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Review article

Environmental, parental and gestational factors that influence the occurrence of hypospadias in male patients

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Summary

Objective
Hypospadias is a congenital defect, which affects normal development of the male urogenital external tract. In this malformation, the urethral orifice of the penis is positioned ventrally, thus interfering with normal urination and creating, in some adults, problems during sexual intercourse. Heritability of hypospadias has been shown in some reports, and the abnormality has been associated with the presence of mutations in one of the genes involved in urogenital development. However, even for patients who were born in families with a higher incidence rate of this defect, no evident genetic alteration could be identified in known genes, indicating that the list of loci involved is still incomplete. To further complicate matters, recent reports also underline that epigenetic changes, without any identifiable gene sequence mutation, may be involved in gene function impairment. Therefore, the inheritance of most hypospadias cases is not evident, suggesting that the genetic background is not the only cause of this malformation; indeed, the majority of hypospadias cases are classified as sporadic and idiopathic.

Materials and methods
Evidence has accumulated highlighting the role of the environment and of its relationships with the genome in the etiology of this abnormality. In particular, the interaction between some chemicals, which are able to mimic endogenous molecules such as sexual hormones — for this reason called endocrine disrupting compounds (EDC) — and specific receptors has been extensively investigated during the pregnancy. Additionally, several articles have shown that parental and gestational factors play a significant role too. Indeed, physiological alterations, such as body weight of the mother and/or of the newborn, mother's diabetes, impaired father fertility, and exposure of one parent to job-related pollutants, show in many cases a direct correlation with hypospadias incidence. The overall prevalence of this condition has been studied in many countries, suggesting that at least in some periods and/or in specific populations there are detectable fluctuations, probably mirroring the different natural environments. However, many articles present data that do not agree with these findings and, consequently, most causes of hypospadias are still highly debated.

Results
In this review, we summarize the developmental steps involved in urogenital tract formation, with a particular emphasis on the genes that most frequently are associated with this condition, or that are subject to environmental stress, or that may be the targets of hormone-like, exogenous molecules. Then, we make an overview of the identified factors able to impair the function of important genes, even in the absence of their mutations, including those for which contradictory reports have been published. Finally, we propose an explanation of sporadic cases of hypospadias that reconciles these contradictions and suggest some steps for moving forward in the research focused on this condition.

Conclusion
We hypothesize that most patients develop hypospadias because of gene–environment interactions acting on polymorphic genes that, in the absence of environmental stimuli, would otherwise cause no developmental anomaly during urogenital development.

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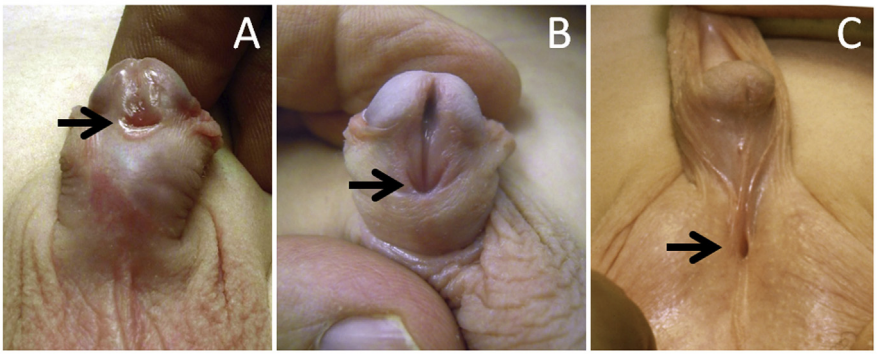

Figure Three cases of boys affected by hypospadias; the arrows indicate the position of the urethral meatus in the distal (A), midshaft (B) and proximal (C) positions.

Introduction

Hypospadias is a relatively common malformation, typically affecting 1 in 300 live male births [1], of the male external urinary tract, which is characterized by defective development of the penile ventral surface. It is defined as a 'hypoplasia of the tissues forming the ventral aspect of the penis beyond the division of the corpus spongiosum' [1]. The morphological characteristics of this condition in hypospadiac children are: (i) a ventral opening of the urethral meatus, (ii) a ventral downward curvature of the penis (also called chordee) and (iii) a ventrally deficient, hooded foreskin [1]. Notably, these three features are not always present at the same time [1]. The urethral meatus may be located in several different positions between the glans and the perineum, thus allowing the classification of hypospadias as distal, medial and proximal. A further classification is derived by intermediate positions of the meatus, as illustrated (Fig. 1) [2].

