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±2.74%) of NSH men; but in 5/16 (31±3%; 99%-CI: 11±6.24%) of complex NSH men and in 13/22 (59±1%; 99%-CI: 33±8.07%) 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.

Funding Research grants from the European Society of Paediatric Endocrinology, the Belgian Society of Pediatrics, the Belgian Society of Paediatric Endocrinology and Diabetology and the Research Foundation Flanders (FWO).

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Keywords: Hypospadias; Fertility; Testicular function; DSD; Andrology; Testicular dysgenesis syndrome

*eBioMedicine 2022;81: 104119
Published online 24 June 2022 https://doi.org/10.1016/j.ebiom.2022.104119

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Introduction

Intrauterine testicular androgen production induces physiological development of the penile urethra. Hence, prenatal factors such as placental insufficiency or exposure to endocrine disruptors that affect testicular function can compromise penile development. 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. 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. 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. 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. Epidemiological evidence has revealed lower paternity rates in men with hypospadias. However, data on semen characteristics are scarce and often do not distinguish mild from severe forms or lack control groups.

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 under-virilisation 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 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; 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; 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 (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.

NHS 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 inhibit B to FSH ratio to predict reduced sperm concentrations.

**Role of funders**

Research grants were received from the European Society of Paediatric Endocrinology, the Belgian Society of Pediatrics, the Belgian Society of Pediatric Endocrinology and Diabetology and the Research Foundation...
Flanders (FWO). MC (1801018N) and EDB (1802220N) are Senior Clinical Investigators of the FWO. Funders were not involved in study design, data collection, data analyses or manuscript writing.

**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 4.4 cm 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

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**Table 1**: Summary of genital phenotype, gestational and birth data.

|                      | 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 | 3/164 (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) |
### Puberty*  
**Multinomial logistic regression**  
Reference category: average onset of puberty

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

### SGA NSH Vs. AGA NSH and typical males

|                      | AGA NSH Vs. typical males |          |          |
| AGA NSH*             | Early puberty N (%)       | Adjusted OR | 99% CI   |
| 28/167 (16.8%)       | 0.69                      | 0.16; 3.99 |
| Typical males$       | 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              | 35/167 (21.0%)            | 0.488     | 0.14; 1.76 |
| Typical males$       | 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              | 0.5 ± 6.5 | 5.54    | 1.72; 9.37 |
| Typical males$       | -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              | 14/152 (9.2%)    | 0.20        | 0.05; 0.77 |
| Typical males$       | 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              | 5/167 (3.0%)       | 0.09        | 0.02; 0.47 |
| Typical males$       | 2/30 (6.7%)        | 0.16        | 0.02; 1.62 |
| SGA NSH              | 7/26 (26.9%)       | -          | -        |

### BMI (kg/m²)*  
**Linear regression**

|                      | AGA NSH Vs. typical males | Mean (IQR) | B-value | 99% CI   |
| Distal hypospadias*  | 21.5 (4.4)               | 0.45       | -1.03; 1.92 |
| Proximal hypospadias*| 21.7 (3.9)               | 0.39       | -1.50; 2.29 |
| Complex hypospadias* | 19.3 (4.9)               | -0.28      | -2.74; 2.18 |
| Typical males        | 22.5 (3.2)               | -         | -        |

|                      | SGA NSH Vs. AGA NSH and typical males | Medain (IQR) | B-value | 99% CI   |
| AGA NSH              | 21.9 (4.4)               | 0.27       | -1.53; 2.08 |
| Typical males$       | 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 | Mean ± SD | B-value | 99% CI   |
| Right testicle       | 12.7 ± 3.8                | -0.84     | -2.60; 0.93 |
| Proximal hypospadias*| 12.9 ± 3.9                | -0.34     | -2.56; 1.89 |
| Complex hypospadias* | 10.2 ± 4.0                | -3.07     | -6.11; 0.03 |
| Typical males        | 13.0 ± 3.9                | -         | -        |
| Adjusted $R^2$       | 0.103                    |           |          |

|                      | Left testicle Mean ± SD | B-value | 99% CI   |
| Distal hypospadias*  | 12.5 ± 3.7               | -0.49    | -2.14; 1.15 |
| Proximal hypospadias*| 12.0 ± 3.1               | -0.83    | -2.90; 1.25 |
| Complex hypospadias* | 9.4 ± 4.9                | -3.31    | -6.16; -0.47 |
| Typical males        | 12.5 ± 3.4               | -        | -        |
| Adjusted $R^2$       | 0.041                    |           |          |

