnosis on clinical workup. Over a third of subjects did not have any genetic testing, even though karyotype analysis and androgen receptor sequencing were both relatively high yield for identifying a genetic etiology. Increased utilization of traditional genetic approaches could significantly improve the ability to find a genetic diagnosis. Furthermore, using whole-exome sequencing on ten 46,XY boys with bifid scrotum identified two novel *NR5A1* variants, both impacting exon 7. One of these variants demonstrated significant genotype-phenotype variability in the setting of a rare instance of paternal inheritance from an unaffected father.

Title: Variation in the Clinical and Genetic Evaluation of Undervirilized Boys with Bifid Scrotum

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Short title: Evaluation of patients with bifid scrotum

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Abbreviations: DSD, disorders of sex development; GU, genitourinary; GWAS, genome wide association study; EMR, electronic medical record.

What's Known on This Subject: Disorders of sex development can cause a spectrum of phenotypes, and not all affected individuals have frank genital ambiguity. It is unclear whether children presenting with these milder phenotypes, such as 46,XY males with bifid scrotum and hypospadias, require a full diagnostic evaluation.

What This Study Adds: Our study demonstrates that, with current practice, there are missed opportunities to establish a genetic diagnosis in mildly undervirilized boys.

Abstract:

Background and Objectives: Bifid scrotum and hypospadias suggest undervirilization, yet boys presenting with these findings often do not receive a genetic diagnosis. Some of these individuals fall on the spectrum toward genital ambiguity, which often has an identifiable genetic etiology. In some cases, identifying an underlying genetic diagnosis can help optimize clinical care. The objective of this study was to characterize current practice for genetic evaluation for patients with bifid scrotum, and to identify approaches with a good diagnostic yield.

Methods: We conducted a retrospective study of the Boston Children's Hospital electronic medical record (1993-2015) using the search term "bifid scrotum." and extracted clinical data.

Results: We identified 110 subjects evaluated in the Endocrinology and/or Urology clinics for bifid scrotum. Genetic testing (karyotype, microarray, or targeted testing) was performed on 64% of the subjects with bifid scrotum, and of those tested, 23% (15% of the total cohort of 110 subjects) received a genetic diagnosis. Karyotype analysis led to a diagnosis in 17% of patients when performed. When sent, androgen receptor gene sequencing identified a pathogenic mutation 20% of the time.

Conclusion: This study demonstrates that the majority of individuals with bifid scrotum do not receive a genetic diagnosis. Over a third of subjects did not have any genetic testing, even though karyotype analysis and androgen receptor sequencing were both relatively high yield for identifying a genetic etiology. Increased utilization of traditional genetic approaches could significantly improve the ability to find a genetic diagnosis, and newer approaches such as whole-exome sequencing may further increase diagnostic yield.

INTRODUCTION:

Disorders of male genital development are congenital malformations of the penis and scrotum and can vary in severity. Fortunately, advances in surgical technique provide functional improvement for patients if referred to an experienced surgical team.[1,2] In many cases, however, the underlying etiology of this developmental abnormality is not known; this lack of a diagnosis may lead to significant distress for patients and their families.[3,4] Androgen receptor mutations provide a clear example of the impact of having a genetic diagnosis. Identification of an androgen receptor mutation allows for targeted clinical care, including the possible use of testosterone, fertility guidance, family testing, and assessment of risk to future children.[5,6]

For this study, we focused on males with bifid scrotum. Bifid scrotum is a midline cleft in the scrotum and can be associated with incomplete fusion of the labioscrotal folds.[7] In the majority of cases, individuals with bifid scrotum also have hypospadias. One potential cause of this phenotype is insufficient testosterone secretion or action: fusion of the labioscrotal folds and urethral localization both take place during the first trimester under the influence of androgens.[8] As such, bifid scrotum with hypospadias may represent a mild disorder of sex development (DSD).

Although such mild undervirilization has been well managed surgically, progress in diagnosis has lagged. In many diseases, genetic testing to determine etiology has led to significant progress in understanding pathogenesis and even to targeted treatment, such as in congenital cardiac disease,[9] intellectual disability,[10] and hyperinsulinism/neonatal diabetes mellitus.[11] Recently, studies have focused on the genetics of disorders of sex development that result in

frankly ambiguous genitalia,[12–15] but relatively few studies have focused on the genetics of milder cases of undervirilization. However, there are recent reports suggesting that a significant number of these milder cases may also have underlying genetic etiologies, including *AR*, *NR5A1*, and *WT1* mutations.[16]

Bifid scrotum was chosen as a proxy for undervirilization in this study to evaluate a group of subjects with a relatively narrow range of phenotypes. Individuals with this phenotype are often seen in Urology and/or Endocrinology clinics and are not consistently classified as having a DSD. We hypothesized that few children received a genetic diagnosis, yet still received comprehensive surgical and medical management. This hypothesis was based upon our clinical experience that only a small fraction of individuals with bifid scrotum, regardless of whether they had additional clinical features, received a diagnosis to explain the underlying mechanism of disease. As syndromic features would potentially point toward possible genetic etiologies, we also hypothesized that those patients with additional clinical findings were more likely to have additional evaluation and workup. To test these hypotheses, we undertook a retrospective chart review of children with bifid scrotum with and without hypospadias to document the characteristics and genetic evaluation of these males at a tertiary care referral center. We also sought to identify the differences in diagnostic evaluation and outcome between those with isolated genitourinary abnormalities and those with syndromic features.