The clinical significance of hypospadias is related to psychological, aesthetical and functional aspects: the abnormal position of the meatus interferes with the normal flux of urine, and the possible presence of a shaft curvature in adults may cause painful erections and difficult penetration during sexual intercourse, caused by chordee and penile hypoplasia, especially in proximal forms [3]. Distal hypospadias impacts patients' lives to a significantly lesser extent.

The etiology of hypospadias is currently an issue in most situations. In a few selected cases of families with a higher incidence rate it is possible to establish a genetic origin of this condition, and several reports are available that this is a multifactorial, non-Mendelian defect [2]. Some cases of hypospadias are associated with syndromes, such as the Smith—Lemli—Opitz syndrome (a defect in cholesterol synthesis that involves mutations of the *DHCR7* gene), Robinow syndrome (that depends on *ROR2*, a gene coding for a tyrosine-protein kinase transmembrane receptor), Klinefelter syndrome (involving the *SRY* gene and affecting males with an XXY karyotype, even in the form of mosaics), Denys-Drash and Frasier syndromes (both characterized by *WT1* mutations) or other complex conditions, such as disorders of sex development (DSD) [1,2]. In many of these cases it was possible to identify some of the genes involved, and consequently to link those genes also to the impairment of urogenital development and its molecular mechanisms. However, the causes of hypospadias remain mostly unknown, as it is estimated that more than 90% of cases are idiopathic [4]. Indeed, there is growing evidence to suggest that non-genetic factors might be evoked to explain at least some of the reported clinical cases.

In this review we focus on the factors that can alter the normal development of the external genitourinary tract even in the absence of specific DNA anomalies and highlight the target genes/proteins whose function is impaired.

Development of the normal external genitourinary tract in male children

There are three main molecular pathways for male external genitalia formation: (i) androgen-independent, (ii) androgen-dependent and (iii) dependent on endocrine and environmental factors [4]. The last point may involve the first two, and it also has genetic and epigenetic ground.

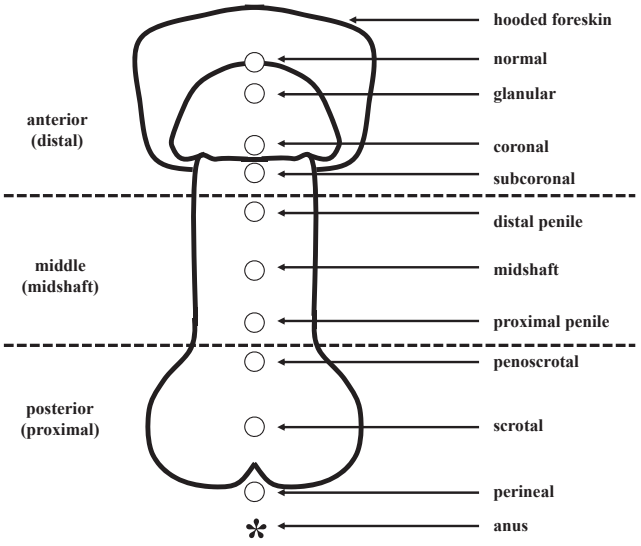

Figure 1 Classification of hypospadias based on the approximate (left side) and precise (right side) localization of the urethral meatus.

These pathways show complex interaction patterns and final normal development relies on all of them.

