How Far Should We Explore Hypospadias? Next-generation Sequencing Applied to a Large Cohort of Hypospadiac Patients

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Abstract

Background: Next-generation sequencing (NGS) is generally used for patients with severe disorders of sex development (DSD). However, NGS has not been applied extensively for patients with hypospadias only, and most affected children do not benefit from an etiological diagnosis.
Objective: To evaluate the clinical usefulness of NGS for patients with hypospadias, regardless of severity.
Design, setting, and participants: Prospective multicenter research included 293 children with glandular to penoscrotal hypospadias (no undescended testis and no micropenis). After excluding likely pathogenic androgen receptor (*AR*) variants by Sanger sequencing, an NGS panel tested 336 genes including unexplored candidates in 284 patients.
Outcome measurements and statistical analysis: The rate of pathogenic and likely pathogenic variants was assessed using REVEL, ClinVar, and in-house tools (Captain-ACHAB, MobiCNV, and MobiDetails).
Results and limitations: Likely pathogenic variants were identified in 16 (5.5%) patients with both Sanger sequencing and NGS taken into account. Some genes were related to DSD (*AR*, *NR5A1*, *HSD17B3*, and *MAMLD1*), but reverse phenotyping revealed two syndromic disorders with midline defects (*MID1*) and alteration in the retinoic acid signaling pathway (*RARA*). Coverage analysis revealed an 18q deletion. Identification of likely pathogenic variants increased with hypospadias severity. Other variants of unknown significance (VUSs) in genes implicated in hypogonadotropic hypogonadism, Noonan syndrome, and genital tubercle development were also identified. Genetic study mainly focused on exonic variants, and most cases remain unexplained.
Conclusions: NGS reveals minor forms of DSD, undiagnosed syndromes, or candidate rare variants in new genes, indicating that even patients with mild hypospadias benefit from advanced sequencing techniques. Early molecular diagnosis would help improve

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follow-up at puberty and medical counseling for initially undiagnosed syndromes. Future studies will improve the diagnosis by investigating the contribution of VUSs.
Patient summary: Next-generation sequencing enables simultaneous testing of numerous genes and should not be limited to disorders of sex development cases. Even patients with mild hypospadias would benefit from early diagnosis of a genetic defect implicated in sex development or other syndromes.

1. Introduction

The disorders of sex development (DSD) include a broad range of congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical [1]. The most frequent situation is 46,XY DSD with severe hypospadias and associated micropenis and/or undescended testis, which poses both therapeutic and pathophysiological challenges. Identification of the cause of DSD provides the rationale for optimal treatment, guides pubertal follow-up, and helps in parental counseling. Thus, patients with DSD usually benefit from extensive hormonal and genetic exploration.

In contrast, patients with hypospadias, especially when it is mild and not associated with other genital abnormality, are underexplored in routine clinical practice. While it is still debated whether isolated mild hypospadias should be termed DSD, genetic testing demonstrates that it may share a common etiology with more severe forms [2–4]. Hypospadias is the second most frequent genital defect (one in 150 to one in 250 boys), with an increasing prevalence over the years in some regions [5]. Hormonal disturbance and pubertal deficiency [6] are possible, but these children are usually not offered multidisciplinary long-term follow-up. Undiagnosed genetic conditions may thus be deleterious in this population.

Various pathways are involved in the development of the genital tubercle, including gonadal determination, steroidogenesis, and several other signaling pathways: androgen receptor (AR), Hedgehog, canonical wingless-type MMTV integration site family (WNT), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP). Although all are suspected of contributing to hypospadias, few genes have unambiguously been linked to the mild phenotype [2,7,8]. The *AR* gene was initially described in partial or complete androgen insensitivity syndromes, but we reported pathogenic/likely pathogenic (P/LP) variants in mild hypospadias without micropenis or undescended testis [3]. This suggests the relevance of genetic explorations including a wider range of genes and a wider range of phenotypes. Among the new candidate genes are those expressed in the genital tubercle, as revealed by gene expression profiling in the mouse urethral plate [9]; those expressed in the testis during the window of masculinization [4]; and those associated with hypospadias in knockout mice models. Evidence of all of them is still lacking in human pathology [7].

Over the last decade, next-generation sequencing (NGS) has emerged as a powerful tool to investigate Mendelian disorders. Targeted NGS panels and whole exome sequencing (WES) are now diagnostic tools in severe DSD [10–20], but their clinical value in mild isolated hypospadias is unknown. In the present prospective study, we designed an NGS panel of 336 known and candidate genes, and explored a cohort of 284 patients with isolated hypospadias of various severities. Based on our findings of rare, likely and possibly deleterious variants, we propose to enlarge the spectrum of phenotypes for which genetic exploration should be offered.

