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HYPOSPADIAS: ANALYSIS OF A COMPLEX GENETIC DISORDER

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We work in the dark- we do what we can- we give what we have.

Our doubt is our passion, and our passion is our task.

The rest is madness of art.

Henry James

ABSTRACT

BACKGROUND: Hypospadias is a common inborn error of the male urethra that involves an abnormally placed urethral opening. Its complex etiology is largely elusive to date. Twin and familial studies highlight a genetic background in hypospadias. Environmental factors have also been identified, particularly the exposure to endocrine disrupters. For most of the cases, pedigree analyses indicate a heterogeneous, complex pattern of inheritance, with several genetic and environmental factors interacting, yielding high heritability indices. Hypospadias is therefore said to be a complex genetic disorder.

HYPOTHESIS: As an inborn error of development, hypospadias may be induced by disturbances in the pathways of urethral development, which comprise genetic programming, cell differentiation, hormonal signaling, enzyme activity, and tissue remodeling; and follows an orderly sequence. We proposed that gene variants in *FGF8*, *FGF10*, *FGFR2* and *BMP7*, important genes in the early urethral development; in *FKBP52*, an androgen receptor co-chaperone; in the estrogen receptor (*ESR*) genes 1 and 2; and *ATF3*, an estrogen responsive gene; may influence the risk to hypospadias.

STRATEGY: Using a candidate gene strategy, we performed comprehensive analysis of these genes in DNA from boys with hypospadias and controls, including sequence analysis, genotyping and association studies; and complementary expression analysis in human tissues.

RESULTS: Our results indicate that gene variants in the sequence of *FGF8*, *FGFR2*, two androgen-regulated developmental genes; and of *ESR2* and *ATF3*, two estrogen related genes, are associated with hypospadias. We have shown that the last two are expressed in the human developing male urethra.

DISCUSSION: The molecular mechanisms involved in the development of external genitalia during fetal life seem to depend on a complex balance between early morphogenetic cell-cell interactions; and between sex steroid hormones. These balances can be disturbed by the exposure to environmental endocrine disruptors. Ethnical differences in the response to such exposures denote that genetic factors also play an important role. The involvement of sequence variants in *FGFR2*, *FGF8*, *ESR2* and *ATF3*, hormonal responsive genes, in hypospadias is reported in this thesis, increasing the understanding on the complex etiology of hypospadias. Further genetic analysis and gene-environment studies are encouraged.

LIST OF PUBLICATIONS

1. Beleza-Meireles A*, Lundberg F*, Lagerstedt K, Zhou X, Omrani D, Frisen L, Nordenskjold A. **FGFR2, FGF8, FGF10 and BMP7 as candidate genes for hypospadias**. *Eur J Hum Genet* 2007, 15(4):405-10. *Equal contribution

2. Beleza-Meireles A, Barbaro M, Wedell A, Tohonen V, Nordenskjold A. **Studies of a co-chaperone of the androgen receptor, FKBP52, as candidate for hypospadias**. *Reprod Biol Endocrinol* 2007, 7;5:8.

3. Beleza-Meireles A, Omrani D, Kockum I, Frisen L, Lagerstedt K, Nordenskjold A. **Polymorphisms of estrogen receptor beta gene are associated with hypospadias**. *J Endocrinol Invest*. 2006, 29(1):5-10.

4. Beleza-Meireles A, Kockum I, Lundberg F, Söderhäll C Nordenskjöld A. **Risk factors for hypospadias in the estrogen receptor 2 gene**. *J Clin Endocrinol Metab* 2007 Jun 19 [Epub ahead of print].

5. Beleza-Meireles A, Töhönen V, Christian Radmayr, Christian Schwentner, Söderhäll C, Kockum I, and Nordenskjöld A. **Activating transcription factor 3: a hormone responsive gene in the etiology of hypospadias**. Submitted

CONTENTS

LIST OF ABBREVIATIONS

ATF3	activating transcription factor 3
AR	androgen receptor
bp	base pair
BMP	bone morphogenic protein
cM	centimorgan
ESR1	estrogen receptor alfa
ESR2	estrogen receptor beta
FGF	fibroblast growth factor
FKBP4	gene coding for FKBP52
FKBP52	FK506 binding protein 4, FKBP4
FSH	follicle stimulating hormone
GT	genital tubercle
HOXA	homeobox A cluster
HOXD	homeobox D cluster
kb	kilobase
LD	linkage disequilibrium
LH	luteinizing hormone
Mb	megabase
SNP	single nucleotide polymorphism
SOX9	SRY box 9
SRD5A2	5-alfa-reductase
SRY	sex determining region on Y chromosome
SSR	simple sequence repeat
STRP	short tandem repeat polymorphism
TNF-beta	transforming factor beta
UPE	urethral plate epithelium

BACKGROUND

The knowledge of normal (…) processes and their genetic control has permitted an understanding of the abnormal and, reciprocally, understanding of the abnormal has increased our understanding of the normal.

Epstein, 1978

This thesis work focused on the genetics of hypospadias, a common inborn error of the genital development of still elusive etiology. The incidence of disorders of the genital development in humans, manifested as hypospadias, micropenis, cryptorchidism, and ambiguous genitalia, is as high as one in every hundred newborn babies (Vilain E, 2002) and are among the leading categories of structural birth defects in man (March of Dimes Perinatal Data Centre, 2000). Despite common, their causes are largely unknown. Studies of human birth defects and developmental disorders may provide a window through which we can understand normal morphogenesis at the molecular level (Donnai D and Read AP, 2001). This thesis aimed to add more knowledge to the etiology of one of these common disorders.

HYPOSPADIAS: AN INBORN ERROR OF THE URETHRAL DEVELOPMENT

From the Greek *Hypo* - meaning under - and *spadon* -meaning a fissure

Hypospadias is a common inborn error of the male genital development. It results from an arrest in the normal development of the urethral, foreskin and ventral aspect of the penis during the gestational weeks 8-16. As consequence, the foreskin in the ventral aspect of the penis and the spongiosum are malformed, with misplacement of the urethral opening, ventrally and proximally from the tip of the *glans* penis (Baskin LS, 2000).

The site of failure of urethral fold fusion will dictate the position of the abnormal urethra meatus, as a continuum along the urethral shaft, from perineal positions proximally, to more distal openings along the penis (Figure 1). The majority of cases are mild, with an aberrant urethral meatus at the subcoronal margin, corona, or on the glans penis. The urethral opening in moderate and severe cases of is located more proximally on the penile shaft, scrotum, or perineum. More severe forms of hypospadias tend to associate with penile curvature as well as penile–scrotal skin transposition and intersex conditions (Baskin LS, 2000; Baskin LS et al, 2004). Surgery is performed to achieve normal urination, to correct the penis deformity, and to ensure normal sexual function.

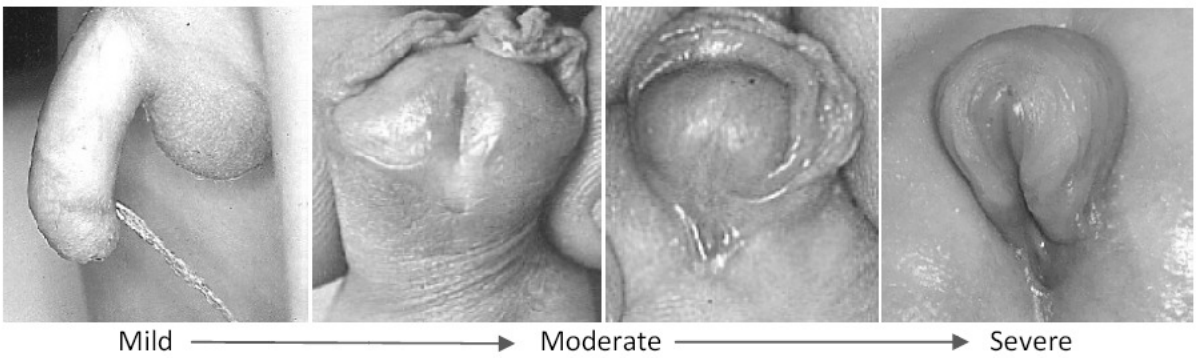

Figure 1: Hypospadias. The anatomical location of the misplaced urethral meatus determines the severity of this inborn error of development.

EPIDEMIOLOGICAL ASPECTS OF HYPOSPADIAS

Hypospadias is a common disorder with an incidence up to 1 in every 250 live male births in western societies (Table 1). It usually occurs sporadically and as an isolated feature (non-syndromic). Less commonly, hypospadias may be associated with other conditions (syndromic), particularly with other genital anomalies such as undescended testis, bifid scrotum, and cryptorchidism (Erickson, JD 2004; Källén B and Winberg H, 1982; Paulozzi L, 1999). In up to 70 % of the cases, no cause is identified.

Furthermore, the incidence of hypospadias worldwide seems to have been increasing in the past decades. In the US, two surveillance studies reported that the incidence had increased from about 1 in 500 total births in the 1970s to 1 in 250 total births in the 1990s (Paulozzi L et al, 1997).

ETHNICITY	*INCIDENCE*	*PHENOTYPE*	*AUTHOR*
Sweden	1.53/1000	75% Mild 2.5% Moderate 12. 5% Severe	Källén, 1998
UK	1.98/1000	85% Mild 14% Moderate 1% Severe	Roberts & Lloyd, 1973
France	2.98/1000	69% Mild 25% Moderate 6% Severe	Stoll 1990
Latin America	0.76/1000	72% Mild 18,5% Moderate 9.5% Severe	Monteneole, 1981
US	4/1000	64.1% Mild 21.9% Moderate to severe	Paulozzi, 1997

Table 1. The incidences of hypospadias in five reports worldwide range from 0.76 to 4 in 1000 male births. Mild hypospadias is the most frequent form in the five studies. The studies were conducted from 1973 to 1998.

EMBRYOLOGY OF HYPOSPADIAS

As an inborn error of development, knowledge of the normal sexual differentiation and, especially, of the male genital and urethral development, may elucidate some of the causes of hypospadias.

The complex processes of sex determination and differentiation, occurs on three levels (Figure 2):

I. The **chromosomal sex**, determined at fertilization, when the 23 chromosomes from the egg combine with the 23 chromosomes from the sperm to produce a zygote, which then has 46 chromosomes. Two of these chromosomes, X and Y, determine sex. The offspring always inherits an X chromosome from the mother. If the father also gives an X chromosome, the offspring will be female or XX. If the father gives a Y chromosome, then the offspring is male or XY.

II. The **gonadal sex**, according to which gonad is formed: one of two organs can develop from the bipotential gonad: a testis or an ovary. In males, the sex-determining factor (SRY), expressed from the Y chromosome, causes the indifferent gonads to develop into testis. Here, testicular cords are formed and contain germ cells and Sertoli cells, while mesenchymal cells migrate into the interstitial space between the cords, giving rise to fetal Leydig cells. The testicular Leydig cells and Sertoli cells produce, respectively, testosterone and anti-Müllerian hormone (AMH or Müllerian inhibiting substance- MIS), two essential hormones for the correct differentiation of both primary and secondary sex characteristics. In the absence of SRY, the bipotential gonad develops into an ovary.

III. The **phenotypic sex**: The development of secondary sex characteristics, including the external genitalia and the male or female ductal systems, depends on whether the gonad develops as a testis or an ovary. In males, the presence of AMH triggers the regression of the Müllerian duct, the anlagen of the female urogenital tract; while testosterone, maintains the Wolffian ducts. Furthermore, testosterone, in some tissues after its reduction to dihydrotestosterone (DHT), stimulates differentiation and growth of the prostate, seminal vesicles, and other parts of the male urogenital tract, including the masculinization of the external genitalia (Reviewed by Hughes IA, 2001). It must be emphasized that, despite considered as default, the ovarian and female pathways, still not very well understood, are likely to be active genetic pathways (Brennan J et al, 2004).

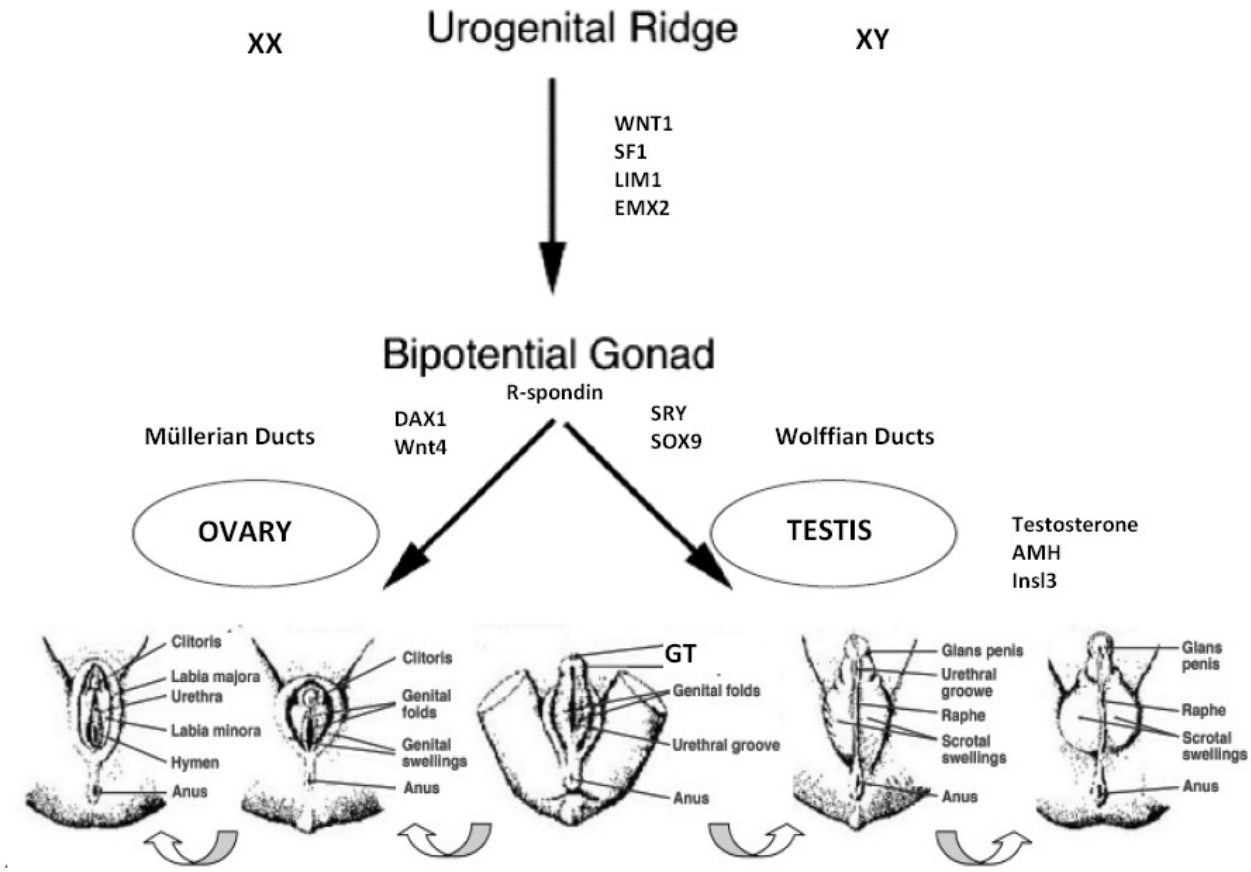

Figure 2. Steps in sex determination and differentiation. The proper sex development is dependent on several steps: The definition of the chromosomal sex; the formation of the gonads; and the apparent, or phenotypic, gonad. Disturbances at any of these complex levels can compromise the sexual differentiation of the new being (Adapted from Hughes IA, 2001).

Disturbances of male sex differentiation lead to a variety of undervirilization phenotypes, ranging from males with hypospadias, individuals with ambiguous genitalia, to complete male pseudo-hermaphrodites. These phenotypes may be either due to insensitivity of the target cells to androgens; or to impaired testicular androgen action or production, as a result of a steroidogenic enzyme defect, such as 17ß-hydroxysteroid dehydrogenase type II, or reduced sensitivity of the Leydig cells to luteinizing hormone (LH), a pituitary secreted hormone that stimulates de production of testosterone (Reviewed by Hughes IA, 2001).