The first stages of human embryogenesis are common in both males and females [2,5]. During the first six weeks the genital tubercle is formed and the precursors of the gonads (genital ridges) develop. At the seventh week of gestation, the urorectal septum fuses with the cloacal membrane; as a consequence, the cloaca is divided into primitive urogenital sinus and rectum, while the cloacal membrane forms the urogenital and anal membranes. Primordial germ cells start their migration at the fifth week, reaching the hindgut-allantois into the genital ridges. At week six, sex cords are formed; at week seven, Mullerian and Wolffian ducts are created. The early patterning of the genital tubercle is androgen-independent and relies on the function of fibroblast growth factor (Fgf) proteins, wingless-type MMTV integration site family member 5A (Wnt5a) and bone morphogenetic proteins (Bmp). Other genes involved in early genital tubercle patterning are *sonic hedgehog* (*Shh*) and *homeobox A13* (*Hoxa13*), which control the expression of Fgf8 and Bmp7 [2,5]. *Shh* plays a crucial role during normal genital growth as it is expressed inside the urethral plate epithelium and is required for patterning and cell survival in the developing genitalia. Indeed, studies performed on mice lacking the *Shh* function show the absence of external genitalia. In this model, genital swellings are initiated, but their outgrowth is not maintained over time. The absence of Shh signaling triggers the downregulation of several genes, including *Fgf8*, *Bmp2*, *Bmp4*, *Fgf10*, and *Wnt5a* [2,5]; besides, genitalia show an increased level of apoptosis [2]. These results identify the urethral epithelium as a signaling center of the genital tubercle. Overall, Shh seems to have a dual function, inducing both genital tubercle outgrowth and differentiation. Other genes involved in this process are *patched1* (*Ptch1*), *msh homeobox 1* (*Msx1*), *homeobox D13* (*Hoxd13*) [2], *GATA-type zinc finger protein 4* (*GATA4*), *friend of GATA2* (*FOG2*) [5], *mastermind-like domain containing 1* (*MAMLD1*, also known as *CXorf6*, which contains a splicing factor 1 (Sf1) target sequence) [2], and *diacylglycerol kinase kappa* (*DGKK*), which was identified as a major risk factor for hypospadias [2].

Gonadal differentiation takes place between the 10th and 12th week of pregnancy. This process is controlled by the expression of the *sex-determining region Y* (*SRY*) gene [6], which starts a cascade of gene interactions involving *SRY-box 9* (*SOX9*) [7]. *SRY* causes the differentiation of both Leydig cells which produce testosterone, and Sertoli cells which secrete the anti-Mullerian hormone (AMH) [2]. AMH secretion is controlled by Sf1 [8] and induces regression of the Mullerian ducts. The human chorionic gonadotropin (HCG) controls the Leydig cell growth and promotes fetal steroidogenesis from cholesterol [2]; in the presence of fetal testicular androgens, the Wolffian ducts persist and continue their development to form epididymis, vas deferens and seminal vesicles. The testosterone produced by the Leydig cells is converted into dihydrotestosterone (DHT) by steroid-5-alpha-reductase (SRD5A) and promotes the formation of the internal reproductive structure, while DHT allows the formation of the external genitalia [7]. Both testosterone and DHT effects are mediated by their binding to the androgen receptor (AR). In the male genital tissue estrogen receptors (ESR) are also present, which underlines the importance of a correct balance between androgens and estrogens in normal subjects [2]. Between the 12th and 14th weeks, the genital tubercle develops into the penis while the labioscrotal swellings fuse to form the scrotum [7,9]. Urogenital folds close starting from the proximal region to form the penile urethra [7,9,10]; failure to fuse penile urethral folds between the 11th and 16th weeks causes hypospadias. The formation of the glandular part of the urethra is still a debated topic. Some authors hypothesize that it is formed after a secondary fusion and luminization [10]; others believe that it is formed by the fusion of the urogenital folds via cellular migration, so that the disruption of these steps leads to hypospadias, because of embryological arrest induced by the absence of the required stimulus for male phenotypic development at the appropriate time [9]. Other authors postulate that the glandular part of the urethra is developed from different folds, or from the invagination of surface cells or even by luminization of the urethral plate [7]. Consequently, the developmental origin of hypospadias is debated also. Posterior hypospadias is characterized by unfused labioscrotal swellings and glans, and distal dysplasia of both urethral plate and corpora. Middle hypospadias shows a steady urethral plate and a failure of the tubularization of the glans, plus an altered penile skin formation. In anterior hypospadias, there are short tubular septate tracts and missing closure of the skin in the midline. The mildest form is called hypospadias sine hypospadias, whose main feature is the absent fusion between the preputial skin on the ventral side and the dorsal hooded foreskin; chordee may also be present [2].