METHODS:

Cohort. A text-based search system (i2b2)[17] was used to identify subjects seen in the Division of Endocrinology and/or Department of Urology at Boston Children's Hospital between 1993 and

2015 using the search term "bifid scrotum." Additional subjects were identified prospectively through review of Endocrine and/or Urology schedules between December 2014 and May 2015. Subject characteristics including gender, race, ethnicity, age at first evaluation, gestational age at birth, severity of hypospadias, genetic evaluation, serum testosterone measurements, and presence of non-genitourinary abnormalities were extracted from review of medical records. Hypospadias severity was dichotomized as proximal/midshaft (perineal, scrotal, penoscrotal and penile) and distal (coronal and glanular). Categories of non-genitourinary abnormalities (syndromic features) included cardiac, renal, neurologic, neuro-developmental, gastrointestinal, musculoskeletal, ophthalmologic, endocrine, otolaryngologic, or growth abnormalities.

Statistical analysis. Study data were collected and managed using REDCap electronic data capture tools hosted at Boston Children's Hospital.[18] This research was approved by the Boston Children's Hospital IRB. Microsoft Excel, SPSS, and SAS 9.4 software were used for statistical analysis. Groups were compared using Fisher's exact test and two tailed t-tests.

Genetic evaluation. We defined genetic evaluation to include any of the following: karyotype, chromosomal microarray, or targeted genetic evaluation, including sequencing and deletion testing. None of the patients had whole-exome or whole-genome sequencing. The vast majority of the targeted genetic testing has taken place since 2005 (only 4 targeted tests sent prior) but karyotypic analysis has been performed throughout the study time period.

RESULTS:

Description of Cohort

A search of the Boston Children's patient database and of the Urology and Endocrine clinic schedules identified 110 subjects bifid scrotum. Nearly half (46%) of the subjects were seen by both Endocrine and Urology providers, while 11% were seen only in Endocrine clinic and 43% were seen only in Urology clinic. A little less than one third of the subjects (31%) had orchidopexy for unilateral or bilateral undescended testes. In Table 1, we show the characteristics of the bifid scrotum cohort as a whole and also of the subset isolated genitourinary abnormalities (62 %) and the subset with additional non-genitourinary abnormalities (38%). Subjects with non-genitourinary abnormalities in addition to their bifid scrotum are referred to through the rest of this publication as syndromic subjects.

Presence and severity of hypospadias

We examined how frequently subjects with bifid scrotum also had hypospadias. For individuals with only GU findings, 93% had more severe proximal/midshaft hypospadias, whereas a smaller majority (76%) of the subjects with syndromic findings had proximal/midshaft hypospadias (Table 2). Only two individuals (3%) with isolated bifid scrotum had mild or no hypospadias, while 8 syndromic subjects (19%) had mild or no hypospadias. In total, the majority (86%) of subjects with bifid scrotum had severe hypospadias.

Genetic evaluation and diagnosis

To determine how often genetic testing led to a diagnosis, we reviewed all genetics tests done for individuals in the cohort (Table 3). We found that 15% of all subjects had a confirmed genetic diagnosis. Of the 64% of subjects who had any type of genetic testing, including karyotype,

microarray, or targeted genetic testing, 23% received a genetic diagnosis. These diagnoses include sex chromosome abnormalities diagnosed by karyotype (n=9) or microarray (n=1), non-sex chromosome abnormalities (n=3), androgen receptor mutations (n=2), and Smith-Lemli-Opitz Syndrome (n=1). Seven of the 16 cases with genetic diagnoses (44%) required orchidopexy to treat unilateral or bilateral undescended testes. Of the 69 subjects who had karyotype testing sent, 17% were found to be abnormal. Of the isolated GU subjects, 22% were seen by a geneticist at least once, while 60% of the syndromic subjects were seen by a geneticist. Of those who saw a geneticist, 90% had genetic testing as part of their evaluation compared to 50% of those who were not seen in Genetics clinic.

Karyotype

Karyotypes were the most frequent genetic study performed and were sent at comparable rates in the syndromic and isolated genitourinary groups (67% and 60%, respectively). Subjects seen only in urologic clinic had karyotypes sent 26% of the time, compared to 83% of the time for those seen only in Endocrine clinic and 92% of the time for subjects seen both in Urology and Endocrinology clinics. Subjects seen by a geneticist had karyotypes performed 88% of the time, compared to 49% of those who did not see a geneticist.