2. Patients and methods

2.1. Patients

A total of 297 boys with isolated hypospadias (no micropenis and no undescended testis) were prospectively included from 2009 to 2013 in a multicenter study (from newborn to 17-yr old; mean age: 3.2 yr, median age: 1.5 yr). This included the remaining *AR*-negative patients of our previous cohort [3]. Clinical diagnosis was made by direct clinical examination by a pediatric urologist and/or endocrinologist. The measurement of the penis was standardized by measuring the dorsal aspect of the penis from its palpable base after skin retraction to the tip of the glans. Patients with known karyotype abnormalities were excluded ($n=4$: two Klinefelter syndrome, one mosaic 45,X/46,X,i(Y)(p10), and one mosaic trisomy 14). The location of the urethral meatus ranged from glandular to perineal (glandular, coronal, and penile anterior: $n=204$; midshaft: $n=64$; penile posterior: $n=18$; and penoscrotal: $n=7$) [21]. Hypospadias was the reason for referral. Patients with extragenital phenotypes such as neurodevelopmental delay or ophthalmic signs were typically not diagnosed on a molecular basis at the time of inclusion and thus were not excluded. Regarding internal structures, ultrasonography has low sensitivity for the detection of Müllerian remnants. Magnetic resonance imaging, which would have required general anesthesia given the age of most of our patients, and laparoscopy were not performed considering their morbidity for mild hypospadiac patients.

This study was approved by the Institutional Review Board of the institution (Centre Protection Personnes Sud-Méditerannée 4, CPPSMIV, ID-RCB-n°2008-A00781-54) and written consent was obtained from parents.

2.2. Next-generation DNA sequencing

Most patients were first screened by Sanger sequencing, and P/LP variants or variants of unknown significance (VUSs) in *AR* were found in nine patients, as reported previously [3]. Then, NGS was performed for the 284 remaining patients. Genes included in the NGS panel were selected using the OMIM database (keywords: hypospadias, genital differentiation, genital tubercle, and disorder of sex development) and PubMed interrogation (keywords: hypospadias gene, external genitalia gene, genital tubercle development, disorder of sex development, midline gene, gonadal determination, and sex differentiation gene). A gene was included in the panel if: (1) variants of the gene were reported to be associated with hypospadias in humans; (2) variants of the gene

were reported in a condition associated with hypospadias; (3) the gene was known to encode for a protein expressed in the genital tubercle, external genitalia, or gonads in animal and human studies; or (4) an animal knockout model for the gene exhibited a genital defect. The panel was not specifically designed to detect syndromes, but some of the genes linked to hypospadias and sequenced in this study may have been reported in some syndromes. The final panel included 336 genes (Supplementary material), representing 1.19 Mb, which were sequenced with 35 181 probes. Details about sequencing, variant calling, and data analysis can be found in the Supplementary material. For each rare sequencing variant (RSV), we took into account whether or not it was found in multiple patients, a control group of 288 healthy children, our in-house database, or the literature using LitVar [22] and VarSome [23]. RSVs located in new candidate genes were retained only if found in patients or already described in the literature. Last, RSVs were classified according to the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology [24] using VarSome [23]. The inheritance mode was not available and therefore was not implemented. We then used reverse phenotyping in cases of P/LP variants or VUS identification, and the patients' medical records were checked for any relevant sign that could have been diagnosed after the management of the hypospadias.

3. Results

3.1. Genes classically involved in genital defects

We first focused on a subpanel of genes known to be involved in 46,XY DSD according to previous studies (see the Supplementary material). We found LP variants in the *NR5A1* gene in three patients and one P variant in the *MAMLD1* gene in another (Table 1). *NR5A1* encodes SF-1, and variants are implicated in gonadal dysgenesis (OMIM #612965). *MAMLD1* variants induce X-linked isolated hypospadias (OMIM #300758). The OMIM data indicate hypospadias as linked to HYSP1 (linked to *AR*) and HYSP2 (linked to *MAMLD1*). We also identified two patients, each with two heterozygous variants in the *HSD17B3* gene (OMIM #264300). These eight variants are rare (variant allele frequency [VAF] range from 0 to 10^{-3}), and seven of them were absent in 288 control boys. Five of these seven variants are reported for the first time in hypospadias.

Interestingly, we found simple heterozygosity for 25 patients in DSD-associated genes usually linked to autosomal recessive inheritance (Table 1), mainly *HSD17B3* and *SRD5A2* (OMIM #264600). VUSs were also identified in genes associated with autosomal dominant or X-linked inheritance in eight patients (Table 1). No potential impact on splicing sites was predicted, except for the *HSD17B3* c.277+4A>T and *DMRT1* c.823-1G>C variants (Table 1).

