Aetiology of hypospadias: A systematic review of genes and environment

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TABLE OF CONTENTS

Introduction

- Prevalence
- Embryology of the male external genitalia
  - Indifferent stage
  - Early patterning
  - Masculinisation
- Aim of this review

Methods

Results

- Aetiology of hypospadias is multifactorial
- Genes implicated in the aetiology of isolated hypospadias
  - Indifferent stage
  - Early patterning
  - Masculinisation
  - Other genes
- The role of environmental factors in the aetiology of hypospadias
  - Introduction
    - Testicular dysgenesis syndrome
    - Estrogen hypothesis
  - Exogenous exposure to estrogens
    - Oral contraceptives
    - Assisted reproductive techniques
  - Endogenous hormone levels
    - Endogenous estradiol levels
    - Foetal hCG provision
  - Clinical factors

Pregnancy complications
Maternal drug use
Maternal intra-uterine DES exposure
Behavioural factors
Parental age
Maternal diet
Other lifestyle factors
Occupational factors
Exposure to pesticides
Other occupational exposures
Living environment
Conclusion

ABSTRACT

Background Hypospadias is a common congenital malformation of the male external genitalia. Most cases have an unknown aetiology, which is probably a mix of monogenic and multifactorial forms, implicating both genes and environmental factors. This review summarizes current knowledge about the aetiology of hypospadias.

Methods Pubmed was used to identify studies on hypospadias aetiology published between January 1995 and February 2011. Reference lists of the selected manuscripts were also searched to identify additional studies, including those published before 1995.

Results The search provided 922 articles and 169 articles were selected for this review. Studies screening groups of patients with hypospadias for single gene defects found mutations in *WT1*, *SF1*, *BMP4*, *BMP7*, *HOXA4*, *HOXB6*, *FGF8*, *FGFR2*, *AR*, *HSD3B2*, *SRD5A2*, *ATF3*, *MAMLD1*, *MID1* and *BNC2*. However, most investigators are convinced that single mutations do not cause the majority of isolated hypospadias cases. Indeed, associations were found with polymorphisms in *FGF8*, *FGFR2*, *AR*, *HSD17B3*, *SRD5A2*, *ESR1*, *ESR2*, *ATF3*, *MAMLD1*, *DGKK*, *MID1*, *CYP1A1*, *GSTM1* and *GSTT1*. In addition, gene expression studies indentified *CTGF*, *CYR61* and *EGF* as candidate genes. Environmental factors consistently implicated in hypospadias are low birthweight, maternal hypertension and preeclampsia, suggesting that placental insufficiency may play an important role in hypospadias aetiology. Exogenous endocrine disrupting chemicals have the potential to induce hypospadias but it is unclear whether human exposure is high enough to exert this effect. Other environmental factors have also been associated with hypospadias but, for most, the results are inconsistent.

Conclusions Although a number of contributors to the aetiology of hypospadias have been identified, the majority of risk factors remain unknown.

KEY WORDS:

Aetiology

Environment

Genes

Hypospadias

Risk factors

INTRODUCTION

Hypospadias is a congenital hypoplasia of the penis, with displacement of the urethral opening along the ventral surface, often associated with dorsal hooded foreskin and chordee. More than 50% of cases have anterior hypospadias, with a small displacement of the meatus in the glandular region (Fredell *et al.*,2002b; van der Zanden *et al.*,2010b). Other patients have more substantial displacements, with middle (penile) or posterior (penoscrotal, scrotal and perineal) openings (Figure I). Hypospadias is usually diagnosed during physical examination of the newborn but localization is best established during surgery, after chordee release. Compared to healthy children, boys born with hypospadias more often have additional congenital anomalies (Latifoğlu *et al.*,1998; Akre *et al.*,1999; Aschim *et al.*,2004a; Nassar *et al.*,2007), an association that appears to be stronger for posterior compared to anterior cases (Latifoğlu *et al.*,1998; Wu *et al.*,2002; Nassar *et al.*,2007). Cryptorchidism in particular and other urogenital anomalies are frequently found with hypospadias (Weidner *et al.*,1999; Nassar *et al.*,2007; Schnack *et al.*,2009; Akin *et al.*,2011).

Even when patients receive surgery in their first two years of life, they may encounter severe medical, social and sexual problems later in life. After long-term follow-up (10 years) of mainly patients with anterior hypospadias who underwent 1-stage repair, different rates of complications in up to 50% of patients were reported, depending on inclusion of different aspects (Nuininga *et al.*,2005). Although most studies conclude that psychosocial development is not seriously altered, patients do suffer from negative genital appraisal, sexual inhibition, and more erection and ejaculation problems (Mieusset and Soulié,2005; Schönbucher *et al.*,2008).

Prevalence

Figures on the birth prevalence of hypospadias vary considerably across countries, ranging from four to 43 cases per 10,000 births (Kurahashi *et al.*,2004; Nassar *et al.*,2007). Hypospadias occurs most frequently in whites, less frequently in blacks, and rates are lowest among Asians and Hispanics (Gallentine *et al.*,2001; Carmichael *et al.*,2003; Yang *et al.*,2004; Porter *et al.*,2005; Nelson *et*

123 *al.*,2005; Meyer *et al.*,2006; Forrester and Merz,2006; Carmichael *et al.*,2007; Nassar *et al.*,2009).
124 There is debate about whether or not the prevalence of hypospadias is increasing. Some researchers
125 reported increasing prevalences in China (Sun *et al.*,2009; Jin *et al.*,2010), Australia (Nassar *et*
126 *al.*,2007), the USA (Paulozzi *et al.*,1997; Nelson *et al.*,2005) and Europe (Lund *et al.*,2009), whereas
127 others did not find an increase in Canada, the USA (Fisch *et al.*,2001; Carmichael *et al.*,2003; Porter *et*
128 *al.*,2005), Europe (Aho *et al.*,2000; Ahmed *et al.*,2004; Abdullah *et al.*,2007) and Japan (Kurahashi *et*
129 *al.*,2004). However, results of different studies are difficult to compare because some are based on
130 hospital discharge registries, including only surgically treated patients or all newborns diagnosed with
131 hypospadias, whereas others are based on birth defects surveillance systems, including all registered
132 hypospadias cases or excluding cases with glandular hypospadias. In addition, the diagnosis and
133 definition of hypospadias may have changed over time.
134
135 **Embryology of the male external genitalia**
136 ***Indifferent stage***
137 Early development of the external genitalia is similar for males and females. The embryonic cloaca,
138 the far end of the hind gut, is separated from the amniotic cavity by the cloacal membrane. Early in the
139 fifth week of development, a swelling develops on both sides of this membrane, the cloacal folds,
140 which meet in the midline anterior to the cloacal membrane, forming the genital tubercle
141 (Schoenwolf,2009) (Figure II). At the same time, the genital ridges, the precursors of the gonads,
142 develop. Studies in mice showed that this process requires Wilms tumour 1 (Wt1) activity, which
143 activates splicing factor 1 (*Sf1*) (Wilhelm and Englert,2002), thus preventing degeneration of the
144 developing gonads (Luo *et al.*,1994). During the seventh week of human development, the urorectal
145 septum fuses with the cloacal membrane, dividing the cloaca into the primitive urogenital sinus and
146 the rectum, and dividing the cloacal membrane into the urogenital and the anal membrane. The
147 swellings next to the urogenital membrane are then called the urogenital folds and a new pair of
148 swellings, the labioscrotal swellings, appear on either side of these folds. In addition, the urogenital
149 membrane breaks down (Schoenwolf,2009).

Early patterning

The genital tubercle (GT) masculinises if exposed to androgens but early patterning is androgen-independent. Studies on genes and proteins involved in this patterning process have mainly been performed in mice and showed that the distal urethral plate epithelium is the signalling centre regulating GT outgrowth (Perriton *et al.*,2002). Fibroblast growth factor protein (Fgf) and wingless-type MMTV integration site family member 5A (*Wnt5a*) signalling have a growth-promoting role in this outgrowth (Yamaguchi *et al.*,1999), whereas bone morphogenetic proteins (Bmps) stimulate apoptosis (Morgan *et al.*,2003; Suzuki *et al.*,2003). Expression of *Fgf8* in the urethral plate is regulated by sonic hedgehog (Shh) and homeobox A13 (Hoxa13) (Haraguchi *et al.*,2001; Perriton *et al.*,2002; Morgan *et al.*,2003), while Hoxa13 also regulates expression of *Bmp7* (Morgan *et al.*,2003). Shh induces, either directly or via Fgf8 or other factors, expression of *Fgf10*, *Bmp2*, *Bmp4*, *Wnt5a*, Patched 1 (*Ptch1*), Msh homeobox 1 (*Msx1*) and *Hoxd13* (Haraguchi *et al.*,2001; Perriton *et al.*,2002). Shh thus modulates the balance between proliferation and apoptosis (Haraguchi *et al.*,2001) and regulates the initiation of GT outgrowth (Perriton *et al.*,2002). Immunohistochemical staining of human foetal penises showed expression of *SHH*, its receptor *PTCH1*, and its downstream genes smoothened, frizzled family receptor (*SMO*) and GLI family zinc finger 1 (*GLI1*) around the time of urethral closure (Shehata *et al.*,2011). Studies in mice showed that Wnt-β-catenin signalling also seems to play a role in GT development, either in early androgen-independent GT development (Lin *et al.*,2008) or as a downstream effector of androgen signalling essential for GT masculinisation (Miyagawa *et al.*,2009).

Masculinisation

Subsequent masculinisation relies on hormones produced by the testes. Expression of the sex-determining region Y gene (*SRY*) induces a cascade of gene interactions, involving SRY-box 9 (*SOX9*) (Schoenwolf,2009), resulting in differentiation of the gonads into the testes (Sinclair *et al.*,1990). SRY leads to the differentiation of Sertoli cells (Schoenwolf,2009), which secrete anti-Müllerian hormone

(AMH). Studies in mice showed that AMH secretion happens under the influence of Sf1 (Giuili *et al.*,1997). AMH causes regression of the Müllerian ducts that would otherwise form part of the female genital structures (Schoenwolf,2009). HCG, produced by the placenta, controls foetal Leydig cell growth and stimulates foetal testicular steroidogenesis, the generation of steroids from cholesterol (Misrahi *et al.*,1998). The enzymatic steps of steroidogenesis, mainly taking place in the Leydig cell, are well documented and expression of key genes in this pathway is dependent on expression of SF1 (Scott *et al.*,2009) (Figure III). Testosterone leaves the Leydig cell and is converted into dihydrotestosterone (DHT) by steroid-5-alpha-reductase (SRD5A). Testosterone promotes formation of the internal reproductive structures from the Wolffian ducts, whereas DHT induces development of the external genitalia (Schoenwolf,2009), both through their effect on the androgen receptor (AR). Expression of estrogen receptors (ESR) in male genital tissue during development suggests that the balance between androgens and estrogens is important as well (Crescioli *et al.*,2003).