Chromosomal microarray

Seventeen subjects had chromosomal microarray testing, which yielded one pathogenic copy number variant. The majority of this testing was sent in the syndromic group (33%; 14 out of 42) rather than in the isolated GU group, (4%; 3 out of 68; $p < 0.0001$). The diagnostic yield of microarray for the entire cohort was 6%, compared to 7% for the sub-group with additional

syndromic findings. None of the non-syndromic subjects received a diagnosis based on microarray results.

Targeted testing

Targeted gene-specific or deletion testing was sent on a number of genes/regions including *AR, SOX9, SRY, DHCR7, NLGN4, FGFD1, MID1, STK9, NLGN3, NR5A1, SRD5A2*, *FMR1*, and 22q11 deletion. About a quarter of the cohort (24%) had single gene or targeted deletion testing, much of which (39%) was androgen receptor sequencing. Androgen receptor (*AR*) sequencing was sent on 10 subjects, with 20% (n=2) receiving an androgen insensitivity diagnosis.

Prematurity

Based on prior reports of an association between prematurity and undervirilization,[19] we examined gestational age at birth within the cohort. Subjects with missing data on gestational age were omitted from this analysis. Of our cohort of 110 subjects, 83 had data on gestational age, and 51% of those 83 subjects (n=42) were born prior to 37 weeks. Of the subjects with isolated GU findings and a known gestational age, 44% (30 of 68 subjects with available data) were born before 37 weeks, while 60% (21 of 35 subjects) with additional syndromic abnormalities were born before 37 weeks (p=0.18). The average gestational age of those with available data across the cohort was 35.9 weeks. The average gestational age for the isolated GU cases was 36.3 weeks, compared to 35.2 weeks for syndromic cases (p=0.23).

Since most subjects with a lack of documented gestational age data were probably full term, we also performed a differential vs. non-differential missingness analysis on our gestational age data

and assumed that the gestational ages of subjects with missing data were full term (40 weeks). Operating under this assumption, 38% of all subjects were born prior to 37 weeks, but the fraction born prior to 37 weeks was not significantly different between groups (31% in the isolated GU group vs. 50% in the syndromic group; p=0.07). The average gestational age across the cohort was 36.9 weeks. The average gestational age of subjects with isolated GU findings was 37.4 weeks, compared to 36.0 weeks in syndromic cases (p=0.08).

DISCUSSION:

The experience at Boston Children's Hospital over the past two decades offers insights into both the characteristics and work-up of male subjects with bifid scrotum, an indicator of potential undervirilization. These subjects were seen by providers with different areas of clinical focus and underwent variable diagnostic evaluations. The variability may reflect the way in which these patients are classified, at times grouped with patients with isolated genitourinary issues such as hypospadias while at other times considered to have mild disorders of sex development (DSD). The review of medical records indicates that the extent of genetic work-up differs depending on whether subjects were seen by both an endocrinologist and urologist when compared to seeing only a urologist. This may reflect differing diagnostic strategies amongst specialties, or it may be due to confounding by clinical presentation, with more significant cases being sent for multidisciplinary evaluation and also meriting a more comprehensive evaluation.

In our cohort, almost two-thirds of subjects had genetic testing and approximately one-quarter of those subjects received a genetic diagnosis. The majority of the diagnoses were made with chromosomal analysis. Androgen receptor sequencing also yielded diagnoses, with two

confirmed cases out of 10 subjects who received such testing. These results indicate that karyotype analysis and *AR* sequencing appear to be high yield tests in individuals with the bifid scrotum phenotype. Relatively few subjects had *AR* sequencing as part of their clinical workup, and, although this would need to be validated, it seems likely that some of the 100 subjects who did not have *AR* sequencing would have had a pathogenic variant identified if it had been performed. Similarly, karyotypic analysis of the 41 untested subjects could potentially have led to additional diagnoses. Both chromosomal abnormalities and androgen insensitivity diagnoses would have the potential to change evaluation and treatment. Individuals with sex chromosome abnormalities such as Turner syndrome variants benefit from cardiac, renal and audiologic evaluations[20] and may also be at risk for gonadoblastoma. Diagnosis of partial or complete androgen insensitivity is important given risk of testicular tumors, effect on fertility, potential need for hormonal replacement, implications for puberty, and risk for family recurrence.[6]

With additional research focused on subjects with bifid scrotum and/or hypospadias, the true yield of genetic testing will likely become clearer over time. Some genital abnormalities may be caused by environmental or epigenetic factors, or random developmental events, but an additional unrecognized fraction may carry diagnoses that could be identified using whole-exome or whole-genome sequencing. Recent exome sequencing studies of DSD associated with more severe undervirilization demonstrate a reasonable diagnostic yield,[12–14] comparable to other rare disorders when modern genomic approaches are used for diagnosis.[21] This may indicate an opportunity to use similar approaches to advance knowledge of the genetics of hypospadias and forms of mild undervirilization. There will likely be overlap with DSD genes, potentially with milder mutations in genes known to be associated with gonadal or genital development such as

