Endocrine outcome and seminal parameters in young adult men born with hypospadias: A cross-sectional cohort study

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Summary

Background Hypospadias affects around 1/200 newborn males. Intrauterine testicular dysfunction may underlie a subset of cases. The long-term endocrine and reproductive outcomes in these men remain largely unknown.

Methods Cross-sectional study in Ghent and Vienna University Hospitals to assess the endocrine and seminal parameters of young adult men (16–21 years) born with non-syndromic hypospadias (NSH) (n = 193) compared to healthy typical males (n = 50). Assessments included physical exam, semen analysis, hormone assays and exome-based gene panel analysis (474 genes).

Findings All participants had experienced a spontaneous puberty, in spite of higher LH and INSL3 levels than typical males. Oligo- or azoospermia was observed in 32/172 (18·6%; 99%-CI: 12·2–27·4%) of NSH men; but in 5/16 (31·3%; 99%-CI: 11·1;62·4%) of complex NSH men and in 13/22 (59·1%; 99%-CI: 33·2–80·7%) of those born small for gestational age (SGA). No (likely) pathogenic coding variants were found in the investigated genes. Suboptimal statural growth affected 8/23 (34·8%; 99%-CI: 15·4–61·0%) of men born SGA with NSH.

Interpretation Spermatogenesis is significantly compromised in NSH men, especially in those born SGA or those with complex NSH. Long-term andrological follow-up is recommended, including end-pubertal semen analysis. No clear monogenic causes could be demonstrated in our cohort even in proximal or complex NSH. Being born SGA with NSH is frequently associated with poor catch-up growth, requiring growth hormone therapy in some.

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Research in context

Evidence before this study

According to the testicular dysgenesis syndrome hypothesis, some men born with hypospadias are at risk of having testicular dysfunction. The cause of this association remains elusive as the diagnostic yield of genetic testing is exceptionally low in non-syndromic hypospadias. However, impaired testicular function, i.e. Leydig cell dysfunction and/or reduced spermatogenesis, has been reported in men born with mainly severe forms of hypospadias. These few, small-scale studies offer little insight into the outcome of minor forms of hypospadias, nor on the impact of patient characteristics other than the severity of hypospadias, hampering counseling of patients and parents.

Added value of this study

This is the first large-scale, controlled study that provides comprehensive assessment of endocrine function and reproductive potential in 193 men born with non-syndromic hypospadias compared to 50 healthy male peers, combined with broad genetic testing to identify the underlying etiology of urethral developmental defects, especially when associated with poor endocrine-reproductive outcomes. Stratification of patients based on severity of undervirilisation and on birth weight reveals new insights and provides specific guidance for clinicians involved in hypospadias counseling and care.

Implications of all the available evidence

Hypospadias is a heterogeneous condition requiring specific follow-up and management depending on specific patient characteristics, such as birth weight. Follow-up of puberty does not seem warranted in any subgroup of non-syndromic hypospadias, whereas growth monitoring is highly recommended in those born small for gestational age. Testicular function is impaired, as reflected by overall subclinical Leydig cell dysfunction and frequently impaired spermatogenesis. Therefore, semen analysis should be considered, especially in men born SGA or with severe or complex hypospadias. Genetic testing should be reserved for cases where an underlying syndrome or difference in sex development is suspected.

Introduction

Intrauterine testicular androgen production induces physiological development of the penile urethra.[1] Hence, prenatal factors such as placental insufficiency or exposure to endocrine disruptors that affect testicular function can compromise penile development.[2,3] The testicular dysgenesis syndrome (TDS) hypothesis states that cryptorchidism, some cases of hypospadias, poor semen quality and testis cancer share a common developmental genetic or environmental etiology.[4] Differences/disorders of sex development (DSD) are an extreme form of this spectrum. Mutations in several DSD-related genes, *e.g.* the *Wilms' tumor 1 gene* (*WT1*), *Steroidogenic factor 1* (*SF1, NR5A1*) and the *androgen receptor* (*AR*) gene have been reported in isolated hypospadias as well as infertility without genital anomalies.[5,6] Approximately 10% of boys born small for gestational age (SGA) have hypospadias, often with a more severe phenotype than boys born appropriate for gestational age (AGA) with hypospadias.[7] These findings suggest a multifactorial etiology of hypospadias, but how this translates to future testicular function is unclear. Some small-scale, uncontrolled studies report impaired Sertoli and Leydig cell function in severe and complex hypospadias.[8–10] Epidemiological evidence has revealed lower paternity rates in men with hypospadias.[11] However, data on semen characteristics are scarce and often do not distinguish mild from severe forms or lack control groups.[11–14]

This study aimed to assess endocrine and reproductive outcome as reflected by growth, puberty, testicular hormone production and seminal characteristics in a large cohort of young men born with non-syndromic hypospadias (NSH) as compared to healthy typical male peers (TM). In addition, associations of the endocrine and reproductive outcomes with the severity of undervirilisation and being born SGA/AGA were sought. Exome-based testing of genes involved in gonadal development and spermatogenesis and for SGA-NSH, genes associated with low birth weight/length and poor growth, was performed.

Methods

Participants

Men aged 16–21 21 years and treated for NSH in the past were recruited between October 2017 and September 2019 at Ghent University Hospital (Belgium) and Vienna Medical University (Austria). In total, 556 men were contacted by mail to participate in this cross-sectional study to assess their endocrine and seminal parameters, with additional information two weeks later by phone. If no phone number was available, one e-mail reminder was sent. SGA was defined as a birth weight or length below -2 SD for gestational age using population-specific references. Classification in subgroups (distal, proximal, complex NSH) was based on the physical exam at first presentation, as retrieved in surgical records. Distal NSH was defined as hypospadias *sine* hypospadias to subcoronal hypospadias; proximal NSH was midshaft to perineal hypospadias, and for both groups no other genital symptoms (cryptorchidism, micropenis, bifid scrotum). Complex NSH was defined as having an additional genital symptom with any form of hypospadias; and micropenis as stretched penile length (SPL) of <25 mm at first presentation. If

measurements were lacking, they were inputted as not having a micropenis. No corrections were performed for severity of chordee.

TM were recruited through flyers and posters in educational institutions and through social media. Fifty men, 16–21 years old, born AGA and without genital anomalies were included.

Ethics

All participants gave written consent prior to participation, and parental consent in those younger than 18 years. The local ethical boards of Ghent and Vienna approved the study (B670201835984 and 1547/2018).

Exams

Questionnaire. Parents were asked to complete a questionnaire regarding circumstances of conception, pregnancy and birth. Participants were asked about the timing of their puberty and if this had required hormone supplementation.

Endocrine and urological assessment. Physical exams, all performed by the same researcher (LT), included pubertal staging (Tanner), height, weight, SPL, examination for varicocele according to World Health Organization (WHO) guidelines and testicular ultrasound screening for parenchymal irregularities. Grade II or higher (palpable or visible without Valsalva) varicocele was considered high-grade. Abnormal ultrasounds were repeated by a senior urologist/andrologist. A uroflowmetry was performed to detect obstructive voiding[15]; blood sampling was between 8:00 and 9:30 AM. Details of hormone assays are presented in Supplemental Table 1a.

Semen analysis. Participants were asked to provide at least two separate semen samples after three days of sexual abstinence. Semen analysis was interpreted according to WHO 2010 diagnostic guidelines[16]; the best sample was selected for data analysis (*i.e.* seminal parameters above lower reference limit or highest sperm concentration) (for details, see Supplemental Table 1b).

Genetic investigations. For details of genetic investigations and investigated genes (total $n = 474$), see Supplemental Table 1d&1e).

