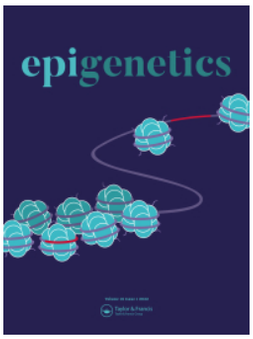

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# Genome-wide methylation analysis in patients with proximal hypospadias – a pilot study and review of the literature

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**ABSTRACT**

In patients with proximal hypospadias, often no genetic cause is identified despite extensive genetic testing. Many genes involved in sex development encode transcription factors with strict timing and dosing of the gene products. We hypothesised that there might be recurrent differences in DNA methylation in boys with hypospadias and that these might differ between patients born small versus appropriate for gestational age. Genome-wide Methylated DNA sequencing (MeD-seq) was performed on 32bp LpnPI restriction enzyme fragments after RE-digestion in leucocytes from 16 XY boys with unexplained proximal hypospadias, one with an unexplained XX testicular disorder/difference of sex development (DSD) and twelve, healthy, sex- and age-matched controls. Five of seven differentially methylated regions (DMRs) between patients and XY controls were in the Long Intergenic Non-Protein Coding RNA 665 (LINC00665; CpG24525). Three patients showed hypermethylation of MAP3K1. Finally, no DMRs in XX testicular DSD associated genes were identified in the XX boy versus XX controls. In conclusion, we observed no recognizable epigenetic signature in 16 boys with XY proximal hypospadias and no difference between children born small versus appropriate for gestational age. Comparison to previous methylation studies in individuals with hypospadias did not show consistent findings, possibly due to the use of different inclusion criteria, tissues and methods.

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## Introduction

Hypospadias is a common form of atypical genitalia, occurring in about 1:300 boys [1,2]. Most forms are distal with the urethra ending at or distal of the corona [3]. There is a strong genetic predisposition with about 1:10 of such cases being familial [4]. In contrast, about 15% of hypospadias are more proximal, from somewhere on the penile shaft to perineal. This is usually associated with chordee, i.e., curving of the penis due to the ventral skin defect. Especially, the proximal forms may be the result of a disorder or difference of sex development (DSD). Genetic analyses may demonstrate pathogenic variants in genes such as *NR5A1, ZFPM2, MAP3K1, SRD5A2, AR, WT1*, and many others [5] or chromosomal abnormalities such as a 45,X/46,XY genotype or a microdeletion of various chromosome regions [6–8]. Still, in the majority of cases no genetic cause can be detected. Oligenic or multifactorial inheritance has been suggested and may explain why, for example, the presence of *MAMLD1* (OMIM # 300758) deletions or sequence variants in XY DSD patients is associated with a variable, broad DSD-phenotype, which may be influenced by concurrent variants in other DSD-related genes [9,10].

It is well known that hypospadias is more often seen in children born small for gestational age (SGA) [5,11]. In discordant male twins, hypospadias usually occurs in the twin with the lowest birth weight [12]. The nature of the association between SGA en hypospadias is still unclear, but

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epigenetic mechanisms could play a role [13–16]. Fetal growth restriction is associated with altered DNA methylation, which has been proposed to mediate the increased risk of cardiometabolic diseases later in life [17]. A hypothesis is that a reduction in the placental hormones early in pregnancy may cause an aberrant timing or dosage of gene transcription and subsequently transcription might be influenced by epigenetic factors [18]. Silver-Russell syndrome (SRS, OMIM # 180860) is a syndrome characterized by fetal growth retardation and is often associated with proximal hypospadias and cryptorchidism. It is most commonly associated with hypomethylation of *H19* and the degree of hypomethylation has been found to correlate with the risk of a genital phenotype [19–21]. Another indication that epigenetic changes may be relevant in the etiology of hypospadias is provided by the finding of an increased risk of hypospadias in grandsons of women who used diethylstilbestrol, an organic compound with estrogenic activity [22]. Prenatal exposure to diethylstilbestrol causes epigenetic changes, which may underly such transgenerational effects [23,24].

To further investigate the role of epigenetic changes in hypospadias, we performed a pilot genome-wide DNA Methylated DNA sequencing (MeD-seq) study using the method developed by Boers et al. [25] on XY individuals with proximal hypospadias, in whom extensive genetic investigations had not identified a genetic cause. DNA from a boy with *H19* hypomethylation and hypospadias served as a positive control. Simultaneously, the same genome-wide methylation study was performed for a male individual with *SRY* negative XX testicular DSD and proximal hypospadias without a genetic diagnosis. The goals were to investigate if 1. an episignature was recognizable in boys with proximal hypospadias, 2. methylation differed between SGA or non-SGA individuals with proximal hypospadias and 3. for individual patients a diagnosis could be established.

## Materials and methods

### Patient and controls inclusion

Inclusion criteria for the study were as follows: 1. proximal hypospadias, 2. no pathogenic copy number variation detected using SNP array, 3. no identified (likely) pathogenic sequence variants with analysis of a WES-based panel of DSD-related genes or larger panel including these genes, 4. DNA isolated from EDTA blood available and 5. informed consent to use DNA for research. Patients were investigated in a diagnostic laboratory. One individual (case 5) with a known *H19* hypomethylation was originally tested as described by Bliek et al. [26] and was included as a positive control. One individual with *SRY* negative, unexplained 46,XX testicular DSD and proximal hypospadias was also included to see if an epigenetic cause could be identified. Two individuals were one of a twin pair (cases 4, 11). In these cases, the twin sibling was not included even if the inclusion criteria were met, only the individual with the most proximal hypospadias of the pair was included. Birth weight standard deviations were calculated using male references [27] and SGA was defined as birth weight <-2 SD. Controls for the XY patients with hypospadias were age-matched boys and for the individual with XX testicular DSD age-matched girls from the Erasmus MC Biobank. The controls did not have any known genetic disorder and had a simple non-genital surgical procedure such as correction of prominent ears, placement of tympanostomy tubes or removal of a benign tumor. Informed consent and DNA were available. For the WES, SNP array and Med-seq analysis, the same batch of peripheral blood lymphocytes extracted DNA was used.