NR5A1.[22] There may also be distinct genes/loci identified, as suggested by a recent genome wide association study of hypospadias that identified 22 loci, many of which do not lie near genes known to be related to DSD.[23]

Our study's findings also support some previously reported observations. It has been noted in the past that there is an association between prematurity and hypospadias/undervirilization, though the pathophysiology is not well understood.[19,24,25] This association may potentially be related to hCG, placental function, and/or LH receptor signaling in the 1st trimester. For those with documented gestational ages in our cohort, the average was 35.9 weeks, approximately 4 weeks earlier than a standard full term pregnancy.

It is also important to recognize some of the limitations of this study. As a retrospective chart review, the information gathered is limited by the information found in the electronic medical record (EMR). Although outside records were reviewed when available, it is possible that some outside records were not uploaded into the EMR and that our analysis may not reflect the full extent of all testing. Another challenge is evaluating genetic testing over a period of time when the availability of testing evolved. Changes over time may lead to an underestimate of current rates of targeted genetic testing and chromosomal microarray, forms of testing used with increasing frequency over time. Given the overall rarity of this patient phenotype, a longer time frame was necessary to gather sufficient numbers of subjects. We note that the use of a karyotype, an approach that has been in routine use for decades, should be less affected by the time frame encompassed by this study.

CONCLUSION: This analysis demonstrates genetic testing yields a diagnosis for many individuals with milder degrees of undervirilization. Identification of sex chromosome mosaicism and partial androgen insensitivity has significant implications on anticipatory guidance for patients and families, as well as on clinical evaluation and management. The results of this study support the use of genetic testing as part of the evaluation of boys with bifid scrotum. Recent advances in technology for genetic evaluation, including whole-exome and whole-genome sequencing could further improve the diagnostic yield and could thereby change the care of these patients in the future.

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Table 1: Bifid scrotum cohort characteristics

	Age at 1st Visit (years)	Race	%	n	Ethnicity	n	#	Gestational age (weeks)*	% born prior to 37 weeks
Total Bifid Cohort	0.43	White	53.6%	59	Hispanic	15.5%	17	35.85	50.6%
		Black or African American	10.9%	12	Non-Hispanic	21.8%	24		
		Asian	7.3%	8	Unknown	62.7%	69		
		American Indian or Alaskan Native	0.9%	1					
		More than one race	4.5%	5					
		Unknown	22.7%	25					
Isolated GU Subjects	0.35[a]	White	57.4%	39	Hispanic	13.2%	9	36.34[b]	42.9%[c]
		Black or African American	7.4%	5	Non-Hispanic	19.1%	13		
		Asian	7.4%	5	Unknown	67.6%	46		
		American Indian or Alaskan Native	1.5%	1					
		More than one race	2.9%	2					
		Unknown	23.5%	16					
Syndromic Subjects	0.56	White	47.6%	20	Hispanic	19.0%	8	35.17	60.0%
		Black or African American	16.7%	7	Non-Hispanic	26.2%	11		
		Asian	7.1%	3	Unknown	54.8%	23		
		American Indian or Alaskan Native	0.0%	0					
		More than one race	7.1%	3					
		Unknown	21.4%	9					

[a] p=0.514
[b] p=0.23
[c] p =0.18
* Missing gestational age data on 27 subjects (20 syndromic, 7 isolated GU)
**Comparisons between isolated genitourinary subjects and syndromic subjects

Table 2: Hypospadias severity

	Proximal/midshaft	n	Distal/none	n	Unknown	n
Total Bifid Cohort	86.4%	95	9.1%	10	4.5%	5
Isolated GU Subjects	92.6%*	63	2.9%	2	4.4%	3
Syndromic Subjects	76.2%	32	19.0%	8	4.8%	2

*p= 0.006 compared to syndromic subjects
Fisher's exact

Table 3: Genetic evaluation

			Total Cohort	n	GU only	n	Syndromic	n	two sided p-value *
Any genetic evaluation			63.6%	70	60.3%	41	69.0%	29	0.42
	Karyotype		62.7%	69	60.3%	41	66.7%	28	0.68
	Chromosomal Microarray		15.5%	17	4.4%	3	33.3%	14	<0.0001
	Gene-specific or targeted deletion testing		23.6%	26	19.1%	13	28.6%	12	0.35
		AR sequencing	9.1%	10	11.8%	8	4.8%	2	0.31
Confirmed Genetic Etiology of group/sub-group			14.5%	16	10.3%	7	21.4%	9	0.16
Confirmed Genetic Etiology of those with any genetic evaluation			22.9%	16	17.1%	7	31.0%	9	0.25
	AR diagnosis of those tested		20.0%	2	25.0%	2	0	0	1

*Comparisons between isolated genitourinary and syndromic subjects using Fisher's exact test

Two unrelated undervirilized 46,XY males with inherited NR5A1 variants identified by whole exome sequencing

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Abbreviated title: *NR5A1* exome sequencing

Key terms: *NR5A1*, DSD, bifid scrotum, undervirilization, exome sequencing

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ABSTRACT:

Background: Undervirilized 46,XY males with bifid scrotum often pose a diagnostic challenge, and the majority of cases typically do not receive a genetic diagnosis. *NR5A1* mutations can be seen in 10-20% of cases and are a relatively common cause of undervirilization.