Whole exome sequencing (WES) was performed in all NSH born SGA or AGA with proximal, complex or familial hypospadias, hypogonadism, reduced sperm concentration or family history of genital anomalies. Withheld variants were classified using the American College of Medical Genetics (ACMG) guidelines[17] (Supplemental Table 1c). In case a variant with an established link with hypospadias and undervirilisation in 46,XY individuals was classified as variant of unknown significance (class 3) or higher (class 4/5, [likely] pathogenic) and had an allele frequency of less than 1/1000 males (gnomAD v2), parental blood samples were requested for segregation analysis by Sanger sequencing. Potential disease-causing oligogenic variant combinations were sought using the Oligogenic Resource for Variant AnaLysis (ORVAL) online platform.[18] Digenic variant combinations predicted to be at least 95%-likely disease-causing were withheld. Further filtering was performed by literature search of gene expression and function in male genital and gonadal tissues.

NSH with a reduced sperm concentration underwent additional testing for Y-chromosome microdeletions, *CFTR* variants and were asked to provide a second blood sample for conventional karyotyping and FISH to screen for sex chromosomal mosaicism. In those who did not provide a second blood sample, the presence of a Y-chromosome was confirmed by assessing the coverage of three Y-linked genes (*SRY, TSPY1* and *PRY*) on the WES data. Of note, this method does not formally exclude sex chromosomal mosaicism.

Statistics

Statistical software package of IBM SPSS© version 27.0 was used. Linear and (multinomial) logistic regressions were performed analyzing subgroups of NSH. First, AGA-NSH who had distal, proximal and complex NSH were compared with TM. Second, SGA-NSH were compared with AGA-NSH and TM. Age at time of the study was a co-variate for all regression models. Correction for body height was performed for varicocele, SPL and testicular volume. In the latter, presence of varicocele grade II or higher was also included as a co-variate. For semen analyses, age, varicocele (grade II or higher) and days of abstinence were used as co-variates. Spearman's rank-order correlation assessed the relationship between hormone levels and sperm concentration and total sperm count. Score intervals for differences in proportions and confidence intervals for single proportions were calculated using R version 4.4.1 and the R PropCIs package, version 0.3-0. Effect sizes were calculated using Cohen's d and Z/sqrt(n) for the non-parametric Mann–Whitney U-test. Receiver operator characteristic (ROC) curves were calculated for inhibin B, FSH and inhibin B to FSH ratio to predict reduced sperm concentrations.

Role of funders

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Results

Participant characteristics

Participants included 193 NSH (AGA: 167 (86·5%); SGA: 26 (13·5%)) and 50 TM (Table 1 and Supplemental Figure 1). Median ages of AGA, SGA-NSH and TM were 18.3, 17.8 and 19.5 years. Eleven (42·3%) NSH were SGA for weight, five (19·2%) for length and ten (38·5%) for weight and length. Proximal and complex NSH were more common in SGA (Table 1). Parental questionnaires were missing or incomplete in three AGA and three SGA-NSH and four TM. Parental height was missing in twelve additional AGA-NSH.

Endocrine and urological assessment

All participants had experienced spontaneous pubertal onset and progression, with similar proportions of self-reported early and late puberty compared to peers.

SGA-NSH were on average 5·5 cm shorter than AGA-NSH and 4·4cm shorter than TM. Approximately 35% of SGA-NSH did not reach their target height, 27% had short stature (<-2SD) (Table 2a).

Three AGA-NSH and one SGA-NSH had only one testicle (AGA: prenatal regression $n = 2$, loss after childhood orchidopexy $n = 1$; SGA: torsion $n = 1$). All other

	Contacted NSH	Participants
Distal hypospadias	407/537 (75.8%)	132/193 (68.4%)
Midshaft hypospadias	69/537 (12.8%)	38/193 (19.7%)
Proximal hypospadias	61/537 (11.4%)	23/193 (11.9%)
Undocumented	19/556 (3.4%)	0/193 (0%)

	AGA NSH	SGA NSH
Distal hypospadias	122/167 (73.1%)	10/26 (38.5%)
Midshaft hypospadias	33/167 (19.8%)	5/26 (19.2%)
Proximal hypospadias	12/167 (7.2%)	11/26 (42.3%)
Complex hypospadias	16/167 (9.6%)	7/26 (26.9%)
Cryptorchidism	11/167 (6.6%)	4/26 (15.4%)
Micropenis	2/167 (1.2%)	1/26 (3.8%)
Bifid scrotum	5/167 (3.0%)	2/26 (7.7%)

	AGA NSH	SGA NSH	Typical males
Age at study visit (years)	18.3 (2.4)	17.8 (1.9)	19.5 (1.8)
Gestational age (weeks)	39.7 (2.0)	39.0 (3.1)	39.1 (2.1)
Preterm birth	19/164 (11.6%)	5/26 (19.2%)	4/48 (8.3%)
Extreme preterm birth	5/164 (3.0%)	2/26 (7.7%)	0/48 (0%)
Assisted reproductive techniques	11/164 (6.7%)	5/26 (19.2%)	3/47 (6.4%)
Insemination	3/164 (1.8%)	2/26 (7.7%)	1/47 (2.1%)
Hormone stimulation	2/164 (1.2%)	0/26 (0%)	0/47 (0%)
IVF	1/164 (0.6%)	1/26 (3.8%)	2/47 (4.3%)
ICSI	5/164 (3.0%)	2/26 (7.7%)	0/47 (0%)
Pregnancy complications			
Severe	9/164 (5.5%)	6/26 (23.1%)	3/47 (6.4%)
Multiple pregnancy	7/164 (4.3%)	4/26 (15.4%)	2/47 (4.3%)
Gestational hypertension	12/164 (7.3%)	3/26 (11.5%)	1/47 (2.1%)
Preeclampsia	4/164 (2.4%)	3/26 (11.5%)	0/47 (0%)
Birth weight (SD)	-0.25 (1.11)	-2,52 (0.98)	-0.54 (1.23)
Birth length (SD)	-0.03 (1.28)	-2.22 (1.61)	-0.04 (1.46)

***Table 1*: Summary of genital phenotype, gestational and birth data.**
AGA: appropriate for gestational age; SGA: small for gestational age; NSH: non-syndromic hypospadias; Distal hypospadias: hypospadias sine hypospadias to subcoronal hypospadias; Midshaft hypospadias: meatus urethrae along the penile shaft; Proximal hypospadias: penoscrotal to perineal hypospadias. Preterm: birth before 37 weeks of gestation; Extreme preterm birth: birth before 33 weeks of gestation; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; Severe pregnancy complication: by parents reported severe complications during gestation.