### Previous investigations

For all patients, except the *H19* hypomethylation individual, WES was performed, followed by analysis of sequence variants in a composite panel of genes, involved in DSD or multiple congenital anomalies (MCA), or a full exome analysis was performed (see additional file 1). In analysis of the DSD panel, WES was performed on DNA extracted from the patients' blood and if applicable, from the parents' blood followed by a trio analysis (MCA panel or open exome) or by singleton analysis (DSD panel) [28]. Variants were filtered and annotated with Alissa Interpret software (Agilent Technologies) on quality (read depth ≥ 10), minor allele frequency (<1% in 200 alleles in dbSNP, ESP6500, the 1000 Genome project, GoNL,

gnomAD or the ExAC) database and location (exonic variants or variants within the exon-flanking intronic regions −10 and + 10). In case of a large panel variants were further selected based on inheritance mode.

Alamut Visual Plus software (interactive Biosoftware, SOPHiA GENETICS) was used for classification of the sequence variants according to the international standard ACMG criteria [29] and only class 4 (likely pathogenic), class 5 (pathogenic) variants and possibly relevant class 3 variants (variants of uncertain clinical significance or VUS) were reported. In cases of doubt whether or not to report a variant, including incidental findings (IF), the variant was discussed in a multidisciplinary team and reported if considered relevant. For the content of the gene panels and sequence details, see https://www.erasmusmc.nl/nl-nl/patientenzorg/laboratoriumspecialismen/klinische-genetica. Individual 5 had a targeted study for hypomethylation of H19 as described by Bliek et al. [26]. Endocrine evaluation generally consisted of measurement of anti-Müllerian hormone (AMH), inhibin B and a steroid profile and, depending on the initial results, sometimes an hCG test was performed. All samples were reanalyzed using the same version of the panel (10.2).

### Snp-array processing and analysis

Next, we determined Copy Number profiles of all patients included in this study using SNP-array and evaluated if there were putative deleterious rare Copy Number Variations (CNVs) or rare CNVs impacting DSD-related genes. High-resolution analyses were performed using the Infinium Global Diversity Array-8 v1 ($n = 15$) or the Infinium Global Screening Array-24 v3.0 ($n = 3$) (Illumina Inc., San Diego, CA, USA). SNP-arrays were processed using the GenomeStudio genotyping module (v2.0, www.illumnia.com) and resulting final reports were loaded and processed and visually inspected using Biodiscovery Nexus CN10.0 (Biodiscovery Inc., Hawthorne, CA, USA). CNVs were classified into artefact, common polymorphism (CNP) and rare Copy Number Variant by confirming if the log-R ratio matched the expected allele frequency shift (Gain) or a Copy Number loss matched the Loss of Heterozygosity (LOH) seen in allele frequency track. Moreover, CNPs/CNVs were classified based size, probe content, quality, and overlap/frequency in a modified version (ie, excluding BAC arrays and small InDels) of the database of genomic variation (http://dgv.tcag.ca/dgv/app/home). More detailed methods have been described previously [28].

### MeD-seq sample preparation

DNA samples were prepared for MeD-seq as previously described [25]. In brief, DNA samples were digested with LpnPI (New England Biolabs, Ipswich, MA, USA) which resulted in fragments of 32bp with the methylated cytosine in the centre. Fragments were purified by Pippin system gel after preparation. The 32bp DNA fragments were prepared for sequencing using a ThruPlex DNA-seq 96D kit (Takara Bio Inc, Kusatsu, Japan) according to the manufacturer's protocol. To include dual indexed barcodes, stem – loop adaptors were blunt-end ligated to repaired input DNA and amplified (4 + 10 cycles) using a high fidelity DNA polymerase. Multiplexed samples were sequenced on Illumina HiSeq2500 systems for single reads of 50bp according to the manufacturer's instructions. Dual indexed samples were demultiplexed using bcl2fastq software (Illumina).

### Data processing and analysis

MeD-seq data were processed and analysed with Python 2.7.5 using specifically created scripts as previously described [25]. In short, before mapping of the reads to the Hg38 genome using bowtie 2.1.0., the raw FASTQ files were subjected to Illumina adaptor trimming and filtered for the presence of LpnPI restriction sites 13-17bp from the 3' or 5' end. For visualization of the mapped reads, BAM files were generated using SAMtools. LpnPI site scores were used to produce read count scores for the transcription start sites (TSS) (1kb before and 1kb after), gene bodies (1kb after the TSS until the transcription end site) and CpG islands. Gene and CpG island annotations were downloaded from UCSC (hg38). To detect DMRs between two data sets, genome-wide read counts

were compared using the Chi-Squared test. Significance was set at $p < 0.05$ and was called with a Bonferroni correction or FDR using the Benjamini–Hochberg procedure. In addition, a genome-wide sliding window was used to detect sequentially differentially methylated LpnPI sites. Statistical significance was called between LpnPI sites in predetermined groups using the Chi-square test with a Bonferroni correction. Neighbouring significantly called LpnPI sites were binned and reported. Overlap of genome-wide detected DMRs was reported for TSS, CpG island or gene body regions using the annotations of UCSC (hg38). Gene enrichment analyses were carried out using Gene Ontology (GO) software (geneontology.org). GO-terms with p-values ≤ 0.05 were considered to be significant. For Quality control data see additional file 2.