Recently, a number of other regulatory factors were described, which affect sexual development (Drews U, 2000; Wilhelm D, 2007; Zarkower D, 2001; Parma P et al, 2006); furthermore, the importance of estrogens and their receptors in male sex development has been increasingly acknowledged, being involved in the male reproductive tract development and functions (Atanassova N et al, 2000; Berensztein EB et al, 2006; Course JF et al, 1999; Crescioli C et al, 2003; Delbes G et al, 2006; Raven G et al, 2006); but have not yet found an exact place in the above canonical scheme of sex determination.

THE MALE GENITAL DEVELOPMENT

During the first 8 gestational weeks (= 6 fetal weeks), genetically male and female embryos develop similarly and show sexually undetermined genital structures. By the end of the first month of gestation, the hindgut and future urogenital system, reach the surface of the embryo at the cloacal membrane on the ventral surface, where the cloacal folds develop, flanking the anogenital membrane. There, the most cephalad structure is the genital tubercle (GT), which will latter differentiate into a penis in males and a clitoris in females (Gilbert's Developmental Biology, 7th Ed).

The sexual dimorphic genital differentiation starts in humans around gestational weeks 9, after the fetal leydig cells start producing testosterone, particularly through its metabolite dihydrotestosterone (DHT) (Aaronson IA et al, 1997; Klonisch T et al, 2004; Wilson JD et al, 1993). In male fetuses this period is characterized by rapid elongation of the GT (Rey R and Picard JY, 1998; Allera A et al, 1995; McPhaul MJ, 2002). In the female fetus, without the influence of testosterone, the genital tubercle develops into the clitoris, and the labioscrotal folds do not fuse, leaving labia minora and majora (Gilbert's Developmental Biology, 7th Ed).

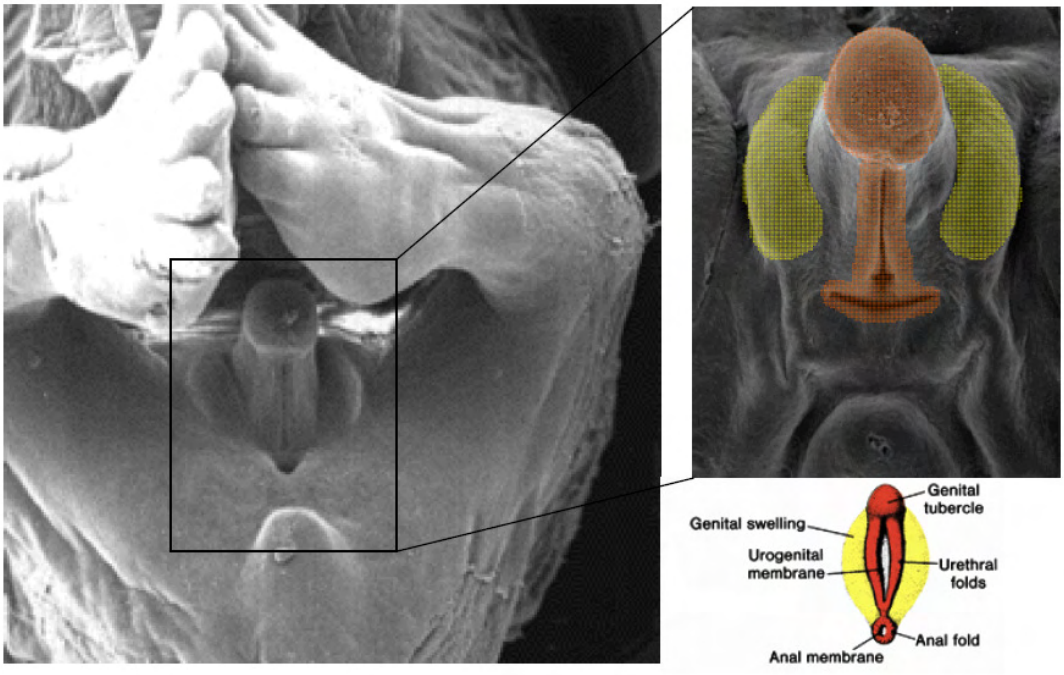

Figure 3: Urogenital structure at week eight of human development. Adapted from: http://www.med.unc.edu/embryo_images/unit-welcome/welcome_htms/contents.htm

As the male GT elongates, the urethral groove appears on its ventral aspect. It is defined laterally by urethral folds that are continuous with the urogenital (late cloacal) folds. The urethra forms by ventral growth and fusion of the urethral folds. This process begins proximally in the perineal region and extends distally toward the glans penis and results from epithelial seam remodeling, comprising internal cell migration and highly focalized apoptotic activity (Baskin LS et al, 2001a).

Distally, the urethral groove terminates in a solid plate of epithelial cells (urethral plate) that extends into the glans penis. The solid urethral plate canalizes and thus extends the urethral groove distally into the glans by endodermal cellular differentiation (Kurzrock EA et al, 1999a; Kurzrock EA et al 1999b).

THE MOLECULAR TEMPUS IN THE GENITAL DEVELOPMENT

The formation of the external genitalia is a developmental process that involves genetic programming, cell differentiation, hormonal signaling, enzyme activity and tissue remodeling (Baskin LS, 2004; Cunha GR et al, 2004). And it can be roughly divided in two stages:

I. An **initial hormone independent period**, about 6 to 9 weeks of gestation, common to males and females, when the formation of the rudiments of the external genitalia and the initial outgrowth of the GT from the embryonic trunk take place, orchestrated by morphogenetic cell-cell interactions mediated via a spectrum of growth factors and other signaling molecules such as sonic hedgehog (SHH), fibroblast growth factors (FGF's), and bone morphogenetic proteins (BMPs), whose actions befall before the onset of the hormonal signaling. (Baskin LS, 2004; Haraguchi R et al, 2000; Kurzrock EA et al, 1999; Minelli A, 2002; Morgan EA et al, 2003; Ogino Y et al, 2001; Perriton CL et al, 2003; Petiot A et al, 2005; Suzuki K et al, 3003, Yucel S et al, 2004).

II. The **sexual dimorphic period**, leading to the development of male or female genitalia, with differentiation of the GT into penis or clitoris, respectively. This period is hormone dependent and starts around gestational week 9 in humans. In male fetuses, the onset of masculinization of the male external genitalia coincides with Leydig cell-derived androgen production. This period also constitute a critical window of development, because a shift in the equilibrium between androgens and estrogens may induce disturbances in, and lead to maldevelopment of the genitalia (Akre O et al, 1999; Baskin LS et al, 2001b; Bay K et al, 2006; Hughes IA et al, 2006; Klonisch T et al, 2004; Panet-Raymond V et al, 2000; Stoll C et al, 1990; Yucel S et al, 2003).

ETIOLOGY OF HYPOSPADIAS

Despite common, and with an increasing incidence (Paulozzi L et al, 1997; Toppari J et al, 2001), the etiology of hypospadias is still largely unknown. In up to 30% of the cases, particularly in severe hypospadias, a cause can be identified (Akre O et al, 1999; Baskin LS et al, 2001b; Utsch B et al, 2004). In the remaining 70% "idiopathic" hypospadias, a complex pattern of inheritance has been proposed (Fredell L et al, 2002; Frisén et al, 2004).

ENVIRONMENTAL AND NON-GENETIC RISK FACTORS TO HYPOSPADIAS

Environmental toxicants have been reported to increase the risk of giving birth to a boy with hypospadias, particularly the exposure to endocrine disrupters, which have been blamed for the increasing incidence of hypospadias (Paulozzi, 1997; Toppari et al, 2001). Furthermore, a maternal vegetarian diet, rich in fitoestrogens, the intake of diethylstilbestrol (DES), a potent estrogen, and loretadine have also been associated with hypospadias, with conflicting results in some studies (Bay L et al, 2006; Klip H et at, 2002; North K et al,2000; Storgaard L et al, 2006).

One of the best-established risk factors for hypospadias is low birth weight (Akre et al. 1999; Chen YC and Woolley PV, 1971; Gatti JM, 2001; Monteleone Neto R et al, 1981; Weidner IS et al, 1999). Fredell et al have suggested that this association is independent from genetic factors in monozygotic twins discordant for hypospadias, where the twin with the lower birth weight is affected with hypospadias. In addition, the birth of boys with hypospadias is more often associated with a higher rate of weak contractions, lower placental weight, induced deliveries and caesarean. And the incidence of hypospadias in monozygotic twins is 8.5-fold that in singletons. Those evidences point to deficits of the fetal-placental-maternal interaction (Avellan L et al, 1977; Calzonari E et al, 1986; Hussain N et al 2002).

Hypospadias is also more common in infants born after *in-vitro* fertilization (IVF), particularly with intra-cytoplasmatic sperm injection (ICSI), (Ericson A and Källén B, 2001, Källén B et al 2005). The association with IVF and hypospadias has been related to paternal subfertility (i.e. by genetic factors) (Wennerholm UB et al 2000), and also to the gonadotropic hormones used during the IVF procedure may interfere with androgen production, as well as progestins administered after embryo transfer (Meijer WM et al 2006, Sorensen HT et al 2005).

GENETIC AND MOLECULAR ASPECTS OF HYPOSPADIAS

Several observations support a strong genetic influence in hypospadias: Familial clustering is seen in up to 25% of cases. A concordance rate of 27% (6 of 22) in monozygotic twins and of 9% (1 of 11) in dizygotic twins was reported in hypospadias (Fredell L et al, 1998), which is in accordance with previous results (Bauer et al, 1979; Calzolari E, et al, 1986; Chen YC et al, 1971; Czeizel A et al, 1979; Monteleone Neto R et al, 1981; Stoll C et al, 1990). Furthermore, a search at the OMIM database finds more than 150 entries for hypospadias. From these, several genetic factors have been identified to be associated both with isolated or syndrome-associated forms of hypospadias.

GENETIC DEFECTS IN HORMONAL PATHWAYS

Since the masculinization of the urogenital structures is an androgen-dependent process (Aaronson IA et al, 1997; Kim KS et al, 2002; Yucel S et al, 2003), it is not surprising that impairment of the androgen biosynthesis and activity can induce hypospadias (McPhaul MJ, 2002). Abnormalities in the androgen receptor (AR) and in 5-alpha-reductase type 2 (SRD5A2), the enzyme that mediates the conversion of testosterone into the more potent androgenic derivative DHT, have been found in hypospadias (Allera A et al, 1995; Silver RI et al, 1999; Wilson J et al, 1993).

Moreover, some authors report endocrine abnormalities in boys with hypospadias, particularly the more severe cases (Aaronson IA et al, 1997; Allen TD, Griffin JE, 1984; Nonomura K et al, 1984; Gearhart JP et al, 1990; Hyun G et al, 2004; Shima H et al, 1986; Silver RI, 2004): Some reports suggest an abnormal androgenic milieu in boys with hypospadias, who present either hypergonadotropic hypogonadism or hypogonadotropic hypogonadism, i.e. that these boys have either an impaired testicular testosterone production or an impaired pituitary gonadotropin production, respectively.

Defects at various steps of the steroid biosynthesis pathway can also induce hypospadias in patients with an XY karyotype (Aaronson IA et al, 1997; Moisan AM et al, 1999; Denes FT et al, 2000). In addition, defects in the anti-Müllerian hormone (Imbeaud S et al, 1995), or in the LH and its receptor (Martens JW et al 1998; Mendonça BB et al, 2000), account for a very small number of hypospadias.

OTHER GENETIC DEFECTS

Some rare genetic syndromes are associated with hypospadias. Examples are the ATR-X syndrome, campomelic dysplasia, and the hand-foot-genital syndrome with mutations in the corresponding transcription factors *XH2* (Gibbons RJ et al, 1995), *SOX9* (Cameron FJ et al, 1997; Wagner T et al, 1994), and *HOXA13* (Mortlock DP et al, 1997; Utsch B et al, 2002), respectively. Chromosome rearrangements in 46,XY individuals with hypospadias have also been reported: 13q and 14q (Kilic N et al, 2005); 6p and 17q (Mansouri MR et al, 2006); 1q and 18q (Frizell ER et al, 1998); 8q and 20p (Tar A et al, 1997); 3p and 4q (Hegmann KM et al 1996); 7q and 18q (Johnson DD et al, 1986). Abnormalities in the sex chromosomes have also been found, such as 46,XX/46,XY chimerism (Benirschke K et al 1972) and mosaicism, such as 45,X/46,XY; 46,XX/47,XXY; 45,X/46,XY/47,XYY (Federman DD et al, 1995; Yordam N et al, 2001).

COMPLEX GENETIC ETIOLOGY

The aforementioned defects, as well as other rare genetic disorders, do not explain the vast majority of the non-syndromic hypospadias cases (Avellan L, 1977; Frydman M et al, 1985; Lowry RB et al, 1976; Martens JW et al 1998; Mendonça BB et al, 2000). For most of the cases, pedigree analyses indicate a heterogeneous, complex pattern of inheritance for hypospadias, with several genetic and environmental factors interacting, a model that yields high heritability indices, ranging from 57% to 74% (Harris EL, 1990; Stoll C et al, 1990). A complex segregation analysis of 2005 pedigrees with at least one member with hypospadias, showed a heritability of 0.99 and evidence for multifactorial inheritance; furthermore, the authors suggested that hypospadias might be due to monogenic effects in a small proportion of the families, but that there is a multifactorial cause for the majority of the cases (Fredell L et al, 2002).

Studies on candidate genes, such as the *AR* and the *SRD5A2*, have evidenced that polymorphisms in these genes may individually increase the risk to hypospadias; but are not sufficient to produce hypospadias. Variants in the *AR*, such as CAG and GGN repeat polymorphisms; and in the *SRD5A2,* such as the A49T, and the V89L gene variants are associated with this inborn error of development (Klonisch T et al, 2004; Sharpe RM, 2006; Silver R et al, 1999).

Frisén et al, in 2004, reported a genome-wide linkage analysis for hypospadias susceptibility genes; and suggestive linkage was found in 4 chromosome regions: 9q22 (in Swedish and Middle Eastern families), 2p11, 10p15 and 10q21 (only in Swedish families), providing a basis for outlining the complex genetic background of hypospadias.

HYPOSPADIAS: ANALYSIS OF A COMPLEX GENETIC DISORDER

Hypospadias is a common complex genetic disorder for most of the cases, as evidenced by Fredell et al in 2002, involving the combination of several genetic and environmental risk factors. Genetically complex traits and disorders are those which are not inherited in an easily determined manner: Although they often cluster in families, no clear-cut pattern of inheritance is identified. This makes it difficult to determine a person's risk of inheriting or passing on these disorders. It is the case of common disorders, such as heart disease, hypertension, diabetes, obesity, several forms of cancer, cleft palate, low IQ or autism, which consist in a serious burden to the society and to the medical resources.

Mapping the genes that contribute to complex diseases has become a major challenge of the post-genome era, since the determination of the genetic variants underlying these disorders may provide new insight into disease etiology, suggesting novel pharmaceutical targets; and could lead to genetic screening to identify individuals at increased risk.

GENETIC ARCHITECTURE UNDERLYING COMMON DISEASES

In contrast with monogenic disorders, which are controlled by genes of large effect and show simple patterns of inheritance within families, the genetic architecture underlying complex diseases is poorly understood: A defining feature of complex phenotypes is that no single locus contains alleles that are necessary or sufficient for disease. For a given disease, an individual's risk probably depends on some unknown function of genetic, environmental/lifestyle and stochastic factors. Some disorders may be explained by the cumulative result of few variants in a few genes; in other cases, the combination of a large number of common genetic variants, each contributing with small effects, influence the risk to a disease. Some disorders may also be determined by the result of an interaction between one or more genetic variants and environmental or non-genetic disease risk factors, including probably also imprinting and epigenetics. Furthermore, we do not yet know whether susceptibility alleles in complex disorders are common or rare, neutral or deleterious, few or many (Weiss et al, 2000; Wright AF et al, 2001).