3.2. Syndromes revealed by hypospadias

We then looked for variants in genes responsible for syndromes where hypospadias is one of the main features. Using reverse phenotyping, we identified two P/LP variants in *MID1* and *RARA* (Table 2), which are, respectively, altered in X-linked Opitz G/BBB syndrome (OS; OMIM #300000) and another recently described syndrome [25]. The patient with the *MID1* variant and anterior hypospadias was ultimately diagnosed with facial asymmetry, a nasoethmoidal dermoid cyst, nasal septum deviation, and a coccygeal dermal sinus. The patient with the *RARA* variant was secondarily diagnosed with dysmorphic features, cervical vertebral fusion and scoliosis, hypotonia, clinodactyly, bronchial atrophy with bronchocele, right sigmoid kidney with fused kidneys, secondary retractile testis, and moderate neurodevelopmental delay.

3.3. New candidate genes

This NGS panel also screened new candidate genes reported in animal studies but not yet reported in human hypospadias. We found several significant RSVs in new relevant genes (Table 2). Most genes are involved in congenital hypogonadotropic hypogonadism (CHH), which has not yet been linked to isolated hypospadias. We also found RSVs in genes implicated in the development of the genital tubercle (*SHH*, *WNT5A*, *BMP2*, and *HOXD13*; Table 2) or Noonan syndrome and related disorders (NSRD; *PTPN11* and *BRAF*).

3.4. Coverage analysis

Using MobiCNV, we checked that each patient had a chromosomal dosage compatible with a 46,XY karyotype. We found evidence for a *CYB5A* heterozygous deletion in a boy with midshaft hypospadias. He was then diagnosed with developmental delay, hypotonia, kidney asymmetry, epicanthus, and clinodactyly. *CYB5A* is located on 18q22.3, and biallelic alterations of *CYB5A* can lead to methemoglobinemia and ambiguous genitalia (OMIM #250790). However, the remaining *CYB5A* allele was normal in our case and we hypothesized a distal 18q deletion, which is known to be associated with hypospadias (OMIM #601808). Subsequent chromosomal microarray analysis confirmed a de novo 12.4 Mb heterozygous deletion (ISCN: arr[GRCh38] 18q22.1q23(63253358_75721820)x1 *dn*, with a SurePrint G3 Human CGH Microarray 4x180K from Agilent Technologies, Santa Clara, CA, USA), which was assumed to be responsible for the whole clinical picture. No pathogenic RSV was found for this patient.

4. Discussion

4.1. Overall performance of NGS in hypospadias

NGS provides higher throughput than Sanger sequencing and new opportunities to look for RSVs. Previous reports have mainly focused on complex DSD with severe phenotypes or uncertain sex, which is expected to be more often linked to monogenic etiology [26]. Some reports have described panel use in hypospadias, but in association with micropenis or undescended testis for the vast majority of patients [8,19]. We report the largest study applying NGS to a cohort of patients with mainly mild forms of hypospadias and no associated genital defects (micropenis or undescended testis). These phenotypes are usually not explored, in line with recommendations [26]. We provided a

Table 1 – Variants found in genes classically involved in DSD: P or LP variants (diagnosis confirmed); a simple P, LP, or VUS variant in a gene classically associated with autosomal recessive inheritance; and VUS in a gene classically associated with autosomal dominant or X-linked inheritance (might be incomplete penetrance)