Puberty*
Multinomial logistic regression
Reference category: average onset of puberty

AGA NSH Vs. typical males			
Early puberty	***N* (%)**	**Adjusted OR**	**99% CI**
Distal hypospadias[a]	23/116 (19.8%)	0.99	0.31; 3.22
Proximal hypospadias[a]	3/35 (8.6%)	0.43	0.07; 2.83
Complex hypospadias[a]	2/16 (12.5%)	0.70	0.07; 6.81
Typical males	10/50 (20.0%)	-	-
Late puberty	***N* (%)**	**Adjusted OR**	**99% CI**
Distal hypospadias[a]	20/116 (17.2%)	1.21	0.36; 4.13
Proximal hypospadias[a]	10/35 (28.6%)	2.07	0.49; 8.82
Complex hypospadias[a]	2/16 (12.5%)	2.66	0.44; 16.31
Typical males	9/50 (18.0%)	-	-

SGA NSH Vs. AGA NSH and typical males			
Early puberty	***N* (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	28/167 (16.8%)	0.69	0.16; 2.99
Typical males[b]	10/50 (20.0%)	0.79	0.14; 4.44
SGA NSH	5/26 (19.2%)	-	-
Late puberty	***N* (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	35/167 (21.0%)	0.488	0.14; 1.76
Typical males[b]	9/50 (18.0%)	0.336	0.07; 1.67
SGA NSH	8/26 (30.8%)	-	-

Delta Midparental Height (cm)*
Linear regression

	Mean ± SD	**B-value**	**99% CI**
AGA NSH[b]	0.5 ± 6.5	5.54	1.72; 9.37
Typical males[b]	-0.3 ± 5.9	4.44	-0.10; 8.98
SGA NSH	-5.3 ± 8.0	-	-
Adjusted R^2		0.059	

Target height*
Logistic regression
Reference category: target height reached

Not reached	***N* (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	14/152 (9.2%)	0.20	0.05; 0.77
Typical males[b]	2/46 (4.3%)	0.10	0.01; 0.94
SGA NSH	8/23 (34.8%)	-	-

Short stature (≤168 cm)*
Logistic regression
Reference category: normal or tall stature

Short stature	***N* (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	5/167 (3.0%)	0.09	0.02; 0.47
Typical males[b]	2/50 (4%)	0.16	0.02; 1.62
SGA NSH	7/26 (26.9%)	-	-

Table 2 *(Continued)*

BMI (kg/m^2)*
Linear regression

AGA NSH Vs. typical males			
	Median (IQR)	**B-value**	**99% CI**
Distal hypospadias[a]	21.5 (4.4)	0.45	-1.03; 1.92
Proximal hypospadias[a]	21.7 (3.9)	0.39	-1.50; 2.29
Complex hypospadias[a]	19.3 (4.9)	-0.28	-2.74; 2.18
Typical males	22.5 (3.2)	-	-
Adjusted R^2		0.030	

SGA NSH Vs. AGA NSH and typical males			
	Median (IQR)	**B-value**	**99% CI**
AGA NSH[b]	21.9 (4.4)	0.27	-1.53; 2.08
Typical males[b]	22.5 (3.2)	-0.11	-2.25; 2.03
SGA NSH	20.5 (3.5)	-	-
Adjusted R^2		0.034	

Testicular volume (mL) (Ultrasound)[$]
Linear regression

AGA NSH Vs. typical males			
Right testicle	**Mean ± SD**	**B-value**	**99% CI**
Distal hypospadias[a]	12.7 ± 3.8	-0.84	-2.60; 0.93
Proximal hypospadias[a]	12.9 ± 3.9	-0.34	-2.56; 1.89
Complex hypospadias[a]	10.2 ± 4.0	-3.07	-6.11; -0.03
Typical males	13.0 ± 3.9	-	-
Adjusted R^2		0.058	
Left testicle	**Mean ± SD**	**B-value**	**99% CI**
Distal hypospadias[a]	12.5 ± 3.7	-0.49	-2.14; 1.15
Proximal hypospadias[a]	12.0 ± 3.1	-0.83	-2.90; 1.25
Complex hypospadias[a]	9.4 ± 4.9	-3.31	-6.16; -0.47
Typical males	12.5 ± 3.4	-	-
Adjusted R^2		0.103	

SGA NSH Vs. AGA NSH and typical males			
Right testicle	**Mean ± SD**	**B-value**	**99% CI**
AGA NSH[b]	12.5 ± 3.9	0.24	-1.96; 2.45
Typical males[b]	13.0 ± 3.9	1.10	-1.47; 3.67
SGA NSH	11.6 ± 3.4	-	-
Adjusted R^2		0.041	
Left testicle	**Mean ± SD**	**B-value**	**99% CI**
AGA NSH[b]	12.1 ± 3.8	0.17	-1.93; 2.26
Typical males[b]	12.5 ± 3.4	0.83	-1.62; 3.29
SGA NSH	11.2 ± 4.0	-	-
Adjusted R^2		0.059	

Stretched penile length (cm)[+]
Linear regression

AGA NSH Vs. typical males			
	Mean ± SD	**B-value**	**99% CI**
Distal hypospadias[a]	13.6 ± 1.8	-0.17	-1.12; 0.78

Table 2 *(Continued)*

Stretched penile length (cm)+
Linear regression

AGA NSH Vs. typical males

	Mean ± SD	B-value	99% CI
Proximal hypospadias[a]	12.6 ± 1.6	-1.00	-2.22; 0.23
Complex hypospadias[a]	11.0 ± 2.3	-2.54	-4.07; -1.01
Typical males	13.8 ± 1.6	-	-
Adjusted R^2		0.157	

SGA NSH Vs. AGA NSH and typical males

	Mean ± SD	B-value	99% CI
AGA NSH[b]	13.2 ± 2.0	1.64	0.46; 2.82
Typical males[b]	13.8 ± 1.6	2.19	0.78; 3.60
SGA NSH	11.1 ± 1.8	-	-
Adjusted R^2		0.159	

Varicocele+
Logistic regression
Reference category: No or low grade varicocele

AGA NSH Vs. typical males

High grade varicocele	*N* (%)	Adjusted OR	99% CI
Distal hypospadias[a]	24/116 (20.7%)	3.89	0.72; 21.05
Proximal hypospadias[a]	3/35 (8.6%)	1.44	0.16; 13.26
Complex hypospadias[a]	2/16 (12.5%)	2.24	0.18; 28.06
Typical males	3/50 (6.0%)	-	-

SGA NSH Vs. AGA NSH and typical males

High grade varicocele	N (%)	Adjusted OR	99% CI
AGA NSH[b]	29/167 (17.4%)	0.57	0.14; 2.32
Typical males[b]	3/50 (6.0%)	0.16	0.02; 1.29
SGA NSH	6/26 (23.1%)	-	-

***Table 2*: Results of the endocrine exam.**
Early puberty: self-reported early pubertal onset compared to peers; Late puberty: self-reported late pubertal onset compared to peers; AGA: appropriate for gestational age; SGA: small for gestational age; NSH: non-syndromic hypospadias; Distal hypospadias: hypospadias sine hypospadias to subcoronal hypospadias without other genital symptoms; Proximal hypospadias: midshaft to perineal hypospadias without other genital symptoms; Complex hypospadias: hypospadias with other genital symptoms (i.e. cryptorchidism, micropenis, bifid scrotum); SD: standard deviation; IQR: interquartile range. A: reference group are typical males; B: reference group are SGA NSH. *: co-variate age; $: co-variates age, body height and varicocele ≥ grade II (yes/no); +: covariates age and body height.

participants had bilateral scrotal testes. Compared to TM, testes were bilaterally 3 mL smaller in AGA-NSH with complex hypospadias (Table 2 and Supplemental Table 2b).