Methods: Whole-exome sequencing was utilized to study ten undervirilized 46,XY subjects with bifid scrotum.

Results: Exome sequencing identified novel *NR5A1* variants, both impacting exon 7, in two of the ten subjects with bifid scrotum. Subject One had a heterozygous frameshift variant, c.1150delC, p.Leu384fsTer1 within the ligand-binding domain inherited from his unaffected father. Subject Two had a novel splice-site variant c.1139-2T>C, affecting the canonical splice acceptor site for exon 7 and also disrupting the ligand-binding domain. Both subjects had serum testosterone within the normal range as infants.

Conclusions: We describe two novel *NR5A1* variants, demonstrating mutations in this gene as a common cause of milder cases of 46,XY undervirilization. Whole-exome sequencing results yielded the diagnosis in 2 out of 10 cases without a previous diagnosis, supporting the value of this approach. Significant genotype-phenotype variability was also noted with subject one's paternal inheritance from his unaffected father.

Established Facts:

- Whole exome sequencing has been used effectively in severe DSD cases to yield a diagnosis.

- Heterozygous mutations in *NR5A1* have been identified as a cause of undervirilization in 46,XY males without adrenal insufficiency.

- The vast majority of inherited *NR5A1* cases reported in the literature in 46,XY males are from carrier mothers.

Novel Insights:

- Whole-exome sequencing performed on ten 46,XY subjects with bifid scrotum led to the identification of pathogenic heterozygous *NR5A1* mutations in two cases.

- One of the heterozygous *NR5A1* variants was inherited from an unaffected father, expanding the phenotypic spectrum associated with *NR5A1* variants reported in the literature.

Introduction

Undervirilized 46,XY males are a challenging group of patients to evaluate, particularly when searching for an underlying genetic diagnosis. The broad phenotype can range from female-appearing external genitalia with or without mild clitoromegaly, to ambiguous genitalia, to typical male-appearing genitalia with hypospadias. While the more severe cases are readily recognized as disorders of sex development (DSD)[1], milder cases of hypospadias and bifid scrotum may not lead to a DSD label. Studies of exome sequencing applied to 46,XY DSD cases with frankly ambiguous genitalia has demonstrated the success of this approach in establishing a diagnosis in nearly one-third of cases, potentially avoiding expensive and extensive diagnostic odysseys[2]. It remains unclear if this diagnostic yield extends to milder cases.

This study applied whole exome sequencing to 46,XY males with mild/moderate undervirilization, a group not previously assessed using exome sequencing. We selected 46,XY subjects with bifid scrotum and hypospadias as a narrowly defined phenotype who undergo an extensive workup and often do not receive genetic diagnoses. For many, the phenotype is sufficiently mild that they do not receive a formal DSD workup; indeed, 40% are seen solely by surgeons in the setting of hypospadias repair and undergo few if any diagnostic tests (manuscript in preparation, Boston Children's Hospital). The initial clinical workup for these patients can vary, but may involve ruling out congenital adrenal hyperplasia (such as 21-hydroxylase deficiency or 3 beta-hydroxysteroid dehydrogenase deficiency), performing ultrasound imaging, obtaining a karyotype, and ordering targeted genetic testing[3]. Partial androgen insensitivity with an androgen receptor (*AR*) mutation is one of the most frequently identified genetic causes of undervirilization[4]. Mutations in *NR5A1*, the gene encoding steroidogenic factor-1 (SF-1), is another cause of undervirilization, seen in as many as 10-20% of cases of undervirilization[5,6]. The phenotypic spectrum of disease with *NR5A1* is quite variable. Early reports of biallelic *NR5A1* mutations were found in patients with complete

gonadal dysgenesis and adrenal failure[7–9]. In recent years, there have been a number of publications focused on the wide phenotypic spectrum associated with monoallelic (heterozygous) *NR5A1* mutations, including cases of undervirilization without adrenal insufficiency[10–12]. The relatively common 46,XY undervirilized phenotype associated with testicular dysgenesis in the setting of heterozygous *NR5A1* mutations appears to be much more common than the rare homozygous cases with associated adrenal insufficiency[5].