Patient (y/o at referral)	Hypospadias	Gene	rsID	HGVS_ transcript	HGVS_ protein	ACMG	Found in control	gnomAD v2 VAF	REVEL score	REVEL interpretation	ClinVar/ARdb interpretation
P/LP variants											
1 (9)	Anterior	*NR5A1*	rs762769507	c.763C>T	p.(Arg255Cys)	LP	–	4.9E-05	0.939	Damaging	Not reported
2 (0.5)	Anterior	*NR5A1*	rs141502483	c.769G>A	p.(Asp257Asn)	LP	–	3.3E-05	0.412	Uncertain	Not reported
3 (0.5)	Anterior	*NR5A1*	rs1478477850	c.779C>T	p.(Ala260Val)	LP	–	4.1E-06	0.308	Uncertain	Not reported
4 (0.5)	Posterior	*MAMLD1*	–	c.885del	p.(Leu296TyrfsTer12)	P	–	0	NA	NA	Not reported
5 (1)	Penoscrotal	*HSD17B3*	–	c.694_698delinsCCCATA			p.				
			rs139084702	c.133C>T							
					(Ser232ProfsTer18)	P	–	0	NA	N/A	Not reported
					p.(Arg45Trp)	LP	2	1.8E-03	0.392	Uncertain	LB/VUS
6 (1.5)	Anterior	*HSD17B3*	rs201115371	c.277+4A>T	p.?	P	–	3.3E-04	NA	NA	P
			rs372430180	c.902C>T	p.(Ala301Val)	LP	–	8.0E-06	0.110	Benign	Not reported
Simple heterozygous variants in genes associated with autosomal recessive inheritance											
7 (1)	Penoscrotal	*HSD17B3*	rs201115371	c.277+4A>T	p.?	P	–	3.3E-04	NA	NA	P
8 (1)	Anterior	*HSD17B3*	rs191153391	c.139A>G	p.(Met47Val)	VUS	–	6.4E-05	0.390	Uncertain	Not reported
9 (1)	Anterior	*HSD17B3*	rs139084702	c.133C>T	p.(Arg45Trp)	VUS	2	1.8E-03	0.392	Uncertain	LB/VUS
10* (2)	Anterior	*HSD17B3*	rs868469733	c.50G>A	p.(Cys17Tyr)	VUS	–	4.0E-06	0.480	Uncertain	Not reported
11 (1.5)	Anterior	*HSD17B3*	rs200961609	c.329A>G	p.(Asp110Gly)	VUS	–	1.1E-04	0.366	Uncertain	Not reported
12 (2.5)	Midshaft	*HSD17B3*	rs772813211	c.883C>T	p.(Leu295Phe)	VUS	–	1.6E-05	0.191	Benign	Not reported
13 (5)	Posterior	*HSD17B3*	rs370264627	c.641A>G	p.(Glu214Gly)	VUS	1	1.2E-05	0.250	Uncertain	Not reported
14 (1)	Anterior	*HSD17B3*	rs116436956	c.304A>T	p.(Ile102Phe)	VUS	–	2.7E-04	0.475	Uncertain	LB
15 (10)	Midshaft	*HSD3B2*	–	c.453del	p.(Thr152LeufsTer22)	P	–	0	NA	NA	Not reported
16 (2.5)	Posterior	*SRD5A2*	rs9332964	c.680G>A	p.(Arg227Gln)	LP	–	4.7E-04	NA	NA	P/LP
17 (1)	Anterior	*SRD5A2*	rs34552434	c.644C>T	p.(Ala215Val)	VUS	–	1.6E-05	NA	NA	Not reported
18 (6)	Anterior	*SRD5A2*	–	c.223C>G	p.(Leu75Val)	VUS	–	0	NA	NA	Not reported
19 (2)	Anterior	*SRD5A2*	–	c.223C>G	p.(Leu75Val)	VUS	–	0	NA	NA	Not reported
20* (0.5)	Anterior	*DHH*	–	c.191A>T	p.(Glu64Val)	VUS	–	0	0.914	Damaging	Not reported
21* (0)	Midshaft	*STAR*	rs34908868	c.361C>T	p.(Arg121Trp)	VUS	–	1.7E-03	0.527	Damaging	B/VUS
22 (2.5)	Anterior	*STAR*	rs550388651	c.157C>G	p.(Arg53Gly)	VUS	–	8.5E-05	0.434	Uncertain	Not reported
23 (5)	Anterior	*STAR*	rs779586809	c.164G>A	p.(Arg55Gln)	VUS	–	1.8E-05	0.239	Uncertain	Not reported
24 (0)	Anterior	*POR*	rs199634961	c.344C>T	p.(Ala115Val)	VUS	–	1.8E-04	0.481	Uncertain	LB
25 (3.5)	Anterior	*POR*	rs367810540	c.1115C>T	p.(Thr372Met)	VUS	–	1.8E-04	0.652	Damaging	Not reported
26 (0.5)	Midshaft	*POR*	rs367810540	c.1115C>T	p.(Thr372Met)	VUS	–	1.8E-04	0.652	Damaging	Not reported
27 (0.5)	Anterior	*POR*	rs782248163	c.1586C>T	p.(Thr529Met)	VUS	–	6.3E-05	0.435	Uncertain	Not reported
28 (2.5)	Anterior	*POR*	rs72557954	c.1820A>G	p.(Tyr607Cys)	VUS	–	1.0E-04	0.752	Damaging	Not reported
29 (1)	Anterior	*POR*	–	c.1988A>G	p.(Asp663Gly)	VUS	–	4.0E-06	0.516	Damaging	Not reported
Variants in genes associated with autosomal dominant or X-linked inheritance											
30 (1)	Posterior	*AR*	rs200390780	c.1424C>T	p.(Ala475Val)	VUS	–	1.4E-03	0.539	Damaging	VUS/MAIS
31 (3)	Anterior	*AR*	rs758361525		c.1368_1370dup		p.			(Gly473dup)	VUS
–	7.2E-04	NA	NA								
				Not reported							
32 (1)	Anterior	*NR5A1*	rs761496130	c.460G>A	p.(Ala154Thr)	VUS	–	2.2E-05	0.270	Uncertain	VUS
21* (0)	Midshaft	*WT1*		c.1479T>G	p.(Ser493Arg)	VUS	–	0	0.252	Uncertain	Not reported
33 (2)	Midshaft	*WT1*	rs138073760	c.830C>T	p.(Thr277Ile)	VUS	–	1.5E-05	0.415	Uncertain	Not reported
34 (1)	Anterior	*DMRT1*	–	c.823-1G>C	p.?	VUS	–	0	NA	NA	Not reported
35 (0)	Anterior	*SOX9*	–	c.92C>T	p.(Ala31Val)	VUS	–	0	0.283	Uncertain	Not reported
36 (2)	Anterior	*WNT4*	rs369780122	c.742C>T	p.(Arg248Cys)	VUS	–	4.0E-05	0.460	Uncertain	Not reported

