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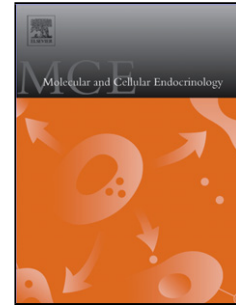
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## Hypospadias: interactions between environment and genetics

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**Abstract**

Hypospadias is one of the most common congenital malformations. **It is considered to be a mild form of the 46,XY disorders of sex development (DSD), but its precise etiology remains to be elucidated. Compromised androgen synthesis or effects can cause this frequent malformation, although the mutational analyses of the genes involved in androgen actions have identified abnormalities in only a very small portion of patients. The overwhelming majority of cases remain unexplained and hypospadias may be a highly heterogeneous condition subject to multiple genetic and environmental factors. We here review the recent advances in this field and discuss the potential interactions between the environment and genetics.**

**Key words**

Hypospadias, etiology; Hypospadias, genetics; Environment; Endocrine disruptor; Polymorphism, single nucleotide.

**Hypospadias is defined as a defect in the development of the ventral aspect of the penis along with an ectopic opening of the urethral meatus. Its incidence ranges from 1/1000 to 1/100 [1], with significant variations according to ethnic origin, making it one of the most frequent developmental defects.** In most cases, the degree of hypospadias is relatively mild and a specific endocrine cause is not sought or is not found[2]. However, four main **elements** are involved in male genital construction and may contribute to this malformation[3,4]: (1) the genetic and endocrine background of the child, principally the genes of phallic development, gonadal steroid synthesis (mainly testosterone and its  $5\alpha$  reduced form, dihydrotestosterone, DHT), and the responsiveness to these hormones. **The genital tubercle thus grows under the influence of androgens and any alteration in androgen production or receptors may produce a hypospadiac penis;** (2) the placenta, which orchestrates the hormonal climate, especially during the first part of gestation; (3) the mother, with her own hormonal production and possible disorders; and (4) the environment of mother and child, which may also interfere in this fine balance[5,6].

Over the last 30 years, male reproductive health has been **altered, with deterioration** in sperm count and an increasing number of cases of undescended testes, testicular cancers and hypospadias[9]. This phenomenon has raised some concerns regarding environmental chemicals such as the **products** of industrial and agricultural development [10,11]. **We here review the** role of the environment in the occurrence of hypospadias and the interaction between environmental factors and genetics.

## I Genetic background

Before evaluating the role of the environment, it should be acknowledged that several arguments are in favor of a predominant role for the genetic background. Familial clustering is seen in about 10% of the cases[12-15], and the recurrence risk in the male siblings of an affected patient is about 15% [16-18]. Seven percent of the fathers of children with hypospadias are also affected[19]. **The risk of recurrence is also found to aggregate in more distant relatives. Using Danish health registers, Schnack et al. identified 5,380 boys diagnosed with hypospadias in a cohort of 1,201,790 boys born in the period 1973-2005. The risk ratios of hypospadias for male first-, second-, and third-degree relatives of a hypospadiac case were, respectively, 11.6%, 3.27%, and 1.33%.** The risk of recurrence for the next male sibling depends on the severity of the hypospadias [16]. Segregation analysis suggests that hypospadias might be due to monogenic effects in a small proportion of the families, whereas a multifactorial mode of inheritance was reported to be more likely in the majority of families[20]. Finally, some of the 200 syndromes that include hypospadias have known genetic bases and shed light on the molecular mechanisms involved in genital development. [21,22]

The environment may act on the genes that contribute to the occurrence of hypospadias at several levels.

1- *Level of phallus development:* **Homeobox genes A (HOXA) and D (HOXD) participate in the development of the phallus since knock-out of these genes in mice induces a malformation in the external genitalia consistent with hypospadias[23]. In humans, the hand-foot-genital syndrome (HFGS)[24,25] is related to mutations of HomeoboxA13 (HOXA13). HOXA13 allows the normal expression of fibroblast growth factor (FGF) 8**

and bone morphogenetic protein (BMP) 7 in the developing urethral epithelium in mice, thus modulating androgen receptor expression and glans vascularization[3]. The FGF gene family, especially FGF10[26], is also implicated in the development of external genitalia in mice[27]. In humans, polymorphisms of FGF8, FGF10 and FGFR2 may be associated with an increased risk of hypospadias[28].