### Literature review

Previous studies of methylation in individuals with hypospadias were found by searching PubMed using the terms 'hypospadias and (epigenetic OR methylation)', leading to a total of 53 manuscripts. Excluded were single-case studies, studies about the effect of endocrine disruptors, animal studies, or studies that reported on targeted screening of, e.g., H19 in a diagnostic work-up only and studies that only mentioned that epigenetic factors could play a role in hypospadias, but did not report original methylation data (see additional file 3). This resulted in selection of six studies, which were used for comparison to the results of the current study [13,15,16,30–32].

### Results: patient characteristics

Sixteen boys with unexplained proximal hypospadias and XY genotype met the inclusion criteria (see Table 1 for a summary of patient characteristics). One boy with XX testicular DSD with hypospadias was also analyzed (patient 18) and a second boy with hypospadias due to SRS was included as a positive control (patient 5, Figure 1). Of all included individuals, nine (cases 1–9) were SGA and nine (including the XX male) had normal birth weight (cases 10–18). Endocrine investigations were performed in 15/18 children and unremarkable. The testosterone/DHT ratio after hCG stimulation was relatively high, >15, in three children (cases 3, 4 and 7) but no (likely) pathogenic variants were identified in *SRD5A2* in these three boys. The ages at collection of DNA used for this study varied from 1 d after birth to 15 y (see Table 1). For the XY controls, the ages varied from 3 months to 12 y; the XX controls were <1 y at the time of DNA collection, matching the age of the XX male patient.

### Standard testing

A WES-DSD gene panel cohort analysis was performed to make sure all patients were evaluated by the same panel, and SNP-array analysis for copy number variants in known DSD genes did not reveal a (likely) explanation for the hypospadias in any of the included patients. Heterozygous pathogenic variants found in *PROKR2* (OMIM # 244200, patient 2) and *GNRHR* (OMIM # 146110 patient 13), both genes involved in hypogonadotropic hypogonadism were not considered explanatory for the phenotype, nor was the variant of uncertain significance identified in the XY-DSD associated gene *CHD7* (OMIM # 214800 patient1), without a phenotype of CHARGE syndrome (Table 1). There were no relevant rare Copy Number Variations impacting DSD genes.

### Methylation analysis

A genome-wide analysis was performed and methylation patterns were compared between the selected individuals with hypospadias and male controls and between patients born SGA and non-SGA. In addition, methylation of promoter regions of relevant genes, i.e., genes known to be associated with hypospadias from our local DSD exome panel, were compared between patients and controls. H19 hypomethylation was confirmed in the positive control individual with known SRS with the MeD-seq data analyzed at patient level (see Figure 1, case 5, this patient was a clear outlier with Z-Score −3,04, $p < 0{,}001$). This confirmed the methods used in the current study are able to identify methylation alterations at the individual level.

**Table 1.** Overview of patient characteristics and relevant genetic test results.

| Case | Age at DNA collection | SGA present (+) or absent (-) Gestational age Birth parameters if known | External genital phenotype | Sequence variants detected using WES and other relevant genetic findings | Other |
|---|---|---|---|---|---|
| 1. | 5 m | + 37w + 1d BW 2185 g (−2.0 SDS), BL43 cm (−2.8 SDS), OFC 32 cm (−1.19 SDS) | Penoscrotal hypospadias, scrotal testes | WES-DSD panel:* VUS: NM_017780.2(CHD7): c.1282C>T, p.(Pro428Ser), heterozygous | |
| 2. | 19 d | + 33w + 4d BW 1315 g (−2.4 SDS), OFC 28.8 cm (−2.6 SDS) | Penoscrotal hypospadias, scrotal testes | WES-DSD panel * NM_144773.3(PROKR2): c.518T>G, p. (Leu173Arg), pathogenic, heterozygous | Morrocan |
| 3. | 16 m | + 28w + 3d BW 660 g (−2.2 SDS) | Scrotal hypospadias. undescended testes | WES DSD panel*: - | |
| 4 | 10 m | + 32w + 1d BW 720 g (−3.6 SDS) (sister 1 kg +) | Perineal hypospadias, undescended testes | WES-DSD panel: -Full exome**: -Normal methylation *KCNQ1OT1, H19*Paternal and maternal chromosome 7 present, no UPD | Twin with sister.Growth hormone therapy for short stature. |
| 5 | 2 m | + 38w + 4d BW 1390 g (−5.3 SDS) OFC 34.5 (0.0 SDS) | Perineal hypospadias, undescended testes. | H19 hypomethylation Silver Russell syndrome (OMIM # 180860) UPD7, *AR, SRD5A2* and *HSD17B3* pathogenic variants not detected | |
| 6 | 8 m | + 37w + 3d BW 2195 g (−2.1 SDS) | Scrotal hypospadias, undescended testes | WES DSD panel*: - | IUI pregnancy |
| 7 | 15 y | + 34w + 4d BW 1350 g (−2.9 SDS) | Perineal hypospadias, bifid scrotum, scrotal testes | WES-MCA panel***: - | Short stature. Maternal uncle hypospadias. |
| 8 | 13 y | + 31 w, BW 833 g (−2.6 SDS) | Scrotal hypospadias, scrotal testes | WES-DSD panel*: - | |
| 9 | 1 d | + 32w + 5d BW 1120 g (−2.4 SDS) | Scrotal hypospadias, testes position at birth unknown | WES-DSD panel*: - | Mother with unspecified uterus malformation |
| 10 | 18 d | -36w + 3d BW 2010 g (−2.0 SDS) OFC 32.5 cm (0.6 SDS | Scrotal hypospadias, bifid scrotum, scrotal testes | WES DSD panel*: - | Neonatal hypoglycemia |
| 11 | 9 m | -38w + 1d BW 2620 g (−1.4 SDS) | Proximale shaft hypospadias, scrotal testes. | WES DSD panel*: - | Twin |
| 12 | 12 y | -35w BW 2 kg (−1.3 SDS) | Proximal shaft hypospadias, penoscrotal transposition, scrotal testes | WES-DSD panel*: - | Maternal cousin with hypospadias |
| 13 | 2 m | -32w BW 1740 g (−0.2 SDS) | Perineal hypospadias, bifid shawl scrotum, scrotal testes | WES-DSD panel*: pathogenic heterozygous NM_000406.2 (GNRHR):c.416 G>A, p. (Arg139His) | |
| 14 | 2 m | -36w + 4d BW 2960 g (+0.2 SDS) | Penoscrotal hypospadias, scrotal testes | WES DSD panel*: - | |
| 15 | 15 y | -39 w BW 3310 g (−0.2 SDS) | Scrotal hypospadias, hypoplastic left hemi-scrotum, undescended testes | WES DSD panel*: - | |
| 16 | 2 m | -39w + 3d BW 3590 g (+0.3 SDS) | Shaft hypospadias, scrotal testes | WES-DSD panel*: - | Father and brother with hypospadias |
| 17 | 2 m | -35w + 3d BW 2035 g (−1.3 SDS) | Penoscrotale hypospadias, scrotal testes | WES-DSD panel*: - | South African. Pre-auricular tag |
| 18 | 6 d | -38w + 5d BW 2630 g (−1.7 SDS), BL 49 cm (−1.8 SDS) | Penoscrotal hypospadias, scrotal testes | WES-DSD panel*: -Karyotyping: 46,XX FISH: ish X(CEPX(DXZ1)×2,LSI-SRYx0). nuc ish(CEPX(DXZ1)×2) | Mother with POI. |