During masculinisation of the external genitalia, between the 12^{th} and 14^{th} week after conception (Schoenwolf,2009), the GT develops into the penis, the labioscrotal swellings fuse to form the scrotum (Ammini *et al.*,1997; Schoenwolf,2009) and the urogenital folds close in a proximal to distal direction to form the penile urethra (Ammini *et al.*,1997; van der Werff *et al.*,2000; Schoenwolf,2009; Yamada *et al.*,2003; Hynes and Fraher,2004b) (Figure II). Several hypotheses have been proposed about formation of the glandular portion of the urethra. One of these states that, while the penile urethra is created by fusion and primary luminisation, the glandular urethra develops by fusion and secondary luminisation (van der Werff *et al.*,2000). According to another hypothesis, the complete urethra arises by fusion of the urogenital folds (Ammini *et al.*,1997; Baskin *et al.*,2001). Still others believe that the glandular portion of the urethra originates from a different set of folds (Hynes and Fraher,2004a), ingrowth of surface cells (Jones,1910) or canalization of the urethral plate (Schoenwolf,2009).

As a result, the development of hypospadias is also controversial. From a clinical point of view, development of the urethra, corpora, glans and penile skin are directly correlated. In posterior

hypospadias, there is non-fusion of the labioscotal swellings with a distal dysplasia of the urethral plate and corpora, as well as non-fusion of the glans and skin in the midline. In middle hypospadias, the distal part of the penis shows a persistence of the urethral plate and non-tubularisation of the glans with disturbed penile skin formation. In glandular hypospadias, there is a dimple or a short tubular tract with a septum in between this tract and the urethral plate or tube and no closure of the skin in the midline. In the most minimal form, hypospadias sine hypospadias, only non-fusion of the preputial skin on the ventral side is seen, with dorsal hooded foreskin with or without some chordee.

Aim of this review

In 30% of the least frequently occurring posterior hypospadias cases a cause can be identified, for example, a complex genetic syndrome, partial androgen insensitivity related to AR mutations, or SRD5A type II deficiency (Albers *et al.*,1997; Boehmer *et al.*,2001). The aetiology of most other hypospadias cases, however, is not yet solved in spite of intensive research. In this review, we will summarize the current knowledge about the causes of the isolated, non-syndromic form of this common birth defect in humans, from both a genetic and an environmental point of view. In addition, we will provide recommendations for further research.

METHODS

Pubmed was used to identify all relevant manuscripts on the aetiology of hypospadias. We searched for papers published between January 1995 and February 2011 in the English language using the following keywords in the title or abstract: "(hypospadia OR hypospadias) NOT surgical NOT surgery NOT reconstruction NOT repair NOT incised NOT procedure". This search provided 922 articles, of which we used the titles and abstracts to identify relevant papers. We focussed our review on the aetiology of isolated hypospadias in humans. Therefore, we excluded all animal studies (N = 99), articles that were not about hypospadias or the aetiology of hypospadias (N = 235), and articles or case-reports that described the phenotype of patients suffering from syndromes including hypospadias, or that investigated or described the most likely cause of the syndrome in these boys (N = 308). To systematically exclude articles with a lesser degree of evidence, we excluded all ecological studies (N = 11). For epidemiologic studies reporting negative findings for environmental factors, we took the power into consideration before reporting that it showed no association. In general, we excluded negative results on environmental factors from studies describing <100 cases, as these have, for example, only 37% power to significantly (P<0.05) detect a two-fold increased risk, assuming a prevalence of 10% (15 studies were completely excluded because of this criterion). To guarantee that all information was included only once in the article, we excluded all reviews and meta-analyses (N = 79). In addition, when a study was supplemented with new data in a later publication (N= 3), we only included the article reporting the most complete data. Finally, all commentaries were excluded (N = 32). Reference lists of the selected manuscripts were searched to identify additional studies, including those published before 1995, although these were only included if they reported results that were not found in one of the more recently published articles (N = 29). This selection process resulted in 169 original articles that were included in this review and are described below.

RESULTS

Aetiology of hypospadias is multifactorial

Hypospadias shows familial clustering, with 7% of cases having affected first, second or third degree relatives (Fredell *et al.*,2002b). Familial occurrence seems to be more common for anterior and middle forms of hypospadias than for posterior types (Fredell *et al.*,2002b; Brouwers *et al.*,2010). The chance that a brother of an affected boy will also have hypospadias is 9 to 17% (Calzolari *et al.*,1986; Stoll *et al.*,1990; Schnack *et al.*,2008). In two family studies and one small twin study, the heritability of hypospadias was estimated to be 57 to 77% (Calzolari *et al.*,1986; Stoll *et al.*,1990; Schnack *et al.*,2008), meaning that 57 to 77% of the phenotypic variability can be attributed to genetic variability. Because hypospadias is equally transmitted through the maternal and paternal sides of the family and recurrence risks for brothers and sons of hypospadias cases are similar, genetic rather than shared environmental factors may play a principal role in familial hypospadias (Schnack *et al.*,2008). Segregation analysis, however, suggested that the majority of cases have a multifactorial aetiology, involving both genes and environmental factors (Fredell *et al.*,2002a).

Genes implicated in the aetiology of isolated hypospadias

Much of the genetic research on hypospadias has been focused on identification of causal mutations. In Table I, we summarize the exonic (including 3'-untranslated and splice acceptor site) mutations found in studies screening candidate genes in groups of patients with hypospadias, ordered according to the different stages of embryonic development. Whether these mutations have functional consequences remains unclear in most cases, as only few studies reported conservation and function of the region in which the mutation is located, or predicted potential influence of the mutation on protein function using bioinformatics. The majority of mutations were found only once and were identified in posterior or penile cases. The latter has contributed to the view that there is a difference in the genetic models underlying posterior versus anterior hypospadias, with posterior cases being more common in monogenic forms of hypospadias and anterior cases having a polygenic or multifactorial aetiology.

The studies investigating associations between genetic polymorphisms and hypospadias are summarized in Table II (following the same order as Table I).

Indifferent stage

All genes involved in the development of the male external genitalia are obvious candidate genes for hypospadias. Because *Wt1* and *Sf1* play major roles in early embryonic development of the kidneys and the urogenital system, mutations in these genes are likely to cause not only hypospadias but also more severe defects. Indeed, *SF1* mutations were found in severe penoscrotal hypospadias cases with cryptorchidism (Köhler *et al.*,2009), while a mutation in *WT1* was described in a boy with penoscrotal hypospadias and micropenis and also in three boys with isolated penile or glandular hypospadias (Wang *et al.*,2004) (Table I).

Early patterning

Genes involved in GT patterning are additional candidates for hypospadias. Mutation screening in hypospadias cases revealed mutations in *BMP4*, *BMP7*, *HOXA4*, *HOXB6*, *FGF8*, and the fibroblast growth factor receptor *FGFR2* (Chen *et al.*,2007; Beleza-Meireles *et al.*,2007c) (Table I), while associations with hypospadias were also observed for polymophisms in *FGF8* and *FGFR2* (Beleza-Meireles *et al.*,2007c) (Table II).

Masculinisation

Expression of the *SRY* gene, located on the Y chromosome, is crucial for development of the testis from the indifferent gonad (Sinclair *et al.*,1990; Gubbay *et al.*,1990). Sex chromosome abnormalities were noticed in four out of 100 patients with hypospadias (Moreno-García and Miranda,2002) but no mutations in *SRY* were found in 90 patients in another study (Wang *et al.*,2004). In addition, screening Yq for microdeletions in 44 cases did not reveal any abnormalities (Tateno *et al.*,2000) and neither did screening the segments of the Y chromosome associated with infertility in 20 cases with middle or posterior hypospadias and cryptorchidism (Castro *et al.*,2004).

Genetic research has been focused on the hormone-dependent stage of sexual development as well. The gene encoding AR in particular was investigated extensively. AR is expressed in the developing human penis and urethra (Kim *et al.*,2002) and several studies reported rare mutations in the gene encoding AR in patients with hypospadias (Hiort *et al.*,1994; Alléra *et al.*,1995; Sutherland *et al.*,1996; Nordenskjöld *et al.*,1999; Wang *et al.*,2004; Thai *et al.*,2005) (Table I). In addition, polymorphisms in *AR* have been investigated for associations with the anomaly and may increase hypospadias risk. For example, expansion of the polyglutamine (CAG) repeat in the N-terminus of *AR*, shown to decrease AR transactivation function (Chamberlain *et al.*,1994), was found to be associated with undermasculinisation (Lim *et al.*,2000). Two studies reported that longer GGN repeat length increased the risk of penile hypospadias (Aschim *et al.*,2004b; Radpour *et al.*,2007) but these two (and one other) studies did not find an association between CAG repeat length and hypospadias (Muroya *et al.*,2001; Aschim *et al.*,2004b; Radpour *et al.*,2007) (Table II). DHT binding capacity of the AR in genital skin fibroblasts was reported to be decreased in some patients with hypospadias (Schweikert *et al.*,1989; Alléra *et al.*,1995), whereas normal binding capacity was found in others (Gearhart *et al.*,1988; Terakawa *et al.*,1990). In addition, AR levels were similar in foreskin samples of hypospadias cases and controls (Bentvelsen *et al.*,1995).

Several proteins are needed for AR function. FK506 binding protein 4, 59kDa (FKBP4, also known as FKBP52), for example, is a component of AR complexes, enhancing AR-mediated transactivation (Cheung-Flynn *et al.*,2005). However, no differences in FKBP4 expression were noted between patients with hypospadias and controls and no mutations in *FKBP4* were observed (Beleza-Meireles *et al.*,2007a).

As normal male urethral development requires testosterone and DHT, defects in steroidogenesis could also account for hypospadias. One article stated that up to 50% of patients with hypospadias have a testosterone biosynthesis defect (Aaronson *et al.*,1997), a conclusion that could not be confirmed in

two other studies that found no enzymatic defects (Feyaerts *et al.*,2002; Holmes *et al.*,2004). Nevertheless, mutations have been found in hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*HSD3B2*) (Codner *et al.*,2004) and SRD5A type II (*SRD5A2*) (Silver and Russell,1999; Wang *et al.*,2004; Thai *et al.*,2005).