2- *Level of testicular determination:* The genes leading to testicular dysgenesis are a cause of hypospadias. Severe hypospadias along with other genital abnormalities[29,30] can reveal heterozygous mutations of Wilms tumor 1 (WT1). SOX9, DMRT1 and GATA4 encode transcription factors acting immediately before the differentiation of the gonad into testis. Mutations of these genes induce testicular dysgenesis and are associated with 46,XY disorders of sex differentiation (DSD), including severe hypospadias [31-34]. Variation in gene dosage, as shown in 46,XX and 46,XX d17 patients with SOX9 duplication, can also induce penoscrotal hypospadias [31].

3- *Level of androgen biosynthesis:* Mutations in the LH receptor gene (inducing a Leydig cell hypoplasia) and the 5 $\alpha$ -reductase gene (inducing a defect of dihydrotestosterone synthesis) induce hypospadias, most often in a severe form with associated cryptorchidism and/or micropenis [32,35,36] [37]. MAMLD1 (mastermind-like domain containing gene) is another candidate gene that seems to modulate the synthesis of testosterone around the critical period of sex differentiation. MAMLD1 is expressed in the male gonad in mice, and it augments testosterone production and contains the SF1 target sequence[38]. Fukami et al.[39] identified three nonsense mutations in four individuals with 46,XY DSD including micropenis, bifid scrotum and penoscrotal hypospadias. Genetic variants of MAMLD1 were further shown to be present in patients

with isolated hypospadias[40], as confirmed by Chen et al. [41], who identified five non-synonymous mutations, some of them as polymorphisms.

4- *Level of androgen action*: Mutations in the androgen receptor gene (AR) have been found in patients with either severe forms of hypospadias[42-44] or other signs of undervirilization, such as cryptorchidism[45] or micropenis[46,47]. Mutation of the AR gene in partial androgen insensitivity syndrome is found only in 20% to 30% of cases and the phenotype remains particularly variable [46,48].

## II Environment

### II-1 Arguments for an environmental contribution

Several findings in both animal and human studies raise suspicion of an environmental contribution to this malformation. Hypospadias, whether associated with micropenis or not, has been reported in numerous wildlife species when the habitat is contaminated by pesticides[49]. The effects of prenatal xenoestrogens on animal male reproductive tract development have been studied by several groups. Male rat pups exposed to DES during gestation (at concentrations similar to those measured in first-trimester human fetal tissues) developed hypospadias[50,51]. Hypospadias was also found in male rodents after maternal treatment with vinclozolin (dose-response effect)[52], and similar findings were recorded for prenatal exposure to polychlorinated biphenyls (PCB), phthalates and dioxin[52-54].

Despite some inaccurate registers which under-evaluate the number of hypospadias cases and the variable geographical distribution of the malformation[55], several reports suggest an increase in hypospadias over the last 20 years[56]. Boisen[57] performed a



prospective cohort study and found a high prevalence (1%) of hypospadias in the Danish population of male newborns, whereas the prevalence was reported to be 0.73% in the Netherlands in a cross-sectional study[58]. A prospective case-control study of 1,442 male newborns identified 16 cases of middle and posterior hypospadias (1.1%) in the south of France[59]. **More recent reports have also described an increased incidence[60,61], but epidemiological studies nevertheless give divergent results about whether the trend of hypospadias is increasing and thus raise questions about temporal trends [62][63]. Aho et al. [64] identified all patients in the national hospital discharge registry who had been born in the period 1970-1986 and surgically treated for hypospadias before the age of 9 years. They calculated the cumulative prevalence by dividing the number of patients by the number of male births and found that the prevalence of hypospadias in Finland remained constant throughout the study period but appeared to be approximately three times higher than previously reported. Improvements in completing the registration forms may account for a substantial proportion of the higher prevalence of hypospadias, according to these authors. Martinez Frias et al. [65] found similar results using the Spanish Collaborative Study of Congenital Malformations (ECEMC) registry to analyze the prevalence in two different periods.**