None of the patients had consanguineous parents. Unless stated otherwise in the column 'other,' the family history was negative for genital anomalies and patients were Caucasian. If concurrent anomalies were present this is stated. For a flow chart of performed genetic tests, see additional file 1.

SGA= Small for gestational age; + = present, - = absent; WES= whole exome sequencing; WES-DSD = WES with analysis of DSD genes; WES-MCA = WES with analysis of multiple congenital anomalies gene panel; VUS = variant of unknown significance; BW = birth weight; BL= birth length, OFC =occipitofrontal circumference; SDS = standard deviation score; w=weeks; d=days; UPD – uniparental disomy; IUI= intrauterine insemination, POI=premature ovarian insufficiency; *DSD gene panel, version 10.2, 25-2-2022; **Full exome V3; ***version 3; https://www.erasmusmc.nl/nl-nl/patientenzorg/laboratoriumspecialismen/klinische-genetica#35d085e6-2dc0.

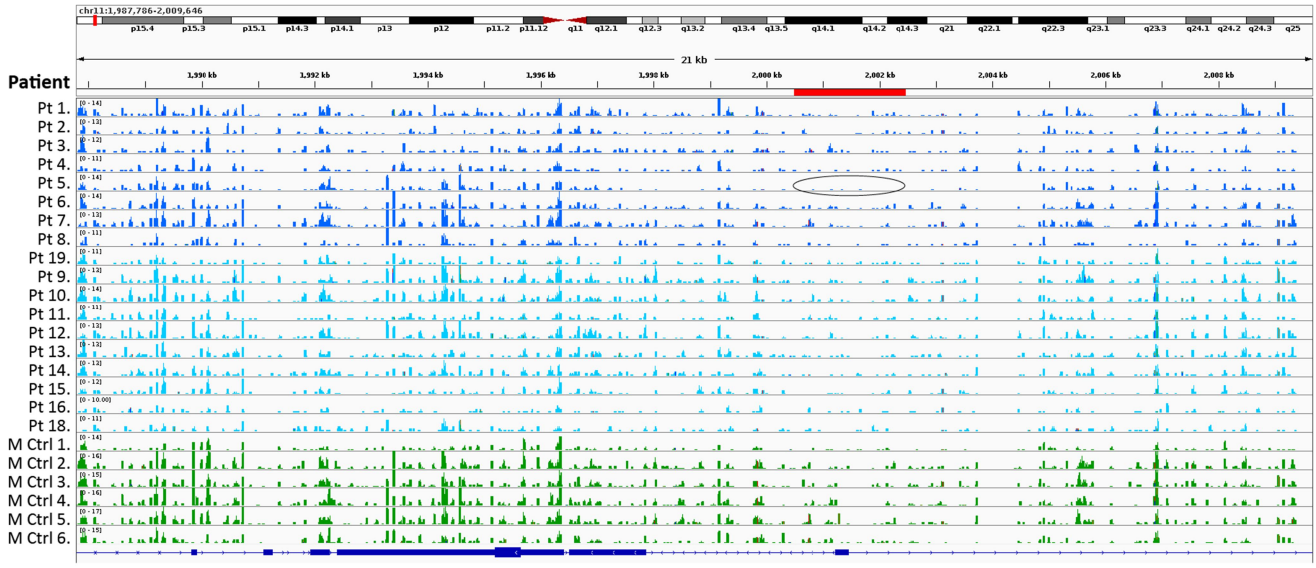


**Figure 1.** Methylation in H19 region, showing hypomethylation in patient 5 (encircled).

The individual with SRS and the XX individual were not included in further analyses unless stated otherwise for the specific test.