One hypothesis about complex genetics architecture proposes that common, *invisible-to-selection*, weakly associated alleles influence the risk to complex disorders. The genetic risk effects would be due to disease loci where there is one, or a very small number of common variants; this is the *common disease–common variant* hypothesis (Lander ES, 1996). Under this hypothesis, one can assume that of there is little allelic

heterogeneity within loci. And therefore it may be possible to map susceptibility mutations through linkage disequilibrium with genotyped markers. Supporting this hypothesis is the fact that susceptibility variants involved in complex diseases have low or medium penetrance, and are probably little affected by selection (Pritchard JK et al, 2002).

In opposition, some authors suggest that few variants of clinical consequence will actually be common (Figure 4). Indeed, modeling of complex diseases taking into account the genetic variation at disease-susceptibility loci, the evolutionary processes, including mutation, genetic drift, and the possibility of selection, has predicted that neutral susceptibility alleles do not contribute much to the genetic variance underlying disease. These neutral alleles tend to be either lost or close to fixation in the population (Pritchard et al, 2001).

It seems more likely that most of the genetic variance underlying complex diseases is due to loci where susceptibility gene variants are mildly deleterious. At such loci, the total frequency of susceptibility mutations may be quite high, meaning a high allelic heterogeneity. Such heterogeneity may be greater in more physiologically complex disorders (Pritchard JK, 2001; Wright AF et al, 2001). Under these assumptions, direct sequence analysis may be needed to identify genetic variants in complex disorders.

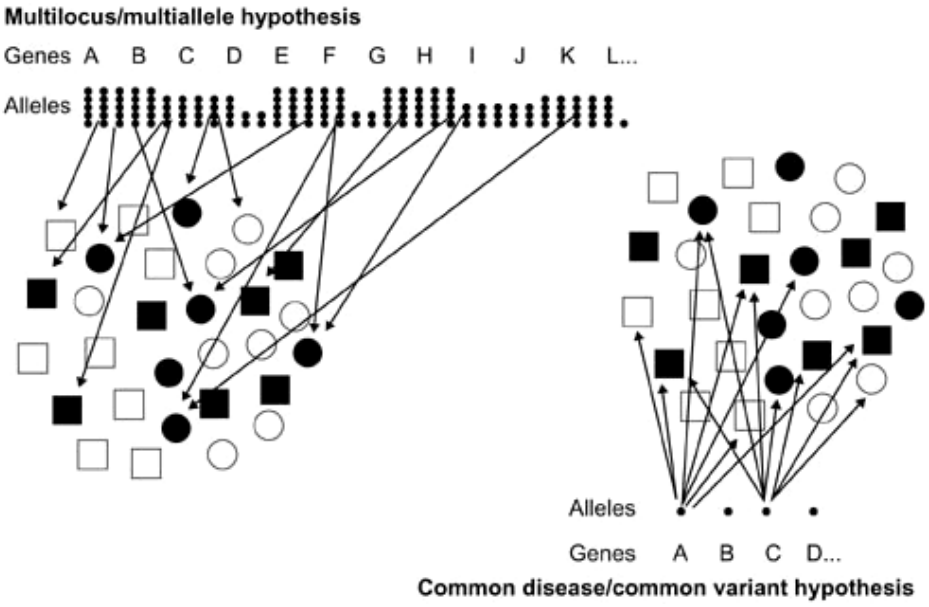

Figure 4: Genetic dissection of complex traits. Common disease-common variant hypothesis proposes that complex traits are the result of the combination of multiple risk gene variants; the multilocus-multiallele hypothesis, suggests that a few tens of heterogeneous disease loci, but not many hundreds, interact in different combinations in different individuals to influence the trait (adapted from Wright AF et al, 2001).

Between these two hypotheses, the answers and paths that define the best approach to map complex diseases will be trait specific. And probably no single approach will be sufficient. An analysis of the trait, by defining the phenotype, and determining an eventual genetic component, through family studies, heritability studies, segregation analysis, twin studies, adoption studies, heritability studies and/or population based risks to relatives and probands, must be consistently done. After a careful experimental design, strategies for genetic mapping are evaluated. The aims of genetic analyses of complex disorders are to identify the genetic risk factors and to model their role, the interactions between them and with environmental factors by statistical and bioinformatic resources. The parameters to consider for designing a genetic study of a complex disease are at different levels:

I. **The knowledge of pathophysiological process:**
   - If specific hypotheses on the role of particular genes are available, the *candidate gene* approach can be applied. It consists in choosing markers near the candidate gene.
   - If unknown, a *genome scan* approach may be set up using markers distributed over the genome.

II. **The methodological strategy most suitable according to the kind of genetic information available:**
   - *Linkage studies* which consist in finding a physical linkage between a marker and a gene. These studies deal with family design.
   - *Association studies* which consist in finding a correlation between several factors and the disease. These studies imply in general unrelated individuals or a population.
   - *Methods testing at the same time association and linkage*: The most popular such method uses the parental genotyping information and is known as the Transmission Disequilibrium Test (TDT); TDT uses healthy relatives as controls, overcoming the problem of population stratification of case-control studies, and tests for association in presence of linkage.

STRATEGIES FOR MAPPING IN COMPLEX GENETICS

LINKAGE ANALYSIS

Linkage mapping, also called positional cloning, is the process of systematically scanning the DNA genomes of various members of families using regularly spaced, highly variable (polymorphic) DNA segments whose position is known (genetic markers). This approach is an unbiased search of the entire genome without any preconceptions about the role of a certain genomic region or gene. Genetic linkage relies on the tendency

for haplotypes to be passed on to the next generation intact, without recombination events. If a marker can be identified that passed down through a family such that it is consistently accompanies the disease of interest, this suggests a gene with functional effect that is located close to that marker. Linkage analysis methods can be applied to both major gene disorders - parametric linkage- and complex diseases - model-free and non-parametric linkage. Successful mapping of complex disorders depend on the ability to adequately model the phenotype, and the genetics underlying it (Forabosco P et al, 2005; Mayeux R, 2005; Risch N, Merikangas K, 1996).

Several potential DNA regions that may contain susceptibility genes for hypospadias have been identified in our lab (Frisén L et al, 2004); and fine mapping of these regions is still ongoing. Linkage analysis was not the focus of this thesis.

ASSOCIATION ANALYSIS

The goal of association studies is to identify patterns of polymorphisms that vary systematically between individuals with different disease states and could therefore represent the effects of risk-enhancing or protective alleles. Association differs from linkage in it relies on shared common ancestry in populations; and therefore is able to detect smaller genetic effects on disease susceptibility. A statistically significant difference in association studies indicates that: 1) the studied polymorphism has a causal role; 2) the polymorphism has no causal role but is in LD with a causal variant; 3) the association is due to some underlying stratification or admixture of the population. To avoid the last, it is necessary to be careful about the matching of cases and controls, for a maximum of potential confounders, such as age and sex, and most importantly, ethnicity, in addition to the status of case or control (Balding DJ, 2006; Forabosco P et al, 2005).

Direct association studies analyze target, polymorphisms. This type of study is the easiest to analyze and the most powerful; and is based on an *educated guess* about the genetic basis of a disorder, on a gene and/or on a putative functional gene variant (Collins et al. 1997; Risch e 1996). Direct association studies may be a good choice for detecting gene variants underlying complex disorders where the risk associated with any given candidate gene is relatively small. The difficulty in direct association studies is the identification of candidate gene variants: Undoubtedly, we do not know enough about the genome nor about complex genetics to predict which variants may have such effects. Thus, direct association studies only have the potential to discover a few of the genetic causes of disease and disease-related traits.

Indirect association studies, in the other hand, rely on linkage disequilibrium (LD) to detect disease-causing variants in the proximity of the tested polymorphisms. This is the most commonly used form of association studies in complex diseases, particularly in the study of candidate gene/ genomic region.

LD refers to the non-independence of alleles at different sites. A given pair of intra-chromosomal markers are said to be in LD if they segregate together more often, and recombine less, than would be expected under independent assortment. Hence, the measure of LD between two markers will indicate the extent to which the genotype of one marker will predict the state of the other (Balding DJ, 2006; Forabosco P et al, 2005).

Recent advances in high throughput genotyping technology mean that screening of the all genome for disease associations is becoming feasible. However, even as whole genome studies are increasingly used, candidate gene studies will continue to play an important role in the study of complex genetic disorders.

ASPECTS OF THE ANALYSIS IN ASSOCIATION STUDIES

Having selected candidate genes or candidate genomic regions, an association study must be appropriately designed and analyzed to optimize the possibility of identifying causal variants, as well as of delivering reliable negative results, which are important to reduce duplication of effort and conflicting reports.

SNP TAGGING

By understanding the LD structure of the genome, frequency differences between affected and unaffected individuals do not need to be measured in all SNPs; but only in a subset of tag SNPs, which serve as proxy for the remaining SNPs and represent the total sequence variation in a target gene or genetic region. *Tagging* refers to the methods to select these SNPs (Carlson CS et al 2004; Zeggini E et al, 2005); and can be done in appropriate softwares, such as the *Haploview* (Barret et al, 2005).

Previously, this was a laborious initial step in study design, implying the typing and analysis of an extensive SNP set in a few individuals. Now with the International HapMap Project, it is possible select tag SNPs on the basis of publicly available data. The population that underlies a particular study will typically differ from the populations for which public data is available. However, recent studies indicate that tag SNPs often transfer well across populations (Gonzalez-Neira A et al, 2006).

PRELIMINARY ANALYSES IN ASSOCIATION STUDIES

The quality of the data should be checked thoroughly before starting the analysis. Testing for Hardy–Weinberg equilibrium (HWE) is one way to do it (Nielsen, DM et al 1998): deviations from HWE can be due to typing errors, such a mutant PCR-primer site, or because of a tendency to miscall heterozygotes as homozygotes, inbreeding, population stratification or selection. Deviations from HWE can also be a symptom of disease association, the implications of which are often under-exploited; and can also arise in the presence of deletion polymorphisms or segmental duplications that could be important in disease causation (Conrad DF et al, 2006; Bailey JA et al, 2006).

Testing for deviations from HWE can be carried out using a Pearson goodness-of-fit test with a $\chi 2$ null distribution (Wigginton JE et al 2000). The *Haploview* (Barret et al, 2005) software performs this test, calculating a HWE p-value, which is the probability that its deviation from HWE could be explained by chance.

MEASURES OF LD AND ESTIMATES OF RECOMBINATION RATES

It is usually of interest to summarize LD over the tested genomic region, since if a causal polymorphism is not genotyped, we can still hope to detect its effects through LD with polymorphisms that are typed. One approach is to compute local averages of pairwise values of D' and r^2, two proposed measures of LD (Devlin B et al, 1995). D', the normalized disequilibrium coefficient, which ranges from 0 to 1, is sensitive to even a few recombinations between the loci. The squared correlation coefficient, r^2, reflects statistical power to detect LD and can be an indicator of the sample size that would have been required to detect the disease association.

The LD values over a region can be illustrated diagrammatically with colors encoding different values (Abecasis GR et al, 2000), such as presented by the *Haploview* software (Barrett JC, 2005). LD maps provide another solution, fitting an exponential decay function to D' values, and the decay parameter provides a measure of local LD (Tapper W et al, 2005).

TESTS OF ASSOCIATION IN SINGLE SNPS

The analysis of SNP genotypes in a case–control study is performed by comparing frequencies between affected and unaffected individuals. Usual methods in epidemiology for case-control design can be used: 2X3 contingency tables with a Pearson test (2-df), considering the null hypothesis of no association. For complex traits, it is widely assumed that contributions to disease risk from individual SNPs will often be roughly additive. The power to detect risks may improve by counting alleles rather than genotypes using a 2X2 table, and a Pearson test (1-df), provided that the frequencies are in HWE (Sasieni PD, 1997). The general statistic tests have reasonable power regardless of the underlying risks. Bayesian methods may be a good option when *a priori* predictions about the nature of disease risks can be explicated (Lunn, D et al 2006).

Regression analysis is another way of analyzing the data. In its simplest form, regression analysis involves finding the best straight line relationship to explain how the variation in an outcome (or dependent) variable, y, depends on the variation in a predictor (or independent or explanatory) variable, X. Once the relationship has been estimated we will be able to use the equation: $y_{ij} = a+bx_{ij}+\varepsilon_{ij,}$ where a is the intercept, b is the slope and ε is the error term. The model picks up the unpredictable part of the response variable y_i. The x's and y's are the data quantities from the sample or population in question (where y_{ij} is the trait value for individual i with genotype j and x_{ij} is the mean effect of genotype *j*); and a and b are the unknown parameters to be estimated from the data (Balding DJ, 2006). The test requires the trait to be approximately normally distributed for each genotype, with a common variance.

Alternatively, logistic regression may be used to, as it makes no assumption about the distribution of the independent variables; they do not have to be normally distributed, linearly related or of equal variance within each group. Furthermore, it offers a flexible tool that can readily accommodate other variables, such as environmental interactions or covariates such as sex or age of onset. Logistic regression applies maximum likelihood estimation after transforming the dependent variable into a logit: logit (y) = log [y/ (1 - y)]. The value of logit is equated to either β0, β1 or β2, according to the genotype of individual (β1 for heterozygotes). The likelihood-ratio test of this general model, against the null hypothesis β0 = β1 = β2, has 2 df. Tests for recessive or dominant effects can be obtained by requiring that $\beta_0 = \beta_1$ or $\beta_1 = \beta_2$ (Balding DJ, 2006).

TESTS OF ASSOCIATION IN MULTIPLE SNPS

Analyzing SNPs one at a time can neglect information in their joint distribution (Waldron ERB et al, 2006). Logistic regression analyses for a set of *n* SNPs in a candidate gene is a natural extension of the single SNP analyses discussed above: there is now a coefficient (β_0, β_1 or β_2) for each SNP, leading to a general test with 2n df. Interactions between SNPs can be investigated, being potentially useful for investigating epistatic effects.

Haplotype-based methods can also be used to capture the correlation structure of SNPs, particularly in regions of little recombination. There are statistical methods, and softwares that implements them such as *Haploview* (Barret et al, 2005) and *Unphased* (Dudbridge F, 2003), for inferring haplotype frequencies from the genotypes of related and unrelated individuals (Marchini J et al, 2006). The simplest analysis involves testing in a 2Xn contingency table, where n denotes the number of distinct haplotypes. Alternative approaches, based on the estimated haplotype proportions among cases and controls, without an explicit haplotype assignment for individuals, compare the product of separate multinomial likelihoods in the two groups with that obtained by combining cases and controls (Tzeng JY et al, 2003).

The haplotype analysis of unrelated individuals has some caveats. One of them is that usually these approaches rely on assumptions of HWE and of near-additive disease risk. Furthermore, haplotypes cannot be known for sure in population based studies. Instead, phase must be inferred with a certain degree of uncertainty. Therefore, when assessing the overall significance of any finding, this must be taken into account, particularly when LD between markers is low. Moreover, the inclusion of many rare haplotypes in analyses can decrease the power to detect association due to too many degrees of freedom. And to combine all rare haplotypes into a *dustbin* category might miss their separate effects. Some methods are emerging to try to overcome this and other aspects (Ke XY et al, 2004; Lin DY, Zeng D, 2006; Schaid DJ et al, 2002). Despite their clear usefulness in genetic association studies, the limitations of haplotype-based methods should be considered when performing the analysis (Browning SR, 2006).

MISSING GENOTYPE DATA

Missing genotypes can be predicted, maximizing the use of the observed data, with appropriate softwares based on the observed genotypes at neighboring SNPs (Little RJA, Rubin DB, 2002). These methods either seek a best prediction of a missing genotype, such as a maximum-likelihood estimate (single imputation), as *Unphased* (Dudbridge F, 2003); or randomly select it from a probability distribution (multiple imputations).

These analyses needs, though, to be made critically (Balding DJ, 2006; Little RJA et al 2002; Souverein OW et al, 2006).

MULTIPLE TESTING

The analysis of association data usually generates a lot of tests, and consequently the problem of multiple testing emerges. The error rate of each test made is defined by $[1 - (1 - \alpha)]^n$, n being the number of independent tests performed. One conservative solution to multiple testing can be provided by the Bonferroni correction, which consists in multiplying the *p*-value by the number of performed tests (Balding DJ, 2006).