This study addresses the diagnostic yield of whole-exome sequencing in a cohort of 10 subjects with bifid scrotum. To our knowledge, this is the first study to apply systematic exome-wide evaluation of mild to moderate undervirilization to better understand the underlying molecular genetics. The findings also have the potential to yield insights into whether these cases share a genetic etiology with more classical DSD presentations.

Methods and Subjects:

Subjects and Clinical History

This study was approved by the Boston Children's Hospital Institutional Review Board. All subjects provided written informed consent and assent (when appropriate). All probands were known to have a 46,XY karyotype and no identified genetic etiology. The majority of cases had physical findings of bifid scrotum with proximal hypospadias, consistent with mild to moderate undervirilization. Presence and location of testes and penile measurements were extracted from medical records. These individuals were all assigned a male gender at birth and had evaluations of hormonal and gonadal function to varying extents depending on clinical providers.

Subject One was born at 39 1/7 weeks gestation by Cesarean section and shortly after delivery was noted to have bifid scrotum, penoscrotal hypospadias, and chordee. The parents were

expecting a female infant based on prenatal imaging. Both testes were palpable in prominent, rugated labioscrotal folds. His stretched phallic length was 2.4 cm and his midshaft diameter was 1.1 cm. An ultrasound confirmed the presence of inguinal testes bilaterally, and revealed absence of Müllerian structures. Laboratory assessment for congenital adrenal hyperplasia due to 21-hydroxylase deficiency and 3β-hydroxysteroid dehydrogenase deficiency ruled out these conditions. He was found to have a 46,XY karyotype. His first testosterone measurement on DOL3 was 21 ng/dL. Labs were remeasured in the setting of an hCG stimulation test at approximately 1 month of age. His baseline and stimulated testosterone levels were 195 (reference 233 +/- 88) and 349 ng/dL respectively, and his dihydrotestosterone levels were 193 (reference 50-600) and 415 pg/mL. He also had a cortisol level of 18.6 mcg/dL and ACTH level of 30 pg/mL (reference <45) included in post-hCG labs. These results were interpreted as demonstrating robust testicular function and normal conversion of testosterone to dihydrotestosterone. After evaluation a male gender was assigned. Subject one also had androgen receptor (*AR*) sequencing completed clinically and this testing did not show any pathogenic variants. He was followed until age 2 without any additional testing. He underwent urologic surgery to repair his hypospadias during this time. At age 2, his penis remained small, and his clinical providers treated him with testosterone enanthate 50 mg IM monthly x 3 doses. This resulted in an increased stretched penile length from 3.2 cm to 4.6 cm, and his midshaft diameter increased from 1.6 cm to 2.1 cm, demonstrating a robust response to testosterone. He was otherwise developmentally appropriate for age.

Subject Two was born at 40 6/7 weeks gestation by vaginal delivery and was also noted to have bifid scrotum, penoscrotal hypospadias, and bilateral 1 mL scrotal gonads. Prenatal ultrasounds had suggested the baby appeared female. His scrotum was rugated and pigmented. His stretched phallic length was 2 cm and his midshaft diameter was 1 cm. He had incomplete scrotal fusion and an anogenital ratio of 0.75. An ultrasound showed normal-

appearing scrotal testes with a blind-ending vaginal pouch and no Müllerian structures. He had an evaluation of adrenal labs that ruled out CAH from 21-OHase deficiency and 3B-HSD deficiency. His karyotype returned as 46,XY. He had a serum testosterone of 48 ng/dL on DOL1 and a stimulated cortisol level of 26.8 mcg/dL at 2 weeks, indicating normal adrenal function. At age 3 weeks, LH was 8.93 IU/L, FSH was 7.97 IU/L, testosterone was 177 ng/dL and dihydrotestosterone was 301 pg/mL. Following 3 days of hCG stimulation, his testosterone rose to 231 ng/dL and dihydrotestosterone to 500 pg/mL, consistent with appropriate testicular function. By one month of age, his penis size increased without treatment to a stretched length of 2.5 cm with a midshaft diameter of 1 cm. This improvement was thought to be related to his endogenous testosterone and, given the appropriate dimensions for age, he did not receive exogenous IM testosterone nor was it recommended that he have androgen receptor sequencing.

Genetic Analyses

Whole-exome sequencing of blood or saliva-derived genomic DNA was performed at the Broad Institute (Cambridge, Massachusetts, USA) on ten probands as well as parents for six of the probands. For hybrid selection, we used the custom Illumina Content Exome capture kit (Illumina, San Diego, California, USA). Sequencing reads were aligned to the hg19 reference genome[13]. We applied the Genome Analysis Toolkit (GATK) for base quality score recalibration and indel (insertion-deletion) realignment[14]. Variant quality score recalibration was simultaneously performed for SNP and indel discovery according to GATK Best Practices recommendations [15,16]. We used SnpEff (http://snpeff.source forge.net/) for functional annotation. We filtered for variants that were novel or found in less than 1% of the reference population based on allele frequencies from the Exome Aggregation Consortium (http://exac.broadinstitute.org/)[17]. Based on the quality standards at the Broad Institute, at least 80% of the exome had 20X coverage. When parental sequencing was performed, we

additionally filtered for de novo variants that were absent in ExAC. Results were reviewed for variants in known DSD related genes previously reported[2] as well as genes near hypospadias GWAS loci[18]. Sanger sequencing was performed to confirm the variants of interest along with *SRY* confirmation when indicated.