### Stretched penile length (cm)+  
**Linear regression**

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

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*Table 2 (Continued)"
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. AGA-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 Testosterone (nmol/L)), LH (U/L) and Free Testosterone (nmol/L) in AGA-NSH compared to typical males and SGA NSH, there were no significant differences in adjusted R² for these variables. (Table 3: Continued)

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: midshaft to perineal hypospadias without other genital symptoms; Complex hypospadias: with other genital symptoms (e.g. 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; $: co-variates age, body height and varicocele grade II (yes/no); +: covariates age and body height.
FSH) levels, few NSH had levels above laboratory thresholds (Table 3 and Supplemental Table 2c). Testicular androgen levels (i.e. total and free testosterone, dihydrotestosterone) were not different in NSH compared to TM. NSH had higher insulin-like factor 3 (INSL3) levels, with no differences between subgroups (Figure 1a). In all participants, LH significantly correlated with INSL3 ($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: $r_s = 0.232, 99\%-CI: 0.157; 0.342$; 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 ng/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 (<18 years: 69/84 (82.1%); >18 years: 103/109 (94.5%). Sperm
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 (ACMG17 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 PRTY).

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 (4-11 U/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)*

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| Distal hypospadias     | 2.8 (1.8)    | -0.47   | -1.07; 0.14 |
| Proximal hypospadias   | 2.7 (2.2)    | -0.26   | -1.04; 0.53 |
| Complex hypospadias    | 2.1 (1.4)    | -1.04   | -2.02; 0.06 |
| Typical males          | 3.2 (1.3)    | -       | -       |
| Adjusted R²            |              | 0.072   |         |

### SGA NSH Vs. AGA NSH and typical males

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| AGA NSH                | 2.6 (1.7)    | 0.15    | -0.65; 0.95 |
| Typical males          | 3.2 (1.3)    | 0.66    | -0.26; 1.58 |
| SGA NSH                | 2.6 (2.7)    | -       | -       |
| Adjusted R²            |              | 0.052   |         |

### Sperm concentration (10⁶/mL)*

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| Distal hypospadias     | 46.25 (78.82)| -6.48   | -32.60; 19.24 |
| Proximal hypospadias   | 52.78 (49.68)| -8.01   | -41.87; 25.86 |
| Complex hypospadias    | 28.00 (38.39)| -52.07  | -94.59; -9.56 |
| Typical males          | 57.0 (66.6)  | -       | -       |
| Adjusted R²            |              | 0.053   |         |

### SGA NSH Vs. AGA NSH and typical males

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| AGA NSH                | 45.6 (62.4)  | 21.98   | -12.65; 35.61 |
| Typical males          | 57.0 (66.6)  | 32.56   | -7.26; 72.36 |
| SGA NSH                | 10.9 (43.4)  | -       | -       |
| Adjusted R²            |              | 0.039   |         |

### Total sperm count (10⁹/ ejaculate)*

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| Distal hypospadias     | 130.90 (181.84)| -53.30  | -135.88; 29.28 |
| Proximal hypospadias   | 138.36 (198.49)| -40.36  | -147.44; 66.71 |
| Complex hypospadias    | 39.29 (48.48) | -200.27 | -334.64; -0.65 |
| Typical males          | 187.4 (234.3)| -       | -       |
| Adjusted R²            |              | 0.085   |         |