Other way to take into account the multiple statistical tests is to correct the p-values by performing permutations. Permutation testing is a nonparametric re-sampling approach to statistically test hypotheses about populations, based on samples of data. It is performed as follows: The trait values are randomly shuffled between subjects a number of times. In each permutation the minimum p-value is compared to the minimum p-value observed in all the analyses in the original dataset. Both *Haploview* and *Unphased* softwares provide a framework for permuting association results in order to obtain a measure of significance corrected for multiple testing biases.

ADDITIONAL REMARKS

Since no test is absolute, and all tests have limitations, the application of different algorithms to the same data set can be useful to increase the degree of evidence. The importance of replication studies is undeniable. And additional evidences, at the physiological and functional level are desirable.

CANDIDATE GENES IN HYPOSPADIAS

In this thesis, a candidate gene strategy was chosen to investigate genes, which take part in the urethral development, and with a potential role in the etiology of hypospadias. Several aspects of the development of the GT and the male genitalia, revealed through the study of model organisms (reviewed by Stadler HS, 2003), and humans (such as Crescioli C et al, 2003; Kim KS et al, 2002; Pelletier G et al, 2000; Shapiro E et al, 2006; Westberg L et al, 2001; Liu Bet al, 2005; Hughes IA, 2000), were the rationale behind the selection of genes: the early developmental genes *FGF8*, *FGF10*, *FGFR2* and *BMP7*; *FKBP4* that codes to a co-chaperone of the AR; the *ESRs*; and *ATF3*, an hormone responsive gene.

EARLY DEVELOPMENTAL GENES

The outgrowth and patterning of the GT start by gestational week 6 in humans, before the sexually dimorphic phase, i.e., in the absence of endocrine signals. This period, as shown in studies of model organisms, is marked by a network of morphogenetic events, coordinated by a series of signaling molecules, particularly fibroblast growth factors (FGF's), sonic hedgehog (SHH)*, HOX13* gene products, and bone morphogenic proteins (BMPs) (Baskin LS, 2004). This process is orchestrated by the urethral plate epithelium (UPE), at the GT, which has polarizing activity (Haraguchi et al, 2000; Kurzrock EA et al 1999a; Murakami and Mizuno, 1986). Moreover, the balance between cell proliferation and apoptosis seems to be important for the genital development, influenced by the interaction of positive growth regulators (FGF family or SHH) and negative growth regulators (such as BMP 2, 4 and 7) (Suzuki K et al, 2003).

The distal UPE region in mice is initially marked by the expression of fgf8 (also called androgen-induced growth factor, AIGF), which regulates the initial outgrowth of the GT outgrowth and the expression of mesenchymal gene expression, such as *fgf10* (Haraguchi et al, 2000). The expression of Shh is also observed in the UPE; and regulates the expression of fgf8, as well as several mesenchymaly expressed molecules, fgf10, and hoxd13 (Perriton CL, et al, 2002). The shh *null* mice (Yucel S et al, 2004) display complete GT agenesis, with increased cell death and reduced cell proliferation, indicating that Shh is essential for the initiation of the GT outgrowth, polarized gene expression and cell surviving. The Gli family of transcription factors, as target of Shh; is also required for the normal development of the GT (Haraguchi et al, 2001; Motoyama et al, 1998). Bmp genes (such as bmp 2, 4 and 7), their antagonists (nog and chordin) and receptors (bmpr1 and bmpr2) are also expressed in the UPE, and in the distal-ventral mesenchyme adjacent to it, acting as negative growth regulators (Suzuki K et al, 2003).

HOX13 gene products also take part in the urethral development. Both *hoxa13* and *hoxd13* are strongly expressed in the mesenchyme of the GT (Warot et al., 1997). Some *hox13* gene mutations affect the development of the distal (cloacal) portion of the hindgut and the GT (Kondo et al, 1996; de Santa Barbara P et al, 2002; Warot X et al, 1997). In a knock-out mouse model, the loss of hoxa13 function (*hoxa13-/-*) affects the differentiation and maturation of the urethral epithelium, (Morgan EA et al, 2003), with a marked reduction of fgf8 and bmp7 expression, which leads to coronal hypospadias. In these mice, both programmed cell death and cell proliferation are reduced in genital-shelf mesenchyme.

Fgf10 is another important morphogen of the most distal mesenchymal GT structures (Haraguchi Ret al 2000). Mutant mice lacking *fgf10* or its receptor, the fibroblast growth factor receptor 2 isoform IIIb (*fgfr2-IIIb*), exhibit normal corporal bodies, but disturbed urethral plate ventral fusion, consistent with hypospadias. In these mice, urethral signaling regions are established, but cell proliferation is prematurely arrested and maturation of the urethral epithelium is disrupted. Interestingly, these genes are downstream targets of AR during external genital development, since the loss of *fgfr2-IIIb* and *fgf10* expression in the urethra, and associated hypospadias phenotype, is observed as an effect of androgen antagonists (Yucel S, et al, 2004; Petiot A et al).

Based on the *fgf10*, *fgfr2-IIIb* and *hoxa13* knock-out mice phenotypes, mutations of the *FGF10, FGFR2, FGF8* and *BMP7* genes could underlie hypospadias in humans. We explored this hypothesis in a set of familial cases of non-syndromic hypospadias.

FKBP52, A CO-CHAPERONE OF THE ANDROGEN RECEPTOR

ANDROGENS IN THE MALE URETHRAL DEVELOPMENT

Androgens start being produced by fetal Leydig cells around gestational week 9 in man, marking the beginning of the sexually dimorphic phase of development. Androgens are crucial for the survival and differentiation of the Wolffian duct. The masculinization of the external male genitalia is dependent on testosterone, and specially its conversion via SRD5A2, to the more potent DHT (Rey R et al, 1998). Moreover, androgens have a direct effect on the fusion of the urethral fold, as demonstrated by an acceleration of urethral fold fusion and a longer urethral tube in mice treated with androgens *in uterus* (Yucel S, 2003). Androgens acts upon the male sexual development through its receptor (AR), which is a hormone induced transcription factor.

The expression of AR and SRD5A2 has been documented in the human developing male urethra (Kim KS et al, on 2001). Between 12 and 14 weeks of gestation, AR is localized on the epithelial cells of the urethral plate in the glans, the tubular urethra of the penile shaft, and stromal tissue surrounding the urethral epithelium. Between 16 and 20 weeks gestation, AR expression is observed in urethral epithelial cells, greater along the ventral aspect of the glandular urethra. The expression of SRD5A2 localizes to the stroma surrounding the urethra, especially along the urethral seam area in the ventral portion of the remodeling urethra (Kim KS et al, 2001). These evidences indicate an important role of androgens in the urethra seam formation.

Abnormalities in the AR and on the androgen biosynthetic pathway have been found in hypospadias (Aaronson IA et al, 1997; Denes FT et al, 2000; Klonisch T et al, 2004; Moisan AM et al, 1999; Sharpe RM, 2006; Silver R et al, 1999). And it is possible that other aspects of the androgen action may also be related with this inborn error of development. One of such aspects is the complex AR regulation. As a steroid receptor, AR's activity is subjected to regulation by co-regulators and general transcription factors, which modulate the transcription of AR target genes. And AR's regulatory molecules can be regarded as possible candidates for androgen related dependent disorders (Hughes IA, 2000).

In fact, hormone-resistance syndromes have already been attributed to disorders of co-regulator proteins. In 1999 MI New et al described two sisters with multiple partial hormone resistance, in which a co-activator defect was proposed as underlying mechanism (New MI et al, 1999). This report was followed by a case of clinical androgen insensitivity described by Adachi et al, 2000, with normal *AR* sequence, but in which an unknown protein that interacts with the AF-1 region was missing (Adachi M et al, 2000). Furthermore, it has been proposed that the dysfunction of one member of the large family of co-regulators is likely to produce mild hormone resistance, since compensatory mechanisms might be activated if one member of these complexes is dysfunctional, a scenario compatible with isolated hypospadias (Hughes IA, 2000). The phenotype of the mice lacking fkbp52 (52KO) supports this concept.

FKBP52, AND THE REPRODUCTIVE PHENOTYPE OF THE 52KO MOUSE

The co-regulator FKBP52, is a 90-kDa heat shock protein (Hsp90)- binding immunophilin found in steroid receptor complexes, which directly controls their transcriptional activity (Pratt WB et al, 1997; Tranguch S et al, 2005; Wochnik GM et al, 2005). Mice lacking all coding exons of *fkbp4* (52KO), the mouse gene encoding fkbp52, were generated in order to assess the physiological importance of this molecular co-chaperone. Surprisingly, 52KO males developed penile hypospadias with 100% penetrance (Cheung-Flynn J et al, 2005; Yong W et al, 2006). Some 52KO mice also showed dysgenesis of anterior prostate and seminal vesicle, infertility and unilateral undescended testis. No abnormalities in testicular histology were observed

in 52KO males; and gross defects in other organs and systems were ruled out. Besides, and contrary to what was expected, no alterations related to other steroid receptors were identified.

Since Fkbp52 is critical to AR transcriptional activity (Cheung-Flynn J et al, 2005), the authors conclude that FKBP52 plays an important physiological role in up-regulating AR activity in some murine male reproductive tissues, mediating the association of the AR with Hsp90, establishing and maintaining hormone binding ability. Therefore, hypospadias can be attributed to the loss of Fkbp52-enhanced AR function during urethral development. Based on the 52KO mouse phenotype, variants of the *FKBP4* sequence and expression could induce hypospadias in humans, underlying defects of the androgen action that are not due to AR gene mutation.

ESTROGEN RECEPTORS, AND THEIR ROLE IN THE MALE REPRODUCTIVE TRACT

Estrogens are steroid hormones, which, through the regulation of the transcriptional activity of the estrogen receptors (ESRs), elicit a myriad of biological responses, with known important roles in development, reproduction, homeostasis, (Ascenzi P et al, 2006; Leung YK et al, 2006) and disease progression (Deroo and Korach, 2006). ESRs, as the AR, belong to the family of nuclear transcription factors. There are two ESR molecules: ESR1 and ESR2, products of separate genes (*ESR1* and *ESR2*) in distinct chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively). These two forms have different affinities and responsiveness, different modulators, different tissue distribution and different gene regulatory sites. However, both share a general domain structure common to ligand-modulated nuclear transcription factors (Figure 5).

ESTROGENS AND THE MALE REPRODUCTIVE TRACT

Traditionally, the male reproductive system was considered to be controlled by both gonadotropins and androgens. Estrogens were, for many years, considered to be the *female hormones*. It was not before the 70ies that testis were acknowledged as a source of estrogens (Kelch et al, 1972). This was followed by evidence of testosterone aromatization into estradiol in the male reproductive tract; as well as of estrogens synthesis in peripheral tissues of normal men (MacDonald et al, 1979; Valladares et al, 1979). More recently, it has been showed that both immature and mature germ cells and spermatozoa are able to produce estrogens (Aquila S, 2002; Carreau S et al, 2001; Carreau S et al, 2002). Further molecular characterization of the ESRs (Evans RM, 1988), together with gene-expression studies on ESR1 (Couse JF et al, 1997) and on ESR2 (Couse JF et al, 1997; Enmark E et al, 1997) have progressively clarified the

relationships between estrogens and male reproductive function, particularly in fertility (Herbst AL et al, 1975; Sharpe RM, 1998; Wilcox AJ et al, 1995).

Active ESRs are found during male reproductive structures (Berensztein EB et al, 2006; Carreau S et al, 2002). In mice, esr1 is abundant in early Leydig cells, as well as in the developing, and mature, efferent ductules. In the seminiferous epithelium and in the epididymis of the male fetus, esr2 expression is higher that esr1, the latter being absent or very low; esr2 is the isoform which prevails in Leydig cells and testis during fetal life, suggesting a major role of esr2 in the development and function of male reproductive structures until birth (Baskin LS et al, 2001b; Couse JF et al, 1999; Dietrich W et al 2003; Jefferson WN et al, 2000).

Functional ESRs are expressed in the human reproductive tract. In the testes, ESR2 immunoreactivity was detected in nuclei of Sertoli and Leydig cells; ESR1 is only expressed in Leydig cells. In the efferent ducts, only ESR2 could be detected. In the prostate, ESR2 is localized in the nucleous of basal and secretory cells in alveoli as well as in stromal cells; no ESR1 is detected (Pelletier G, El-Alfy M, 2000). Similar studies were performed in the penis (Dietrich W et al 2003): ESR2 is strongly expressed in the corpus cavernosum, corpus spongiosum, penile and urethral epithelia; while ESR1 is only found sporadically in the penile and urethral epithelia.

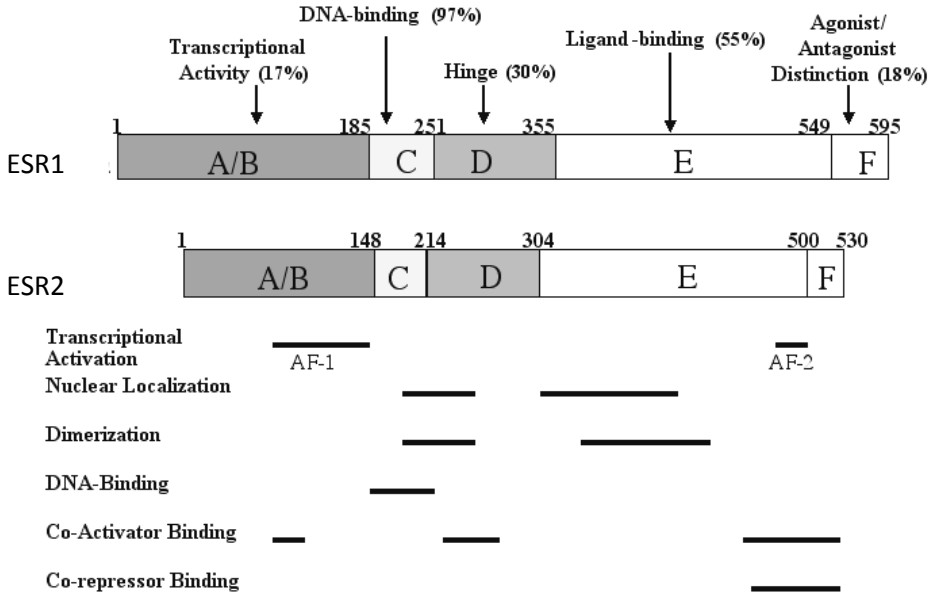

Figure 5 Comparison of domain structures of human ESR1 and ESR2. The estrogen receptors are members of the nuclear receptor superfamily and share a domain structure, which is depicted schematically. The ESRs have six domains, A–F, and the number of amino acids in these domains, as well as the functions associated with these domains, are indicated for each form of ESR. AF-1 and AF-2 refer to regions that mediate the transcriptional activation functions of the ERs (adapted from Ascenzi P et al, 2006; Kuiper GG et al, 1997; and Nilsson S et al, 2002).

ESRs IN THE MALE URETHRAL DEVELOPMENT

The importance of estrogens in male genito-urinary development has been increasingly acknowledged and corroborated by the presence of active ESRs in the developing male urethra. In humans, the ESR2 is expressed ubiquitously in the male external genitalia as early as 9 weeks of gestation; and expression remains intense at 22 weeks, being the predominant subtype. ESR1 expression is found only after 16 weeks of gestation, localized to the squamous epithelium of the distal dorsal urethra and the ventral junction of the glans epithelium and inner prepuce (Shapiro, E, et al, 2006). Moreover functional estrogen receptors localize at the same structures as the androgen receptors in the developing murine male external genitalia, indicating interactions between effects exerted by the two steroid hormones (Crescioli C, 2003).

Studies in both animal models and humans suggest that the inappropriate exposure to estrogens *in utero* and during the neonatal period impairs the urethral development (Herbst AL et al, 1975; Sharpe RM, 1998; Wilcox AJ et al, 1995). An excess of estrogens causes inhibition of cell proliferation in a dose-dependent manner in the male developing urethra (Yucel S et al, 2003). And exposure to synthetic estrogens during pregnancy induces hypospadias in a mouse model (Kim KS et al, 2004). These aspects indicate that estrogens, particularly through ESR2, have an important role in the normal and abnormal development of the male genitalia (Crescioli C, 2003).