B = benign; LB = likely benign; LP = likely pathogenic; MAIS = variant linked to mild androgen insensitivity syndrome in the McGill *AR* database (ARdb: http://androgendb.mcgill.ca/); NA = not applicable; P = pathogenic; RSV = rare sequencing variant; VAF = variant allele frequency; VUS = variant of unknown significance; y/o = years old.
Only VUSs of potential significance are reported. Patients presenting with multiple RSVs are shown with an asterisk (*).

diagnosis for at least 16/293 patients (5.5% compared with 0/25 in a previous study [19]) based on P/LP variants only. If the next series confirm the role of VUSs identified in our patients, whatever the mode of inheritance, NGS performance may reach 24% in the future (Table 3 and Fig. 1). The role of simple heterozygosity in high candidate recessive genes (Table 1) may also be underevaluated. A dose effect of gene expression or an associated variant located in an unexplored region of the gene or on the second allele is highly possible. Molecular diagnosis is known to influence the outcome of patients with complex genital defects [27], and genetic findings may also influence the outcome of hypospadiac patients [6]. For example, an LP variant of the *AR* gene may justify close follow-up of penile growth during puberty and evaluation of fertility in the future. RSVs in genes involved in CHH and *HSD17B3* also raise the question

Table 2 – Variants found in syndromic and candidate genes: LP variant (diagnosis confirmed) and variants in candidate genes