### SGA NSH Vs. AGA NSH and typical males

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| AGA NSH                | 123.0 (184.1)| 54.33   | -52.05; 160.71 |
| Typical males          | 187.4 (234.3)| 118.48  | -3.80; 240.77 |
| SGA NSH                | 43.0 (122.1) | -       | -       |
| Adjusted R²            |              | 0.071   |         |

### Progressive motility (%)*

|                        | Mean ± SD | B-value | 99% CI  |
|------------------------|-----------|---------|---------|
| Distal hypospadias     | 39.27 ± 16.77 | -3.54  | -12.11; 5.03 |
| Proximal hypospadias   | 37.33 ± 15.91 | -5.44  | -16.55; 5.67 |
| Complex hypospadias    | 31.52 ± 19.33 | -11.13 | -25.08; 2.82 |
| Typical males          | 43.2 ± 20.7  | -       | -       |
| Adjusted R²            |           | 0.000   |         |

### Sperm morphology (%)*

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| Distal hypospadias     | 4.0 (2.0)    | 2.17    | 0.72; 3.61 |
| Proximal hypospadias   | 4.0 (3.0)    | 2.55    | 0.89; 4.21 |
| Complex hypospadias    | 4.0 (3.0)    | 2.08    | -       |
| Typical males          | 51.4 ± 20.8  | 20.39   | 7.40; 33.38 |
| Adjusted R²            |              | 0.059   |         |

### Sperm concentration*

|                        | N (%) | Adjusted OR | 99% CI  |
|------------------------|-------|-------------|---------|
| < 15.106/mL AGA NSH    | 9/104 | 0.75        | 0.26; 1.43 |
| Typical males          | 5/30  | 2.37        | 0.96; 4.43 |
| SGA NSH                | 5/16  | 2.37        | -       |
| Adjusted R²            |       |            | 0.013   |

### Total motility (%)*

|                        | Median (IQR) | B-value | 99% CI  |
|------------------------|--------------|---------|---------|
| Distal hypospadias     | 4.0 (2.0)    | 0.55    | 0.28; 0.81 |
| Proximal hypospadias   | 4.0 (3.0)    | 1.00    | 0.48; 1.51 |
| Complex hypospadias    | 4.0 (3.0)    | -       | -       |
| Typical males          | 51.4 ± 20.8  | 20.39   | 7.40; 33.38 |
| Adjusted R²            |              | 0.059   |         |

### Sperm concentration* Logit regression

|                        | N (%) | Adjusted OR | 99% CI  |
|------------------------|-------|-------------|---------|
| < 15.106/mL AGA NSH    | 9/104 | 0.37        | 0.13; 0.95 |
| Typical males          | 5/30  | 2.37        | 0.96; 4.43 |
| SGA NSH                | 5/16  | 2.37        | -       |
| Adjusted R²            |       |            | 0.013   |

Table 4 (Continued)
growth in the overall SGA population. These results corroborate data from our recent retrospective study using the I-DSD registry, in which 31/104 (29%) undervirilized SGA boys had insufficient catch-up growth and underscore the importance of follow-up using the I-DSD registry, in which 31/104 (29%) of growth and consideration of growth hormone treatment in undervirilized boys born SGA.21 These results corroborate data from our recent retrospective study revealing need for penile re-interventions in 39% of AGA NSH Vs. typical males

| Motility* | Logistic regression |
|----------|---------------------|
| Reference category: Progressive motility ≥32% or total motility ≥40% | |
| AGA NSH Vs. typical males N (%) | Adjusted OR | 99% CI |
| Progressive ≥32% and total motility ≥40% | |
| Distal hypospadias* | 28/104 (26.9%) | 1.46 | 0.46; 4.69 |
| Proximal hypospadias* | 9/30 (30%) | 1.63 | 0.39; 6.86 |
| Complex hypospadias* | 6/15 (37.5%) | 2.12 | 0.39; 11.38 |
| Typical males | 9/50 (18%) | - | - |

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 AGA NSH. *: co-variates age, varicocele ≥ grade II (yes/no) and days of abstinence.