ENDOCRINE DISRUPTERS AND DISORDERS OF THE REPRODUCTIVE TRACT

The hormonal dependence of masculinization renders this process inherently susceptible to disruption by factors that interfere with hormone production, bioavailability, metabolism or action (Gray LE Jr et al, 2004; Steinhardt GF, 2004). Several reports indicate that exposure to environmental toxicants, particularly those in the environment and in food, during pre- and post-natal development may be detrimental to the reproductive tract development and function. Examples are pesticides (DDT, methoxycholor, vinclozolin, trichlorfon, atrazine), plastics (phtalates), and a range of xenoestrogens, which directly or after being metabolized, act as endocrine disrupters, and elicit their actions on the developing reproductive structures (Hughes IA et al, 2006; Manson JM et al, 2003).

Xenoestrogens and xenobiotics induce changes in the mRNA expression profile, as well as in the binding response, of estrogen receptors in developing reproductive structures (Thuillier R et al, 2003). Furthermore, estrogenic potency of phytoestrogens is significant, especially for ESR2, and they may trigger many of the biological responses that are evoked by the physiological estrogens (Kostelac D et al, 1993; Kuiper GG et al, 1998).

Exposure to environmental components with estrogenic activity *in utero* induces a range of disorders of the male reproductive tract, such as lower sperm quality, cryptorchidism, hypospadias and testicular cancer (Bay K et al 2006, Delbes G et al, 2006). Boys born of women treated during their pregnancy with DES, a potent estrogenic compound indicated to prevent miscarriages, presented a higher rate of defects of the reproductive tract development. The most frequent structural and functional abnormalities reported were: epididymal cysts, meatal stenosis, microphallus, cryptorchidism and hypospadias (Klip HB et al 2002; Wilcox AJ et al, 1995). These findings were reproduced in laboratory animals, after the exposure to DES during fetal and neonatal live. Moreover, early transplacental administration of DES induces accelerated testicular development, abnormal differentiation of Sertoly cells, hyperplasia of fetal Leydig cells, and changes in the steroidogenic factor-1 in rats (Perez-Martinez C et al, 1996; Majdic G et al, 1997).

Experiments *in vitro* and with animal models have indeed been very illustrative the negative effects of excessive estrogens on testicular development and functions (gametogenesis and steroidogenesis), which is essential to the proper masculinization of the male reproductive tract. Probably the most extreme case is in the marsupial tammar wallaby, in which high doses of estrogens can inhibit early testicular development, and testis determination, causing sex reversal (Coveney et al 2001).

Although total sex reversal is not observed in mammals, estrogens can inhibit the development of gonocytes, Leydig and Sertoli cells in rat fetal testis (Lassurguere J et al, 2003). In mice, the spermatogenic lineage is able to independently respond to estrogens, where it induces ESR2-mediated inhibition of the development, and promotes apoptosis, of male germ cell line (Delbes G et al 2004). Moreover, lowered fertility rates are observed wildlife populations as a consequence of exposure to agents with estrogenic activity (Colborn T et al, 1993).

Estrogens and *estrogen-like* chemicals can also disturb testicular steroidogenesis (Fukuzawa NH et al, 2003), and neonatal exposures induce disturbances of puberty onset (Atanassova N et al, 2000). Furthermore, high doses of estrogenic compounds during fetal life increase the risk to hypospadias and cryptorchidism in laboratory animals (reviewed by Sharpe RM, 2003). These evidences indicate processes which can be affected in mammal male reproduction by the exposure to environmental estrogens.

The overall incidence of disorders of the male reproductive tract in newborns (hypospadias and cryptorchidism), young men (low sperm count and testicular cancer) and older men (prostate cancer), has increased, paralleling an increased exposure to endocrine disrupters (Baskin LS et al, 2001b; Bay K et al, 2006), which suggests common etiological factors. It has been proposed that a maldevelopment of the fetal testis could underlie these disorders, comprising the testicular digenesis syndrome (TDT). The different steps of testis development may be disturbed to a greater or lesser extent by the exposure to endocrine

disruption, and result in digenesis, which will give rise to abnormal hormone production and trigger downstream aberrations of hormone dependent processes (Bay K et al, 2006; Delbes G et al, 2006).

BALANCE BETWEEN ANDROGENS AND ESTROGENS

Not only estrogens and androgens, but also the balance between the two is important to the development of the male genitalia. Estrogens modulate androgen levels and can inhibit testosterone production (Lassurguere J et al, 2003; Tsai-Morris CH et al, 1996). This modulation can occur at multiple levels, including the negative feedback exerted by estradiol on the hypothalamus-pituitary-gonadal axis (Gooren L, 1989; Raven G et al, 2006); or the competition for the same binding protein (Joseph DR, 1994). Furthermore, an excess of estrogens alters the responsiveness to androgens, by down-regulation of AR by proteosome degradation pathway and by interfering with the Leydig cell function and testosterone production （Woodham C et al, 2003).

The co-localization of ESRs and ARs in the developing male urethra and the evidence that estrogens and androgens have a direct effect on the fusion of the urethral folds (Yucel S et al, 2003), proposes that the urethral development depends on a delicate balance between these hormones (Aranda A et al, 2001; Sharpe RM et al, 2006). This hypothesis was circumstantiated by the prevention of the reproductive tract abnormalities induced in DES-treated in male rats when testosterone is co-administrated, demonstrating that the abnormalities induced by estrogenic compounds are, at least partially, a result of a disturbed androgen-estrogen balance (Rivas A et al, 2003). In addition, increased AR mRNA levels have been observed in male GTs as a response to *in utero* ethinyl estradiol exposure, possibly as an adaptive mechanism (Agras K et al, 2006).

GENES VS ENVIRONMENT

If environmental influences are detrimental to the regulation of the hormonal processes during development, ethnic – genetic - differences are also important in the susceptibility to environmental toxicants (Giwercman A et al, 2006; Nabholz CE, von Overbeck J, 2004). Interestingly, association with *ESR2* gene variants has been documented in some *environmental sensitive* male reproductive tract disorders, such as low sperm count (Aschim EL et al, 2005) and prostate cancer risk (Thellenberg-Karlsson C et al, 2006) in Caucasians. Furthermore, gene variants in *ESR2* gene may influence serum levels of testosterone, as individuals with longer (CA)n variants in this gene display lower serum androgen levels (Westberg L et al,

2001). These indications propound that variants in the *ESR* genes could also be involved in the etiology of hypospadias.

ACTIVATING TRANSCRIPTION FACTOR 3, A HORMONE RESPONSIVE GENE

Due to the importance of estrogens and ESRs in human reproduction and in urethral development, estrogen responsive genes can also be regarded as candidates for hypospadias. Indeed, gene profile from twin brothers with and without hypospadias, and comparison of microarray data of normal and hypospadic human tissue, evidence that the estrogen responsive genes *ATF3*, *CTGF* and *Cyr61* are strongly up-regulated in hypospadiac tissue (Wang Z et al, 2007). These findings provide support for the concept that estrogenic responsive compounds have a role in the etiology of hypospadias and their up-regulation may correlate with an increased risk to this disorder of the urethral development.

ATF3 is a member of the ATF/CREB (cAMP responsive element binding) family of transcription factors; it contains a basic region and leucine zipper (bZIP) motif characteristic of the bZIP superfamily of transcription factors, which includes members of the CCAAT/enhancer-binding protein family, the Jun/Fos family and ATF/CREB family (Fan F et al, 2002). Two main isoforms have been found for this gene. Isoform 1, the major and longest isoform, with 2049 bps represses rather than activates transcription from promoters with ATF binding elements. Isoform 2, also called deltaZip2, contains an additional exon in the 3' coding region, which includes a termination codon; this isoform lacks the leucine zipper protein-dimerization motif and it stimulates transcription presumably by sequestering inhibitory co-factors away from the promoter. It is possible that alternative splicing of the ATF3 gene may be physiologically important in the regulation of target gene (Chen BP et al, 1994).

Recently, *ATF3* started being studied in the context of sex development and hypospadias. These studies evidence that ATF3 expression is up-regulated during murine sexual differentiation and that, in GTs of estrogen-exposed mice fetuses, ATF3 mRNA levels are significantly increased (Liu B et al, 2006). Furthermore, Liu B et al, 2005, compared the expression of ATF3 in tissues from 28 children with hypospadias compared and in 20 normal penile skin tissue samples from elective circumcision. Eighty-six percent of the hypospadias samples were immunohistochemically positive, compared with only 13% of normal tissue samples. Moreover, 75% of hypospadias samples were positive from *in situ* hybridization, compared with 1% of circumcision samples. These findings indicate that ATF3 is up-regulated in the penile skin tissues of boys with hypospadias, and suggests a role for this transcription factor in the development of this abnormality.

AIMS

I. **TO ACCESS WHETHER GENE VARIANTS IN THE EARLY DEVELOPMENTAL GENES *FGFR2*, *FGF8*, *FGF10* AND *BMP7* CAN CAUSE OF HYPOSPADIAS**

II. **TO INVESTIGATE IF THE ANDROGEN RECEPTOR'S CO-CHAPERONE FKBP52 IS INVOLVED IN THE ETIOLOGY OF HYPOSPADIAS**

III. **TO EVALUATE THE ROLE OF THE ESTROGEN RECEPTOR GENES – *ESR1* AND *ESR2*- IN HYPOSPADIAS**

IV. **TO EXAMINE WHETHER *ATF3*, AN ESTROGEN REGULATED GENE, IS IMPLICATED IN HYPOSPADIAS**

METHODS

The protocols for the research project have been approved by the Ethics Committee of the institutions where the work was undertaken; and it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Subjects and/or their parents gave informed consent and patient anonymity has been preserved.

PATIENTS AND CONTROLS

For sequence analysis, DNA from boys with non-syndromic hypospadias of different severities, both familial and sporadic cases, and of both Swedish and non-Swedish ethnicities, selected through medical records in Sweden, was analyzed. As control group we used DNA from an anonymous sample constituted of 380 healthy voluntary blood donors at Karolinska University Hospital, Sweden. Genomic DNA was extracted from EDTA-preserved blood, using standard protocols.

For expression analysis, human foreskin samples from prepubertal boys were obtained after surgery at the Karolinska University Hospital. The samples were prepared according to standard procedures. Through collaboration with the Department of Pediatric Urology at the University of Innsbruck, Austria, fetal samples, obtained following miscarriage, with no signs of maceration or macroscopic abnormality were studied. The age of the fetuses was estimated from crown-rump length, as defined by standard protocols. The ano-genital structures were removed en bloc from fresh fetuses and fixed in formalin. The specimens were routinely embedded in paraffin, processed into 4 µm serial transverse sections and dried at 60◦C overnight.

DNA ANALYSIS

PCR

Polymerase chain reaction (PCR) is an enzyme-mediated reaction which is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. Optimized PCR protocols were used: DNA to be amplified has been put in solution with short DNA primers which bind to the 3' ends of the DNA, the 4 nucleotide bases (dNTPs), magnesium chloride, DMSO as needed; and DNA polymerase. In our experiments high-fidelity DNA polymerases working at very high temperature were

used: AmpliTaq Gold®, Applied Biosystems; and Finnzymes - DyNAzyme™ EXT DNA Polymerase, which contains a proofreading enzyme. Normally, about 30 cycles of amplification are carried out, each with three steps 1) DNA is heated to 95^{o} C – the double-stranded DNA denatures; 2) the solution is then cooled to 55^{o} -64^{o} C - primer annealing to template DNA; 3) and then the temperature is raised to 72^{o} and the DNA polymerase causes the synthesis of new complementary single strands in solution - primer extension. The cycling process of PCR is automated and completed in a few hours in a thermocycler. In 30 cycles at 100% efficiency, close to 1 billion copies of targeted DNA region are created.

PCR was carried out in 96 or 384 wells plates, in total volumes of 25 and 5 µl respectively; and blank controls were added (water instead of genomic material) as contamination control.

DHPLC WAVE

Denaturing high-performance liquid chromatography (DHPLC) -WAVE was used for mutation detection and SNP discovery. It is an automated and high-throughput method. The principle of DHLPC's fragment separation lies in the ability to separate two or more chromosomes as a mixture of previously denatured and reannealed PCR amplicons, revealing the presence of a mutation by the differential retention of homo- and heteroduplex. DHPLC was carried out on an automated WAVE instrument equipped with a DNASep column (Transgenomic Inc., Omaha, NE). Column temperature was selected for optimal heteroduplex separation, using the WAVERMAKER 3.4 software.

Temperature determines sensitivity; and its optimum for each DNA fragment has been predicted by computation. The DNA is eluted from the column as an increasing concentration of acetonitrile flows across the matrix. Heteroduplexes formed in heterozygous samples will elute before homoduplexes, appearing as two or more peaks in electropherograms, instead of only one. Single-nucleotide substitutions, deletions, and insertions have been detected successfully by on-line UV or fluorescence monitoring within 2-3 minutes in unpurified amplicons as large as 1.5 Kb. Sensitivity and specificity of DHPLC consistently exceed 96%. (Xiao W et al, 2001).

DHPLC was carried out on an automated WAVE instrument equipped with a DNASep column (Transgenomic Inc., Omaha, NE). Column temperature was selected for optimal heteroduplex separation, using WAVERMAKER 3.4 software. The reactions were carried out in 96 well plates, and each well contained denaturated PCR product. Direct sequence analysis was used to confirm DHPLC results.

SEQUENCING

Direct capillary DNA sequencing of PCR products became the method of choice for sequence analysis, allowing unambiguous identification the target sequence, the detection of allele variants and mutations, resolution of difficult heterozygous combinations, and recognition of new alleles. Direct sequencing was performed using BigDye Terminator v3.1. sequencing kit (Applied Biosystems Foster City, CA). This method essentially involves amplifying a single stranded piece of DNA many times. Normally, when DNA is amplified, new deoxy-nucleotides (dNTPs) are added as the strand of DNA grows. Special bases called dideoxy-nucleotides (ddNTPs) are added to the reaction. These are similar to dNTPs, except for two important differences: they have fluorescent tags attached to them (a different tag for each of the 4 ddNTPs) and are missing a crucial atom that prevents new bases from being added to a DNA strand after a ddNTPs has been added. Thus, once a ddNTP is inserted into a growing DNA strand, synthesis of that strand is stopped (BigDye Terminators). After many repeated cycles of amplification, all the possible lengths of DNA are represented and every piece of synthesized DNA containing a fluorescent label at its terminus.

To increase the sequencing quality, PCR product cleaning was performed before every sequence reaction using ExoSAP-IT, which removes any unconsumed dNTPs and primers remaining in the PCR mixture by utilizing two hydrolytic enzymes, exonuclease I and shrimp alkaline phosophatase. Thereafter, sequencing reaction was carried out in 96 well plates, each with 2µl of cleaned PCR product and 3pmol of the sequencing primer were used in a 20µl sequencing reaction. The sequencing reaction mixes were subjected to 25 cycles in a thermal cycler. Each cycle consisted of 10'' 95°C, 5'' 50°C and 4' 60°C. Capillary sequencing was performed in ABI Prism 3730 Sequencer (PE Applied Biosystems,), with post analysis with the program SeqScape v2.5 (Applied Biossystems). The primers used for sequencing were the same as for the PCR reaction; and bidirectional sequencing was performed.

SIZE FRAGMENT ANALYSIS

Microsatellites (sometimes referred to as a variable number of tandem repeats or VNTRs) are short segments of DNA that have a repeated sequence, such as (CA)n and (TA)n in *ESRs*, found in most genomes; and they tend to occur in non-coding DNA. These polymorphisms show exceptional variability, making them the genetic marker of choice for the number of applications, including the analysis of genetic structure and parentage testing, and to investigate evolutionary links between species and populations. Microsatellites are particularly useful as markers in genetic mapping.