Results

The clinical characteristics and laboratory measurements for the ten subjects with bifid scrotum who had exome sequencing performed are detailed in Table 1. Most subjects had severe hypospadias in addition to bifid scrotum. Laboratory measurements during the first few months of life, when the gonadotropin axis is active, were available for half of the subjects.

Exome sequencing identified two subjects with presumed pathogenic mutations in known DSD-associated genes – both in *NR5A1* (Figure 1), which had not been sequenced as part of the clinical evaluation in any of the 10 subjects. Subject One was found to have a heterozygous frameshift variant, c.1150delC, p.Leu384fsTer1 within the ligand binding domain. This frameshift variant is predicted to result in premature cessation of translation, with nearly 80 fewer amino acids than the wild-type protein product. This variant was not present in any of the 60,706 exomes in ExAC. A nearly identical frameshift variant, c.1151del, p.Leu384Argfs*7, was recently reported in a 46,XY female with clitoromegaly and was shown to have significantly decreased activity of the protein product SF-1 on functional assays[19]. Sequencing of Subject One's parents revealed the same variant in the subject's father. The presence of *SRY* was confirmed in Subject One's father to ensure that parental samples were not switched. Evaluation of the father's exome sequencing results did not show any evidence of somatic mosaicism. The father denied any history of genital abnormalities, known hormonal abnormalities, or surgeries.

Subject Two was found to have a heterozygous novel splice-site variant, c.1139-2T>C. The variant disrupts a canonical splice acceptor site and is predicted to perturb the normal splicing of exon 7, which encodes a portion of the ligand binding domain of SF-1. Subject Two's mother was found to have the same *NR5A1* variant. As shown in Table 1, there were no obvious clinical differences between the subjects found to have *NR5A1* variants as compared to the rest of the cohort.

No other pathogenic or likely pathogenic variants in known DSD genes[2], including *AR*, were identified in the cohort of 10 subjects.

Discussion

Establishing genetic diagnoses in individuals with undervirilization has the potential to improve the care of these patients. As next generation sequencing costs continue to decrease, more comprehensive sequencing approaches have already started to supplant targeted single-gene testing. Prior exome-based studies have focused on classic DSD cases with ambiguous genitalia[2]. To our knowledge, this study is the first to apply whole-exome sequencing to a cohort of cases with bifid scrotum and hypospadias but not frank genital ambiguity.

In our study of bifid scrotum cases, 2 of the 10 patients were found to have *NR5A1* variants that are likely to be pathogenic. This finding is consistent previous reports that found pathogenic *NR5A1* variants in 10-20% of DSD cases with varying degrees of undervirilization without adrenal insufficiency[5]. In a related study, we found that androgen receptor mutations were identified in 2 of 10 patients with bifid scrotum during clinical evaluation (Swartz et al., manuscript in preparation). Thus, either whole-exome sequencing or targeted sequencing of *AR* and *NR5A1* may provide a diagnosis in a significant proportion of 46,XY cases with bifid scrotum. This information can guide understanding of recurrence risk in a family and help

inform expected progression of symptoms, appropriate dose of hormonal supplementation (if needed), tumor risk, and fertility.

A number of NR5A1 mutations have been previously described in the literature. Heterozygous or homozygous *NR5A1* mutations can cause DSD, and its gene product SF-1 plays a role in both gonadal and adrenal development as well as steroidogenesis. SF-1 is an orphan nuclear receptor with a central role as a transcription factor for regulation of adrenal and gonadal developmental genes including *SOX9*, *NR0B1*, and *AMH* as well as many genes encoding enzymes essential for steroid hormone synthesis[20]. Previous reports have noted that the degree of undervirilization appears greater than the degree of testicular dysgenesis in many of the described cases[20].

The observation of paternal Inheritance of the *NR5A1* mutation in Subject One is an unusual finding from this study. To date, the vast majority of familial *NR5A1* mutations have been inherited maternally[21,22], as in the case of Subject Two. There is a report of a missense *NR5A1* variant inherited from an unaffected father with normal external genitalia and maintained fertility, but in that case the father was believed to have low level somatic mosaicism[21]. Our finding of a frameshift variant inherited from Subject One's unaffected father is very unusual in the *NR5A1* literature, especially given the lack of evidence of somatic mosaicism on sequencing results. This provides an example of the incomplete penetrance in *NR5A1* cases. The splice site variant is Subject Two is also relatively uncommon in the *NR5A1* literature, with only a few reports of splice site variants in *NR5A1* published to date [12,23].