Patient	Hypospadias	Gene	rsID	HGVS_ transcript	HGVS_ protein	ACMG	Found in control	gnomAD v2 VAF	REVEL score	REVEL interpretation	ClinVar interpretation
P/LP variants											
37 (4.5)	Anterior	*MID1*	rs149482288	c.1561C>T	p.(Arg521Cys)	LP	–	5.1E-04	0.396	Uncertain	LB
38* (2)	Anterior	*RARA*	rs786205678	c.826C>T	p.(Arg276Trp)	LP	–	0	0.886	Damaging	Not classified
Simple heterozygous variants and other VUSs											
39 (2.5)	Midshaft	*RARA*	rs373673287	c.742G>A	p.(Gly248Ser)	VUS	–	4.0E-05	0.782	Damaging	Not reported
40* (0.5)	Posterior	*RARA*	–	c.72C>G	p.(Tyr24Ter)	VUS	–	0	NA	NA	Not reported
41* (5)	Anterior	*PTPN11*	rs397507521	c.455G>A	p.(Arg152His)	VUS	–	1.8E-05	0.727	Damaging	VUS
42 (3)	Midshaft	*BRAF*	–	c.16_40del	p.?	VUS	–	0	NA	NA	Not reported
43 (9.5)	Posterior	*WNT5A*	–	c.487G>A	p.(Gly163Ser)	VUS	–	0	0.605	Damaging	Not reported
44 (0.5)	Anterior	*WNT5A*	–	c.895C>A	p.(Leu299Met)	VUS	–	0	0.817	Damaging	Not reported
45 (8.5)	Anterior	*WNT5A*	–	c.65C>T	p.(Ser22Phe)	VUS	–	0	0.303	Uncertain	Not reported
46 (5.5)	Anterior	*WNT5A*	rs781527130	c.907G>A	p.(Asp303Asn)	VUS	–	4.0E-06	0.416	Uncertain	Not reported
47 (7)	Posterior	*SHH*	rs746239519	c.1373C>A	p.(Ala458Glu)	VUS	–	0	0.473	Uncertain	Not reported
48 (0.5)	Midshaft	*SHH*	–	c.935G>C	p.(Gly312Ala)	VUS	–	0	0.932	Damaging	Not reported
40* (0.5)	Posterior	*SHH*	–	c.581A>G	p.(Lys194Arg)	VUS	–	0	0.687	Damaging	Not reported
49 (1)	Anterior	*SHH*	–	c.247G>A	p.(Asp83Asn)	VUS	–	0	0.834	Damaging	Not reported
50 (1.5)	Posterior	*WDR11*	–	c.86G>A	p.(Trp29Ter)	VUS	–	0	NA	NA	Not reported
38* (2)	Anterior	*WDR11*	rs745856006	c.2243A>G	p.(His748Arg)	VUS	–	8.0E-06	0.413	Uncertain	Not reported
51 (1)	Anterior	*WDR11*	rs745952055	c.3331C>T	p.(Arg1111Trp)	VUS	–	1.2E-05	0.835	Damaging	Not reported
52 (1)	Posterior	*WDR11*	–	c.3518-38_3530dup	p.?	VUS	–	0	NA	NA	Not reported
53 (1)	Anterior	*GNRHR*	rs104893837	c.785G>A	p.(Arg262Gln)	VUS	1	1.8E-03	0.726	Damaging	P
54 (8)	Midshaft	*FGFR1*	–	c.455C>T	p.(Ala152Val)	VUS	–	0	0.421	Uncertain	Not reported
55 (4)	Anterior	*KAL1*	rs375767556	c.1921G>A	p.(Gly641Arg)	VUS	–	4.4E-05	0.730	Damaging	LB
56 (1.5)	Anterior	*PROKR2*	rs149992595	c.868C>T	p.(Pro290Ser)	VUS	–	1.3E-04	0.939	Damaging	Not reported
57 (0)	Midshaft	*PROKR2*	–	c.747G>C	p.(Glu249Asp)	VUS	–	8.0E-06	0.287	Uncertain	Not reported
58 (2.5)	Midshaft	*PROKR2*	rs149396342	c.403C>T	p.(Arg135Cys)	VUS	–	5.0E-05	0.266	Uncertain	VUS
20* (0.5)	Anterior	*CHD7*	–	c.3202G>A	p.(Gly1068Ser)	VUS	–	0	0.828	Damaging	Not reported
10* (2)	Anterior	*CHD7*	rs369550114	c.7591C>T	p.(Arg2531Trp)	VUS	–	2.5E-05	0.558	Damaging	Not reported
41* (5)	Anterior	*CHD7*	rs187751757	c.8740G>A	p.(Gly2914Arg)	VUS	–	2.9E-04	0.399	Uncertain	LB
59 (0)	Anterior	*HHAT*	–	c.498C>G	p.(Tyr166Ter)	VUS	–	0	NA	NA	Not reported
60 (0.5)	Anterior	*PROP1*	rs121917842	c.218G>A	p.(Arg73His)	VUS	–	8.0E-06	0.763	Damaging	P
61 (10)	Anterior	*BMP2*	rs549080568	c.551C>G	p.(Ser184Trp)	VUS	–	8.0E-06	0.398	Uncertain	Not reported
62 (0)	Anterior	*HOXD13*	rs1377323943	c.218G>T	p.(Gly73Val)	VUS	–	5.3E-06	0.333	Uncertain	Not reported

LB = likely benign; LP = likely pathogenic; NA = not applicable; P = pathogenic; RSV = rare sequencing variant; VUS = variant of unknown significance.
In the absence of consistent evidence of pathogenicity (eg, clinical data, functional analyses), variants in candidate genes were all classified as VUSs. Only VUSs of potential significance are reported. Patients presenting with multiple RSVs are shown with an asterisk (*).

Table 3 – Number of confirmed diagnoses (with pathogenic and likely pathogenic variants only), according to hypospadias severity

	Patient number	Diagnosis by Sanger	Diagnosis by NGS	Overall diagnoses	Overall yield (%)
Anterior	204	4	6	10	4.9
Midshaft	64	2	1	3	4.7
Posterior	18	1	1	2	11.1
Penoscrotal	7		1	1	14.3
Total	293	7	9	16	5.5

NGS = next-generation sequencing.

of an endocrine evaluation and potential support for pubertal development. Long-term studies are thus needed.

4.2. *No genotype-phenotype correlations in hypospadias*

The diagnosis rate increased with hypospadias severity (Table 3). We showed that *AR* variants may be related to a milder phenotype than expected [3]. We extended the phenotype associated with *NR5A1* and *RARA* variants and described VUSs in new candidate genes. We applied strict criteria for considering a variant as pathogenic in our series, which may have resulted in an underestimation of the performance of the genetic screening. Incomplete penetrance can lead to a wide spectrum of phenotypes and the misinterpretation of real P variants such as VUSs. Examples in the *AR* gene include p.(Ala475Val) and p.(Pro492Ser), which are relatively frequent in the general population (ie, VAF of 0.001 and 0.004, respectively). The corresponding phenotypes may be isolated infertility or hypospadias of varying severity [3,12,28,29]. The ACMG-AMP guidelines