The gene encoding SRD5A2 is particularly interesting because this enzyme is expressed during male genital development around the ventral part of the remodelling urethra and it converts testosterone to the more potent androgen DHT, which induces formation of the external genitalia (Kim *et al.*,2002). Two single nucleotide polymorphisms (SNPs) in this gene seemed to be associated with hypospadias in some but not all studies (Silver and Russell,1999; Wang *et al.*,2004; Thai *et al.*,2005; Sata *et al.*,2010; van der Zanden *et al.*,2010b) (Table II). One of these SNPs (rs523349) causes a valine to leucine substitution (V89L), resulting in a decrease in enzyme activity by approximately 30% (Makridakis *et al.*,1997; Makridakis *et al.*,2000), whereas the other SNP (rs9282858) results in an alanine to threonine replacement (A49T), which causes an increase in enzyme function (Makridakis *et al.*,2000). Another SNP that seems to be associated with hypospadias and to have functional consequences is rs2066479 in *HSD17B3*. The glycine to serine substitution (G289S) caused by this SNP results in reduced *HSD17B3* mRNA expression levels *in utero* (Sata *et al.*,2010).

Other genes

Not only steroidogenesis but also the balance between androgens and estrogens appears to be important in development of the male external genitalia. The estrogen receptors ESR1 and ESR2 are expressed in the developing human male GT (Crescioli *et al.*,2003) and associations have been reported between hypospadias and several SNPs in the genes encoding these receptors, as well as with the CA-repeat in *ESR2* (Beleza-Meireles *et al.*,2006; Watanabe *et al.*,2007; Beleza-Meireles *et al.*,2007b; Ban *et al.*,2008; van der Zanden *et al.*,2010b) (Table II). One of the SNPs in *ESR1*, rs9340799, was shown to increase enhancer activity of ESR1 (Maruyama *et al.*,2000).

Some additional genes are also suggested to be involved in development of hypospadias. Activating transcription factor 3 (*ATF3*) is an estrogen-responsive gene showing strong up-regulation in hypospadias (Liu *et al.*,2005; Wang *et al.*,2007; Kalfa *et al.*,2008a; Gurbuz *et al.*,2010). Studies focusing on the relation between this gene and hypospadias found mutations and associations with several SNPs (Beleza-Meireles *et al.*,2008; Kalfa *et al.*,2008a) but not all associations could be replicated (van der Zanden *et al.*,2010b) (Tables I and II). Recently, mastermind-like domain containing 1 (*MAMLD1*, previously known as *CXorf6*) was identified as a causal gene for hypospadias. *MAMLD1* contains the SF1 target sequence (Fukami *et al.*,2008) and mutations and polymorphisms in *MAMLD1* have been found in patients with hypospadias (Fukami *et al.*,2006; Kalfa *et al.*,2008b; Chen *et al.*,2010) (Tables I and II). A recent genome-wide association study using pooled DNA samples identified diacylglycerol kinase, kappa (*DGKK*) as a major risk gene for hypospadias (van der Zanden *et al.*,2010a). An intronic SNP was associated with a 2.5 times increased hypospadias risk, while *DGKK* expression in preputial skin was shown to be lower in boys carrying the risk allele. In the van der Zanden *et al.* (2010a) study, additional candidate genes i.e. peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*), glutamate receptor, ionotropic, delta 1 (*GRID1*) and *KIAA2022* were also identified but these still need to be confirmed. One study investigated *MID1* in relation to hypospadias and found mutations in patients with hypospadias as well as a SNP in this gene to be associated with the disorder (Zhang *et al.*,2011) (Tables I and II). Insulin-like 3 (*INSL3*) mutations have been found in patients with cryptorchidism but no alterations were detected in 94 hypospadias cases (El Houate *et al.*,2007) (Table I).

Expression studies have also identified some candidate genes. Using prepuce samples of patients with hypospadias and controls, Wang *et al*. (2007) not only found *ATF3* to be upregulated in patients but also connective tissue growth factor (*CTGF*) and cysteine-rich, angiogenic inducer, 61 (*CYR61*), two other estrogen-responsive genes. In addition, epidermal growth factor (EGF) staining in prepuce showed lower expression of *EGF* within the penile skin adjacent to the urethra in patients with hypospadias compared to controls (el-Galley *et al.*,1997).

A balanced translocation in a man with hypospadias and other congenital anomalies indicated basonuclin 2 (*BNC2*) as a candidate gene. This gene is expressed in developing human periurethral tissue and mutations were found in 6 out of 48 patients with hypospadias but also in 2 out of 23 controls (Bhoj *et al.*,2011) (Table I).

As exposure to environmental toxicants has also been suggested to cause hypospadias, and cytochrome P4501A1 (CYP1A1) and glutathione S-transferases (GSTM1 and GSTT1) are involved in the metabolism of various toxicants, two studies evaluated the effect on hypospadias risk of polymorphisms in the genes encoding these enzymes. One study found an association with hypospadias for concomitant deletion of *GSTM1* and *GSTT1* (Yadav *et al.*,2011) (Table II). The other study investigated associations between maternal smoking, maternal SNPs in the genes and the risk of hypospadias in offspring. They found an association between a SNP in *CYP1A1* and hypospadias, which was not modified by smoking behaviour (Kurahashi *et al.*,2005).

One genome-wide linkage analysis in 69 families with at least 2 members with hypospadias found suggestive linkage at 9q22, 2p11, 10p15 and 10q21 (Frisen *et al.*,2004), while another linkage study in a three-generational family showing autosomal dominant inheritance of hypospadias found a peak on 7q32.2-q36.1 (Thai *et al.*,2008). Mutation analysis of two genes in this region, *AKRID1* and *PTN,* failed to reveal any mutations (Thai *et al.*,2008).

Screening 17 isolated patients with hypospadias and 12 patients with associated anomalies for copy number variants (CNVs) revealed clinically significant CNVs in 3 patients with isolated hypospadias (5p15, 12p13 and Xq28) and in 2 patients with an associated anomaly, which were cryptorchidism (2q22) and cleft palate (16p11) (Tannour-Louet *et al.*,2010).

The role of environmental factors in the aetiology of hypospadias

406 ***Introduction***

407

408 While genes involved in the aetiology of hypospadias have received a considerable amount of
409 attention, research on environmental factors has been even more extensive. Despite the large number
410 of studies, however, clear evidence for causal environmental factors is still lacking, although some
411 consistent associations have been reported. Table III gives a summary of environmental factors
412 investigated in relation to hypospadias.

413

414 *Testicular dysgenesis syndrome*

415 In 2001, Skakkebæk *et al.* suggested that poor sperm quality, testicular cancer, undescended testes and
416 hypospadias are symptoms of one underlying entity, the Testicular Dysgenesis Syndrome (TDS)
417 (Skakkebæk *et al.*,2001). They were convinced of its existence because countries with high incidences
418 of testicular cancer also had high prevalence rates of hypospadias, cryptorchidism and poor sperm
419 quality (Virtanen *et al.*,2005). Other researchers question whether TDS actually exists as there is little
420 evidence of shared causes (Akre and Richiardi,2009), only a few patients display all features, and
421 incidences of the four components of the syndrome did not increase over time at the same rate (Thorup
422 *et al.*,2010). Although testicular germ cell cancer risk was increased in patients with hypospadias or
423 undescended testis, risk was not increased in their family members. This does not support the
424 hypothesis of shared heritability (Schnack *et al.*,2010). Recently, Skakkebæk *et al.* concluded that
425 TDS does exist but that it encompasses only a fraction of hypospadias and impaired spermatogenesis
426 cases (Jørgensen *et al.*,2010).

427

428 *Estrogen hypothesis*

429 In 1993, Sharpe and Skakkebæk hypothesized that the increasing incidence of reproductive
430 abnormalities in males may have a common cause, namely increased estrogen exposure *in utero*,
431 leading to disturbances in AMH secretion or impairment of Leydig cell development (Sharpe and
432 Skakkebæk,1993). Ten years after the introduction of this hypothesis, Sharpe concluded that evidence

for foetal estrogen exposure inducing TDS had strengthened (Sharpe,2003). New pathways were identified through which estrogens could induce TDS, including suppression of testosterone production, AR expression and insulin-like 3 secretion. Whether increased estrogen exposure will turn out to be an important aetiologic factor for TDS is not so certain, however.

The initial 'estrogen hypothesis' was superseded by a more refined definition of endocrine disrupting chemicals (EDCs), suggesting that chemicals may act on the endocrine systems in a plethora of ways (Fisher,2004). In 2008, Sharpe and Skakkebæk highlighted the central role of deficient androgen production or action during foetal testis development in the origin of the downstream disorders of TDS (Sharpe and Skakkebæk,2008). However, the question remains whether levels of exposure to EDCs are sufficient to influence male reproductive health (Fisher,2004) and several reviews concluded that there is little evidence for a role of environmental EDCs (Raman-Wilms *et al.*,1995; Safe,2000; Chia,2000; Vidaeff and Sever,2005; Storgaard *et al.*,2006; Martin *et al.*,2008).

Exogenous exposure to estrogens

Oral contraceptives

Although oral contraceptives probably provide the strongest estrogen exposure that humans can experience, an association between hypospadias and use of oral contraceptives for some time during pregnancy was not found in most studies (Morera *et al.*,2006; Wogelius *et al.*,2006; Brouwers *et al.*,2007; Akre *et al.*,2008; Nørgaard *et al.*,2009; Brouwers *et al.*,2010).

Assisted reproductive technology

Assisted reproductive technologies (ART) frequently involve hormonal stimulation and some studies showed an increased risk of hypospadias with ART (Carmichael *et al.*,2007; Brouwers *et al.*,2007; Brouwers *et al.*,2010). More specifically, ICSI increased hypospadias risk in most (Wennerholm *et al.*,2000; Ericson and Källén,2001; Pinborg *et al.*,2004; Källén *et al.*,2005; Fedder *et al.*,2007; Funke *et al.*,2010) but not all studies (Bonduelle *et al.*,2002; Källén *et al.*,2010), whereas studies on IVF did

not report increased risks or were inconclusive (Ericson and Källén,2001; Bonduelle *et al.*,2002; Morera *et al.*,2006; Funke *et al.*,2010; Källén *et al.*,2010), except for one study that did not report whether ICSI was excluded (Silver *et al.*,1999). In one study, increased hypospadias risk was associated with hormonal stimulation (Carmichael *et al.*,2005a) but this was not confirmed in other studies (Källén *et al.*,2002; Sørensen *et al.*,2005b; Morera *et al.*,2006; Meijer *et al.*,2006).

Other authors assumed that the increased hypospadias risk may be explained by reduced maternal or paternal fertility. Fathers of hypospadias cases were reported to have lower sperm concentration, sperm count (Asklund *et al.*,2007) and sperm motility, as well as a higher proportion of abnormal sperm morphology (Fritz and Czeizel,1996). In addition, several studies reported a prolonged time-to-pregnancy (TTP) for parents of patients with hypospadias (Källén,2002; Pierik *et al.*,2004; Asklund *et al.*,2007; Brouwers *et al.*,2010) and only one study did not confirm these results (Akre *et al.*,1999). The fact that ICSI, rather that IVF, and sperm quality are associated with hypospadias supports the idea that paternal fertility problems in particular play a role in hypospadias (Brouwers *et al.*,2007; Brouwers *et al.*,2010).