Average SPL was 1 and 2·5 cm shorter in proximal and complex AGA-NSH, compared to TM. SGA-NSH had smaller SPL than AGA-NSH (1·6 cm) after correction for body height, irrespective of penile surgery frequency (median 0(IQR:1) *vs.* 1(IQR:2); effect size:0·13). Despite higher average gonadotropin (both LH and

LH (U/L)*
Linear regression

AGA NSH Vs. typical males

	Mean ± SD	B-value	99% CI
Distal hypospadias[a]	5.32 ± 2.08	0.50	-0.45; 1.44
Proximal hypospadias[a]	5.50 ± 1.78	0.70	-0.52; 1.91
Complex hypospadias[a]	6.73 ± 3.87	2.00	0.42; 3.58
Typical males	4.86 ± 1.55		
Adjusted R^2		0.067	

SGA NSH Vs. AGA NSH and typical males

	Mean ± SD	B-value	99% CI
AGA NSH[b]	5.55 ± 2.22	-0.58	-1.75; 0.59
Typical males[b]	4.86 ± 1.55	-1.28	-2.67; 0.11
SGA NSH	6.12 ± 2.37		
Adjusted R^2		0.050	

Testosterone (nmol/L)*
Linear regression

AGA NSH Vs. typical males

	Mean ± SD	B-value	99% CI
Distal hypospadias[a]	18.7 ± 5.9	1.01	-1.53; 3.55
Proximal hypospadias[a]	19.5 ± 6.3	1.75	-1.51; 5.02
Complex hypospadias[a]	18.8 ± 4.3	1.20	-3.04; 5.43
Typical males	18.1 ± 4.4	-	-
Adjusted R^2		-0.005	

SGA NSH Vs. AGA NSH and typical males

	Mean ± SD	B-value	99% CI
AGA NSH[b]	18.9 ± 5.8	0.78	-2.22; 3.78
Typical males[b]	18.1 ± 4.4	-0.48	-4.05; 3.09
SGA NSH	17.9 ± 4.9	-	-
Adjusted R^2		0.004	

Free Testosterone (nmol/L)*
Linear regression

AGA NSH Vs. typical males

	Mean ± SD	B-value	99% CI
Distal hypospadias[a]	0.37 ± 0.12	0.01	-0.04; 0.06
Proximal hypospadias[a]	0.38 ± 0.11	0.02	-0.04; 0.09
Complex hypospadias[a]	0.40 ± 0.09	0.04	-0.04; 0.13
Typical males	0.37 ± 0.09	-	-
Adjusted R^2		-0.005	

SGA NSH Vs. AGA NSH and typical males

	Mean ± SD	B-value	99% CI
AGA NSH[b]	0.38 ± 0.12	0.02	-0.04; 0.08
Typical males[b]	0.37 ± 0.09	0.00	-0.07; 0.07
SGA NSH	0.36 ± 0.09	-	-
Adjusted R^2		0.004	

Table 3 (Continued)

Insulin-like factor 3 (μg/L)*
Linear regression

AGA NSH Vs. typical males

	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	0.67 (0.37)	0.16	0.03; 0.30
Proximal hypospadias[a]	0.63 (0.35)	0.15	-0.02; 0.32
Complex hypospadias[a]	0.58 (0.38)	0.20	-0.02; 0.41
Typical males	0.49 (0.19)	-	-
Adjusted R^2		0.032	

SGA NSH Vs. AGA NSH and typical males

	Median (IQR)	B-value	99% CI
AGA NSH[b]	0.66 (0.36)	-0.06	-0.21; 0.09
Typical males[b]	0.49 (0.19)	-0.21	-0.40; -0.02
SGA NSH	0.68 (0.24)	-	-
Adjusted R^2		0.036	

FSH (U/L)*
Linear regression

AGA NSH Vs. typical males

	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	3.27 (2.14)	0.78	-1.15; 2.71
Proximal hypospadias[a]	3.44 (2.67)	1.04	-1.43; 3.52
Complex hypospadias[a]	5.19 (4.25)	6.04	2.83; 9.26
Typical males	2.69 (2.03)	-	-
Adjusted R^2		0.090	

SGA NSH Vs. AGA NSH and typical males

	Median (IQR)	B-value	99% CI
AGA NSH[b]	3.42 (2.31)	-0.51	-2.85; 1.83
Typical males[b]	2.69 (2.03)	-1.80	-4.58; 0.98
SGA NSH	3.80 (3.94)	-	-
Adjusted R^2	0.004		

Inhibin B (ng/L)*
Linear regression

AGA NSH Vs. typical males

	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	206.8 (90.9)	-15.01	-46.61; 16.59
Proximal hypospadias[a]	237.8 (92.6)	-1.16	-41.76; 39.45
Complex hypospadias[a]	159.3 (82.5)	-79.55	-132.24; -26.87
Typical males	217.0 (92.6)	-	-
Adjusted R^2		0.063	

SGA NSH Vs. AGA NSH and typical males

	Median (IQR)	B-value	99% CI
AGA NSH[b]	207.1 (94.2)	3.66	-37.57; 44.89
Typical males[b]	217.0 (92.6)	19.38	-29.63; 68.39
SGA NSH	201.9 (109.9)	-	-
Adjusted R^2		-0.004	

InhibinB/FSH (ng/U)*
Linear regression

AGA NSH Vs. typical males

	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	61.95 (67.78)	-21.33	-55.35; 12.68
Proximal hypospadias[a]	70.95 (72.41)	-20.07	-63.78; 23.64
Complex hypospadias[a]	31.75 (38.58)	-64.33	-121.04; -7.61
Typical males	71.60 (77.50)	-	-
Adjusted R^2		0.022	

SGA NSH Vs. AGA NSH and typical males

	Median (IQR)	B-value	99% CI
AGA NSH[b]	60.17 (69.27)	-26.46	-83.20; 30.27
Typical males[b]	71.60 (77.50)	-5.48	-72.93; 61.96
SGA NSH	59.2 (72.53)	-	-
Adjusted R^2		0.000	

***Table 3*: Results of the hormone assays.**
AGA: appropriate for gestational age; SGA: small for gestational age; NSH: non-syndromic hypospadias; Distal hypospadias: hypospadias sine hypospadias to subcoronal hypospadias without other genital symptoms; Proximal hypospadias: midshaft to perineal hypospadias without other genital symptoms; Complex hypospadias: hypospadias with other genital symptoms (i.e. cryptorchidism, micropenis, bifid scrotum); SD: standard deviation; IQR: interquartile range. A: reference group are typical males; B: reference group are SGA NSH. *: co-variate age.

FSH) levels, few NSH had levels above laboratory thresholds (Table 3 and Supplemental Table 2c). Testicular androgen levels (*i.e.* total and free testosterone, dihydrotestosterone) were not different in NSH compared to TM. NSH had higher insulin-like factor 3 (INSL3) levels, with no differences between subgroups (Figure 1a). In all participants, LH significantly correlated with INSL3 (r_s = 0·237, 99%-CI: 0·064;0·395) and with total testosterone (r_s = 0·187, 99%-CI:0·018;0·345). INSL3 correlated slightly stronger with androgens (total testosterone: r_s = 0·260, 99%-CI:0·089;0·416; free testosterone: r_s = 0·323, 99%-CI:0·157;0·472; DHT: r_s = 0·256, 99%-CI:0·085;0·412), but not with BMI (r_s = 0·107; 99%-CI:-0·070;0·278). Inhibin B was on average 79 6ng/L lower in AGA-NSH with complex hypospadias; seven (4·2%) had levels below laboratory thresholds.