Genes involved in any of the steps of male genitalia formation may cause hypospadias, if they are malfunctioning. The non-differentiated gonad of the early embryo relies heavily on the function of *WT1* and *Sf1* genes for development of the urogenital apparatus; indeed, in some severe forms of hypospadias, mutations in these two genes have been identified [2]. Genes participating in the next phase, for example genital tubercle development, were similarly identified during genetic screenings of affected patients; these genes include, among others, members of the Bmp family of proteins, various homeobox genes such as *HOXA4* and *HOXB6*, Fgf family members and their receptors [2]. Of course, genes involved in the masculinization phases are involved as well, as they are responsible for control of the above mentioned steps of genital tubercle tubularization and closure. *SRY* alterations, especially of its copy number (as in Klinefelter syndrome) were identified as risk factors for hypospadias [11]. Similarly, the involvement of mutations in the *AR* gene, as well as of the genes involved in testosterone and DHT metabolism (*SRD5A2*, *HSD3B2*), has been reported [2]. Estrogen receptors *ESR1* and *ESR2* also play a role in this abnormality, probably because of the importance of the androgen/estrogen balance during male genitalia formation. Notably, other genes responsive to this balance (*ATF3*, *CTGF*, *CYR61*) are involved in hypospadias as well [2].

Environmental factors related to hypospadias occurrence

One of the clues suggesting an effect of the environment on this condition is the apparently increasing hypospadias

prevalence in the past years. This fact has been reported by numerous authors and in many countries, including large areas of Europe, the USA, Japan, China and Australia [12,13]. A deeper analysis, however, reveals that this trend is not supported by the literature [14] and was detected only for relatively limited time periods. Moreover, some later studies either contradicted previous findings or showed that there was a different diagnostic approach over time, which significantly impacted on data analysis. In other cases it was possible to reveal the inaccuracy of medical registers, which resulted in under-evaluation of this problem. Despite these considerations raising doubts on the real effect of pollutants on hypospadias causation, the study over time of its prevalence is still considered important to unveil the possible role and long-term effects of chemicals.

Scientific evidence shows that the prenatal age is the most vulnerable phase of the genitourinary tract development, and that gene function changes induced by pollutants and chemicals may result in morphological anomalies. Results obtained so far are still largely inconclusive because of (i) the low number of patients examined, (ii) the few types of molecules considered (notably, some chemically related substances may have opposite effects, being inhibitors of antagonistic hormone pathways), (iii) the missing evaluation of the individual metabolic response to any given compound and its metabolites and (iv) the unsatisfactory analysis of non-occupational sources of pollutants [12]. Most of these molecules (i) act during the androgen-dependent phase, as they mimic the structure of these hormones, (ii) may operate in a dose-dependent manner and (iii) can be agonists or antagonists at the receptor and post-receptor level. These chemical compounds can interfere also with synthesis, transport and metabolism of endogenous hormones and, for this reason, are called xenoestrogens or endocrine disrupting compounds (EDC) [15]. Pollutants may enter the body in several ways including ingestion, inhalation and adsorption [13,16—18]. Polychlorinated compounds may be rapidly adsorbed by the gastrointestinal tract and accumulate in the liver and the adipose tissue; these substances may also reach the placenta [13]. Furthermore, most of them are lipophilic and may remain in the body fat for many years; they can also be retrieved from the breast milk and the amniotic fluid [13]. For example, phthalates are known anti-androgenic substances affecting the developing male reproductive system by decreasing testosterone biosynthesis. These chemicals are detectable inside the plasma of mothers [19,20] and are positively related to waist circumference, total fat mass and trunk fat mass in women [21], which indicates that relatively large amounts of EDCs can be stored in the human body in variable quantities, eventually affecting the health of newborns and mimicking a chronic exposure.