### *Comparison of differentially methylated regions (DMRs) between boys with hypospadias born SGA, non-SGA and XY controls*

In the genome-wide analysis, 92 DMRs were identified, seven of which with a fold change ≥2, when comparing the 16 XY boys with unexplained hypospadias to six XY controls. Hierarchical clustering using DMRs with a fold change ≥2 did not show complete separation of individuals with hypospadias from controls (Figure 2), although visually most controls clustered together, as did most patients. No specific methylation pattern for the individuals with proximal hypospadias was identified when compared to the controls. Furthermore, there was no difference in methylation pattern between patients that were SGA and those that were non-

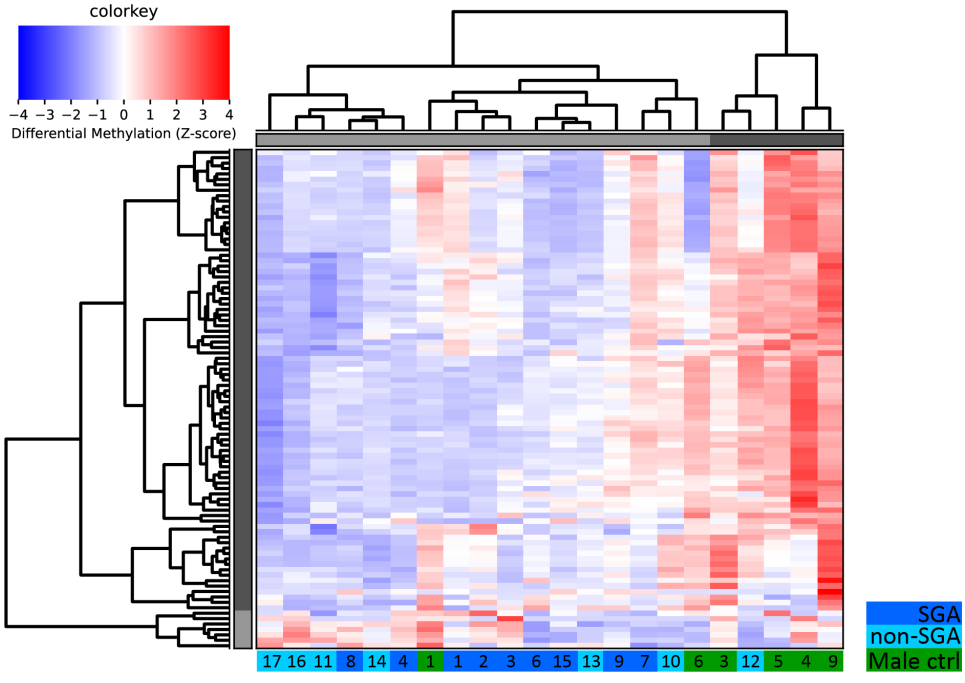


**Figure 2.** Hierarchical clustering of all DMRs (including XY) of XY patients and controls. Patients in blue, M ctrl= male control.

SGA as is also illustrated in Figure 2, so for further analyses all patients with XY genotype were combined into one group unless stated otherwise.

Although none of the seven DMRs with ≥ 2-fold change were located in or around known OMIM genes, five were in CpG24525, overlapping with a long intergenic non-protein coding RNA665 (LINC00665) (Z-score range −1,6 to + 1,4) (additional file 4); the other two were in CpG35888 and PRR20C.

### Methylation of the promoter sites of known dsd-genes at an individual level

We did not only study group differences in genome-wide DMRs but investigated methylation of promoter sites of genes known to be involved in DSD at an individual level as well. The genes of interest were selected from the local DSD panel (version 10.2) which contains 93 genes of which 35 are associated with XY DSD. The range of methylation in XY controls was determined for each promoter (additional file 5).

For XY patients with hypospadias, hypermethylation three times higher than the highest control was observed in the promoter region of *MAP3K1* (OMIM # 613762) in three individuals (2, 8, 11). Hypomethylation three times less than the lower end of the range in controls was observed in *CYB5A* (OMIM # 250790) in individual 8 and for promoters in several genes where no methylation was observed in several patients: *GATA4* (OMIM # 615542) in one individual (2), *AR* in four (OMIM # 300068, 1, 7, 8, 12) *CBX2* in four (OMIM # 613080, 1, 7, 10, 12) and *SOX3* in three (OMIM, # 300833, 1, 7, 12). However, in most patients methylation was comparable to that in controls.

### Methylation analysis of individual with XX testicular DSD

Methylation in the individual with 46,XX testicular DSD was compared to both male and female controls. Hierarchical clustering using all autosomal DMRs with a fold change >2 resulted in separation of most male controls and female controls (Figure 3). The XX male patient showed a methylation pattern more similar to, but not completely following the methylation pattern of the female controls. One male control showed an intermediate methylation pattern and one male clustered together with the individual with XX testicular DSD and the female controls. This male control (Figure 3, second from the right) was a phenotypic male, without a known genetic disorder. Array results showed a normal XY pattern.