This phenomenon has raised some concerns regarding environmental chemicals, such as industrial and agricultural products. Two epidemiological studies reported a possible relationship between exposure to pesticides and hypospadias. Kristensen reported a moderate increase in the odds ratio (OR) for hypospadias in individuals exposed to farm chemicals (OR = **1.51, 95% confidence interval, 1.00-2.26**)[66], and Weidner[67,68] concluded that maternal farming or gardening led to a slightly increased risk of hypospadias (**OR = 1.27, 95% confidence interval, 1.14-2.47**). Residence in the vicinity of hazardous waste-disposal

sites has been associated with a high incidence of hypospadias[69,70]. Similarly, an increased rate of hypospadias was reported in boys from parents exposed to dioxin after the Seveso industrial accident[71]. A vegetarian diet in pregnant women was reported to carry a significant risk of hypospadias[72] (**OR = 4.99, 95% confidence interval, 2.10-11.88**). A study in Western Minnesota[73] found a higher rate of congenital abnormalities in infants conceived in spring when herbicides are usually widely used. Birth rates with urogenital abnormalities, as well as abnormalities in other systems, were significantly increased in high-use areas. Another study[74] also suggested a possible gene-environment interaction at work in this agricultural region. A total of 22% of the families in which the father applied the herbicides had more than one child with a birth defect.

Although most investigations of congenital anomalies have focused on major structural defects, **recent epidemiology** finds subtle developmental defects in genital masculinization. Anogenital distance, a reflection of male reproductive tract development which is reduced in hypospadiac patients[75], was found to be reduced in cases of prenatal exposure to phtalates[75,76].

Last, some of these substances, like DES (diethylstilbestrol), were observed to increase the hypospadias incidence in the second generation, suggesting a transgenerational effect[77].

## II-2 Substances

Several substances in the environment may potentially interfere with male genital development because of their similarity to hormones. Although there is a long list of suspicious substances contained in herbicides, fungicides, insecticides, and industrial by-

products or end-products (plasticizers, cosmetics, paints, etc.), none of them has been clearly identified as responsible for **hypospadias**. Various pollutants potentially involved in the abnormal development of the genital tubercle include: chlorinated pesticides (DDT, Lindane), polychlorinated biphenyl, methoxychlor, phenolic derivatives, nonylphenol, endosulfan, atrazine, phthalates, dioxine, furans[78], xenoestrogens, phytoestrogens, and mycoestrogens [79].

### II-3 Contamination routes

Humans are in constant contact with many of these substances[73,80] as they are found in water, soil, food and air[81,82]. These pollutants enter the body either by ingestion, inhalation, or adsorption, or they may be conveyed through the placenta. Individual exposure varies with dietary habits, life style, and work. Most of these pollutants are lipophilic and are stored in body fat for a lifetime[83]. They are also found in breast milk[84] and in the amniotic fluid. Since most of these chemicals use the same pathways as natural hormones, they have been named xenoestrogens and/or **endocrine disrupting chemicals** (EDC).

## III Interactions between environment and genetics

### III-1 Types of action of environmental pollutants

Environmental pollutants exhibit several genomic and non-genomic actions. They bind to the nuclear receptors **such as** estrogen receptors  $\alpha$  and  $\beta$  (ER $\alpha$ /ER $\beta$ ), inducing transcription activation (or repression) of specific gene expression[85,86]. Non-genomic actions are mediated by a plasma membrane estrogen or androgen receptor. Xenoestrogens have both

estrogenic and antiandrogenic actions and compete with natural androgens for the ligand-binding domain (LBD) of the AR gene[87]. **They also induce more potent estrogenic metabolites. In addition to these receptor-mediated actions, EDCs may affect synthesis, metabolism, excretion, and binding of endogenous hormones to SHBG, and they have the capacity to inhibit the transcription of androgen-dependent genes[88,89].** Finally, an epigenetic action has been demonstrated[90].

A major point regarding the action of environmental **toxicants** in inducing hypospadias is the cumulative effects of multiple low-dose exposures. The cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development has previously been demonstrated[91]. In this study, the complex mixture behaved in a dose-additive manner, and compounds that acted by disparate mechanisms of toxicity displayed cumulative effects when present in combination. This situation could reflect real environmental conditions, in which several chemicals that do not act via a common cellular mechanism of action are present together and disrupt fetal tissues during sexual differentiation in a dose-additive manner[92,93].