Some reports published in the 1990s show an increased risk of genital malformations for greenhouse workers and farmers [16—18], hairdressers [22] and military personnel [23]. So far, data about the association between hypospadias and (i) tractor spraying equipment, (ii) grain, (iii) pesticides and (iv) insect repellents are contradictory, with only some reports supporting a positive correlation [24]. This correlation may be explained by recalling that most of these chemicals interact with hormone receptors but with a higher dissociation constant compared with their natural counterparts [25]. This effect may be strong in the presence of high single pollutant doses, but also in the case of multiple low-dose pollutants. Studies performed on rats [26,27] demonstrate that mixtures of low-concentration EDCs have additive effects and are able to impair normal fetal tissue formation. Additionally, gene polymorphisms seem to play a role too; an example that needs further validation comes from the gene *steroid-5-alpha-reductase, alpha polypeptide 2* (*SRD5A2*), whose product converts testosterone into dihydrotestosterone, which is required for normal development of the male external genitalia [28]. Also, allelic variations of *ESR2* and *AMP-dependent transcription factor 3* (*ATF3*) [2] are strongly associated with hypospadias because of their responsiveness to estrogens or estrogen-like compounds. Other examples of gene polymorphisms associated with hypospadias come from *FGF8*, *FGFR2* and *MAMLD1* [2]. As many pollutants are metabolized by detoxifying enzymes, also polymorphisms of their encoding genes, which are able to interfere with their catalytic efficiency, may play a noteworthy role in the urogenital tissue formation. Indeed, some alleles of *cytochrome P450, family 1, member A1* (*CYP1A1*) are protective against hypospadias [2]. Therefore, it is likely that in everyday life each chemical is not sufficient per se to induce this condition, but a cocktail of them (as happens in agricultural use) has the potential to cause abnormalities. Notably, not only these molecules, but also their metabolites, may alter the normal function of endogenous receptors, and in some cases these derived compounds might have stronger effects because of higher affinity, longer persistence in the human body, or both [29]. Chemicals identified as toxic in the urogenital tract include dichlorodiphenyltrichloroethane (DDT), lindane, atrazine, furans, phytoestrogens, mycoestrogens, epichlorohydrin, ethylene dibromide, kepone and dioxin [13]; many of them also show anti-androgenic effects through their metabolites [30]. DDT and its metabolites have estrogenic effects in males because they block androgen receptors. Linuron, a urea-based pesticide, is an anti-androgen and reduces testosterone production by acting on expression of the luteinizing hormone (LH) receptor. Dioxin suppresses the steroidogenic acute regulatory protein (StAR) and cytochrome P450, 17 (CYP17), probably by lowering the LH secretion. Interestingly, a vegetarian diet in pregnant women, who were also adding iron to their food, was identified as a cause of hypospadias [31]. The authors suggest that this may be because of greater exposure to phytoestrogens of these subjects compared with omnivore mothers, either with or without iron supplement; although it is also possible that this higher incidence depends on the pesticides and/or herbicides used on the farms. Giordano and co-workers found that consumption of pesticide-contaminated food by mothers increases the probability that their children have genital defects, including hypospadias and cryptorchidism [32]. Notably, however, even iron supplements alone in non-vegetarian women have been suggested as a possible risk factor [24]. A similar conclusion can be drawn for frequent consumption of fish, because they are potential vehicles of lipophilic contaminants of the food chain [19], but data about this issue are not conclusive. Living in proximity to hazardous waste-disposal sites has been linked to this condition, but the risk was described as minor, at least in some cases [33].

Hypospadias might be one of the consequences of reduced fetal growth [24,34], a typical characteristic of babies born from smoking mothers [35], although this issue is still debated [34]. Indeed, several bibliographic sources report a positive correlation between smoking habits and hypospadias occurrence [22,24]. Nevertheless, some articles highlight a negative association between smoking and this condition [36]. While the positive association with smoking may be caused by some chemicals in tobacco smoke, less clear is the negative correlation. Some authors explain this outcome by invoking the anti-estrogenic effects of tobacco smoke that is dependent on the 2-hydroxylation pathway of estrogen metabolism [37].

Hypospadias may also be a consequence of occupational exposure of the parents. The anogenital distance, a recognized metric of the effects of androgenic hormones, decreases in some hypospadias patients; several studies report a shorter distance in cases of prenatal exposure to phthalates [22]. A similar effect has been attributed to bisphenol A, a weak estrogenic compound present in plastic [38]. Sons of mothers working in the leather industry [39] seem to have a higher prevalence of hypospadias; some evidence exists that hairspray also may play a role in this malformation [22]. Interestingly, sons of mothers serving in the Gulf War [23] show an increase in hypospadias prevalence, although the cause of this abnormality has not been identified. As for the fathers, risk factors were identified in jobs such as vehicle mechanics or manufacturers [40], police officers or fire fighters [40], and men exposed to dust produced by grinding metals [41], even though the real risks often seem to be modest. Interestingly, however, paternal involvement raises the chances that an epigenetic mechanism also is involved in this condition. Indeed, a recent report [42] illustrates that in foreskin samples of hypospadiac children, the *AR* methylation is higher than in control samples, independently of the number of CAG repeats inside exon 1; this would result in a lower expression of this gene. This DNA modification is probably mediated by the DNA methyltransferase DNMT3A. A later report [43] on genome-wide DNA methylation profiling of CpG islands in hypospadiac patients demonstrated that several genes have an altered methylation pattern compared with controls, and allowed identification of new, candidate sequences involved in this malformation.