Of the 1275 DMRs between the male patient with XX testicular DSD and six female controls of similar age, seven had a fold change >20. These DMRs were not located in or around genes known to be associated with DSD, but included two hypermethylated DMRs in *UPK3B* (OMIM *611887) and *ADAM3A*, (OMIM *601533) genes with known expression in the urogenital tract in humans or other species (see additional file 6). Another 25 DMRs had a fold change >10 but <20, including DMRs in *SDHB, KIAA1429* and *ADA*, (OMIM *185470, * 616447, * 608958) genes expressed in the reproductive system. Among the DMRs with a fold change between 2 and 10, two were in genes associated with DSD, a 4,7 fold increase in methylation at the transcription start site of *SRD5A2* (OMIM *6073060 and a 4.6 fold increase in methylation of *GLI2* (OMIM *165230, additional file 7). Gene ontology (GO) enrichment analysis of the DMRs with >2 fold change showed a 4.26 fold enrichment for genes involved in hemophilic cell adhesion via plasma membrane adhesion molecules, including a cluster of protocadherins-alpha genes (*PCDHA1-10*, OMIM 604,966).

### Review of the literature

Literature review yielded 53 manuscripts describing patients with hypospadias and (epigenetic OR methylation) and six previous comparable methylation studies in hypospadias were selected as described in the methods section and additional file 3. An overview of these previous studies [13,15,16,30–32] and their findings is shown in Table 2.

All used cultured preputial skin fibroblasts obtained during surgery, one also blood [31].

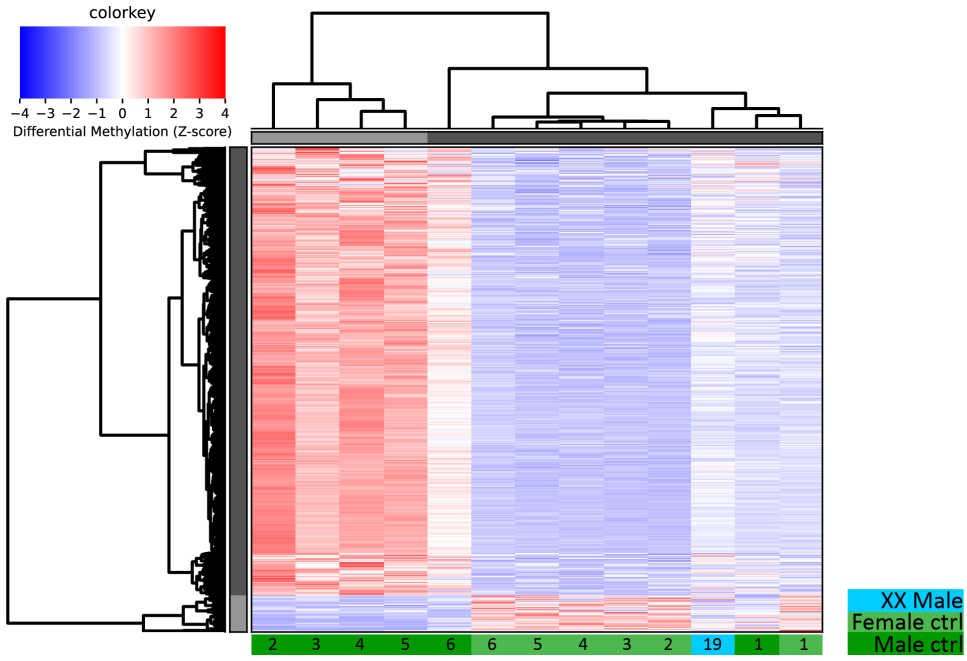


**Figure 3.** Hierarchical clustering based on autosomal DMRs: comparison of methylation of the XX male individual with male and female age-matched controls.

Most performed a genome-wide methylation analysis with different techniques, and specifically addressed genes known to be involved in hypospadias. None of the DMRs identified in previous studies were confirmed in the current study. Vottero et al. [32] studied the methylation of the *AR* gene specifically and found higher *AR* methylation in patients with hypospadias than in male controls, but this was not confirmed in our cohort.

## Discussion

Our first aim was to investigate if an episignature was recognizable in boys with proximal hypospadias. However, when analyzing the genome-wide data for the whole group methylation patterns did not discriminate between boys with hypospadias and controls, i.e., no distinct episignature for proximal hypospadias was identified.

Next, we hypothesized that differences might exist in gene methylation, specifically in boys with proximal hypospadias born SGA, as proximal hypospadias is seen more frequently in boys born SGA and the underlying mechanism could be through methylation, similar to *H19* hypomethylation. However, boys with hypospadias born SGA did not show a different methylation pattern from those born with normal birthweight either. There are several possible explanations as to why a specific episignature for hypospadias or discrimination between boys that were SGA and those that were non-SGA was not demonstrated. One explanation could be heterogeneity of the group of boys with hypospadias. Pathogenic variants in many genes are known to give a similar genital phenotype [5]. The individuals in our study may all have a different cause for their hypospadias, rather than one common specific methylation abnormality. Furthermore, methylation is tissue and age-specific. We studied methylation in postnatally collected leukocytes, whereas genital development takes place prenatally. Possibly, methylation alteration is no longer detectable after birth, or only detectable in gonadal or genital tissue. However, the use of foreskin may not be optimal either, as stated by Kaefer et al. [30], as proximal hypospadias is often associated with a lack of foreskin. Furthermore, Aiba et al. [31] found more differentially methylated CpGs in blood as compared to foreskin. Alternatively, although we had a higher number of individuals with proximal hypospadias compared to previous