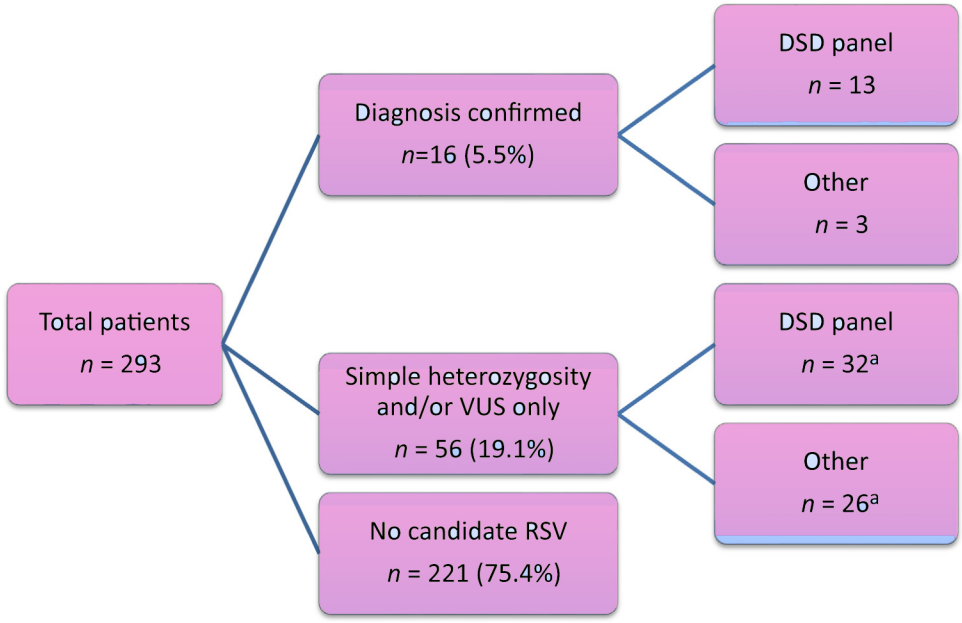

Fig. 1 – Summary of findings of our genetic analyses (NGS and Sanger, combined). Data are separated by result and by type of gene. DSD = disorders of sex development; NGS = next-generation sequencing; RSV = rare sequencing variant; VUS = variant of unknown significance. [a] Two patients had only VUS and/or simple heterozygosity in both DSD panel and other genes.

suggest that these variants are VUSs, but these guidelines might be too stringent for mild disorders because they do not take into account incomplete penetrance, variable expressivity, and a wide phenotypic spectrum. Some endocrine or fertility defects may indeed be revealed later in life [30] and may be directly related to the variants. Considering the young age of the patients, we were unable to address this point.

Similarly, biallelic *HSD17B3* variants are usually reported in severe genital defects; yet, it is conceivable that a moderate alteration in gene dosage would explain the residual activity of the enzyme and the mild genital defect. Six out of eight patients with *HSD17B3* single heterozygosity had minor hypospadias in our series. RSVs of *HSD17B3*, which is implicated in androgen biosynthesis, thus raise the question of the potential impact of mild haploinsufficiency in hypospadias.

4.3. *Syndromes*

Hypospadias is reported in >200 syndromes, but very few children are diagnosed at the time of hypospadias referral. In our series, no patient had a diagnosis of a syndrome at the time of inclusion. In some patients, secondary associated defects in accordance with our genetic findings were revealed over time. Others benefited from a complete workup following our results. NGS for minor genital defects could thus be of interest early in life to optimize diagnosis and propose a multidisciplinary follow-up of syndromic boys.

The *MID1* p.(Arg521Cys) variant was published once in a Chinese family with OS [31]. This midline genetic disorder, caused by alteration of the E3-ubiquitin ligase Midline-1, is mainly characterized by hypertelorism, hypospadias, and laryngotracheoesophageal abnormalities (OMIM #30000) and is consistent with our patient's phenotype. The *RARA* p.(Arg276Trp) variant was described only recently in a girl with various abnormalities, including coloboma, minor dysmorphic features, enlarged pulmonary trunk, additional spleen, and ectopic left kidney [25]. Although incomplete, the overlap with our patient harboring the same variant is notable. This strong genotype-phenotype correlation is probably linked to the functional impact of Arg276 substitution on retinoic acid binding [32]. Undoubtedly, early diagnosis would have helped provide this patient with better care. Regarding the other RSVs we found in *RARA* (Table 2), functional analyses or larger studies would help evaluate their contribution to hypospadias.