Higher INSL3 levels were observed mainly in complex NSH. INSL3 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, inhibit B and sperm concentration. Overall, 18-6% of SGA 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 Nординvall 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 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.30-32

Table 4: Results of the semen analysis.

| Morphology* | Logistic regression |
|-------------|---------------------|
| Reference category: ≥4% normal morphology | |
| AGA NSH Vs. typical males N (%) | Adjusted OR | 99% CI |
| Progressive ≥32% and total motility ≥40% | |
| Distal hypospadias* | 32/104 (30.8%) | 0.85 | 0.31; 2.31 |
| Proximal hypospadias* | 12/30 (40.0%) | 1.29 | 0.36; 4.56 |
| Complex hypospadias* | 7/16 (43.8%) | 1.50 | 0.31; 7.18 |
| Typical males | 17/50 (34%) | - | - |

| SGA NSH Vs. AGA NSH and typical males N (%) | Adjusted OR | 99% CI |
| AGA NSH* | 51/150 (34.0%) | 0.11 | 0.03; 0.52 |
| Typical males* | 17/50 (34%) | 0.11 | 0.02; 0.60 |
| SGA NSH | 18/22 (81.8%) | - | - |

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 in-utero growth restriction, hypothesized to result from impaired hCG secretion due to placental insufficiency.2,10 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
### AGA NSH

| N° | Phenotype | Gene | Segregation analysis | Transcript | HGVS c. and p. notation | rsID | REVEL score | gnomAD v2VAF | gnomAD v2max VAF | gnomAD v2Homozygotes |
|----|-----------|------|----------------------|------------|------------------------|------|-------------|--------------|----------------|-------------------|
| 1  | M, Cb     | BNC2 | De novo              | NM_017637.5| c.1022A>G              | -    | 0.156       | -            | -              | -                 |
|    |           |      |                      |            | p.(Asn341Ser)          |      |             |              |                |                   |
| 2  | M         | CHD7 | Paternal/ de novo    | NM_017780.3| c.3730A>G              | -    | 0.722       | -            | -              | -                 |
|    |           |      |                      |            | p.(Thr1244Ala)         |      |             |              |                |                   |
|    |           |      |                      |            | rs900214501            |      | 0.180       | 0.00001593   | 0.00001965     | 0.00005953        |
|    |           |      |                      |            |                       |      |             |              |                |                   |
| 3  | D         | ESR2 | Maternal             | NM_001437.2| c.1123G>C              | -    | 0.866       | -            | -              | -                 |
|    |           |      |                      |            | p.(Glu375Gln)          |      |             |              |                |                   |
|    |           |      |                      |            | rs121908601            |      | 0.329       | 0.002691     | 0.002755       | 0.004557          |
|    |           |      |                      |            |                       |      |             |              |                |                   |
| 4  | D         | ZFPM2| NA                   | NM_012082.3| c.89A>G                | -    | -           | 0.0007869    | 0.0007224      | 0.001398          |
|    |           |      |                      |            | p.(Glu30Gly)           |      |             |              |                |                   |
|    |           |      |                      |            |                       |      |             |              |                |                   |
| 5  | D, Mic, B | DGRX | NA                   | NM_001013742.3| c.255,290del          | -    | -           | 0.0007869    | 0.0007224      | 0.001398          |
|    |           |      |                      |            | p.(Ser91_Ala102del)   |      |             |              |                |                   |
| 6  | M         | GATA4| NA                   | NM_0002052.4| c.1037C>T              | -    | 0.592       | 0.001520     | 0.001545       | 0.002640          |
|    |           |      |                      |            | p.(Ala346Val)          |      |             |              |                |                   |
| 7  | P         | LHCGR| NA                   | NM_000233.3| c.1046C>T              | -    | 0.596       | 0.000007958  | 0.000007363    | 0.0001760         |
|    |           |      |                      |            | p.(Ala349Val)          |      |             |              |                |                   |
| 8  | P         | ESR2 | NA                   | NM_005235.2| c.2444C>T              | -    | 0.756       | 0.000007953  | 0.000007358    | 0.00001758        |
|    |           |      |                      |            | p.(Ile815Thr)          |      |             |              |                |                   |