**Table 2.** Review of methylation studies in hypospadias.

| Reference | Kaefer et al., 2023 | Richard et al., 2020 | Aiba et al. 2019 | Ohsako et al., 2018 | Choudhry et al., 2012 | Vottero et al., 2011 | Present study 2023 |
|---|---|---|---|---|---|---|---|
| Method targeted or genome wide | Genome-wide (>95%) epigenetic analysis Genomic features of the DMRs identified | Epigenome-wide association study, case/control and case only | Methylated-site display-amplified fragment length polymorphism (MSD-AFLP) | Methylation of *CYP1A1, CYP1B1, AR, SRD5A2* | Case control study, genome wide methylation Illumina chip. Also compared methylation status at 25 known DSD gene sites: | CAG repeats length, methylation status, and expression of the *AR* Also study of *DNMT* family at the protein level,sequencing *DNMT3A* | Genome wide methylation study with compared methylation at 93 known DSD gene sites |
| Population and numbers | Patients 30 with distal midshaft and 6 proximal hypospadias, 15 circumcision controls | 45 patients with hypospadias including 9 with proximal hypospadias, 45 healthy male controls | Three patients with hypospadias; three phimosis controls | 23 Patients with unspecified hypospadias, 16 controls with phimosis | Patients with isolated hypospadias: 8 mild and 4 severe; 8 healthy circumcision controls | 20 Patients with isolated glandular hypospadias; and 20 circumcion controls | 16 XY and 1 XX patients. SGA and non-SGA with proximal hypospadias; 6 healthy male, 6 female controls and one H19 hypomethylation positive control |
| Ethnicity | Caucasian | Unknown | Unknown | Asian | Unknown | Unknown | Caucasian |
| Material | Preputial skin | Preputial skin | Preputial skin and blood | Preputial skin | Preputial skin | Preputial skin | Blood |
| Results: with regard to methylation in proximal hypospadias | DMRs in severe hypospadias: *WDR4P2/ FBXO48; ABCB11; NCK1; LRBA; TERT; LOC100506990/ LOC728732/ RPS3AP34; FUT10; LOC112268031/ TMEM65; LHPP; DIO2-AS1; TNFAIP2/ NDUFB4P11; FA2H; LOC105372158; OR4G3P/OR4G1P/ OR4F17; UPRT* | CpGs differentially methylated in severe compared to moderate hypospadias: *ATP1A4; ENAH-SRP9; SATB1-KCNH8; DAAM2; TNKS-MSRA; CHST1* | In foreskin: DMRs in 27 CpG, five with large differences (nearest genes): hypermethylation of CpG in/near *MYH11* and *NACC2*; hypomethylation of CpGs in/near *C7orf27; RP4-706G24.1; PLA2G15* In blood: DMRs in 369 CpGs, six listed as disease-specific: hypomethylation CpGs (nearest genes):*SGCD1; AL390816.1; TEK-2; ZNK302; CTD-2194F4.2; IQSEC3* | No methylation of *CYP1A1* and *CYP1B1* or *AR* in patients or controls, no difference in methylation of *SRD5A2* between patients and controls | 887 differentially methylated CpGs, 14 most significantly different between patients and controls: near or in *SCARB1; MYBPH; SORBS1; LAMA4; HOXD11; MYO1D; EGFL7; C10orf41; LMAN1L; SULF1*; Hypometylation of CpG sites in *HOX11D*, *C10orf41*, hypermethylation of others. | *AR* hypermethylation in foreskin compared tocontrols; lower AR expression DNMT3A protein level significantly higher in patients DHT and T reduced *AR* methylation and DNMT3A expression in vitro and increased AR expression. | No DMRs were identified between SGA and non-SGA patients. Compared to controls DMRs in CpG24525, CpG35888. At individual level hypermethylation of the promoter site of *MAP3K1*was found in three patients |

DMR = differentially methylated region, DHT= dihydrotestosterone, SGA = small for gestational age, *T*= testosterone.

studies [13,15,16,30–32], the number of individuals may still have been too small to detect an underlying change in methylation. Figure 2 shows no clear separation of the patients with proximal hypospadias and controls, however visually there seemed to be some clustering which may become more obvious with larger cohorts of patients and controls.

The recurrent CpG24525 DMR that was identified in the XY hypospadic individuals overlaps with the Long Intergenic Non-Protein Coding RNA 665 (LINC00665). Long non-coding RNAs (lncRNAs) are highly expressed in testicular and neural tissues and may play a role in gonadal development [33]. Examples of lncRNAs thought to be important in sex determination are those in a region called RevSex, upstream of the locus of SOX9 [34] or lncRNA in the DMR region regulating DMRT1 expression [35]. So far their exact roles are still unclear. In a Chinese soft-shell turtle, an RNA-sequencing study led to the prediction of lncRNAs involved in regulation of *Dmrt1, Sox9, Cyp19a, Sox3* and *Sox8* [36].

Studying lncRNA expression profiles in mouse testes, Sun et al. showed many differentially expressed lncRNAs close to or overlapping with genes, e.g., transcription factors known to be important for testis function including spermatogenesis [37]. Another lncRNA, which was a regulator of steroidogenesis in the Leydig cells of mouse testes, was described by Otsaku and coworkers [38]. Further research will have to clarify if LINC00665 plays a role in sex development and possibly in hypospadias.

Next, we studied if for individual patients a diagnosis could be established. We used sex chromosome matched controls to compare to our patients, as Singmann and coworkers showed in different European cohorts that over a 1000 CpG distributed over all autosomes were differentially methylated in men compared to women. Gene expression of some of these loci differed as well and genes known to be subject to imprinting were enriched in the DMRs in their study [39].