ART may be associated with genomic imprinting disorders (Laprise,2009). This possible interference with epigenetic regulation is another mechanism by which ART could increase hypospadias risk. A very recent study indicated that alterations in the methylation pattern of *AR*, leading to abnormal expression of the gene in foreskin tissue from patients, may contribute to the development of hypospadias (Vottero *et al.*,2011).

Endogenous hormone levels

Endogenous estradiol levels

Endogenous levels of free estradiol increase with increasing BMI and are elevated in women with an early age at menarche (Apter and Vihko,1983; Emaus *et al.*,2008). Several studies found associations between hypospadias and mothers being overweight ($25 \leq BMI < 30$ kg/m^2) (Waller *et al.*,2007) or

severely overweight or obese (BMI>29 or 30 kg/m^2) (Waller *et al.*,2007; Akre *et al.*,2008; Giordano *et al.*,2010; Blomberg and Källén,2010) but one study did not (Brouwers *et al.*,2010). Another study found increased risks for underweight but not for overweight or obese women (Rankin *et al.*,2010). The results for early age at menarche were inconsistent (Morera *et al.*,2006; Giordano *et al.*,2010). Estradiol levels are also higher in first pregnancies and twin pregnancies (Kappel *et al.*,1985; Bernstein *et al.*,1986), which were both repeatedly investigated for their association with hypospadias. Most studies showed that women in their first pregnancy (Akre *et al.*,1999; Weidner *et al.*,1999; Hussain *et al.*,2002; Källén,2002; Carmichael *et al.*,2003; Aschim *et al.*,2004a; Sørensen *et al.*,2005a; Meyer *et al.*,2006; Morera *et al.*,2006; Carmichael *et al.*,2007; Nassar *et al.*,2009; Jin *et al.*,2010) or with a twin or triplet pregnancy (Akre *et al.*,1999; Fredell *et al.*,2002b; Morera *et al.*,2006; Brouwers *et al.*,2007; Carmichael *et al.*,2007; Sun *et al.*,2009; Nassar *et al.*,2009; Brouwers *et al.*,2010; Funke *et al.*,2010; Jin *et al.*,2010) were at increased risk of having a son with hypospadias but a few studies could not replicate the findings for primiparity or for multiple pregnancies (Weidner *et al.*,1999; Carmichael *et al.*,2003; Aschim *et al.*,2004a; Sørensen *et al.*,2005a; Ghirri *et al.*,2009). The latter may be caused by overadjustment for birthweight in some studies. As only early-onset intrauterine growth restriction (IUGR) could be a risk factor for hypospadias, it is more likely that low birthweight and hypospadias share an underlying cause rather than low birthweight being a risk factor for hypospadias.

Foetal hCG provision

Placental hCG stimulates foetal testicular steroidogenesis before the foetus's own pituitary-gonadal axis is established. Placental insufficiency may result in inadequate foetal hCG provision and IUGR, possibly explaining the association between hypospadias and low birthweight or being small for gestational age (SGA) that was consistently reported, although not always statistically significant (Weidner *et al.*,1999; Akre *et al.*,1999; Gatti *et al.*,2001; Hughes *et al.*,2002; Hussain *et al.*,2002; Fredell *et al.*,2002b; Carmichael *et al.*,2003; Pierik *et al.*,2004; Aschim *et al.*,2004a; Boisen *et al.*,2005; Chong *et al.*,2006; Morera *et al.*,2006; Brouwers *et al.*,2007; Akre *et al.*,2008; Giordano *et al.*,2008; Sun *et al.*,2009; Nassar *et al.*,2009; Ghirri *et al.*,2009; Brouwers *et al.*,2010; Funke *et*

514 *al.*,2010; Jin *et al.*,2010; Giordano *et al.*,2010). However, because hCG levels were similar in maternal
515 serum samples of hypospadias cases and controls, this is unlikely to be related to decreased maternal
516 hCG production (Kiely *et al.*,1995). IUGR was also found more often in the affected twin of same-sex
517 twin pairs discordant for hypospadias (Fredell *et al.*,1998; Chambers *et al.*,2006). Direct proof of a
518 link between placental insufficiency and hypospadias was provided by research showing an
519 association between hypospadias and low placental weight (Stoll *et al.*,1990), an increased frequency
520 of placental infarction among extremely low birthweight boys with hypospadias (Fujimoto *et al.*,2008)
521 and a high rate of early-onset IUGR related to placental insufficiency among SGA newborns with
522 hypospadias, with the more posterior cases having more severe IUGR (Yinon *et al.*,2010). The
523 association with low birthweight also seems to be stronger for more posterior forms of hypospadias
524 (Carmichael *et al.*,2003; Carlson *et al.*,2009; Ghirri *et al.*,2009; Brouwers *et al.*,2010).
525 Nausea in early pregnancy may be caused by the early surge of hCG (Furneaux *et al.*,2001),
526 suggesting that placental insufficiency may cause absence of nausea. Indeed, vomiting and nausea
527 during early pregnancy were shown to decrease hypospadias risk (Carmichael *et al.*,2007; Akre *et*
528 *al.*,2008). Maternal hypertension during pregnancy (Morera *et al.*,2006; Akre *et al.*,2008; Caton *et*
529 *al.*,2008; Sun *et al.*,2009; Brouwers *et al.*,2010) and preeclampsia (Akre *et al.*,1999; Aschim *et*
530 *al.*,2004a; Sørensen *et al.*,2005a; Chong *et al.*,2006; Morera *et al.*,2006; Sun *et al.*,2009; Brouwers *et*
531 *al.*,2010) were consistently associated with hypospadias, and both factors may be associated with
532 placental dysfunction, possibly by compromising uteroplacental perfusion (Caton *et al.*,2008). Preterm
533 delivery may be associated with late placental dysfunction and several studies demonstrated an
534 association with hypospadias (Pierik *et al.*,2004; Meyer *et al.*,2006; Akre *et al.*,2008; Sun *et al.*,2009;
535 Nassar *et al.*,2009; Funke *et al.*,2010; Jin *et al.*,2010; Giordano *et al.*,2010; Akin *et al.*,2011) while
536 others could not confirm this (Akre *et al.*,1999; Weidner *et al.*,1999; Carmichael *et al.*,2003; Aschim
537 *et al.*,2004a; Chong *et al.*,2006; Ghirri *et al.*,2009), again possibly because of overadjustment for
538 birthweight in some studies.
539
540 ***Clinical factors***

Pregnancy complications

In a few studies, associations were investigated between hypospadias and complications during pregnancy, such as maternal bleeding, which seemed to be more prevalent among cases (Aschim *et al.*,2004a; Jin *et al.*,2010). The amount of weight gain was not associated with hypospadias (Morera *et al.*,2006; Meyer *et al.*,2006). Complications during labour, such as labour induction and Caesarean section, occurred more frequently among mothers of hypospadias cases (Aschim *et al.*,2004a; Meyer *et al.*,2006), indicating that pregnancies affected by hypospadias are associated with other difficulties that make them prone to these complications. Diabetes has been another focus of research, but most studies were too small to draw conclusions (Hussain *et al.*,2002; Sørensen *et al.*,2005a; Morera *et al.*,2006; Sun *et al.*,2009; Brouwers *et al.*,2010). One study found maternal gestational and pre-existing diabetes not to be associated with occurrence of hypospadias (Aschim *et al.*,2004a), whereas others reported an increased risk for pre-existing but not for gestational diabetes (Åberg *et al.*,2001; Porter *et al.*,2005). Results were inconsistent for thyroid disease (Aschim *et al.*,2004a; Browne *et al.*,2009) and fever during pregnancy (Stoll *et al.*,1990; Jin *et al.*,2010). Women with gynaecological diseases (ovarian cysts or benign uterine tumours) (Giordano *et al.*,2008), those who are carriers of hepatitis B antigen (Sun *et al.*,2009) and women experiencing a viral infection or influenza in the first trimester of pregnancy (North and Golding,2000; Morera *et al.*,2006) seem to be at increased risk of giving birth to a son with hypospadias but evidence was derived from only one study. Urinary infections and anaemia do not seem to increase hypospadias risk (Aschim *et al.*,2004a).

Maternal drug use

Most therapeutic drugs, such as corticosteroids, antibiotics, antipsychotics, antifungal and anti-asthmatic drugs, do not seem to be associated with hypospadias, although some studies may suffer from under reporting (Czeizel and Rockenbauer,1997; Czeizel *et al.*,2001; Brouwers *et al.*,2007; Källén and Otterblad,2007; Carter *et al.*,2008; Carmichael *et al.*,2009a; Brouwers *et al.*,2010). Based on data from the Swedish Medical Birth Register 1995-2001, Källén *et al.* reported 15 hypospadias cases in 2780 infants born after maternal use of loratadine, an antihistamine, during pregnancy (Källén

568 and Olausson,2001) but in 2001-2004 only two cases were identified among 1911 infants exposed to
569 loratadine, indicating that the primary finding occurred by chance (Källén and Olausson,2006). Other
570 studies also failed to find an association between loratadine and hypospadias (CDC 2004; Pedersen *et*
571 *al.*,2008). Results for progestogens/progestins used for threatened abortion vary (Katz *et al.*,1985;
572 Calzolari *et al.*,1986). Use of loperamide (Källén *et al.*,2008), antiretroviral therapy (Watts *et*
573 *al.*,2007), antihypertensive drugs (Caton *et al.*,2008; Brouwers *et al.*,2010), nystatin (Czeizel *et*
574 *al.*,2003) or paroxetine (Reis and Källén,2010) during early pregnancy may increase hypospadias risk,
575 while codeine (North and Golding,2000) may decrease the risk but most of these associations were
576 reported only once. In contrast, use of anti-epileptic drugs was linked to hypospadias several times
577 (Arpino *et al.*,2000; Hunt *et al.*,2008; Rodríguez-Pinilla *et al.*,2008; Bánhidy *et al.*,2010; Jentink *et*
578 *al.*,2010). Most studies showed no effects of folate (Källén,2007; Carmichael *et al.*,2009b; Brouwers
579 *et al.*,2010) or iron supplementation (Morera *et al.*,2006; Brouwers *et al.*,2010) on hypospadias risk,
580 although one study showed a reduced risk of folate (Ormond *et al.*,2009) and two others an increased
581 risk of iron supplementation (North and Golding,2000; Brouwers *et al.*,2007).
582
583 *Maternal intrauterine DES exposure*
584 In 2002, Klip *et al.* reported a 21 times increased hypospadias risk among sons of women exposed to
585 diethylstilbestrol (DES) *in utero* in a cohort of women with fertility problems (Klip *et al.*,2002).
586 Thereafter, other studies were consistent in showing an increased risk for sons of DES-daughters,
587 although less strong (Palmer *et al.*,2005; Pons *et al.*,2005; Brouwers *et al.*,2006; Brouwers *et*
588 *al.*,2010). This transgenerational effect may have been related to genetic or epigenetic changes in
589 primordial oocytes, which were transmitted to the next generation, or in somatic cells of the DES-
590 daughter, resulting in disturbed hormonal balance in adult life (Klip *et al.*,2002). Another explanation
591 would be that pathology of the DES-daughter's reproductive structures interferes with normal foetal
592 development (Brouwers *et al.*,2006).
593
594 ***Behavioural factors***