Diethylstilbestrol (DES) is an anti-abortive pharmaceutical that is able to bind estrogen receptors (ESR) alpha and beta. Recently, evidence has been found that ESR alpha mediates the detrimental actions of neonatal DES exposure in the murine reproductive tract, with an increased hypospadias incidence in exposed mothers [24]. Remarkably, this anomaly is present also in the second generation, thus suggesting a transgenerational effect [44], and it is known that DES interferes with histone methylation [45]. An epigenetic mechanism via histone methylation has been suggested also for regulation of the *enhancer of Zeste homolog 2* (*EZH2*) on ESR-mediated signaling [46].

Other factors that influence the development of hypospadias

Assisted reproductive technologies, that is in vitro fertilization (IVF), make extensive use of hormone-mediated stimulation. Data are still contradictory, but some studies support the hypothesis that intracytoplasmatic sperm injection (ICSI), but not standard procedures, increases risk of hypospadias [44,47,48].

In some cases, paternal sub-fertility with a specific genetic background of fathers has been invoked [24,47,48]. According to some recent works, IVF may also act epigenetically by altering the methylation pattern of the *AR* gene and inducing its abnormal expression. Endogenously altered hormone levels able to cause hypospadias were reported also in women with high body mass index (BMI) (BMI $\geq$ 25 kg/m^2) and those who are severely overweight/obese (BMI above 29 kg/m^2) [2]. Primiparous obese mothers older than 35 years show an increased risk of delivering hypospadiac sons [49]. It is possible that obesity interferes with maternal free circulating estrogens by altering the binding to receptors of these sex hormones [50]; however, it is also possible that greater fatty tissue masses may store larger quantities of chemicals, which interfere with fetal development, as explained before. Nevertheless, being underweight also has been reported as a possible cause of hypospadias [51]. Instead, early age at menarche, first or twin/triplet pregnancy, gestational diabetes, fever episodes, viral infections and influenza in the first trimester of pregnancy [31], parental age, and paternal low sperm motility [48] could not be associated with an increased risk [2]. A debated topic in the 1970s was the possible seasonal variations of hypospadias. According to those studies, climate, temperature or even daylight hours would be associated with an increased risk; however, more recent investigations did not find any indication supporting this hypothesis [2]. Finally, the association between low birth weight and hypospadias is high, especially for the more posterior (i.e. proximal) forms (Fig. 1) [19,24]. Indeed, a significantly decreased risk of hypospadias per 100 g of body weight increase of the new-born has been demonstrated [19]. Some authors hypothesize that the small body size at birth may be caused by impaired intrauterine growth, which, in turn, is a consequence of androgen function failure [52]. Moreover, there are chances that a malfunctioning placenta may lower chorionic gonadotropin production and consequently influence normal external genitalia development [53]. Therefore, placental insufficiency may impair the proper supply of nutrients, cause growth retardation and induce low birth weight as final outcome. However, a clear relationship between newborn body weight and hypospadias has not been elucidated yet.

Finally, maternal hypertension during pregnancy, preeclampsia, and preterm delivery are all consistently associated with this condition, probably because of placental dysfunction [2].

Conclusions

Hypospadias may or may not be associated with other malformations. In the former case, this anomaly may be considered as one of the many features that characterize medical syndromes. The global picture of hypospadias risk factors and of their onset during embryological development is complex and likely largely incomplete (Fig. 2). Notably, for this condition it is very difficult to discriminate