This study highlights that normal testosterone levels during infancy, either at baseline or after hCG stimulation, do not rule out *NR5A1* mutations. Most prior reports found low levels of testosterone in undervirilized *NR5A1* cases, though there have been some reports of normal

androgen biosynthesis in such patients [6,11,12,24]. The evidence of undervirilization at birth indicates decreased androgen signaling during early fetal development, but this signaling abnormality may not be evident on later testing. This raises a yet-unanswered question about the role of SF-1 in steroidogenesis during early fetal life compared to postnatal life. This may be an indication that intra-individual variability on the impact of SF-1 variants on steroidogenesis at different developmental times (e.g., in utero vs postnatal life) may be as relevant as inter-individual variability seen within families with the same *NR5A1* variants[22].

This study also demonstrates the utility of genetic testing in patients with bifid scrotum. Exome sequencing was shown to be high-yield in more severe DSD cases, with a likely genetic diagnosis being identified in about one-third of patients with 46,XY DSD [2]. Our study suggests that exome sequencing is similarly high yield in milder cases of undervirilization. There is evidence that a significant fraction of bifid scrotum cases may have an identifiable genetic mutation that will improve disease understanding as well as be important for families interested in having additional children. These findings have significant implications for families and their management in the clinical setting. Based on our findings, we feel it is reasonable to perform a genetic evaluation for all children with bifid scrotum, even those with a normal hormonal profile, as the genetic evaluation is reasonably likely to establish a diagnosis, which in turn can guide counseling about risk for future siblings, treatment, and prognosis.

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Figure 1

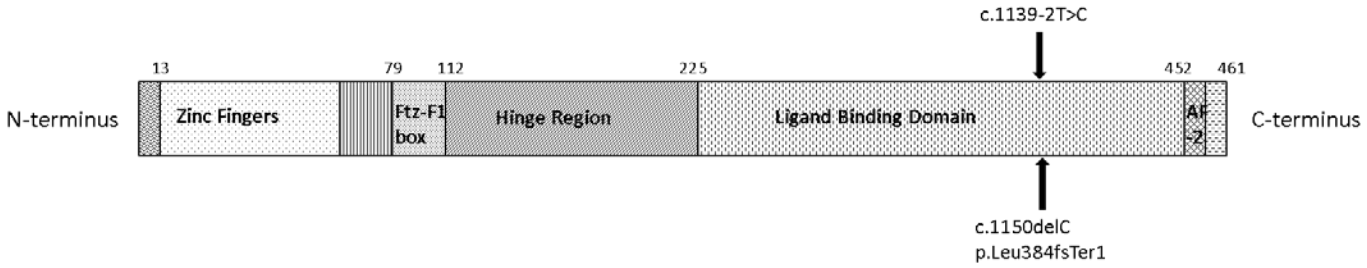

Figure 1: *NR5A1* gene structure, with location of heterozygous mutations in Subjects One and Two. Both mutations involved exon 7 in the ligand binding domain. (adapted from Camats, JCEM, 2012) AF-2: activation function domain 2
*Figure not according to scale.

Table 1

ID	GA	Clinic Visit	Phallic Stretched Length*	Phallic Midshaft Diameter*	Hypospadias Degree	Gonadal Location	Testosterone ** (ng/dL)	DHT** (pg/mL)	LH** (IU/L)	FSH** (IU/L)	AR Sequencing	Non-GU Abnormalities
1	39.1	Both	2.1	0.9	Penoscrotal	Scrotal	195	193	4.34	NR	Y, no dx	None
2	40.9	Both	2	1	Perineal	Scrotal	177	301	8.93	7.97	N	None
3	40.0	Urology	NR	NR	No hypospadias	Scrotal	NR	NR	NR	NR	N	Tethered cord, anteriorly placed anus, renal abnormalities
4	33.0	Both	2.5	1.1	Penoscrotal	Scrotal	132	462	3	3.34	N	Intrauterine growth restriction
5	41.0	Both	3.2	1.1	Perineal	Inguinal	NR	NR	NR	NR	N	None
6	34.7	Both	NR	NR	Scrotal	Scrotal	207	71	NR	NR	Y, no dx	None
7	37.0	Both	NR	NR	Scrotal	Scrotal	NR	NR	NR	NR	N	Global developmental delay
8	35.3	Urology	NR	NR	Penoscrotal	Scrotal	NR	NR	NR	NR	N	None
9	39.1	Urology	NR	NR	Glanular	Scrotal	NR	NR	NR	NR	N	Ventricular Septal Defect
10	39.0	Both	2	1.1	Perineal	Scrotal	85.9	29	5.23	4.62	N	None

GA, Gestational age in weeks
NR, not reported
Clinic visit: Endocrinology, Urology or both (at any point in the EMR)
*In cm, initial measurement reported between birth and 3 months of age)
** Baseline measurement between 2 weeks and 3 months