595 *Parental age*

596 Women become pregnant at different ages but, overall, maternal and paternal age at time of
597 conception did not seem to increase the risk of having a son with hypospadias (Akre *et al.*,1999;
598 Weidner *et al.*,1999; Källén,2002; Hussain *et al.*,2002; Aschim *et al.*,2004a; Sørensen *et al.*,2005a;
599 Morera *et al.*,2006; Meyer *et al.*,2006; Brouwers *et al.*,2007; Akre *et al.*,2008; Materna-Kiryluk *et*
600 *al.*,2009; Sun *et al.*,2009; Nassar *et al.*,2009; Ghirri *et al.*,2009; Lund *et al.*,2009; Brouwers *et*
601 *al.*,2010). However, some studies reported a higher maternal age (Fisch *et al.*,2001; Hussain *et*
602 *al.*,2002; Carmichael *et al.*,2003; Reefhuis and Honein,2004; Porter *et al.*,2005; Carmichael *et*
603 *al.*,2007; Fisch *et al.*,2009; Akin *et al.*,2011) or lower or higher paternal age (McIntosh *et al.*,1995;
604 Materna-Kiryluk *et al.*,2009) to increase hypospadias risk.
605
606 *Maternal diet*
607 In 2000, North and Golding reported a five times increased risk of a hypospadias-affected son for
608 women with a vegetarian diet (North and Golding,2000), a finding that was confirmed in one study
609 (Akre *et al.*,2008) but not in others (Brouwers *et al.*,2007; Ormond *et al.*,2009; Brouwers *et al.*,2010).
610 However, all of these results were based on case-control studies with relatively few exposed cases and
611 controls (<15) except for a study in England reporting no association in more than 75 cases and
612 controls who were vegetarian (Ormond *et al.*,2009). The suggestion that an increased risk might be
613 related to intake of phytoestrogens was refuted by a small study involving phytoestrogen-specific
614 questionnaires that did not find an association (Pierik *et al.*,2004). Another dietary factor found to be
615 associated with hypospadias in two small studies is the frequent consumption of fish, possibly
616 associated with the bioaccumulation of contaminants in fish (Giordano *et al.*,2008; Giordano *et*
617 *al.*,2010). However, a larger case-control study found a decreased hypospadias risk for frequent fish
618 consumption (Akre *et al.*,2008).
619
620 *Other lifestyle factors*

Alcohol consumption during pregnancy was consistently found not to be associated with hypospadias (Hussain *et al.*,2002; Meyer *et al.*,2006; Brouwers *et al.*,2007). For maternal smoking, most studies showed no association (Akre *et al.*,1999; Källén,2002; Hussain *et al.*,2002; Carmichael *et al.*,2005b; Morera *et al.*,2006; Meyer *et al.*,2006; Brouwers *et al.*,2007; Akre *et al.*,2008; Brouwers *et al.*,2010). One small study found maternal cocaine use to be associated with hypospadias (Battin *et al.*,1995).

Occupational factors

Exposure to pesticides

Occupational exposures have been a major focus in hypospadias research, especially exposure to pesticides, with contradicting results. Paternal exposure to pesticides before pregnancy does not seem to be associated with hypospadias (Weidner *et al.*,1998; Brouwers *et al.*,2007; Nassar *et al.*,2009; Brouwers *et al.*,2010), although one small study reported a possibly increased risk (Giordano *et al.*,2008). In addition, an increased risk was found among farmers who were indicated as exposed to pesticides in a register-based study (Kristensen *et al.*,1997). Most studies showed no association with maternal occupational exposure to pesticides (Weidner *et al.*,1998; Vrijheid *et al.*,2003; Brouwers *et al.*,2007; Nassar *et al.*,2009; Brouwers *et al.*,2010; Morales-Suarez-Varela *et al.*,2011) but being involved in agricultural activities (Sun *et al.*,2009) or using insect repellents (Dugas *et al.*,2010) seemed to increase hypospadias risk in two studies. Maternal serum levels of dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethane (DDE) during pregnancy were not associated with hypospadias (Longnecker *et al.*,2002; Bhatia *et al.*,2005) but maternal serum hexachlorobenzene (HCB) concentrations approximately one year after birth were more often above the median of all subjects among hypospadias cases than among controls (Giordano *et al.*,2010).

Other occupational exposures

Boys conceived to mothers employed in the leather industry (García and Fletcher,1998) and post-war to mothers who served in the Gulf war (Araneta *et al.*,2003) seemed to have a higher prevalence of hypospadias. Most other maternal occupational exposures were not associated with hypospadias,

although results for EDCs, heavy metals and phthalates vary, while exposure to hairspray increased the risk in one study (Vrijheid *et al.*,2003; Brouwers *et al.*,2007; Ormond *et al.*,2009; Nassar *et al.*,2009; Brouwers *et al.*,2010; Giordano *et al.*,2010; Morales-Suarez-Varela *et al.*,2011). For fathers, being a vehicle mechanic or manufacturer (Schnitzer *et al.*,1995; Irgens *et al.*,2000), police officer or fire fighter (Schnitzer *et al.*,1995), and occupational exposure to dusts from grinding metals (Brouwers *et al.*,2010) seemed to increase the risk of having a son with hypospadias. Results on heavy metals vary (Nassar *et al.*,2009; Morales-Suarez-Varela *et al.*,2011) but most other paternal occupational exposures were not associated with hypospadias (Brouwers *et al.*,2007; Nassar *et al.*,2009; Brouwers *et al.*,2010; Morales-Suarez-Varela *et al.*,2011).

Living environment

Results on living in rural or (sub)urban areas are contradictory (Sun *et al.*,2009; Nassar *et al.*,2009), whereas living close to a landfill site seemed to be associated with an increased hypospadias risk (Dolk *et al.*,1998). Maternal serum levels of polychlorinated biphenyls (PCBs) were elevated during pregnancies affected by hypospadias in two small studies but these results were not statistically significant (Carmichael *et al.*,2010; Giordano *et al.*,2010). Another study found marginally increased PCB levels in serum samples of women pregnant with a hypospadias-affected son, but the study samples were collected in the 1960s, when PCB exposure was substantially higher than nowadays (McGlynn *et al.*,2009). Maternal exposure to water disinfection by-products was also suggested to increase hypospadias risk but most studies provided little evidence for this association (Källén and Robert,2000; Luben *et al.*,2008; Iszatt *et al.*,2011).

In one study, the prevalence of hypospadias seemed to be higher in areas of intensive pesticide use or in agricultural areas (Morera *et al.*,2006). Another study showed an increased risk of hypospadias for living in an area where diclofopmethyl was applied but a decreased risk for alachlor and permethrin, or for pesticide application in aggregate (Meyer *et al.*,2006).

675 In some older studies, a seasonal trend for hypospadias was identified (Wehrung and Hay,1970;
676 Roberts and Lloyd,1973; Avellan,1977), which was attributed to factors such as hours of daylight,
677 climate or temperature, whereas more recent studies did not find seasonal variation (Skriver *et*
678 *al.*,2004; Morera *et al.*,2006; Jin *et al.*,2010).
679
680

CONCLUSION

Most hypospadias cases have an unknown aetiology, which is likely to be a mix of monogenic and multifactorial forms, implicating both genes and environmental factors. Several mutations have been found that might cause hypospadias but most investigators are convinced that single mutations are not likely to be the cause for the majority of isolated hypospadias cases. Neverteheless, studies screening patients with hypospadias for single-gene defects found mutations in the genes *WT1*, *SF1*, *BMP4*, *BMP7*, *HOXA4*, *HOXB6*, *FGF8*, *FGFR2*, *AR*, *HSD3B2*, *SRD5A2*, *ATF3*, *MAMLD1*, *MID1* and *BNC2*. Association studies found polymorphisms in *FGF8*, *FGFR2*, *AR*, *HSD17B3*, *SRD5A2*, *ESR1*, *ESR2*, *ATF3*, *MAMLD1*, *DGKK*, *MID1*, *CYP1A1*, *GSTM1* and *GSTT1* to be risk factors for hypospadias. In addition, gene expression studies indentified *CTGF*, *CYR61* and *EGF* as candidate genes.

Additional evidence for the involvement of genes can be derived from syndromes commonly associated with hypospadias, which were not reviewed in this article. For example, additional evidence for the involvement of *WT1* comes from the fact that *WT1* mutations cause syndromes such as Denys-Drash and Frasier syndromes, characterized by progressive nephropathy, intersex and predisposition to develop genitourinary tumours (Morrison *et al.*,2008). Male cases having hypospadias were reported for both syndromes (Sherbotie *et al.*,2000; Melo *et al.*,2002; Kaltenis *et al.*,2004). Syndromes which are commonly associated with hypospadias can also help in the indentification of new candidate genes. One example is hand-foot-genital syndrome, which is caused by mutations in *HOXA13* (Mortlock and Innis,1997; Goodman and Scambler,2001). *Hoxa13* mutant mice also exhibited hypospadias (Morgan *et al.*,2003) and expansion of a polyalanine tract in *HOXD13* found in synpolydactyly families also seems to be associated with hypospadias (Goodman *et al.*,1997; Tüzel *et al.*,2007). Mutations in zinc finger E-box binding homeobox 2 (*ZEB2*) cause Mowat-Wilson syndrome, which is associated with hypospadias in more than 50% of affected males (Mowat *et al.*,2003; Zweier *et al.*,2005; Adam *et al.*,2006; Garavelli and Mainardi,2007; Garavelli *et al.*,2009).

Additional candidate genes for hypospadias aetiology include genes for which mutations were described in case reports, such as *CYP11A1* (Rubtsov *et al.*,2009), *CYP17A1* (Sherbet *et al.*,2003) and *HSD17B3* (Lee *et al.*,2007).

Animal studies also provide some additional candidate genes, such as the genes encoding the cell-surface molecules ephrins and their receptors, EPH receptor B2 (EphB2) and Ephrin-B2 (Efnb2) (Lorenzo *et al.*,2003; Dravis *et al.*,2004). *EFNB2* has been suggested as the gene underlying genital malformations in patients with a 13q33-34 deletion (Garcia *et al.*,2006; Walczak-Sztulpa *et al.*,2008; Andresen *et al.*,2010).