4.4. *New candidate genes*

Given the limited number of genes directly linked to human hypospadias [7], we investigated new candidate genes and report novel RSVs predicted as deleterious. Some are in genes mediating Hedgehog signaling (Table 2). *SHH*, a gene altered in holoprosencephaly with incomplete penetrance and variable expressivity, is central in the development of the genital tubercle and involves *WNT5A* as a transcriptional target [7,9]. P variants of *WNT5A* and subsequent alterations of the Hedgehog pathway may be associated with hypospadias, as in Robinow syndrome (OMIM #180700). We identified several variants of *WNT5A* in patients with hypospadias only. Whether mildly deleterious RSVs in *WNT5A* could trigger hypospadias as an isolated sign of Robinow syndrome is intriguing.

We also found RSVs in genes involved in CHH (Table 2). Hypospadias usually does not belong to the spectrum of

CHH, but it is well documented that some patients with isolated hypospadias ultimately reveal endocrine dysfunction at puberty, including hypogonadotropic hypogonadism [33]. More recently, two series of Indonesian and Han Chinese boys with various degrees of undervirilization, including hypospadias, reported variants in CHH genes, such as *PROKR2*, *WDR11*, *CHD7*, or *FGFR1*, and not in DSD genes [34,35]. In our series, several patients exhibited heterozygous *PROKR2* variants, and one of them should be considered an LP variant, according to VarSome. The definitive demonstration that RSVs in CHH genes induce hypospadias would nevertheless require long-term follow-up during puberty.

Genes usually involved in NSRD (*PTPN11* and *BRAF*) may also explain the incomplete forms of Noonan syndrome that include hypospadias. Patient 41 with anterior hypospadias harbored the *PTPN11* p.(Arg152His) variant, which is located in a domain where missense variants are not expected to be responsible for NSRD [36]. This may explain a partial form of the syndrome limited to the genital defect. The rarity of this variant—absent from gnomAD—and its prediction as damaging are indeed intriguing. The same questions can be raised about loss-of-function variants such as the *BRAF* deletion in a midshaft hypospadiac patient, as no similar case has yet been documented.

Overall, since the bioinformatic processing and molecular interpretation of NGS are dependent on the phenotype, any disturbance in pubertal development or fertility or any secondarily diagnosed extragenital phenotype will require a new read of the NGS data, with a focus on the genes included in these specific factors. Meanwhile, determination of pathogenicity of these variants is pending validation in functional studies.

4.5. Chromosome number, structure, and copy number variations

In addition to screening for RSVs, we were able to detect copy number variations (CNVs) and identified a patient with a distal 18q deletion inferred from a *CYB5A* deletion. Cytogenetic analyses characterized the chromosomal abnormality and enabled appropriate genetic counseling. We did not replicate previous findings regarding five pathogenic CNVs described in hypospadiac patients [37]. We also did not find candidate RSVs in genes overlapping with these regions. Targeted NGS may nevertheless miss balanced chromosomal rearrangements (except if a breakpoint is accurately sequenced with consistent coverage) and abnormalities not covered by the probe design.

4.6. Challenging variants previously reported in the literature

Over the years, the suspicion has been that many variants contribute to hypospadias. Our results challenge previous findings. For instance, the p.(Ala215Thr) variant was described in *AKR1C3* [38], a gene involved in backdoor androgen synthesis. In our cohort, we found no pathogenic missense variant but four truncating or frameshift-stop variants in this gene, two in patients and two in controls. This is consistent with *AKR1C3* haploinsufficiency being most likely benign (probability of being loss-of-function intolerant pLI in the ExAC database = 0). Another typical example is the p.(Cys154Alafs*62) variant on the *CBX2* isoform 2 (NM_032647.3:c.460del). This variant was previously described in two 46,XY DSD patients, using WES [39]. We also found it in five of 285 patients. However, we identified this variant in six of 288 controls and in only one of two affected brothers. This finding is consistent with gnomAD (VAF = 0.003), and this variant is likely benign on its own.

5. Conclusions

In conclusion, we report the application of NGS in a large cohort of boys with isolated, mostly mild hypospadias. We were able to identify in our series minor forms of DSD (*HSD17B3, AR,* and *NR5A1*), undiagnosed syndromes (*RARA* and *MID1*), and candidate rare variants in new genes (Hedgehog pathway, and CHH and NSRD genes). These results challenge the commonly accepted principle of exploring only patients with severe genital defects and illustrate the relevance of genetic exploration even in mild phenotypes. We thus suggest a reconsideration of the genital defects that are likely to benefit from NGS [40]. NGS may help determine which patients would benefit from pubertal follow-up, later fertility evaluation, early diagnosis of syndrome, and optimal parental counseling. Nevertheless, the question of the cost effectiveness of screening hypospadiac patients remains to be studied [40]. Specific economic studies, declining cost of NGS in the upcoming years, and replication studies to focus on the most worthwhile genes will help define the exact place of NGS in clinical routine.

***Author contributions*:** Nicolas Kalfa had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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