### SGA NSH

| N° | Phenotype | Gene | Segregation analysis | Transcript | HGVS c. and p. notation | rsID | REVEL score | gnomAD v2VAF | gnomAD v2max VAF | gnomAD v2Homozygotes |
|----|-----------|------|----------------------|------------|------------------------|------|-------------|--------------|----------------|-------------------|
| 9  | M         | BNC2 | Maternal             | NM_017637.5| c.2618C>T              | -    | 0.494       | -            | -              | -                 |
|    |           |      |                      |            | p.(Pro873Leu)          |      |             |              |                |                   |
| 10 | P         | ESR2 | NA                   | NM_001437.2| c.64A>G                | rs76299711 | 0.269       | 0.0006192    | 0.0006134      | 0.001124          |
|    |           |      |                      |            | p.(Ile22Val)           |      |             |              |                |                   |
| 11 | P, B      | LHCGR| NA                   | NM_000233.3| c.1435C>T              | rs757229517 | -           | 0.000003981  | 0.00004619     | 0                 |
|    |           |      |                      |            | p.(Arg479Ter)          |      |             |              |                |                   |
| 12 | P, Cb     | NRS51| NA                   | NM_0004959.4| c.374C>T              | rs780952265 | 0.240       | 0.00002207   | 0.00002675     | 0.0003290         |
|    |           |      |                      |            | p.(Pro125Leu)         |      |             |              |                |                   |
| 13 | D         | ZNF53| NA                   | NM_032173.3| c.925T>A               | rs769697204 | 0.090       | 0.00001227   | 0.00002254     | 0.00002676        |
|    |           |      |                      |            | p.(Ser309Thr)         |      |             |              |                |                   |
| 14 | P         | LHCGR| NA                   | NM_000233.3| c.1847C>T              | -    | 0.866       | -            | -              | -                 |
|    |           |      |                      |            | p.(Pro161Phe)         |      |             |              |                |                   |
| 15 | D         | EP300| NA                   | NM_001429.3| c.5869G>T              | rs1301322622 | 0.217       | 0.00001415   | 0.000006522    | 0.00003099        |
|    |           |      |                      |            | p.(Ser195Ser)         |      |             |              |                |                   |
|    |           |      |                      |            | rs78851986           |      | 0.794       | 0.0002047    | 0.002156       | 0.003582          |

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 allele 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).
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. 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, 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 4.11 U/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, MAML2, NR3A1, SRD5A2 and WT1. 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 approach, do not support the hypothesis of rare genetic variants in DSD-related genes as a common cause of hypospadias.

**Figure 2.** a. Scatterplot of sperm concentration and FSH levels. The horizontal line represents the threshold of 12.4 U/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.
pipeline, do not support the recent recommendation of Ea et al. 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.

| Patient factors | NSH normal sperm concentration | NSH oligozoospermia | Effect size/score interval (99% CI) |
|-----------------|-------------------------------|---------------------|----------------------------------|
| 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 | SGA NSH normal sperm concentration | SGA NSH oligozoospermia | Effect size (99% CI) |
|------------------|-------------------------------|---------------------|---------------------|
| 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) |

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.
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/1ixHD0lVfDci1Pr2pKyg0dpKtBZvKwmg4?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.

Acknowledgements
We thank the statistics department of the faculty of Medicine and Health Sciences of Ghent University for their guidance on data analysis. We thank all participants for their time and willingness to provide multiple blood and semen samples. LT received research grants from the European Society of Paediatric Endocrinology, the Belgian Society of Pediatrics, the Belgian Society of Pediatric Endocrinology and Diabetology and the Research Foundation Flanders. MC and EDB are supported by senior clinical investigator grants from the Research Foundation Flanders.

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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