In conclusion, many candidate genes have been suggested for hypospadias. Although some associations with hypospadias were found, none of these associations were replicated consistently, with the possible exception of *DGKK*. Therefore, we suggest that a genome-wide association study using individual genotyping of a large group of cases and controls is the way forward to generate more knowledge about the genetic factors underlying isolated hypospadias. In addition, the novel exome or even whole-genome sequencing techniques generate new opportunities. Currently, the high costs make these techniques only suitable for identification of causes of monogenic forms of hypospadias but with falling prices, the techniques may also be applied to large cohorts of patients with isolated hypospadias in the future.

As for environmental factors, development of the male external genitalia is dependent on the balance between androgens and estrogens. The fact that maternal exposure to synthetic estrogens can induce hypospadias in murine models (Kim *et al.*,2004) and that antiandrogens acting as inhibitors of steroid hormone synthesis or AR antagonists can induce male reproductive abnormalities in animal models (Gray *et al.*,2001) suggests that EDCs have the potential to induce hypospadias. However, because of considerable species differences and markedly different estrogen levels in humans compared to rodent pregnancy, it is debatable whether EDCs also induce hypospadias in humans. Phthalates inhibit

steroidogenesis in the foetal rat testis but this does not occur *in vitro* with human foetal Leydig cells (van Gelder *et al.*,2010). The question remains as to whether exposure levels in humans are high enough to exert an effect on the occurrence of hypospadias. Given that even exposures to high levels of exogenous hormones, such as in case of hormonal stimulation used to induce pregnancy and use of oral contraceptives while pregnant, do not show consistent associations with hypospadias, we suggest that exogenous hormones and EDCs may not be as important in the aetiology of hypospadias as has previously been assumed.

The consistent association of hypospadias with low birthweight, maternal hypertension and preeclampsia suggests that placental insufficiency may be a major risk factor for hypospadias, possibly through inadequate provision of hCG to the foetus. A role for endogenous hormones is suggested by free estradiol levels linked to high maternal BMI, primiparity and multiple pregnancies that appear to contribute to susceptibility to hypospadias.

In addition, maternal intrauterine DES exposure, use of anti-epileptic drugs, pre-existing diabetes, prolonged TTP and pregnancies resulting from ICSI have been associated with hypospadias in most studies. Other potential environmental risk factors were not, or not consistently, associated with hypospadias or studied too infrequently to draw conclusions.

In our opinion, the lack of replication of results for both genetic and environmental factors associated with hypospadias may be related to subtle isolated effects of factors that may have larger influences in combination with other factors (e.g. gene-gene or gene-environment interactions). While a different genetic background of a population may affect its vulnerability to an environmental exposure, different environmental exposures may influence the effect of a genotype. Therefore, we think that the challenges for future research in disentangling the pathogenesis of hypospadias mainly lie in studies focussing on gene-gene or gene-environment interactions.

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TABLES

TABLE I. Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Heterozygosity	Remarks	Reference
Indifferent stage of development									
WT1	**11p13**	35	-	different ethnicities	-				(Nordenskjöld *et al.*,1999)
		90	276	Chinese	N130N	• penoscrotal, micopenis	hetero		(Wang *et al.*,2004)
						• penile	hetero		
					A131T	• penile	hetero		
					S159S	• glandular, also has *BMP7* mutation	hetero		
WTAP	**6q25-q27**	37	20	controls are Caucasian, ethnicity cases ?	-				(Utsch *et al.*,2003)
SF1	**11q13**	60[a]	100	?	Q107X	• penoscrotal, bilateral cryptorchidism	hetero	• showed that variant impairs transcriptional activity	(Köhler *et al.*,2009)
					c.103-3C>A[b]	• scrotal, micropenis, bilateral cryptorchidism	hetero		
					E11X	• penoscrotal, micropenis, bilateral cryptorchidism	hetero	• showed that variant impairs transcriptional activity	
Early patterning stage of development									
BMP4	**14q22-q23**	90	190	Chinese	H207D	• penoscrotal, micropenis	hetero		(Chen *et al.*,2007)
					R223H	• penoscrotal	hetero		
					H251Y	• penile, micropenis	hetero		
BMP7	**20q13**	60[c]	96	different ethnicities	-				(Beleza-Meireles *et al.*,2007c)
		90	190	Chinese	R303C	• glandular, also has *WT1* mutation	hetero		(Chen *et al.*,2007)
					Q199Q	• penile	hetero		
					c.1465T>A[d]	• penoscrotal, micropenis	hetero		
					c.1567A>G[d]	• penile	hetero		
HOXA4	**7p15.2**	90	190	Chinese	G129C	• penoscrotal, micropenis	hetero		(Chen *et al.*,2007)
					S290C	• penile, bifid scrotum, cryptorchidism	hetero		
						• penile, micropenis, also has *SRD5A2* mutation	hetero		

TABLE I. (continued) Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Heterozygosity	Remarks	Reference
HOXB6	**17q21.3**	90	190	Chinese	P42T	• scrotal, micropenis, bifid scrotum, cryptorchidism, also has *SRD5A2* and *MID1* mutations	hetero	• mother heterozygous	(Chen *et al.*,2007)
					C123R	• penile	hetero		
HOXA13	**7p15.2**	37	20	controls are Caucasian, ethnicity cases ?	-				(Utsch *et al.*,2003)
FGF8	**10q24**	60 [c]	96	different ethnicities	c.590C>G[e]	• ?	homo	• Swedish patient	(Beleza-Meireles *et al.*,2007c)
FGF10	**5p13-p12**	60 [c]	96	different ethnicities	-				(Beleza-Meireles *et al.*,2007c)
FGFR2	**10q26**	60 [c]	96	different ethnicities	M186T[f]	• midpenile	hetero	• Swedish patient	(Beleza-Meireles *et al.*,2007c)
					c.2454C>T[e]	• ?	hetero	• Swedish patient	
Masculinisation stage of development									
SRY	**Y**	90	276	Chinese	-				(Wang *et al.*,2004)
SOX9	**17q23**	90	276	Chinese	-				(Wang *et al.*,2004)
AR	**Xq12**	21	90	?	V870A	• penoscrotal, bilateral cryptorchidism	hemi		(Hiort *et al.*,1994)
		9[g]	-	?	G566V	• perineal	hemi	• family history suggested familial component	(Alléra *et al.*,1995)
		40[h]	-	?	P546S	• distal penile shaft	hemi		(Sutherland *et al.*,1996)
		35	-	different ethnicities	F725V	• hypospadias and cryptorchidism, clinically diagnosed with PAIS based on sparse body hair, gynaecomastia and heredity for intersex malformations	hemi		(Nordenskjöld *et al.*,1999)
					S597T	• severe hypospadias, cryptorchidism, bifid scrotum	hemi		
		21	100	Japanese	-				(Muroya *et al.*,2001)

TABLE I. (continued) Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Heterozygosity	Remarks	Reference
AR	**Xq12**	90	276	Chinese	I664T	• glandular, gynecomastia	hemi		(Wang *et al.*,2004)
					R840H	• perineal, micropenis, bifid scrotum	hemi	• mother heterozygous, variant previously described in ambiguous genitalia patient and shown by others to reduce androgen-binding affinity and transcriptional activity	
					I842T	• scrotal, micropenis, bifid scrotum	hemi	• mother heterozygous	
					R855H	• perineal, micropenis, bifid scrotum	hemi	• mother heterozygous, uncle has same mutation and phenotype, variant previously described in 2 brothers with perineal hypospadias, bilateral cryptorchidism and micropenis	
					L859L	• penile	hemi		
		37[i]	-	different ethnicities	Q798E	• scrotal	hemi	• variant previously described in various genital defects and shown by others to affect AR transactivation function	(Thai *et al.*,2005)
		92	190	Iranian	-				(Radpour *et al.*,2007)
FKBP4	**12p13.33**	91	-	different ethnicities	-				(Beleza-Meireles *et al.*,2007a)
HSD3B2	**1p13.1**	90[g]	101	?	S213T	• scrotal, bilateral cryptorcidism	hetero	• mother and brother heterozygous, showed that variant reduces enzyme activity	(Codner *et al.*,2004)
					S284R	• midshaft	hetero	• de novo, showed that variant reduces enzyme activity	
					A238A	• midshaft	hetero		
					T259T	• proximal penile, micropenis and Wilms' tumour (no *WT1* mutation)	hetero	• de novo	

TABLE I. (continued) Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Heterozygosity	Remarks	Reference
HSD3B2	**1p13.1**	90[g]	101	?	T320T	• subcoronal	hetero	• father heterozygous, has bifid preputium and a wide meatus	(Codner *et al.*,2004)
HSD17B3	**9q22**	19[j]	-	different ethnicities	-				(Thai *et al.*,2005)
SRD5A2	**2p23**	35	-	different ethnicities	-				(Nordenskjöld *et al.*,1999)
		81[k]	100	different ethnicities	L113V H231R	• penoscrotal • scrotal	hetero hetero	• variant previously described in 5α-reductase deficiency	(Silver and Russell,1999)
		90	276	Chinese	R227Q	• penile, bifid scrotum, also has *HOXA4* mutation • scrotal, micropenis, bifid scrotum • glandular	homo homo hetero	• variant previously described in patient with scrotal hypospadias, bifid scrotum and micropenis and shown by others to inhibit NADPH binding, reduce testosterone binding, and reduce enzyme half-life	(Wang *et al.*,2004)
					R246Q	• scrotal, bifid scrotum, cryptorchidism	homo	• variant previously described in 2 patients with perineoscrotal hypospadias, micropenis and cryptorchidism and shown by others to reduce enzyme activity	
					Q6X	• scrotal, micropenis, bifid scrotum, cryptorchidism • scrotal, micropenis, bifid scrotum, cryptorchidism, also has G203S variant (also found in controls) and *HOXB6* and *MID1* mutation	homo hetero	• father heterozygous	
					L224H	• scrotal, micropenis, bifid scrotum, also has G203S variant	hetero	• father heterozygous, 2 brothers of patient have same genotype and phenotype as patient	
					656delT	• perineal, micropenis, bifid scrotum, cryptorchidism	hetero		