Looking at methylation of promoters of genes known to be associated with a phenotype of XY DSD at an individual level, hypomethylation of *GATA4*, *AR*, *SOX3* and *CBX2* was found in several patients compared to XY controls. Hypomethylation would be expected to result in increased expression of these genes, while several studies of, e.g., expression of AR or GATA4 indicate that a decreased expression or function of these genes is associated with hypospadias [40–42]. Hypermethylation of *MAP3K1* was identified in three individuals. This may have resulted in decreased expression of this gene which could have contributed to the development of hypospadias. However, the pathophysiology of MAP3K1 variants that affect sex development has not been fully elucidated yet. A few variants have been analysed *in vitro* and were shown to result in increased MAPK signaling but several other variants have not been studied *in vitro* and their pathogenicity has been assumed based on *in silico* predictions [43,44]. The finding of altered methylation of *MAP3K1* in boys with hypospadias needs to be confirmed in other, larger, cohorts to further determine its relevance.

The individual with XX testicular DSD had, as expected, a methylation profile more comparable, but not identical to XX female controls. This probably reflects the difference in methylation patterns between those with an XY versus an XX genotype (see Figure 3), rather than that it explains the testicular DSD resulting in his hypospadias.

Furthermore, methylation profiling did not identify altered methylation of genes known to be associated with XX testicular DSD. Seven DMRs, all located on autosomal chromosomes, showed a >20 fold change, compared to the female controls. Two of these DMRs involved genes with some relation to sexual development. UPK3B is an important component of the urothelium [45]. In rodents Upk3b is expressed in tissues of the male and female urogenital tract at all postnatal stages and embryonic expression was demonstrated in developing gonads as well [46,47]. In mice, Adam3 was shown to be important for sperm-egg adhesion, but so far no direct link to hypospadias or gonadal dysgenesis can be made [48,49].

Among the genes with DMRs with a 10–20 fold change *SDHB*, *KIAA1429* and *ADA* are expressed in the reproductive system, although none of these genes are linked to DSD [50–53]. ADA deficiency leads to severe combined immune deficiency (SCID), but patients also seem more prone to having undescended testes and females to having an early onset of puberty [54].

Only two genes known to be involved in DSD had a fold change >2, *SRD5A2* and *GLI2*; both were hypermethylated in the individual with XX testicular DSD. *SRD5A2* encodes steroid 5-alpha-reductase. which catalyzes the testosterone to dihydrotestosterone conversion. When this conversion is hampered, this can lead to an autosomal recessive condition characterized by atypical genitalia including proximal hypospadias in XY individuals. However, it does not lead to a XX testicular form of DSD [55,56]. GLI2 is a transcription factor important in Hedgehog signaling, a system essential in organogenesis, with Desert Hedgehog (DHH) as a regulator of gonadal development, steroidogenesis and gametogenesis. In mice, *Gli2* is expressed in the genital tubercle from which the clitoris or penis is formed, a process that does not take place if the Sonic Hedgehog pathway is blocked [57,58]. Testicular development in XX Gli2 knockout mice has not been described [59]. No DMRs were found in or around the genes known to be associated with XX testicular DSD, i.e., *NR5A1, RSPO1, WNT4, NR2F2, SOX9, SOX3* or *WT1*.

Gene ontology enrichment analysis of the DMRs showed enrichment for homophilic cell adhesion via plasma membrane adhesion molecules. This was largely due to the PCDHA gene family of at least 15 genes that were differentially methylated (hypermethylation). In case of a gene cluster this may reflect a true DMR, a copy number variation or an artefact, caused by mapping issues. These PCDHA genes are expressed in neurons and are not associated with DSD [60].

Our study is not the first methylation study in the field of reproduction or DSD. However, as apparent from Table 2, differences in tissues used and differences in cohorts and methods make it hard to compare results and may explain the lack of consistent findings. Our study compares best with the recent study of Kaefer and coworkers [30]. They studied genome-wide DMRs in XY individuals with mild, moderate or proximal hypospadias and compared these groups separately and together to controls. A substantial difference is that we used DNA extracted from blood and they used preputial skin. They observed far more DMRs in the mild and moderate hypospadias cases than in the severe group, which they argued could be due to agenesis of the affected foreskin. We used blood instead which, although it is not the affected tissue, is frequently used for methylation studies and may be appropriate [31,61,62]. Furthermore, we had a larger sample size of proximal hypospadias patients and we had excluded cases that had a genetic diagnosis, which is more often established in patients with proximal hypospadias than in patients with milder forms. Familial recurrence risk is higher in mild hypospadias as compared to proximal hypospadias without a known cause [63,64] and etiology of distal and proximal hypospadias may not be the same. Comparing our DMRs with those found in other studies showed no overlap. No DMRs were found that may be responsible for a substantial proportion of unsolved proximal hypospadias in males from previous small cohorts [13,15,16,30,31] and our larger cohort of individuals with proximal hypospadias.

## Summary and conclusions

No recognizable episignature in our 46,XY patient cohort with proximal hypospadias was found, but a DMR overlapping with LINC00665 was identified and three individuals showed hypermethylation of *MAP3K1* which may play a role in the etiology of proximal hypospadias, although this needs further study. No substantial difference in methylation was found between individuals with hypospadias that were SGA and those that were not. Studies with larger numbers are needed as well as a better understanding of regulation of sex development, e.g., by lncRNA. Individual patients, in whom regular diagnostic methods do not show a cause for the proximal hypospadias may benefit from methylation studies or other newer techniques not yet implemented in standard diagnostic genetic evaluation, such as whole-genome sequencing or long read sequencing.

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## Disclosure statement

RB, JB, WvIJ, and JG report being shareholder in Methylomics B. V., a commercial company that applies MeD-seq to develop

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## Data availability statement

Our institution does not allow for sharing raw or processed DNA data.

## Other declarations

All participants gave their written consent for this study.

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