Microsatellite analysis was carried out by PCR amplification of the microsatellite loci, using fluorescently labeled primers; followed by analysis detection of the alleles according to size (Dearlove AM, 2002) on an ABI Prism 3730 Analyzer, a capillary electrophoresis platform that provides accurate size calling, consistent band intensities and lower run-to-run migration variations than ever before. The Applied BioSystems 3730 DNA Analyzer has the ability to determine the size of DNA fragments by using a fluorescence-based detection system. As the DNA migrates through the detection cell, the 48 capillaries are simultaneously illuminated from both sides of the array by an argon-ion laser. A fluorescent size standard in each capillary eliminates variability. The fluorescent emissions are spectrally separated by a spectrograph and focused onto a charged couple device, which are then converted to digital information that is processed by the collection software, the GeneMapper™ Version 3.7 software. The reactions were carried out in 96 well plates, and each well contained 1µl of PCR product, 0.5µl of size standard and filled to 10µl with hi-Di formamide.

SNP TYPING/ ALLELE DISCRIMINATION BY TAQMAN

Allelic discrimination using the TaqMan 5' Nuclease Assay with fluorogenic probes provides a rapid and sensitive method for detecting known mutations or polymorphisms. This method combines PCR and mutation detection in a single step. A hybridization probe is cleaved by the 5' nuclease activity of Taq DNA polymerase only if the specific sequence is successfully amplified. Two TaqMan (PE Applied Biosystems) probes are used, one for each allele. Each probe consists of an oligonucleotide with a 5' reporter dye and a 3' quencher dye. The reporter dyes used are FAM and VIC; and TAMRA is used as the quencher dye. Initially, the proximity of the quencher suppresses the fluorescent signal given by the reporter. The TaqMan probe hybridizes to a smaller 20- to 24mer sequence, which includes the polymorphism. AmpliTaq Gold (PE Applied Biosystems) enzyme then cleaves the probe with its 5'-3' nuclease activity. Thus, the reporter dye and quencher dye become separated, causing an increase in the fluorescence intensity of the reporter dye. This florescence can then be detected to discriminate the alleles of each sample. If there is fluorescence from the VIC reporter for the wild-type allele, then the sample is typed as a homozygous for the wild-type allele; fluorescence from only the FAM reporter represents the homozygosity for the mutant allele; intermediate fluorescence from both reporters represents the heterozygous population (Shi MM, 2002). Reactions were conducted using standard protocols in 384 well plates.

EXPRESSION ANALYSIS

IMMUNOHISTOCHEMISTRY

IHC is a widely used method to localize proteins in cells of a tissue section, exploiting the principle of antibodies binding specifically to antigens in biological tissues. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as FITC, rhodamine, or Texas Red.

DIRECT AND INDIRECT IMMUNOHISTOCHEMISTRY

There are two strategies used for the immunohistochemical detection of antigens in tissue, the *direct* method and the *indirect* method. The direct method is a one-step staining method, and involves a labeled antibody reacting directly with the antigen in tissue sections. While the procedure is simple and rapid, it is in less common use than indirect methods because it can suffer problems with sensitivity due to little signal amplification.

The indirect method, used in the ATF3 and FKBP52 studies, involves an unlabeled primary antibody (first layer) which reacts with tissue antigen, and a labeled secondary antibody (second layer) which reacts with the primary antibody. (The secondary antibody must be against the IgG of the animal species in which the primary antibody has been made). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labeled with a fluorescent dye or an enzyme.

ANTIBODIES

The antibodies used in the ATF3 and FKBP52 studies were polyclonal antibodies, obtained commercially. Polyclonal antibodies are made by injection of animals with peptide antigens together with an immuno-stimulus, after which antibodies from whole serum can be isolated. Thus, polyclonal antibodies are a heterogeneous mix of antibodies that recognize several epitopes of the same protein.

TISSUE PREPARATION

Genital foreskin samples obtained after surgery and genital fetal tissue recovered following miscarriage, were analyzed for FKBP52 or for ATF-3 expression. In the case of FKBP52, human prostate was used as positive control. Prostate tissue was obtained from a patient surgically treated for benign prostatic hyperplasia. The tissues were fixed for 5 to 7 hours in 4% formalin, washed 10 minutes in PBS four times and kept in 70% EtOH until paraffin embedding.

PROCEDURE

The paraffin sections were dewaxed, rehydrated and treated for antigen retrieval by heating at 98°C in 0.1M Tris ph 9.0 for 20 minutes. The slides were allowed to cool to room temperature. Endogenous peroxidase activity was blocked by treating the sections with 1M H_2O_2 for 15 minutes in dark. To block nonspecific antibody binding, sections were pre-incubated in 10mg/ml BSA (Sigma) containing 10% goat non-immune serum (Vector) for 40 min. Affinity-purified polyclonal anti-FKBP52/ anti-ATF-3 was applied to the sections at a 1:100 dilution in 10mg/ml BSA and allowed to incubate at 4°C overnight in a humid chamber. Control sections were incubated without primary antibody. The sections were incubated with the biotinylated secondary goat anti rabbit antibody (Vector) diluted in buffer containing 1% BSA and 10% goat serum followed by enzyme conjugate application (ABC, Vector) and chromogen development (AEC, Vector). All sections were counterstained with hematoxylin (Merck) for 15 sec before mounting in Kaiser's glycerin gelatin (Merck). Images of immune-stained tissues were captured using a Zeiss microscope. Background controls were performed similarly using only the secondary antibody.

GENOTYPING AND GENETIC STATISTICS

POLYMORPHISMS SELECTION

In the study of FKBP52, two SNPs in the *FKBP4* gene were selected from the dbSNP Homepage at NCBI: 1) the SNP rs1062478 (His> Arg), the only non synonymous polymorphism in the gene with known frequency (heterozygozity of 0.039); and 2) the intronic SNP rs3021522 (C>G) (heterozygozity of 0.109), the only polymorphism that was found by sequencing among the initially 91 screened patients. In the *ESR2* gene, four haplotype-tagging SNPs from the dbSNP database (htSNP: rs2987983, rs1887994, rs1256040, and rs1256062) have been chosen, as they capture 99.6% of the haplotype variation in the Swedish population

(Thellenberg-Karlsson C et al, 2006). Two additional SNPs (rs10483774 and rs1271572) were added, since they map to a region of predicted intense transcription factor binding in the putative promoter region. The (CA)n repeat polymorphism was also included in this study. In *ATF3*, eight haplotype-tagging SNPs were selected from the dbSNP Homepage at NCBI using a tagger SNP software, *Haploview* version 3.32 (Barret et al, 2005), with the HapMap data for Caucasian population data, with a LOD cut-off can of 2.0 and a r^2 >0.8, including SNPs with a minor allele frequency of 2% (Table 2).

GENOTYPING

SNP typing was performed with a 5'-nuclease TaqMan assay together with fluorescently labeled probes using standard protocols (Applied Biosystems). The samples were analyzed on an ABI 7900HT. The lengths of the (CA)n repeat were analyzed in an ABI 3730 DNA analyzer (Applied Biosystems). The lengths of the (CA)n containing fragments were ascertained by two different PCRs.

STATISTIC ANALYSIS

After DNA analysis, genotyping quality was tested by checking the conformance with Hardy–Weinberg equilibrium with *Haploview* version 3.32 (Barret et al, 2005). All gene variants were in Hardy–Weinberg equilibrium.

To identify potentially associated polymorphisms, we performed global tests of allele and genotype frequency differences among the cases and the controls. We initially performed χ^2 and Fisher exact tests. Odds ratios (OR) and OR's 95% confidence intervals (OR 95% CI) were estimated. The analysis was conducted using standard statistic packages. Further single locus and multi-marker haplotype association tests were performed with two different softwares, *Haploview* (Barret et al, 2005) version 3.32 and *Unphased* (Dudbridge F, 2003) version 3.0.3, thus providing complementary validity to our results. The calculations were performed both with and without missing data estimation. The threshold for rare haplotypes frequency identification was 0.005. The p-values were corrected for multiple testing by performing permutations as follows: The trait values were randomly shuffled between subjects 1000 times. In each permutation the minimum p-value is compared to the minimum p-value observed in all the analyses in the original dataset. Pair wise linkage disequilibrium (LD) between polymorphisms was measured by estimating D', (the normalized disequilibrium coefficient, which ranges from 0 to 1); and the squared correlation coefficient r^2. Hardy–Weinberg (HW) equilibrium of the genotype frequencies at each SNP in controls and cases was accessed with *Haploview* version 3.32.

To determine whether the associated risk gene variants were independent, logistic regression analysis was performed on the SAS computer program (SAS Institute Inc, Cary, NC) where the *proc logistic* command was used. This resulted in a standard logistic regression model in which the regression coefficients were logarithms of the odd ratios. Models were compared using the log likelihood ratio test which approximates to a $\chi 2$ distribution with 1 df (the difference in number of factors in the models). Initially, different genetic models for each marker against each other were tested (dominant, recessive and co-dominant models). The analysis presented in the table 4 only includes data for the best model (co-dominant). In order to get OR estimates for the different genotypes, variables were re-coded into design variables.

BIOINFORMATICS AND COMPUTATIONAL BIOLOGY

Bioinformatics and computational biology are multidisciplinary subjects that integrate information and computer technology with biotechnology and biological sciences. The main functions of these tools consist in the recording, annotation, storage, and search/retrieve of nucleic acid sequence (genes and RNAs), protein sequence and structural information; as well as to provide reliable computational interpretation of a wide range of experimental data, and the detailed understanding of the molecular apparatus behind cellular mechanisms of sequence information.

These tools were of great importance for this work: for sequence identification; to design primers; to predict the function and structure of proteins and to investigate how sequence variants could change that function and structure; to predict the promoter sequences, and to identify transcription factor binding spots; to compare the sequence of different organisms and to calculate the homology between those sequences; to search for known mutations in the gene; to analyze data or consult and retrieve biological information. For each task, different databases and bioinformatics resources were used, in order to obtain a more accurate prediction.

The next paragraphs present a brief description of the used genetic databases and of the available bioinformatics and computational tools.

GENETIC DATABASES

A biological database is a large, organized body of persistent data, usually associated with computerized software designed to update, query, and retrieve components of the data stored within the system. As an

easy method to access to the information, it became an essential resource for life sciences. Different databases serve different purposes.

PRIMARY DATABASES

Primary databases are the primary data repositories, usually type specific (e.g. sequence, microarray data, SNPs), freely available to the community. The data is usually not deeply integrated with other biological knowledge or attributes. Examples of these databases are:

- **The GDB Human Genome Database** is the official central repository for genomic mapping data resulting from the Human Genome Initiative.
- **GenBank:** The National Institutes of Health (NIH)'s genetic sequence database, an annotated collection of all publicly available DNA sequences
- **EMBL:** The European Molecular Biology Laboratory Nucleotide Sequence Data Library constitutes Europe's primary nucleotide sequence resource. Main sources for DNA and RNA sequences are direct submissions from individual researchers, genome sequencing projects and patent applications.

CURATED DATABASES

Curated databases are derivative databases, which often provide added value to data from repositories by eliminating redundancy, providing standard nomenclature, and often serving specific research communities. Examples of these databases are:

- **NCBI:** The National Centre for Biotechnology Information created a public database, with access to multiple resources: OMIM, Human Genome Resources, Entrez Gene, Search BLAST using Sequence data, BLAST against mouse trace data, Search PubMed, Search Nucleotide Database, Search Protein Database, Unigene Database, as well as other. Its Reference Sequence (RefSeq) collection is of particular interest as it provides a comprehensive, integrated, non-redundant set of sequences, including genomic DNA, transcript (RNA), and protein products, for major research organisms.
- **SWISS-PROT/TrEMBL:** The SWISS-PROT database contains high-quality annotation, is non-redundant and cross-referenced to many other databases. Together with UniProtKB/TrEMBL, it constitutes the UniProt Knowledgebase, one component of the **Universal Protein Resource (UniProt)**, allowing easy access to all publicly available information about protein sequences. It links to the **E**xpert **P**rotein **A**nalysis **Sy**stem (ExPASy).

- **PDB-Protein Data Bank:** Provides a variety of tools and resources for studying the structures, determined by X-ray crystallography and NMR, of biological macromolecules and their relationships This site offers tools for browsing, searching, and reporting that utilize the data resulting from ongoing efforts to create a more consistent and comprehensive archive.
- **MGI:** The Mouse Genome Informatics provides integrated access to data on the genetics, genomics, and biology of the laboratory mouse.

GENOME BROWSERS

Genome browsers are another type of databases, designed for graphical display of genome content; and to relate genome data from multiple sources. Examples of these databases are:

- **ENSEMBL:** A joint project between EMBL- European Bioinformatics Institute (EBI) and the Wellcome Trust Sanger Institute (WTSI) to develop a software system which produces and maintains automatic annotation on selected eukaryotic genomes. The Ensembl project provides accurate, automatic analysis of genome data.
- **UCSC Genome Browser:** This site contains the reference sequence and working draft assemblies for a large collection of genomes. This tool has a number of very useful features. The Genome Browser zooms and scrolls over chromosomes, showing the work of annotators worldwide. The Gene Sorter shows expression, homology and other information on groups of genes that can be related in many ways. Blat quickly maps your sequence to the genome. The Table Browser provides convenient access to the underlying database. VisiGene provides a large collection of *in situ* mouse and frog images to examine expression patterns. Genome Graphs allows to upload and display genome-wide data sets.

SHIFTED DATABASES

Another type of databases is the shifted databases, which combine information from different highly curated databases into new views of the data or biological knowledge. The best well known examples are the:

- **GeneCards**, an integrated database of human genes that includes automatically-mined genomic, proteomic and transcriptomic information, as well as orthologies, disease relationships, SNPs, gene expression, gene function, and service links for ordering assays and antibodies.; from the Weismann Institute of Science, Rehovot, Israel.

- **GENATLAS** gene databases, contains relevant information with respect to gene mapping and genetic diseases. The application is developed and maintained at the Paris René Descartes University, Paris, centre of bioinformatics. GENATLAS is structured in three databases: Genes database, Phenotypes database, Citations database. Information about a gene/ protein/ phenotype is links to other databases, such as NCBI and ENSEMBL.

COMPUTATIONAL BIOLOGY TOOLS

A large range of bioinformatic tools are available freely online. The most commonly used tools in this thesis work are presented below.

SEQUENCE ANALYSIS AND STRUCTURE PREDICTION TOOLS

- **BCM search launcher**: The Baylor College of Medicine Search Launcher is an on-going project to organize molecular biology-related search and analysis services available on the WWW with a single point-of-entry for related searches. It provides several tools, such as multiple sequence alignment, molecular biology-related search and analysis services on the web.
- **CBS Prediction Servers**: Provided by the Centre for Biological Sequence Analysis at the Technical University of Denmark, includes whole genome visualization and analysis, gene finding and splice sites, analysis of DNA microarray data, protein sorting, post-translational modifications, immunological features, protein function and structure
- **ExPASy-Tools**: The ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server of the Swiss Institute of Bioinformatics (SIB) is dedicated to the analysis of protein sequences and structures. It performs DNA to Protein similarity searches, protein identification and characterization, topology prediction, post-translational modification prediction, protein structure analysis (Incl. PHRYE), sequence alignments and phylogenetic analysis.

PROMOTER AND SPLICING PREDICTION AND ANALYSIS PROGRAMS

- **RAVEN- Regulatory analysis of Variation in Enhancers**: Created at CGB, Karolinska Instituted, RAVEN search for putative regulatory genetic variation in selected genes. SNPs (from dbSNP and user defined) are analyzed for overlap with potential transcription factor binding sites and phylogenetic foot printing using human and mouse sequences.

- **GeneID- gene prediction tools:** a program from the Grup de Recerca en Informàtica Biomèdica Universitat Pompeu Fabra, Barcelona, that predict genes in anonymous genomic sequences designed with a hierarchical structure.
- **NNSPLICE**: splice site prediction through the analysis of the structure of donor and acceptor sites using a separate neural network recognizer for each site. Accessible at Berkeley Drosophila Genome Project web page.
- **Genscan**: Developed GENSCAN was developed in the Department of Mathematics, Stanford University, predicts exon-intron structures of genes in several organisms.
- **Primer3:** A widely used program for designing PCR primers, developed at Whitehead Institute and Howard Hughes Medical Institute, many different input parameters and options that can be controlled.