### III-2 Candidate genes implicated in the susceptibility to environment

The dialogue between genes and the environment may include variations in gene expression and variations in receptivity. Genetic variants (polymorphisms) may modulate the individual susceptibility to the external environment.

#### III-2-1 Dysregulation of gene expression

The expression of gonadal development genes varies according to the exposure to estrogen-like substances. Experimental studies in both rats and mice have demonstrated that

estrogens can directly inhibit testicular steroidogenesis in the fetus[94]. Consistent with this finding, studies have reported an increase in the incidence of hypospadias in male mice that were exposed in utero to DES or ethinyl estradiol[95]. In addition to this mechanism, a complete loss of androgen receptor protein expression was found to be a more severe effect of these substances[96].

Xenoestrogens also act as endocrine disruptors. Molecular analyses in fetal rat testes after in utero exposure to phthalates have shed light on the potential mechanisms via which phthalates suppress testicular testosterone production. Several key genes involved in steroidogenesis were disrupted after in utero exposure to monobutyl phthalate and monoethyl hexyl phthalate, such as StAR, HMG-CoA synthase, SRB1 and the steroidogenic enzymes Cyp11a, 3beta HSD and Cyp 17 [97] [98,99]. Linuron, a urea-based pesticide, acts as an antiandrogen. It antagonizes rat and human AR, inhibits androgen-induced gene expression, and reduces testosterone production by effects on LH receptor expression [100] [101]. Dioxin also suppresses StAR and CYP17 mRNA expression later in gestation, possibly through suppression of LH secretion[102] [103].

Three estrogen-responsive genes are suspected to be at the crossroads of environment and genetics in hypospadias for several reasons: ATF3, ER $\alpha$  and TGF- $\beta$ 1. **ATF3 is the most upregulated estrogen-dependent gene in the foreskin of hypospadiac patients [104]. Immunohistochemical analysis on human foreskin confirmed that the majority of the hypospadiac samples were positive for expression of ATF3[105]. Both animal and in vitro studies confirmed these findings. In vitro exposure to ethinyl estradiol increases expression and promoter activity of ATF3 in human foreskin fibroblasts [106]. In a murine model, fetal exposure to estrogen increases the level of ATF3 messenger RNA [107], whereas fetal exposure to DEHP (Di-(2-ethylhexyl) phthalate, a common plasticizer, activates the transcription and transduction of the ATF3 gene [108]. Since**

**ATF3 is implicated in cell cycle suppression, its upregulation may interfere with urethra formation[109], as suggested by the dysregulated apoptosis in the murine model described above [108].**

Exogenous administration of estrogens also results in an increased expression of ER $\alpha$  (but not ER $\beta$ )[110]. Expression of TGF- $\beta$ 1 is also modulated by endocrine disruptors. Reverse-transcription polymerase chain reaction (RT-PCR) and Western blot studies have shown that the expression of TGF- $\beta$ 1 is upregulated in DEHP-treated mice along with a significant inhibition of male fetal genital tubercule[108].

### III-2-2 Gene polymorphisms

Polymorphisms of steroid receptors may modulate the response to toxic substances. An increased GGN trinucleotide repeat in the AR gene has been found to reduce its transcriptional activity in hypospadiac patients[111,112]. **The role of amplification of the CAG repeats remains to be determined [113] and may be associated with undermasculinized genitalia, including hypospadias[114]. For some authors, the V89L variant of the SRD5A2 gene is a risk factor for hypospadias [115], whereas for others it is not [116].** Polymorphisms of the estrogen receptor may also facilitate the deleterious effects of xenoestrogens since their effects are mainly mediated through this receptor. The AGAGA haplotype of the estrogen receptor 1 (ESR1) gene is strongly associated with hypospadias[117]. The ESR1 C-A haplotype, for ESR1 XbaI and ESR2 2681-4A>G, respectively, increases the risk of malformation, as well[118]. An increased number of CA repeats (and subsequently increased ER activity) also augments the risk of malformation[119], and more recently we identified ATF3 polymorphisms in patients with isolated hypospadias[120]. **The data regarding the association of polymorphisms with**

hypospadias should nevertheless be interpreted with caution. In a large series of 620 Caucasian hypospadiac patients, Van der Zanden et al. [116] failed to confirm the association of single nucleotide polymorphisms (SNPs) in SRD5A2 and ESR1 with hypospadias. The SNPs in ESR2 and ATF3 were even found to be associated in the opposite direction compared with earlier publications. These divergent results confirm that genetic association approaches need to be replicated in very large samples.