Seminal parameters

In total, 172/193 (89·1%) NSH provided at least one semen sample; 15/21 refused and sampling failed in six. Those who produced a sample were older, with highest success rates in those over 18 years (<18years: 69/84 (82·1%); >18years: 103/109 (94·5%)). Sperm

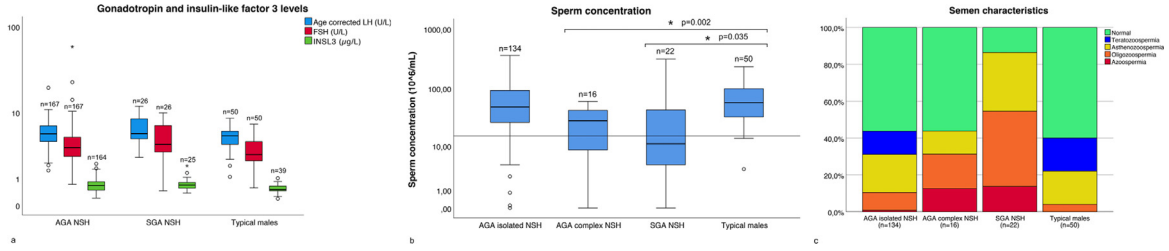

Figure 1. a. Box-plot of gonadotropin and insulin-like factor 3 levels in AGA, SGA-NSH and typical males. Y-axis in logarithmic scale. b. Box-plot of sperm concentration in AGA-NSH with isolated and complex hypospadias, SGA-NSH and typical males. The horizontal line represents the WHO threshold of 15.10^6/mL; *: significant difference. Y-axis in logarithmic scale. c. Summary of semen characteristics. Azoospermia: no sperm cells found after centrifugation on two separate samples; Oligozoospermia: sperm concentration $<15.10^6$/mL; Asthenozoospermia: normal sperm concentration and progressive motility <32% and total motility <40%; Teratozoospermia: normal sperm concentration, motility and normal sperm morphology of less than 4% of sperm cells.

concentrations ($<15.10^6$/mL) were reduced in 12·7% of AGA, 59·1% of SGA-NSH and 4% of controls. Odds of reduced sperm concentrations were higher in AGA complex NSH and SGA-NSH compared to TM (Figure 1b & c; Table 4). Impaired sperm motility was more likely in SGA-NSH, but not AGA complex NSH compared to TM.

Genetic investigations

Exome-based gene panel sequencing was performed in 99 NSH (AGA: $n = 74$; SGA: $n = 25$) to assess the coding regions of genes implicated in gonadal development and function and spermatogenesis ($n = 241$), and in SGA and poor growth ($n = 233$). No (likely) pathogenic variants (ACMG[17] Class 4 and 5) were identified. Interesting variants of unknown significance (class 3) were found in 15 NSH; segregation analysis, performed in three AGA and one SGA-NSH (Table 5), supported a potential link with the phenotype, however, the variants remained class 3 according to ACMG criteria. None of the digenic variant combinations that were predicted by ORVAL to be at least 95%-likely disease-causing were withheld after filtering on gene expression and gene function as none could be linked to the phenotype of NSH. In all 32 NSH who had reduced sperm concentrations, Y-chromosome microdeletions were excluded. Karyotyping excluded sex chromosomal mosaicism in all ten NSH who provided an additional blood sample; in the remaining 22, presence of a Y-chromosome was confirmed through the WES data (coverage of *SRY*, *TSPY1* and *PRY*).

Predictors of reduced sperm concentrations

The sensitivity of FSH and inhibin B (9.4% and 21.9%, respectively) to detect reduced sperm concentrations ($<15.10^6$/mL) was very low, albeit with a specificity of 100% and 97·9% (Figure 2a & b, Supplemental Table 4). ROC curve analysis confirmed the clinical validity of both assays to predict a sperm concentration of less than 15.10^6/mL (Figure 3a & b). However, criterion values for optimal sensitivity and specificity were approximately half and double of the clinical thresholds for FSH (4·11U/L) and inhibin B (196·40 ng/L) respectively.

Median INSL3 levels were higher in NSH with reduced sperm concentrations ($<15.10^6$/mL), however, with very small effect size (Table 6). ROC curve analysis for INSL3 showed criterion value of 0·705 µg/L to predict reduced sperm concentrations (Figure 3c). In complex NSH, similar rates of reduced sperm concentration were seen in NSH with cryptorchidism and NSH with bifid scrotum and/or micropenis (5/14(35·7%) *vs.* 2/7 (28·6%), respectively; 99%-score interval:-0·474;0·522). There were no differences in obstructive voiding, varicocele, lifestyle or prenatal factors between NSH with a normal *versus* a reduced sperm concentration. In addition, birth weight and length SD, SPL, adult height or delta midparental height did not differ between SGA-NSH with normal or reduced sperm concentration (Table 6).

Discussion

The adult testicular function in men born with atypical genital features outside the context of DSD is currently unknown, hampering long-term counseling of parents of a newborn with hypospadias. This study explored the endocrine outcome and reproductive potential in adolescents and young adult men born with NSH compared to TM, and aimed at identifying the genetic causes of a suboptimal outcome, severe and/or complex and familial hypospadias and the frequent SGA-hypospadias association.

Given the spontaneous onset and progression of puberty in all cases, endocrine follow-up of testicular function during puberty in NSH seems only warranted upon strict clinical indication. However, thirty-five percent of SGA-NSH had not reached their target height and 27% had short stature, which corresponds to twice the previously reported 10–15% absence of catch-up

Semen volume (mL)*
Linear regression

AGA NSH Vs. typical males	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	2.8 (1.8)	-0.47	-1.07; 0.14
Proximal hypospadias[a]	2.7 (2.2)	-0.26	-1.04; 0.53
Complex hypospadias[a]	2.1 (1.4)	-1.04	-2.02; -0.06
Typical males	3.2 (1.3)	-	-
Adjusted R^2		0.072	

SGA NSH Vs. AGA NSH and typical males	Median (IQR)	B-value	99% CI
AGA NSH[b]	2.6 (1.7)	0.15	-0.65; 0.95
Typical males[b]	3.2 (1.3)	0.66	-0.26; 1.58
SGA NSH	2.6 (2.7)	-	-
Adjusted R^2		0.052	

Sperm concentration (10^6/mL)*
Linear regression

AGA NSH Vs. typical males	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	46.25 (78.82)	-6.48	-32.60; 19.64
Proximal hypospadias[a]	52.78 (49.68)	-8.01	-41.87; 25.86
Complex hypospadias[a]	28.00 (38.39)	-52.07	-94.59; -9.56
Typical males	57.0 (66.6)	-	-
Adjusted R^2		0.053	

SGA NSH Vs. AGA NSH and typical males	Median (IQR)	B-value	99% CI
AGA NSH[b]	45.6 (62.4)	21.98	-12.65; 56.61
Typical males[b]	57.0 (66.6)	32.56	-7.26; 72.36
SGA NSH	10.9 (43.4)	-	-
Adjusted R^2		0.039	

Total sperm count (10^6/ejaculate)*
Linear regression

AGA NSH Vs. typical males	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	130.90 (181.84)	-53.30	-135.88; 29.28
Proximal hypospadias[a]	138.36 (198.49)	-40.36	-147.44; 66.71
Complex hypospadias[a]	39.29 (48.48)	-200.20	-334.64; -65.77
Typical males	187.4 (234.3)	-	-
Adjusted R^2		0.085	

SGA NSH Vs. AGA NSH and typical males	Median (IQR)	B-value	99% CI
AGA NSH[b]	123.0 (184.1)	54.33	-52.05; 160.71
Typical males[b]	187.4 (234.3)	118.48	-3.80; 240.77
SGA NSH	43.0 (122.1)	-	-
Adjusted R^2		0.071	

Progressive motility (%)*
Linear regression

AGA NSH Vs. typical males	Mean ± SD	B-value	99% CI
Distal hypospadias[a]	39.27 ± 16.77	-3.54	-12.11; 5.03
Proximal hypospadias[a]	37.33 ± 15.91	-5.44	-16.55; 5.67
Complex hypospadias[a]	31.52 ± 19.33	-11.13	-25.08; 2.82
Typical males	43.2 ± 20.7	-	-
Adjusted R^2		0.000	

Table 4 *(Continued)*

SGA NSH Vs. AGA NSH and typical males	Mean ± SD	B-value	99% CI
AGA NSH[b]	38.0 ± 16.9	16.20	5.21; 27.18
Typical males[b]	43.2 ± 20.7	21.00	8.37; 33.62
SGA NSH	21.2 ± 18.3	-	-
Adjusted R^2		0.079	