HOMOLOGY PREDICTION

- **HomoloGene**: One of NCBI's resources; it offers a system for automated detection of homologues among the annotated genes of several completely sequenced eukaryotic genomes.
- **ClustalW**: Produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylograms.

SNP SEARCH AND ASSOCIATION STUDIES SETUP

- **Genetic Association Database**: An archive of human genetic association studies of complex diseases and disorders.
- **dbSNP**: public-domain archive for a broad collection of simple genetic polymorphism, incorporated into NCBI's system.
- **HGVbase**: Provides an accurate, high utility and fully comprehensive catalogue of normal human gene and genome variation, useful as a research tool to help define the genetic component of human phenotypic variation. All records are curated and annotated, ensuring maximal utility and data accuracy.
- **HapMap**: all processed data from the HapMap project.
- **Packages for deriving haplotype tagging SNPs**: Haploview, SNPtagger, ClusTag, HapBlock, htSNP, htSNPer, Tagntell.

RESULTS

In order to explore each one of the aims of this thesis, a series of analysis were performed. In this section, the results of these analyses are presented.

I. STUDY OF *FGFR2*, *FGF8*, *FGF10* AND *BMP7* IN BOYS WITH HYPOSPADIAS

The analysis of the DNA from 60 patients with non-syndromic familial hypospadias, of different severities, revealed several gene variants in the *FGF8* and FGFR2 genes (Figure 6).

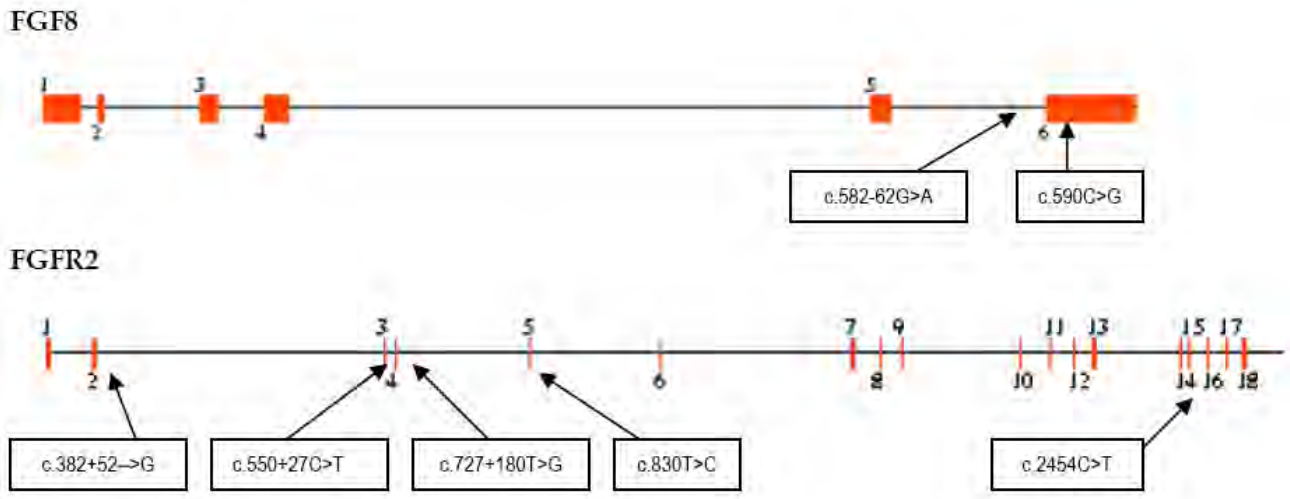

Figure 6: Gene variants in *FGF8* and *FGFR2* detected in our hypospadias patients.

The analysis of the *FGF8* sequence (NM_033164; Ensembl Transcript ID: ENST00000344255) revealed, in 4 Swedish patients, two heterozygous sequence variants in the segment constituted by the exon 6 and adjacent intronic sequences. Three of those boys presented the polymorphism rs3218238 (c.582-62G>A/IVS6-62G>A). This polymorphism was not detected in a control population of 96 individuals, thus resulting in a statistically significant difference. Another patient of Swedish origin presented a synonymous c.590C>G sequence variant, which was not present among controls. This gene variation may represent a private mutation.

Analysis of the *FGFR2* (NM_022976, Ensembl Transcript ID: ENST00000358487) sequence revealed several single nucleotide variations found in Swedish patients: rs755793 (p.Met186Thr), c.382+52->G (IVS2+52->G),

c.550+27 C>T (IVS3+27C>T), c.727+180 T>G (IVS4+180 T>G) and c.2454C>T. None of them were found in the control population. The *FGFR2* gene variant c.727+180 T>G (IVS4+180 T>G) was present in 3 non-related boys (P<0,05). The variants c.382+52->G, c.550+27C>T, and c.2454C>T, found uniquely in unrelated boys, may represent private mutations. None of these sequence variants is predicted to cause any splicing alteration or protein structure modifications by the previously mentioned methods. Other gene variants were found in this gene: rs1047100, rs4647915, rs1047101, rs3135802, rs1613776, rs3135806 and c.2575+15C>T (IVS17+15C>T); but all are in the same frequency as the control population.

The SNP c.830T>C, which converts the amino acid 186 from a hydrophobic (Methionine) to hydrophilic (Threonine) amino acid, was the only non-synonymous polymorphism found in *FGFR2* in our cohort. The affected patient presented a mid-penile type of hypospadias. This gene variant is located in the Ig-like C2-type 1 extracellular domain of the protein (UniProtKB/Swiss-Prot entry P21802), a crucial element to ligand binding functions. However, neither homology nor protein structure predictions suggest any functional implications of this gene variant.

No sequence variants were found in *BMP7* or in *FGF10*.

II. ANALYSIS OF THE *FKBP4* SEQUENCE AND EXPRESSION IN HYPOSPADIAS

SEQUENCE ANALYSIS

The 10 exons and intronic/exonic borders of *FKBP4* (NM_002014.2, Ensembl Transcript ID: ENSP00000001008), were bi-directionally sequenced in 93 boys with hypospadias, both sporadic and familial cases, from different ethnical groups, and with several phenotypic severities. No mutation was found. The intronic sequence variation rs3021522 (c.915C > G), in intron 6, was detected in five boys with hypospadias.

Typing of rs3021522 was performed in 333 (including the initial 91) non-syndromic hypospadias patients and 380 controls. The SNP rs1062478, which converts the aminoacid Histidine, a polar and weakly basic aminoacid, into Arginine, a polar and strongly basic aminoacid, and the only reported non-synonymous SNP in this gene, was also typed in the same case and control groups. Genotyping yielded a 95% success rate. Analysis of allele and genotype frequencies in cases and controls evidenced no genotype and allele frequency differences between the two groups.

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We looked at the expression of FKBP52 in human penile foreskin from hypospadias patients and controls (operated for circumcision). As in the mice, FKBP52 shows expression in luminal epithelial cells of human prostate, which was used as positive control.

FKBP52 immuno-staining is observed in the epidermis of prepubertal male genital foreskin. Some staining is also observed in cells in the dermis, mostly consisting of fibroblasts and smaller blood vessels. Similar to the prostate where FKBP52 and AR are co-expressed in luminal epithelial cells, AR is also observed in the epidermal region of the foreskin, mostly localized to nuclei whereas FKBP52 staining is predominantly cytoplasmic. We could not observe any apparent differences in the level of FKBP52 staining between mild or severe hypospadias patients. Besides, the healthy control individuals show similar pattern and intensity of FKBP52 expression in the epidermal and dermal region.

III. EVALUATION OF THE ESTROGEN RECEPTOR GENES-*ESR1* AND *ESR2*-IN HYPOSPADIAS

MUTATION SCREENING IN ESR1

No coding mutations were found in a set of 60 boys with hypospadias in *ESR1* (NM_000125.2, Ensembl Transcript ID: ENST00000206249). The SNP rs1801132 (1337G>C), located in exon 4, was detected in this patients' group; this SNP was typed in 30 additional patients and a control population of 96 healthy individuals. The heterozygozity was 0.44 in patients and 0.46 in controls, similarly to data published in the public databases. No significant different was detected between cases and controls (X^2=0; p>0,05).

(TA)N IN ESR1

Forty-five patients and forty-five controls were analyzed. The two groups have normal distribution and similar variances. The length of the segment containing the (TA)n repeat polymorphism in *ESR1* ranged from 181 to 217 base-pairs, and no statistical difference was found between the two groups (T tested for paired samples; normal distribution verified; P>0,05). The results were similar to those published by Westberg et al, 2001.

MUTATION SCREENING IN ESR2

No coding mutations were found in *ESR2* (NM_001437.2, Ensembl Transcript ID: ENST00000341099), in a same set of 60 boys with hypospadias. However, a sequence variation rs944050 in the 4th base pair upstream of exon 9 was detected in 3 unrelated boys. Therefore 30 additional patients were analyzed, together with a control population of 96 individuals. Among the 90 tested patients, the sequence variation was found in six individuals with a familial type of hypospadias, five from Sweden and one of Iranian origin. Three of them had moderate to severe hypospadias; the other three presented a mild, glandular hypospadias. Only one individual from the control population presented this variation (P<0,05).

(CA)N IN ESR2

Forty-five patients and forty-five controls were analyzed. The two groups have normal distribution and similar variances. The patients' group present longer alleles than controls (Figure 7: T-test for paired samples; normal distribution verified; P<0,05).

FURTHER STUDIES ON ESR2

To clarify the effect of *ESR2* gene variants on the risk to hypospadias, the genotypes from 354 boys with non-syndromic hypospadias and 380 healthy voluntary blood donors was analyzed. Initially, 4 haplotype tagging SNPs, rs2987983, rs1887994, rs1256040, rs1256062 (Thellenberg-Karlsson C et al, 2006) and the (CA)n repeat polymorphism were typed. All loci were in HWE. Single marker analysis revealed significant association with the SNP rs2987983 ($p<0.01$), in the putative promoter region. And association with the (CA)n repeat polymorphism ($p<0.02$), in intron 6 was confirmed. The estimated p-values after correction for multiple comparisons with 1000 permutations remain below 0.05.

The comparison of (CA)n lengths in patients and controls, with a T-test assuming equal variances, confirmed that longer variants are more common in patients (Figure 8), yielding a p-value < 0.002. Using the fragment length of 159 bp (Westberg L et al, 2001) as threshold above which a fragment is considered long (fragments <159bp S= short; fragments ≥159bp L= long), the L allele is more common among patients than in controls, with an OR of 1.43 (OR 95% CI 1.1-1.8), $p<0.004$. The comparison of SS vs SL vs LL genotype frequencies of the (CA)n polymorphism in patients and controls reveals that the LL genotype is more common among patients than controls with an OR for the LL genotype of 1.48 (OR 95% CI 1.1-2.0), $p<0.02$.

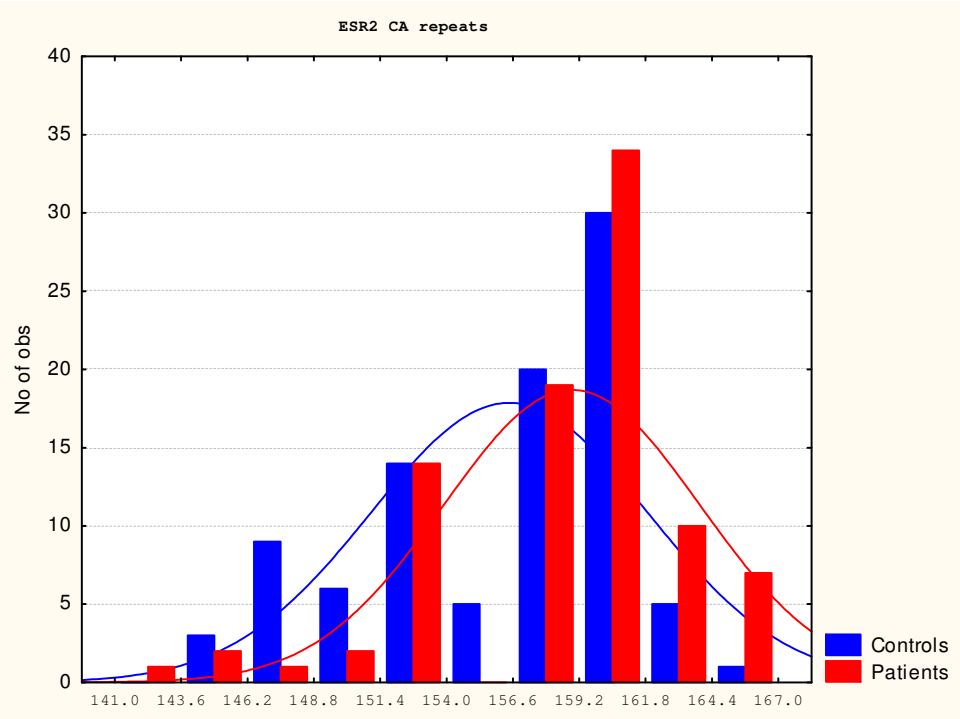

Figure 7: Initial *ESR2* study: Distribution on (CA)n length across the patients and controls (Controls: N = 45, Mean: 156,4; Patients: N = 45, Mean: 158,8). T value assuming equal variances: 2,873 (confidence interval of 95%); p value<0,01.

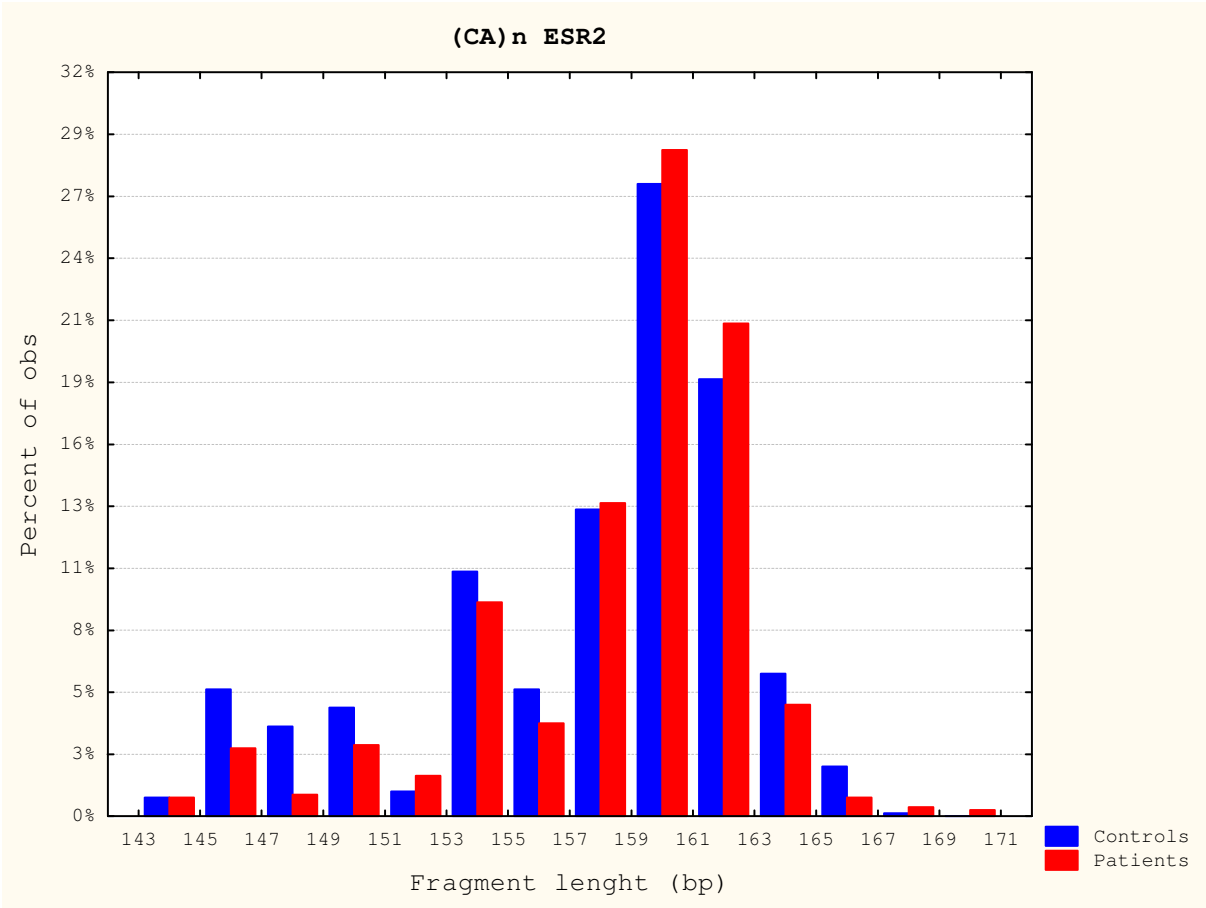

Figure 8: Further studies on *ESR2*: Distribution on (CA)n length across the patients and controls (Controls: N = 380, Mean: 158.9; Patients: N = 354, Mean: 159.7). T value assuming equal variances = -3; p<0.002.