### III-2-3 Xenoestrogen and epigenetics

Changes in the epigenetic background induced by synthetic estrogens could be a significant factor in the susceptibility to disease development. The epigenetics appear to involve altered DNA methylation. The primordial germ cells undergo demethylation during migration and early colonization of the embryonic gonad, followed by remethylation starting at the time of sex determination in a sex-specific manner [121][122,123]. The exposure of the pregnant mother at the time of fetal sex determination may be sufficient to alter the remethylation of the germ line in the male fetus and permanently reprogram the imprinted pattern of DNA methylation in boys[124].

Bredfeldt et al. [90] recently showed that 17beta-estradiol (E2) and the xenoestrogen diethylstilbestrol (DES) reduce levels of trimethylation of the lysine residue 27 on histone H3. Interestingly, the modulation of methylation by these substances is mediated by membrane-activated estrogen receptor signaling—through phosphatidylinositol 3 and kinase/protein kinase B—and occurs during windows of uterine development that are susceptible to developmental reprogramming. Activation of this nongenomic pathway has also been shown to reprogram the expression profile of estrogen-responsive genes in uterine myometrial cells. This mechanism for

**developmental reprogramming caused by early-life exposure to xenoestrogens may contribute to the modulation of the epigenetic machinery during tissue development.**

#### IV Limitations and questions

Although numerous studies point toward a major role for the environment in hypospadias, two limitations should be considered before attempting to draw definitive conclusions. First, caution should be exercised when extrapolating from murine experiments to humans. In these experiments, xenoestrogens induced hypospadias in male offspring exposed in utero, but the doses given to animals may not be comparable to environmental exposure. **Second, several epidemiological studies have reported contradictory results, as noted above** [62,64,65]. Epidemiological studies on maternal exposure are also inconclusive. Contrary to previously cited studies [67] [125], some reports did not confirm any significant risk of hypospadias when mothers were exposed to DTT[62,126]. Moreover, the critical level of exposure to EDCs was not assessed in any of the epidemiological studies implicating the environment[67] [125]. No relationship was identified between polybrominated biphenyl (PBB) [127] or polychlorinated biphenyl (PCB) exposure[128] and hypospadias. A recent meta-analysis indicated only a modestly increased risk of hypospadias associated with pesticide exposure[129].

#### **Conclusion**

The study of hypospadias is of interest for several reasons. It is an easily diagnosed malformation and, although minor, it recapitulates the pathophysiology of the disorders of sex development; the investigation of hypospadias thus offers insight into the mechanisms of sex



determination and differentiation. Moreover, because hypospadias is at the crossroads of genetics and environment, it is a model for exploring genetic and environmental interactions (Figure 1). The environmental data to date, however, should be interpreted with caution. Indisputable proof of the detrimental effects of the environment is still pending and no single EDC has been identified as a cause of hypospadias in humans. Hypospadias nevertheless remains a sentinel of the effects of the environment through genetics, both at the present time and for the next generation.

## Legend

Table 1: Summary of genetic variants associated with an increased risk of hypospadias

Figure 1: Schematic view of the intrication between environment and actors of genital masculinisation.

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**Table 1**

Gene	Variants
AR	- CAG/GGN repeat length polymorphism
SRD5A2	- A49T, - L113V - H231R - V89L
HSD17B3	- S232L
ESR1	- G allele containing variants of ESR1 Xbal
ESR2	- G allele containing variants of ESR2 2681-4A>G - (CA) <sub>n</sub> polymorphism in intron 6
ATF3	- Specific “TTC” haplotype in intron 1 - c.536A > G(R90) - 817C > T in the 3'-UTR. - L23M, - C53070T, - C53632A, - Ins53943A
MAMLD1/CXorf6	- V432A - CAG10 > CAG13

Figure