Total motility (%)*
Linear regression

AGA NSH Vs. typical males	Mean ± SD	B-value	99% CI
Distal hypospadias[a]	53.06 ± 20.42	-4.74	-14.83; 5.36
Proximal hypospadias[a]	51.11 ± 20.70	-6.73	-19.82; 6.36
Complex hypospadias[a]	41.831 ± 22.06	-15.88	-32.31; 0.56
Typical males	58.1 ± 22.4	-	-
Adjusted R^2		0.009	

SGA NSH Vs. AGA NSH and typical males	Mean ± SD	B-value	99% CI
AGA NSH[b]	51.4 ± 20.8	20.39	7.40; 33.38
Typical males[b]	58.1 ± 22.4	26.80	11.87; 41.74
SGA NSH	30.6 ± 22.0	-	-
Adjusted R^2		0.090	

Sperm morphology (%)*
Linear regression

AGA NSH Vs. typical males	Median (IQR)	B-value	99% CI
Distal hypospadias[a]	4.0 (2.0)	-0.29	-1.43; 0.86
Proximal hypospadias[a]	4.0 (3.3)	-0.18	-1.67; 1.31
Complex hypospadias[a]	4.0 (3.8)	-1.30	-3.17; 0.57
Typical males	4.0 (3.0)	-	-
Adjusted R^2		-0.013	

SGA NSH Vs. AGA NSH and typical males	Median (IQR)	B-value	99% CI
AGA NSH[b]	4.0 (2.0)	2.17	0.72; 3.61
Typical males[b]	4.0 (3.0)	2.55	0.89; 4.21
SGA NSH	2.0 (2.0)	-	-
Adjusted R^2		0.059	

Sperm concentration*
Logistic regression
Reference category: ≥15.10^6/mL

AGA NSH Vs. typical males			
<15.10^6/mL	N (%)	Adjusted OR	99% CI
Distal hypospadias[a]	9/104 (8.7%)	0.77	0.26; 18.37
Proximal hypospadias[a]	5/30 (16.7%)	1.57	0.48; 47.58
Complex hypospadias[a]	5/16 (31.3%)	2.37	0.96; 120.32
Typical males	2/50 (4%)	-	-

SGA NSH Vs. AGA NSH and typical males			
<15.10^6/mL	N (%)	Adjusted OR	99% CI
AGA NSH[b]	19/150 (12.7%)	0.10	0.03; 0.40
Typical males[b]	2/50 (4%)	0.03	0.00; 0.29
SGA NSH	13/22 (59.1%)	-	-

Table 4 *(Continued)*

Motility* *Logistic regression* Reference category: Progressive motility ≥32% or total motility ≥40%			
AGA NSH Vs. typical males			
Progressive <32% and total motility <40%	**N (%)**	**Adjusted OR**	**99% CI**
Distal hypospadias[a]	28/104 (26.9%)	1.46	0.46; 4.69
Proximal hypospadias[a]	9/30 (30%)	1.63	0.39; 6.86
Complex hypospadias[a]	6/16 (37.5%)	2.12	0.39; 11.58
Typical males	9/50 (18%)	-	-
SGA NSH Vs. AGA NSH and typical males			
Progressive <32% and total motility <40%	**N (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	43/150 (28.7%)	0.15	0.04; 0.58
Typical males[b]	9/50 (18%)	0.10	0.02; 0.48
SGA NSH	16/22 (72.7%)	-	-
Morphology* *Logistic regression* Reference category: ≥4% normal morphology			
AGA NSH Vs. typical males			
<4%	**N (%)**	**Adjusted OR**	**99% CI**
Distal hypospadias[a]	32/104 (30.8%)	0.85	0.31; 2.31
Proximal hypospadias[a]	12/30 (40.0%)	1.29	0.36; 4.56
Complex hypospadias[a]	7/16 (43.8%)	1.50	0.31; 7.18
Typical males	17/50 (34%)	-	-
SGA NSH Vs. AGA NSH and typical males			
<4%	**N (%)**	**Adjusted OR**	**99% CI**
AGA NSH[b]	51/150 (34.0%)	0.11	0.03; 0.52
Typical males[b]	17/50 (34%)	0.11	0.02; 0.60
SGA NSH	18/22 (81.8%)	-	-

***Table 4*: Results of the semen analysis.**
AGA: appropriate for gestational age; SGA: small for gestational age; NSH: non-syndromic hypospadias;; Distal hypospadias: hypospadias sine hypospadias to subcoronal hypospadias without other genital symptoms; Proximal hypospadias: midshaft to perineal hypospadias without other genital symptoms; Complex hypospadias: hypospadias with other genital symptoms (i.e. cryptorchidism, micropenis, bifid scrotum); SD: standard deviation; IQR: interquartile range. A: reference group are typical males; B: reference group are SGA NSH. *: co-variates age, varicocele ≥ grade II (yes/no) and days of abstinence.

growth in the overall SGA population.[19] These results corroborate data from our recent retrospective study using the I-DSD registry, in which 31/104 (29·8%) undervirilized SGA boys had insufficient catch-up growth[20] and underscore the importance of follow-up of growth and consideration of growth hormone treatment in undervirilized boys born SGA.[21]

SGA-NSH had a smaller SPL, which was unrelated to their height or number of penile surgeries. Other studies have reported micropenises in 25–30% of fetuses and infants following intrauterine growth restriction, hypothesized to result from impaired hCG secretion due to placental insufficiency.[2,22] Our data suggest that prenatally impaired penile growth is not compensated after birth.

Subclinical compensated Leydig cell dysfunction (higher LH, unaffected testosterone) was seen in NSH born SGA or AGA with complex hypospadias, in line with scarce studies in mainly severe hypospadias, where higher LH levels and sometimes impaired androgen production have been reported.[8–10]

Higher INSL3 levels were observed mainly in complex NHS. INLS3 has recently been proposed as a reliable marker of Leydig cell function, not affected by body composition and/or SHBG levels and therefore probably better reflecting Leydig cell functionality than testosterone.[23] Interestingly, higher amniotic INSL3 levels were also reported in pregnancies of boys with hypospadias and cryptorchidism.[24] We hypothesize that the higher INSL3 levels in our study may reflect increased compensatory LH stimulation and perhaps relative Leydig cell hyperplasia in complex NSH. INSL3 has also been shown to correlate with spermatogenesis and to support male germ cell survival.[25] However, no relationship between seminal parameters and serum INSL3 have been found so far.[26] In this study, higher INSL3 levels were found in participants with reduced sperm concentrations, but this did not reach statistical significance.

Sertoli cell dysfunction was more frequent in NSH, as reflected by FSH, inhibin B and sperm concentration. Overall, 18·6% of NSH had oligozoospermia or azoospermia, with the highest rates in those born SGA or AGA with complex NSH (approximately 60% and 30%, respectively). In addition, 8·7% and 16·7% of NSH with isolated mild and severe hypospadias had reduced sperm concentrations, which is still two and four times higher than in TM (4%). We previously reported the urological outcome of this cohort, which revealed need for penile re-interventions in 39·2% and diverse ejaculatory problems in 12%.[15] The role of these observations in the suboptimal semen characteristics of these men remains unclear and warrants further studies, taking into account the more recent and improved surgical techniques.[27] Other studies have also reported lower sperm concentrations and paternity rates in proximal and complex but not distal hypospadias.[12–14] Lower paternity rates and more frequent use of assisted reproductive techniques in men born with – mainly proximal – hypospadias have been recently reported by Nordenvall et al.[11] Semen quality in men born SGA has not been investigated so far. Some but not all studies report an inverse correlation between birth weight and sperm concentration, however these studies include few or no participants born SGA and do not provide details of congenital genital anomalies.[28,29] Therefore, it remains unclear if all men born SGA are at risk of having reduced sperm concentrations, or only those with associated hypospadias. We hypothesize that the combination of being born SGA with NSH indicates a susceptibility for impaired genital and gonadal development due to prenatal factors that may also impair statural growth. These individuals are thus more likely to have