TABLE I. (continued) Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Heterozygosity	Remarks	Reference
SRD5A2	**2p23**	37[i]	-	different ethnicities	G196S	• scrotal	hetero	• mother heterozygous, variant previously described in homozygous form in 8 patients with scrotal hypospadias and micropenis and shown by ohers to partly disrupt NADPH binding	(Thai *et al.*,2005)
SRD5A1	**5p15**	10[l]	49	?	-				(Tria *et al.*,2004)
Other genes									
ESR1	**6q25.1**	60	94	different ethnicities	-				(Beleza-Meireles *et al.*,2006)
ESR2	**14q23.2**	60	94	different ethnicities	-				(Beleza-Meireles *et al.*,2006)
ATF3	**1q32.3**	93	96	different ethnicities	A90G	• moderate	?	• Swedish patient	(Beleza-Meireles *et al.*,2008)
					c.817C>T[d]	• moderate/severe	?	• Middle Eastern patient	
						• moderate/severe	?	• Swedish patient	
		41	30	?	L23M	• anterior	hetero		(Kalfa *et al.*,2008a)
MAMLD1	**Xq28**	166	460	different ethnicities	E124X	• penoscrotal, cryptorchidism, bifid scrotum	hemi	• Japanese patient, mother heterozygous, maternal half-brother has same mutation and similar phenotype	(Fukami *et al.*,2006)
					Q197X	• penoscrotal, micropenis, bifid scrotum	hemi	• Japanese patient	
					R653X	• penoscrotal, micropenis, cryptorchidism, bifid scrotum	hemi	• Japanese patient, mother heterozygous	
		41	30	different ethnicities	V432A[m]	• proximal penile	hemi		(Kalfa *et al.*,2008b)
					L121X	• proximal penile, cryptorchidism	hemi		
						• penoscrotal	hemi	• de novo	
					p.531ins3Q[n]	• coronal	hemi		
		99[o]	95	?	Q529K	• severe, bilateral cryptorchidism	hemi		(Chen *et al.*,2010)
					D686D	• ?	hemi		

TABLE I. (continued) Mutations found in studies screening candidate genes in groups of patients with hypospadias

Gene	Locus	N cases	N controls	Ethnicity	Mutation	Phenotype	Hetero-zygosity	Remarks	Reference
MID1	**Xp22**	114	95	?	E238X	• penoscrotal, hypertelorism	hemi	• mother heterozygous, brother has same mutation and phenotype, variant previously describeded in Opitz syndrome	(Zhang *et al.*,2011)
					K560R	• penoscrotal, hypertelorism	hemi		
INSL3	**19p13.2-p12**	94	270	Moroccan	-				(El Houate *et al.*,2007)
BNC2	**9p22.2**	48[p]	23	different ethnicities	A923V	• distal	hetero	• Caucasian patient	(Bhoj *et al.*,2011)
						• distal	hetero	• African-American patient	
					L414V	• distal	hetero	• Caucasian patient	
					P306A	• distal	hetero	• Caucasian patient	
					P579L	• distal	hetero	• Caucasian patient	
					E240G, R283G, & Q152R	• distal	hetero hetero hetero	• Caucasian patient	

All studies included in this table screened patients with hypospadias for mutations in specific genes. Most studies checked whether mutations were present in healthy controls. The table includes only exonic (including 3'-UTR and splice acceptor sites) mutations that were not found in healthy controls, were not previously reported polymorphisms, and were not described as a polymorphism by the authors of the article. Results from functional analyses, either performed by the study reporting the mutation or performed by earlier studies and referred to by the study reporting the mutation, are included in the table. Most studies included patients with different degrees of hypospadias or information about phenotype was not reported. Most studies excluded syndromal patients, but did not exclude patients with cryptorchidism, micropenis, bifid scrotum, or other associated anomalies, or information about associated anomalies was not reported. Most studies did not exclude patients with affected relatives or information about affected relatives was not reported. Family members carrying the same mutation were unaffected, unless indicated differently.

N, number; hetero, heterozygous; homo, homozygous; hemi, hemizygous; PAIS, partial androgen insensitivity syndrome; [a]only DSD (disorders of sex development) patients with severe penile to penoscrotal hypospadias included; [b]splice acceptor site; [c]only patients with at least one affected relative included; [d]3'-UTR; [e]synonymous variant, not mentioned in which amino acid; [f]variant is known as rs755793, but with allele frequency of 0% in Caucasians; [g]only patients with severe hypospadias included; [h]only patients without other genitourinary abnormalities included; [i]only patients with severe hypospadias or a familial form included; [j]only patients from families contributing most to a linkage peak in the vicinity of *HSD17B3* included; [k]patients with cryptorchidism, intersex condition, or endocrine abnormalities excluded; [l]only patients with elevated testosterone/DHT ratios without mutations in *AR* or *SRD5A2* included;

[m]variant was later found in 2 more patients and in 2 controls (Chen *et al.*,2010); [n]variant was later found in 3 more patients and in 1 control (Chen *et al.*,2010); [o]only sporadic patients included; [p]only patients with distal hypospadias included.

TABLE II. Genetic association results for hypospadias

Gene	Locus	SNP	N cases	N controls	Controls	Ethnicity	Genotypes / alleles associated with increased risk ($P < 0.05$)	Reference
Early patterning stage of development								
FGF8	**10q24**	**rs3218238 or rs3218233**[a]	60[b]	96	healthy voluntary blood donors	different ethnicities	A allele[c]	(Beleza-Meireles *et al.*,2007c)[d]
FGFR2	**10q26**	**c.382+52→G** **c.550+27T>C** **c.727+180T>G**	60[b]	96	healthy voluntary blood donors	different ethnicities	G allele of c.382+52→G[e] C / T allele of c.550+27T>C[e] G allele of c.727+180T>G[c]	(Beleza-Meireles *et al.*,2007c)[d]
Masculinisation stage of development								
AR	**Xq12**	**CAG repeat**	78[f]	425	anonymous females	?	longer repeat	(Lim *et al.*,2000)
			21	100	boys with short stature and normal external genitalia and fertile males	Japanese	no association	(Muroya *et al.*,2001)
			51	210	males from military service, no history of hypospadias or cryptorchidism	cases are Caucasian, controls have Swedish mothers	no association	(Aschim *et al.*,2004b)
			92	190	fertile males	Iranian	no association	(Radpour *et al.*,2007)
		GGN repeat	51	210	males from military service, no history of hypospadias or cryptorchidism	cases are Caucasian, controls have Swedish mothers	longer repeat[g]	(Aschim *et al.*,2004b)
			92	190	fertile males	Iranian	longer repeat[g]	(Radpour *et al.*,2007)
FKBP4	**12p13.33**	**rs1062478** **rs3021522**	333	380	voluntary blood donors	different ethnicities	no association	(Beleza-Meireles *et al.*,2007a)
HSD17B3	**9q22**	**rs4743709** **rs2066476** **rs2066474** **rs2066480** **rs2066479**	89[h]	291	male newborns without malformations	Japanese	A allele of rs2066479 AA genotype of rs2066479	(Sata *et al.*,2010)
SRD5A2	**2p23**	**rs9282858**	81[i]	100+	normal controls	different ethnicities	T allele	(Silver and Russell,1999)[j]

TABLE II. (continued) Genetic association results for hypospadias

Gene	Locus	SNP	N cases	N controls	Controls	Ethnicity	Genotypes / alleles associated with increased risk ($P < 0.05$)	Reference
SRD5A2	**2p23**	**rs523349**	90	87	normal males	Chinese	G allele CG and GG genotypes	(Wang *et al.*,2004)
			158	96	unaffected persons	cases have different ethnicities, controls are Caucasian	G allele CG and GG genotypes	(Thai *et al.*,2005)
			89[h]	281	male newborns without malformations	Japanese	CG genotype[k]	(Sata *et al.*,2010)
			620	596	unaffected males	Caucasian	no association	(van der Zanden *et al.*,2010b)[l]
Other genes								
ESR1	**6q25.1**	**rs6932902[m]**	43	135	boys with short stature and normal external genitalia and fertile males	Japanese	A allele AA genotype	(Watanabe *et al.*,2007)
			620	596	unaffected males	Caucasian	A allele	(van der Zanden *et al.*,2010b)[l]
		TA repeat rs1801132	90	94	voluntary blood donors	different ethnicities	no association	(Beleza-Meireles *et al.*,2006)
		rs2234693 rs9340799	59[h]	286	boys without malformations	Japanese	A allele of rs9340799	(Ban *et al.*,2008)
ESR2	**14q23.2**	**CA repeat**	90	94	voluntary blood donors	different ethnicities	longer repeat	(Beleza-Meireles *et al.*,2006)
			354	380	healthy voluntary blood donors	different ethnicities	longer repeat	(Beleza-Meireles *et al.*,2007b)
		rs1887994 rs1256040 rs1256062 rs10483774 rs1271572	354	380	healthy voluntary blood donors	different ethnicities	G allele of rs10483774 AG genotype of rs10483774	(Beleza-Meireles *et al.*,2007b)
		rs944050	90	94	voluntary blood donors	different ethnicities	AG genotype[n]	(Beleza-Meireles *et al.*,2006)
			59[h]	286	boys without malformations	Japanese	AG genotype[o]	(Ban *et al.*,2008)

TABLE II. (continued) Genetic association results for hypospadias

Gene	Locus	SNP	N cases	N controls	Controls	Ethnicity	Genotypes / alleles associated with increased risk ($P < 0.05$)	Reference
ESR2	**14q23.2**	**rs2987983**	354	380	healthy voluntary blood donors	different ethnicities	G allele GG genotype	(Beleza-Meireles *et al.*,2007b)
			620	596	unaffected males	Caucasian	AG genotype[o]	(van der Zanden *et al.*,2010b)[l]
		rs1256049 **rs4986938**	51	186	control males from military service without genital anomalies and with sperm concentrations $>5\times10^6$ spermatozoa/ml	cases are Caucasian, controls have Swedish parents	no association	(Aschim *et al.*,2005)
ATF3	**1q32.3**	**rs11119982**	330	380	healthy voluntary blood donors	different ethnicities	C allele CC genotype	(Beleza-Meireles *et al.*,2008)
			620	596	unaffected males	Caucasian	T allele TT and CT genotypes	(van der Zanden *et al.*,2010b)[l]
		rs2137424 **rs3125289** **rs1877474** **rs10735510** **rs9429889** **rs12070345** **rs10475**	330	380	healthy voluntary blood donors	different ethnicities	T allele of rs3125289 TT genotype of rs3125289 T allele of rs1877474 TT genotype of rs1877474 strongest association for combination of risk alleles: rs3125289 (T), rs1877474 (T) and rs11119982 (C)	(Beleza-Meireles *et al.*,2008)
MAMLD1	**Xq28**	**rs61740566**	370	380	healthy voluntary blood donors	?	no association	(Chen *et al.*,2010)
		rs41313406 **rs2073043**	370	418	male healthy voluntary blood donors	?	T allele of rs41313406 G allele of rs2073043	(Chen *et al.*,2010)
DGKK	**Xp11.22**	**rs1934179** **rs7063116**	436[p] 133[p] 266[p]	449 133 402	healthy control males mothers[q] male healthy voluntary blood donors	Caucasian	A allele of rs1934179 A allele of rs7063116	(van der Zanden *et al.*,2010a)[r]
MID1	**Xp22**	**rs16986145**	366	405	male controls	?	A allele[s]	(Zhang *et al.*,2011)
CYP1A1 ***GSTM1*** ***GSTT1***	**15q24.1** **1p13.3** **22q11.23**	**?** **gene deletion** **gene deletion**	31[t]	64	mothers of boys without any malformation	Japanese	heterozygous *CYP1A1* genotype[o]	(Kurahashi *et al.*,2005)
CYP1A1 ***GSTM1*** ***GSTT1***	**15q24.1** **1p13.3** **22q11.23**	**?** **gene deletion** **gene deletion**	80	120	age-matched boys	?	concomitant deletion of *GSTM1* and *GSTT1*	(Yadav *et al.*,2011)

Most studies were association studies with a case-control design. Most studies included patients with different degrees of hypospadias. Most studies excluded syndromal patients, but did not exclude patients with cryptorchidism, micropenis, bifid scrotum, or other associated anomalies or information about associated anomalies was not reported. Most studies did not exclude patients with affected relatives or information about affected relatives was not reported. Deviations from these statements are included in the specified footnotes.