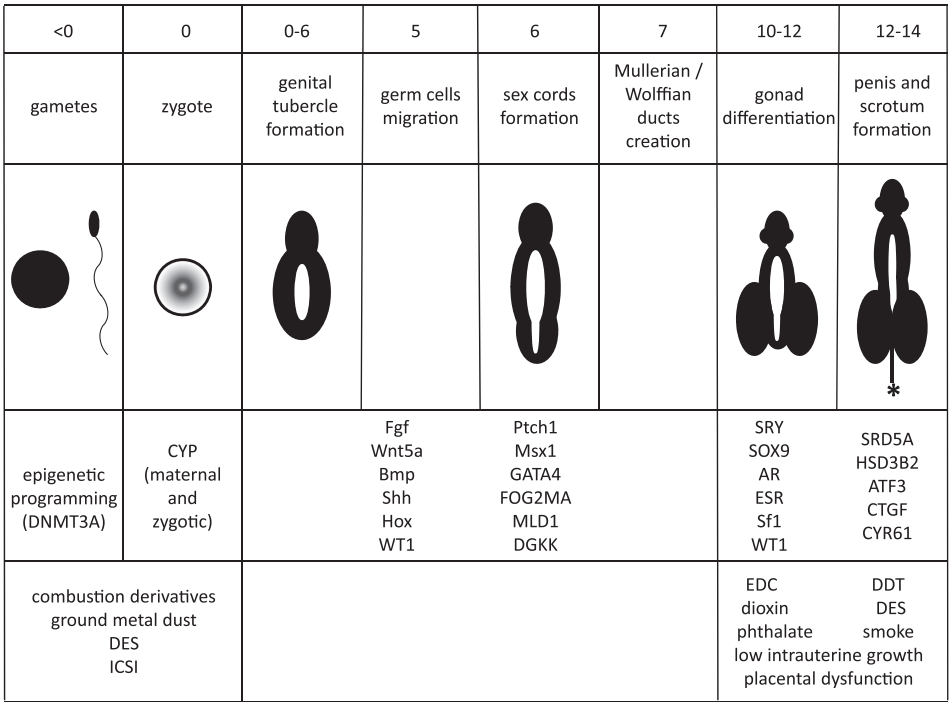

Figure 2 Diagram illustrating the interactions between genotype and environment in relation to the urogenital development. Line 1: gamete, zygote and development stages measured as weeks from fertilization. Line 2: description of the above stages or main embryological event(s). Line 3: schematic illustration of the events listed in Line 2. Line 4: identified genetic causes of hypospadias; it is shown that the action of these genes may influence multiple developmental steps. Line 5: main environmental causes of hypospadias and their time of action. Hypospadias formation occurs during the time frame 11—16 weeks from fertilization.

the genes specifically involved in the urogenital formation from those having a general role in the development and showing an epistatic effect on genital tubercle growth. Sporadic hypospadias is instead more frequent than syndromic hypospadias [2,4], but its etiology is still largely unknown. Many of the genes involved in urogenital tract formation have been identified; however, only in a few cases was it possible to find evident mutations of these genes in affected patients [2].

Interestingly, it has been demonstrated that some gene polymorphisms are associated with an increased risk of hypospadias. This suggests that some polymorphisms may cause hypospadias only occasionally. One possible explanation for this phenomenon is to hypothesize an interaction between genotype and environment. For example, it is possible to speculate that some alleles code for hormone receptors that have dissociation constants higher than the wild-type form. These alleles would be detected phenotypically only in the presence of compounds mimicking endogenous hormones, such as some pesticides [16—18,24]; otherwise, patients harboring such alleles would be normal. This point raises a more general question: what is "normal" and what is not when we look for the causes of this birth defect? Based on what we have described, it is not possible to associate "normality" with the absence of a specific phenotype; yet, certain polymorphisms might cause this defect only in the presence of specific environmental factors and conditions and would not induce it by themselves. The general picture, which comes out from the literature, is that many reports are in contradiction; namely, the same environmental stimulus is described either as associated or not with increased hypospadias risk. We believe that these contradictions may be only apparent and be explained by analyzing in depth the genomic/environmental interactions. Genetic polymorphisms are frequently population-specific, but not all populations are exposed to the same exogenous stresses.

Consequently, it becomes crucial to identify such alleles, correlate them to specific risk factors, and calculate the probability that these interactions may cause the selected anomalies. This goal can be accomplished only using a multidisciplinary approach based on genetics, genomics, physiology, biochemistry and embryology, and relying on proper biostatistical and epidemiological methods; this will likely be the next challenge for the scientific community in order to prevent hypospadias.

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None.

Conflict of interest

None.

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