AGA NSH

N°	Phenotype	Gene	Segregation analysis	Transcript	HGVS c. and p. notation	rsID	REVEL score	gnomAD v2VAF	gnomAD v2Male VAF	gnomAD v2max VAF	gnomAD v2 Homozygotes
1	M, Cb	*BNC2*	*De novo*	NM_017637.5	c.1022A>G p.(Asn341Ser)	-	0.156	-	-	-	-
2	M	*CHD7*	Paternal/ *de novo*$^{+}$	NM_017780.3	c.3730A>G p.(Thr1244Ala)	-	0.722	-	-	-	-
		NR5A1	Paternal/ *de novo*$^{+}$	NM_004959.4	c.629C>T p.(Pro210Leu)	rs900214501	0.180	0.00001593	0.00001965	0.00005953	0
3	D	*ESR2*	Maternal	NM_001437.2	c.1123G>C p.(Glu375Gln)	-	0.866	-	-	-	-
4	D	*ZFPM2*	NA	NM_012082.3	c.89A>G p.(Glu30Gly)	rs121908601	0.329	0.002691	0.002755	0.004557	5
5	D, Mic, B	*DGKK*	NA	NM_001013742.3	c.255_290del p.(Ser91_Ala102del)	-	-	0.0007869	0.0007224	0.001398	53*
6	M	*GATA4*	NA	NM_002052.4	c.1037C>T p.(Ala346Val)	rs115372595	0.592	0.001520	0.001545	0.002640	0
7	P	*LHCGR*	NA	NM_000233.3	c.1046C>T p.(Ala349Val)	rs758729322	0.596	0.000007958	0.000007363	0.0001760	0
8	P	*ERBB4*	NA	NM_005235.2	c.2444T>C p.(Ile815Thr)	rs1264168721	0.756	0.000007953	0.000007358	0.00001758	0

SGA NSH

	Phenotype	Gene	Segregation analysis	Transcript	HGVS c. and p. notation	rsID	REVEL score	gnomAD v2VAF	gnomAD v2Male VAF	gnomAD v2max VAF	gnomAD v2 Homozygotes
9	M	*BNC2*	Maternal	NM_017637.5	c.2618C>T p.(Pro873Leu)	-	0.494	-	-	-	-
10	P	*ESR2*	NA	NM_001437.2	c.64A>G p.(Ile22Val)	rs76299711	0.269	0.0006192	0.0006134	0.001124	0
11	P, B	*LHCGR*	NA	NM_000233.3	c.1435C>T p.(Arg479Ter)	rs757225917	-	0.000003981	0	0.00004619	0
12	P, Cb	*NR5A1*	NA	NM_004959.4	c.374C>T p.(Pro125Leu)	rs780952265	0.240	0.00002207	0.00002675	0.00003290	0
13	D	*ZNRF3*	NA	NM_032173.3	c.925T>A p.(Ser309Thr)	rs769697204	0.090	0.00001227	0.00002254	0.00002676	1
14	P	*LHCGR*	NA	NM_000233.3	c.1847C>T p.(Ser616Phe)	-	0.866	-	-	-	-
15	D	*EP300*	NA	NM_001429.3	c.5869C>T p.(Pro1957Ser)	rs1301322622	0.217	0.00001415	0.000006522	0.00003099	0
		ESR2	NA	NM_001437.2	c.661A>G p.(Arg221Gly)	rs78851986	0.794	0.002047	0.002156	0.003582	0

***Table 5*: Withheld variants of unknown significance (class 3) in 15 individuals born with non-syndromic hypospadias.**
M: midshaft hypospadias, Cb: bilateral cryptorchidism, D: distal hypospadias, Mic: micropenis, B: bifid scrotum, P: proximal hypospadias, NA: not assessed; REVEL score: rare exome variant ensemble learner; VAF: variant allel frequency; max VAF: highest variant allele frequency in any subpopulation; Homozygotes: reported number of homozygotes or hemizygotes (*); +: No DNA was available of the deceased father (who had hypospadias at birth).

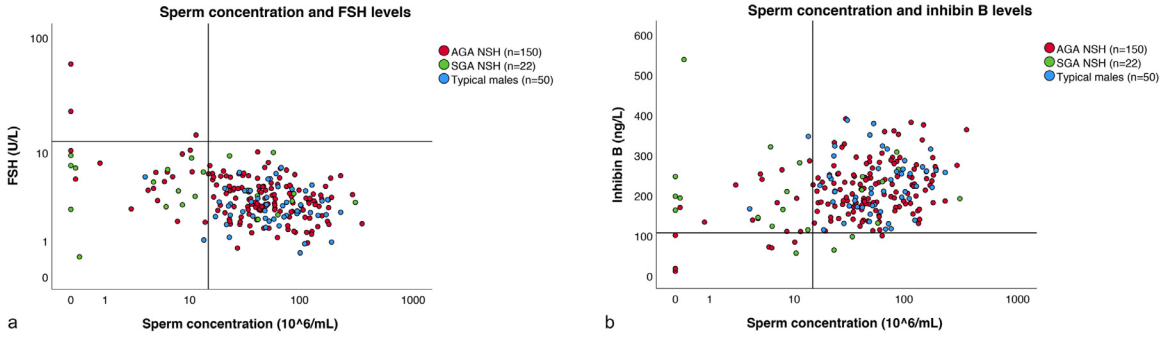

Figure 2. a. Scatterplot of sperm concentration and FSH levels. The horizontal line represents the threshold of 12.4U/L; the vertical line represents the threshold of 15.10^6/mL. X and Y-axis in logarithmic scale. b. Scatterplot of sperm concentration and inhibin B levels. The horizontal line represents the threshold of 105 ng/L; the vertical line represents the threshold of 15.10^6/mL. X and Y-axis in logarithmic scale.

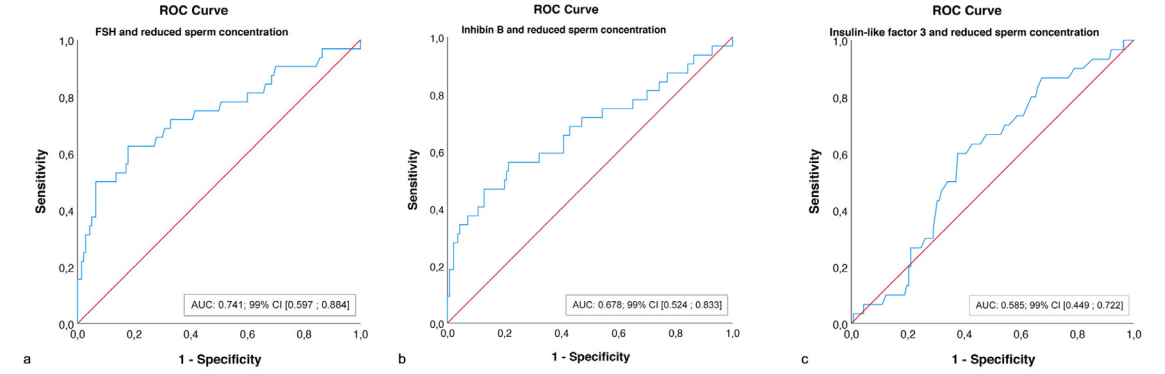

Figure 3. a. ROC-curve of FSH to detect a reduced sperm concentration in NSH; b. ROC-curve of Inhibin B to detect a reduced sperm concentration in NSH; c ROC-curve of INSL3 to detect a reduced sperm concentration in NSH.

impaired spermatogenesis compared to those born TM and SGA. Further studies are needed to confirm this hypothesis, and should also assess testicular function in undervirilized men without hypospadias.