N, number; [a]the SNP reported in the text was different from the SNP reported in the table; [b]all patients have at least one affected relative; [c]this SNP was found in heterozygous form in 3 patients, while it was not found in controls; [d]this was not an association study, but a study screening *FGF8* and *FGFR2* for mutations; [e]these SNPs were found in heterozygous form in 1 patient, while they were not found in controls. For c.550+27T>C it is not clear whether T or C is the risk allele because the SNP reported in the text was different from the SNP reported in the table (c.550+27T>C and c.550+27C>T); [f]undermasculinized patients, most of them with perineoscrotal openings and unfused or partially fused scrotum; [g]only penile patients have longer repeats; [h]patients with affected family members excluded; [i]patients with cryptorchidism, intersex condition, or endocrine abnormalities excluded; [j]this was not an association study, but a study screening *SRD5A*2 for mutations. This SNP was found in homozygous form in 2 patients and in heterozygous form in 3 patients, while it was not found in controls. In another study, this SNP was found in 1 out of 37 patients, but as that study did not genotype controls to perform an association analysis, it was not included in the table (Thai *et al.*,2005); [k]only associated with severe hypospadias; [l]this was an association study with a case-parent triad design analyzed using the transmission disequilibrium test; [m]SNP tagged the 'AGATA' haplotype of rs926779, rs3020364, rs6932902, rs3020371 and rs3020375; [n]all six patients with this genotype had affected family members, and the SNP was inherited from the affected line twice; [o]associated with decreased risk; [p]only patients with anterior and middle hypospadias included; [q]this part of the study was an association study with a case-parent triad design analyzed using the transmission disequilibrium test, but as this in an X-chromosomal SNP, only mothers were taken into account; [r]this was a genome wide association study with a case-control design, suggesting more associations with hypospadias than reported in this table; [s]four cases were familial. Two affected relatives carried the variant and one did not. Five of the nine cases with the variant had at least one parent born in North Africa, where the A allele is more prevalent; [t]mothers of patients with hypospadias.

Table III. Clinical, behavioural, occupational and environmental factors investigated for their association with hypospadias in more than one study.

FACTORS FREQUENTLY INVESTIGATED	
Factors with consistent results in all studies	
Factors consistently associated with hypospadias	***Factors consistently not associated with hypospadias***
Low birthweight / being small for gestational age	Gestational diabetes
Placental insufficiency	Maternal alcohol consumption
Maternal hypertension	
Preeclampsia	
Maternal intrauterine diethylstilbestrol exposure	
Factors with consistent results in most studies	
Factors associated with hypospadias in most studies	***Factors not associated with hypospadias in most studies***
Use of ICSI	Use of oral contraceptives during pregnancy
Prolonged time-to-pregnancy	Use of IVF
High maternal BMI	Use of hormonal stimulation to induce pregnancy
Primiparity	Maternal medication use:
Multiple pregnancy	Loratadine
Pre-existing maternal diabetes	Maternal folate supplementation
Maternal medication use:	Paternal age
Anti-epileptic drugs	Maternal smoking
	Maternal exposure to water disinfection by-products
Factors showing inconsistent results	
Preterm delivery	Maternal occupational exposure to:
Maternal iron supplementation	Endocrine disruptors
Maternal age	Heavy metals
Maternal vegetarian diet	Phthalates
Maternal fish consumption	Maternal serum levels of polychlorinated biphenyls
Maternal and paternal exposure to pesticides	Seasonal trend
FACTORS NOT FREQUENTLY INVESTIGATED	
Factors that seem to be associated with hypospadias	***Factors that do not seem to be associated with hypospadias***
Paternal subfertility	Amount of weight gain during pregnancy
Absence of nausea and vomiting in early pregnancy	Maternal medication use:
Bleeding during pregnancy	Corticosteroids
Complications during labour	Antibiotics
Maternal medication use:	Most maternal and paternal occupational exposures
Antihypertensive drugs	
Father being a vehicle mechanic or manufacturer	
Factors showing inconsistent results	
Early age at menarche	Use of progestogens / progestins for threatened abortion
Maternal thyroid disease	Paternal occupational exposure to heavy metals
Fever during first trimester of pregnancy	Living in rural or urban areas

FIGURES

Figure I Hypospadias subgroups

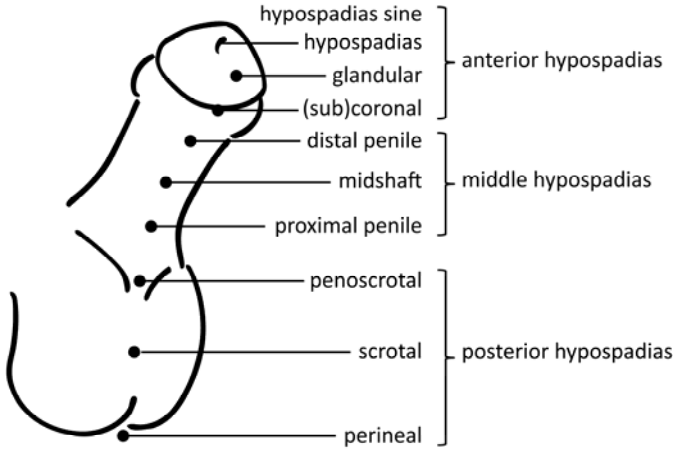


Figure II Simple schematic drawing of the normal embryology of the human male external genitalia, which is disturbed in case of hypospadias development.

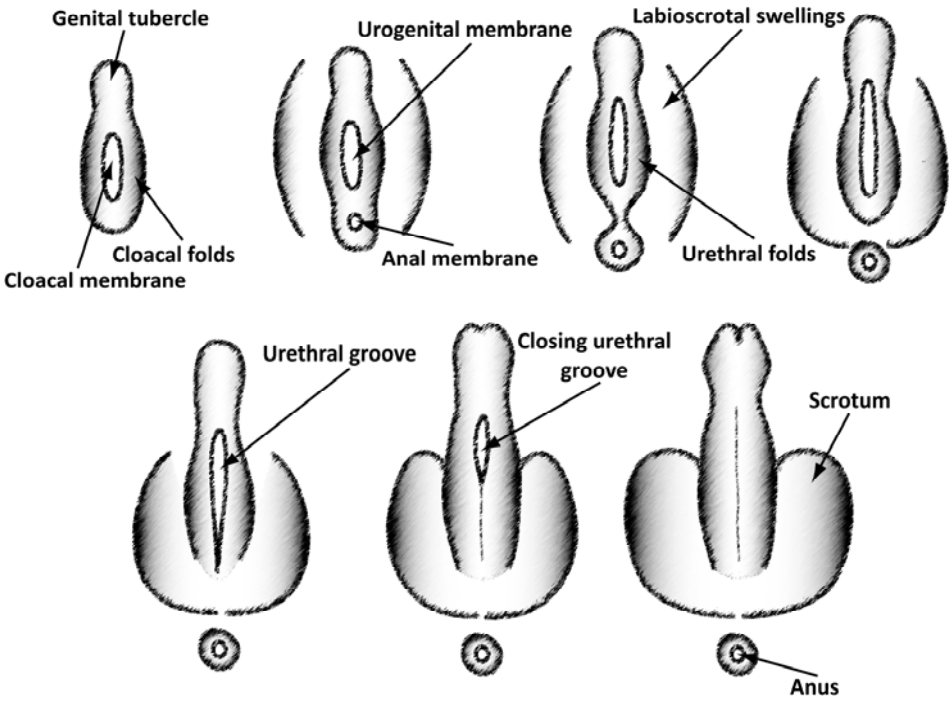
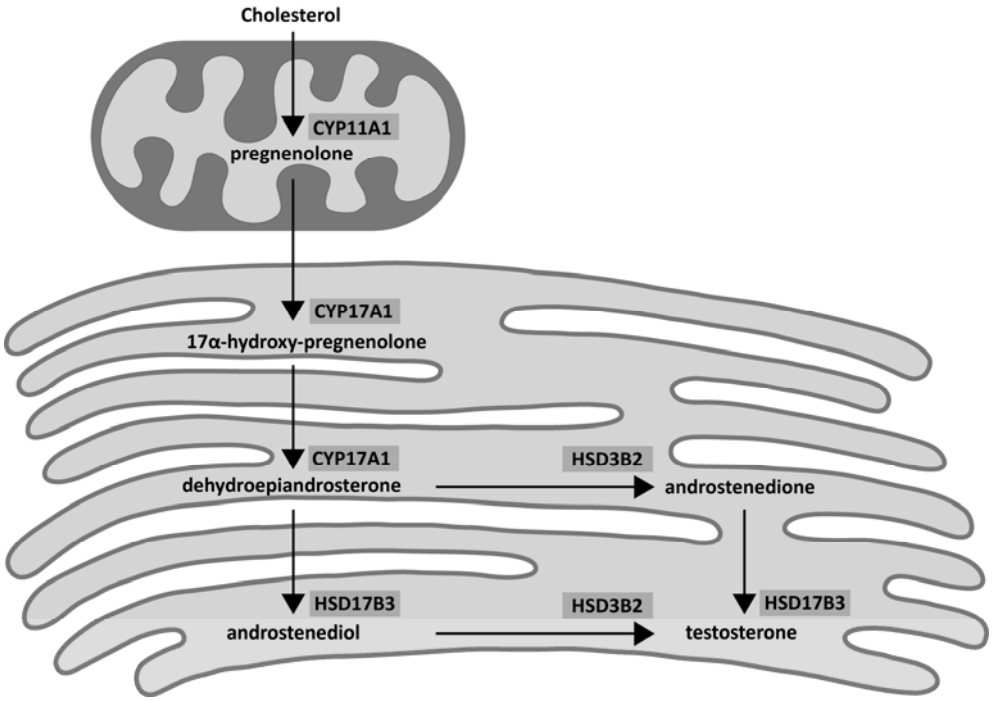


Figure III Steroidogenesis in the mitochondrium (top) and smooth endoplasmic reticulum (bottom) of the foetal Leydig cell



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