In the overall male population, 10–15% have been reported to have oligo- or azoospermia.[30] The lower rate in our TM can be explained by the exclusion of men with factors that could impair spermatogenesis such as cryptorchidism, varicocele, or prenatal risk factors, e.g. low birth weight.

Reproductive hormone assessment is sometimes proposed as a surrogate for semen analysis in the pediatric endocrine setting. However, semen analysis is feasible and reliable in boys who have reached at least Tanner stage 3,[31] and we found that conventional laboratory thresholds for FSH and inhibin B are unreliable to screen for reduced sperm concentrations, with an unacceptably high number of false negatives. Modifying the thresholds to 4·11U/L for FSH and 196·40 ng/L for inhibin B could improve the detection rate, but to what extent these suggested thresholds are age-specific remains to be determined. As 94·5% of young adult men were willing and able to provide a semen sample after careful counseling, we recommend to refer boys over 18 years directly for semen analysis when fertility assessment is sought.

Prior to the introduction of massively parallel sequencing approaches, the genetic basis of hypospadias remained mostly unexplained. Nonetheless, rare complex and even isolated hypospadias cases have been attributed to pathogenic variants in DSD-related genes, such as *AR, HSD17B3, MAMLD1, NR5A1, SRD5A2* and *WT1*.[5] Although some promising variants of unknown significance were identified, our large-scale genetic screening did not reveal pathogenic (class 4 or 5) variants in any known DSD gene, nor in any gene previously implicated in male sub- and infertility or in any gene related to SGA birth or poor growth. In addition, digenic variant combinations predicted by ORVAL to be at least 95%-likely disease-causing, could not be linked convincingly to the case's phenotypes. Therefore, our data, analyzed with a more stringent bioinformatics

	NSH normal sperm concentration*	NSH oligozoospermia*	Effect size/score interval (99% CI)
Patient factors			
Insulin-like factor 3 (µg/L)	0.63 (0.36)	0.73 (0.32)	0.111
Obstructive voiding	30/136 (22.1%)	9/32 (28.1%)	-0.061 (-0.305;0.128)
Varicocele grade II or higher	26/140 (18.6%)	9/32 (28.1%)	-0.096 (-0.334;0.094)
Smoking (>10/day)	8/140 (5.7%)	3/32 (9.4%)	-0.037 (-0.249;0.069)
Alcohol (>14/week)	14/140 (10.0%)	2/32 (6.3%)	0.038 (-0.168;0.145)
Drug use	19/140 (13.6%)	3/32 (9.4%)	0.042 (-0.174;0.168)
Prenatal factors			
Maternal smoking	5/137 (3.6%)	3/32 (9.4%)	-0.057 (-0.270;0.039)
Gestational hypertension	15/137 (10.9%)	4/32 (12.5%)	-0.016 (-0.240;0.112)
Pre-eclampsia	5/137 (3.6%)	1/32 (3.1%)	0.005 (-0.186;0.081)
Exposure to chemicals	20/137 (14.6%)	6/32 (18.8%)	-0.042 (-0.277;0.112)
Industrial	18/137 (13.1%)	4/32 (12.5%)	0.006 (-0.219;0.138)

	SGA NSH normal sperm concentration*	SGA NSH oligozoospermia*	Effect size (99% CI)
Birth weight (SD)	-2.46 (1.02)	-2.57 (0.96)	0.050
Birth length (SD)	-2.47 (1.40)	-2.35 (2.20)	0.064
Adult height (cm)	173.6 ± 9.6	173.2 ± 4.7	0.052 (-1.066;1.168)
Delta midparental height (cm)	2.5 ± 9.8	6.2 ± 6.2	-0.461 (-1.629;0.719)
Stretched penile length (cm)	11.1 ± 2.5	11.1 ± 1.3	-0.006 (-1.203;1.191)

***Table 6*: Predictors of reduced sperm concentrations.**

Normal sperm concentration: $>15.10^6$ sperm cells per mL; Oligozoospermia: $<15.10^6$ sperm cells per mL, including azoospermia; Obstructive voiding: based on visual interpretation of the uroflowmetry curve and flow index; Smoking: more than 10 cigarettes per day; Alcohol: more than 14 alcoholic consumptions per week; Drug use: use of any illegal substance (including marihuana); Varicocele: grade II or higher; Gestational hypertension: clinically documented hypertension during pregnancy (including pre-eclampsia); Exposure to chemical: direct occupational exposure to chemicals; Industrial: living near an industrial site during the pregnancy; SD: standard deviation; CI: confidence interval.

* *N* (%), median (interquartile range) or mean ± standard deviation, as appropriate.

pipeline, do not support the recent recommendation of Ea *et al.*[32] to perform gene panel testing in a routine diagnostic setting in all forms of hypospadias. We recommend reserving this for cases where an underlying syndrome or DSD condition is strongly suspected, or in a specific research context.

Our data confirm the hypothesis that hypospadias, in the vast majority of NSH, do not result from Mendelian causes, even in the most severe forms, or in those with intra-familial recurrence, and in those cases that are associated with sub- or infertility. On the other hand, our findings are in line with the hypothesis that especially complex NSH is part of the TDS, based on the unexpectedly high number of men with oligo- and azoospermia. Taken together, our data lend support to a multifactorial etiology in most NSH, even when associated with a severe or complex phenotype and/or spermatogenic failure.

Strengths of this study include the recruitment of men with various forms of NSH and healthy TM and a holistic approach in assessing their endocrine and reproductive outcome at adult age and the use of mainly objective outcome measures. Limitations include the cross-sectional study design which does not allow detection of causal relationships, the small number of men born SGA and AGA with complex NSH, the proportionally smaller control group of TM and the use of many statistical tests which increases the odds of type I errors. Penile measurements at first presentation were often missing, likely causing some to be misclassified as not having complex NSH. In addition, data regarding penile chordees were missing. The majority of participants were of Caucasian descend, warranting studies of different populations to confirm our data.

In conclusion, pubertal assessment of NSH is not recommended. Given their high rates of reduced sperm concentration, semen analyses can be offered to all young adult men born SGA or AGA with proximal or complex NSH, after counseling. However, in the absence of longitudinal data on the evolution of sperm concentrations in men at risk of developing spermatogenic failure, the role of early sperm cryopreservation is undetermined. Our findings show increased FSH, impaired spermatogenesis and subclinical Leydig cell dysfunction indicating testicular dysfunction in NSH, especially in those SGA or with complex NSH. Mono- and oligogenic causes of hypospadias and testicular dysfunction were not found in our cohort, suggesting an epigenetic and/or environmental etiology. Growth should be monitored and growth hormone therapy considered where appropriate in those born SGA with NSH.

Contributors

LT and MC were responsible for funding acquisition. All authors contributed to the conceptualization and methodology. Investigations were performed by LT, AFS, AS, SR, UT, JW, MH, PH, EV, JVDV, KT and AM. LT, MC, MH, AFS, EDB, HV, JA and AJ were responsible for formal analysis and data curation. Verification of the underlying data was performed by LT, MC, AFS and HV. All authors participated in the writing and critical review of the manuscript. All authors approved this manuscript and approved submission for publication. MC was responsible for overall coordination of the study. The data were accessed and verified by LT, MC, AFS and HV.

Data sharing statement

The SPSS file containing clinical, hormonal and semen data can be accessed through the following link: https://drive.google.com/drive/folders/1ixHDtOIuDC1lPR2pkYqgdpKtBZvKwnw4?usp=sharing. The results of the genetic tests can be shared by contacting the corresponding author.

Declaration of interests

The authors report no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104119.

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