MARKER		PATIENTS VS CONTROLS					Statistics
		ALLELES		GENOTYPES			
		T	C	TT	CT	CC	
RS2987983	P	435	261	142	151	55	p-VALUE <0.01 OR (CC) 1.96 (OR 95% CI 1.2-3.1)
	C	526	228	182	162	33	
		A	G	AA	AG	GG	
RS10483774	P	671	13	329	13	0	p-VALUE <0.01 OR (AG) 7.47 (OR 95% CI 2.1-27)
	C	756	2	378	2	0	
		G	T	GG	GT	TT	
RS1271572	P	370	288	107	156	66	N.S.
	C	401	355	106	189	83	
		G	T	GG	GT	TT	
RS1887994	P	611	53	279	53	O	N.S.
	C	683	59	312	59	4	
		T	C	TT	TC	CC	
RS1256040	P	676	293	105	151	71	N.S.
	C	414	342	112	190	76	
		S	L	SS	SL	LL	
(CA)N	P	170	510	20	130	196	p-VALUE <0.02 OR (LL) 1.48 (OR 95% CI 1.1-2.0)
	C	241	505	43	155	175	
		A	G	AA	AG	GG	
RS1256062	P	589	99	258	73	13	N.S.
	C	658	100	286	86	7	

Table 2: Patients (P) vs. Controls (C) study for single locus analysis: Three of the studied polymorphisms in ESR2 associated with hypospadias: the C allele and the CC genotype in rs2987983, in the *ESR2* promoter; the G allele and the AG genotype in rs10483774, in ESR2 promoter; and longer (CA)n in intron 6. The estimated p-value after correcting for multiple comparisons by performing 1000 permutations remains below 0.05. NS= Not significant.

The C allele and the CC genotype in the promoter SNP rs2987983 are associated with hypospadias, with an OR of 1.38 (OR 95% CI 1.1-1.7) and of 1.96 (OR 95% CI 1.2-3.1) respectively, yielding p-values < 0.01 (Table 2). To further study the promoter region, we performed analysis to detect SNPs that affect transcription factor binding sites. The analysis revealed that the SNP rs2987983 is located in a region of intense transcription factor binding, mapping to two other SNPs, rs1271572 and rs10483774. We typed these two additional SNPs, and observed that the G allele of rs10483774 in the *ESR2* promoter is also associated to hypospadias with an OR for the AG genotype of 7.47 (95% CI 2.1-26.8), p<0.01. However, this SNP is very uncommon and therefore much less informative than SNP rs2987983. Since the two are in LD (D' of 1), we focused our analysis on the latter. No association was found with the SNP rs1271572.

Global D' for all tested polymorphisms is 0.846. This high value is mostly due to a high LD between the SNPs rs2987983 and rs1256040, defining an approximately 1700 bp long haplotype block. LD is much lower to and between the remaining SNPs. D' between rs2987983 and (CA)n is low (0.35), indicating that these two risk polymorphisms in *ESR2* gene are not necessarily inherited together as a haplotype.

Combined analysis of the SNP rs2987983 and the (CA)n polymorphism shown that the combination C-L is over-represented in patients ($p<0.01$), while T-S is over-represented in controls ($p<0.01$). Furthermore, the OR in the presence of the two risk genotypes (C/C)–(L/L) (38 patients vs 20 controls) is of 1.93 (OR 95% CI 1.1-3.2), $P<0.01$, when compared with any other genotype. To test if the risk effects of the SNP rs2987983 and the (CA)n repeat polymorphism were independent from each other, we used logistic regression analysis. For both the rs2987983 SNP the (CA)n, the co-dominant model performed better than the other tested models. We therefore used co-dominant coding to compare the fit of models containing the rs2987983 SNP and the (CA)n polymorphism.

Three models were tested, one with only rs2987983, one with only the CA microsatellite and one with both polymorphisms. The model containing only the (CA)n data had the lowest p-value ($p<0.003$), followed by the one with both polymorphisms ($p<0.004$), the worst fit was observed for the model containing only rs2987983 ($p<0.03$). The SNP rs2987983 is not independently associated when the CA microsatellite is included in the model (Table 5). The significance of a model containing rs2987983 and the (CA)n is significantly better ($p<0.02$) than the significance of a model only containing rs2987983, while a model containing the CA repeat is not improved significantly by adding the rs2987983 marker.

The results suggest that the two polymorphisms are not independently associated; of the studied polymorphisms the primary risk factors for hypospadias in the ESR2 sequence is a longer length of the (CA)n polymorphism.

IV. ANALYSIS OF SEQUENCE AND EXPRESSION OF ATF3 IN HYPOSPADIAS

MUTATION SCREENING

We started the study of the *ATF3* gene by performing sequencing of its coding sequence in 93 unrelated boys with hypospadias. Both sporadic and familial cases, mild to severe phenotypes, and boys from different ethnical groups were included. Several genetic variants were found in this screening.

A missense mutation in exon 3, c.536A>G, causing an aminoacid substitution, Arg90Gly, was found in one Swedish boy with a sporadic, moderate to severe form of hypospadias. An 3'UTR gene variant, c.817C>T, was found in 2 unrelated patients, one Swedish patient with a sporadic form of hypospadias, and one boy from Middle East with a familiar form, both moderate to severe phenotypes. These gene variants were not found in the control population.

Homology analysis of the ATF3 mRNA revealed that the 536A>G gene variant is located in a highly conserved locus. Furthermore, protein structure prediction suggests that this variant induces a change in the protein structure (Figure 9). These evidences indicate that the variant 536A>G is likely to have a pathogenic consequence. Analysis of the c817C>T locus has shown that is not conserved. However, is predicted to be a highly regulatory sequence (data not shown).

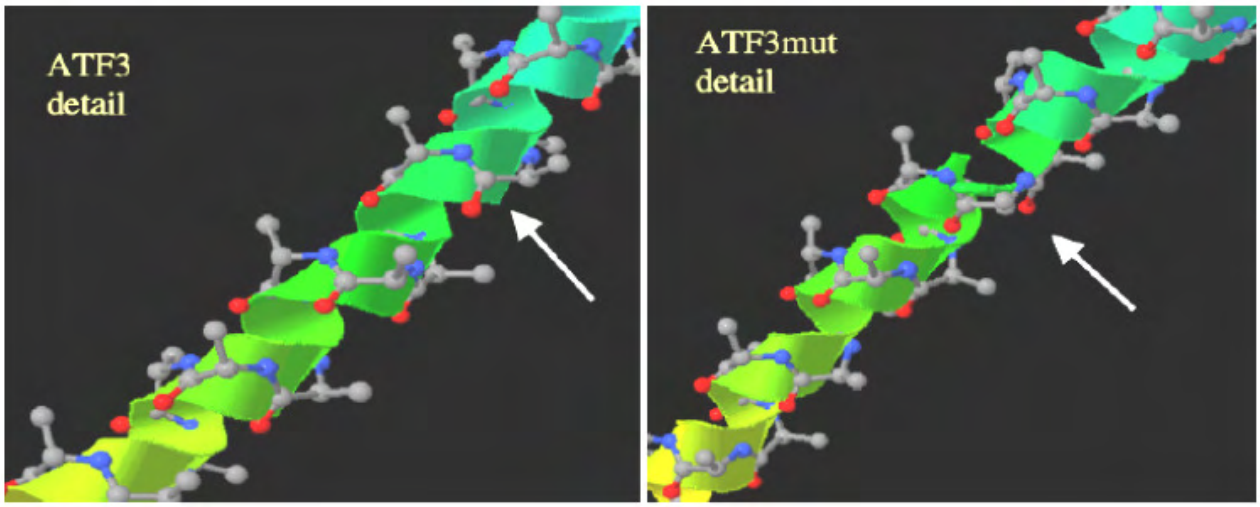

Figure 9: Protein prediction to ATF3 and ATF3 carrying the mutations 536A>G (ATF3 mut). Structure prediction: ATF3 (Estimated precision: 95%) and ATF3mut (Estimated precision: 95%). Predicted at **P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine/ Structural Bioinformatics Group Imperial College London.

A new polymorphism was found in the promoter region, g.210848306delC, in heterozygous form in 7 out of 93 patients and 8 out of 96 controls, indicating a frequency of the rarer allele of 4% in our population. This polymorphism is not associated to hypospadias (p=0.3).

GENOTYPING

To investigate further involvement of *ATF3* in hypospadias, DNA from 354 boys with non-syndromic hypospadias and 380 healthy voluntary blood donors was genotyping for 8 haplotype tagging SNPs.

All loci were in HWE. Single locus analysis of patients and controls revealed that four of the studied polymorphisms in *ATF3* were preliminarily associated with an increased risk to hypospadias: The T allele in rs3125289, the T allele in rs1877474, the C allele in the rs11119982 and the C allele in the rs10735510 are associated with an increased risk to hypospadias. The estimated p-value after correction for multiple comparisons with 1000 permutations remained below 0.05 only for rs1877474 and rs11119982 (Tables 3 and 4).

rs2137424	Controls	Patients	SUM
CC	166	134	300
CT	152	124	276
TT	47	34	81
SUM	365	292	657
P=	0.89		

rs3125289	Controls	Patients	SUM
CC	83	77	160
CT	189	113	302
TT	101	53	154
SUM	373	243	616
P=	0.001		

rs1877474	Controls	Patients	SUM
CC	122	134	256
CT	171	157	328
TT	81	46	127
SUM	374	337	711
P=	0.003		

rs11119982	Controls	Patients	SUM
CC	146	88	234
CT	168	160	328
TT	61	81	142
SUM	375	329	704
P=	0.0003		

rs10735510	Controls	Patients	SUM
CC	127	91	218
CA	179	158	337
AA	68	75	143
SUM	374	324	698
P=	0.08		

rs9429889	Controls	Patients	SUM
CC	285	235	520
CT	86	90	176
TT	6	10	16
SUM	377	335	712
P=	0.07		

rs12070345	Controls	Patients	SUM
AA	115	85	200
AG	174	170	344
GG	75	79	154
SUM	364	334	698
P=	0.04		

rs10475	Controls	Patients	SUM
CC	252	221	473
CT	116	96	212
TT	11	16	27
SUM	379	333	712
P=	0.18		

Table 3: Patients (P) vs. Controls (C) study for single locus analysis: Analysis of the genotypes for the 8 analyzed SNPs. SNPs rs3125289, rs1877474 and rs11119982 are associated with hypospadias, when genotype frequencies are individually analyzed.

Study of haplotype effects, allowing for missing data such as uncertain phase and missing genotypes, has identified that the combination of the three risk alleles in the SNPs rs3125289 (T), rs1877474 (T) and rs11119982 (C) yield the higher effects; and the combination T-T-C is the more strongly associated with hypospadias in our study ($P=4.6X10^{-14}$), suggesting a positive interaction of the three alleles in the risk to hypospadias. The estimated p-value after correcting for multiple comparisons by performing 1000 permutations remains significant. Similarly, the allele combination C-C-T yields a significant p value as a protective haplotype. The D', the normalized disequilibrium coefficient, is very low (0.06) evidencing that the alleles are not in LD. Other marker combinations were not statistically significant.

		SNP1 RS2137424	SNP2 RS3125289	SNP3 RS1877474	SNP4 RS11119982	SNP5 RS10735510	SNP6 RS9429889	SNP7 RS12070345	SNP8 RS10475
		C>T	C>T	C>T	C>T	C>A	C>T	A>G	C>T
Cases	Allele1	192	219	249	326	340	110	328	128
	%	33	45	37	49	52	84	49	19
	Allele2	392	267	425	336	308	560	340	538
	%	67	55	63	51	48	16	51	81
Ctrls	Allele1	246	391	333	290	427	98	324	138
	%	34	52	45	38.5	58	87	45	18
	Allele2	484	355	415	460	309	656	404	620
	%	66	48	55	61.5	42	13	55	82
Case-Ctrl study	OR OR 95% CI	0.96 0.75-1.24	**0.74** 0.60-0.94	**0.73** 0.59-0.90	**1.54** 1.25-1.90	**0.8** 0.65-0.99	1.2 0.98-1.75	1.2 0.98-1.47	1.07 0.8-1.44
	p YATES	0.752	**0.014**	**0.004**	**0.000**	**0.043**	0.078	0.094	0.655
	Perm.P*	NS	NS	0.031	0.001	NS	NS	NS	NS

Table 4: Patients (P) vs. Controls (C) study for single locus analysis: Four of the studied polymorphisms in *ATF3* are associated with hypospadias: rs3125289, rs1877474, rs11119982, rs10735510. The estimated p-value after correcting for multiple comparisons by performing 1000 permutations* remains below 0.05 for rs1877474 and rs11119982. Permutation P-value calculated with *Unphased* and *Haploview* softwares allowing for missing data such as uncertain phase and missing genotypes. The 3 affected boys where the mutations 536A>G and c817C>T were found are heterozygous for the risk alleles in rs1877474 and in the rs11119982.

Model fit	Intercept only	No interaction assumption	Assumption of two-by-two interaction	Assumption of all SNPs interacting
AIC	811.989	782.056	775.322	770.718
SC	816.393	799.671	788.533	779.526
-2logL	809.989	774.056	769.322	766.718
Likelihood ratio		35.9328	40.6668	43.2707
p		**7.7374E-08**	**1.47678E-09**	**4.76668E-11**

Table 5: Logistic regression modeling to evaluate the interaction between risk SNPs: rs3125289, rs1877474 and rs11119982, shows that there is an interaction between the three markers to cause susceptibility

Logistic regression was performed to evaluate the apparently positive interaction between the three polymorphisms rs3125289, rs1877474 and rs11119982. The analysis revealed that the risk is higher if two susceptibility SNPs, rather than only one, are present. And the risk increases in the presence of an additional risk SNP, since the model that best fits is with the interaction term between all three markers; i.e. there is an interaction between the three markers to cause susceptibility.

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ATF3 PROTEIN EXPRESSION IN HUMAN PRE-PUBERTAL SAMPLES

ATF3 protein expression is observed at the nuclei of stromal cells and the vascular endothelium in foreskin tissue samples from pre-pubertal boys with hypospadias and in healthy controls. The number of positive cells or the staining intensity did not differ notably between the patient group and the controls. In a few samples from patients and controls, some positively stained cells could be observed in the epidermal region. The ATF3 staining was then seen in both nuclei and cytoplasm in these cells of different origins (Figure 10)

ATF3 PROTEIN EXPRESSION IN HUMAN FETAL SAMPLES

Human fetal sections from gestational ages 9 to 15 weeks were analyzed for ATF3 expression by immunohistochemistry. ATF3 expression is seen in urethral wall at 15^{th} weeks of gestation, but in earlier stages, particularly in the epithelium, localizing in the nucleus, preferentially located adjacent to the nuclear membrane (Figure 18). A similar pattern of expression was observed in the developing vagina, at every observed stage. Nuclear AFT3 expression was observed consistently in the rectum walls in both male and female fetuses

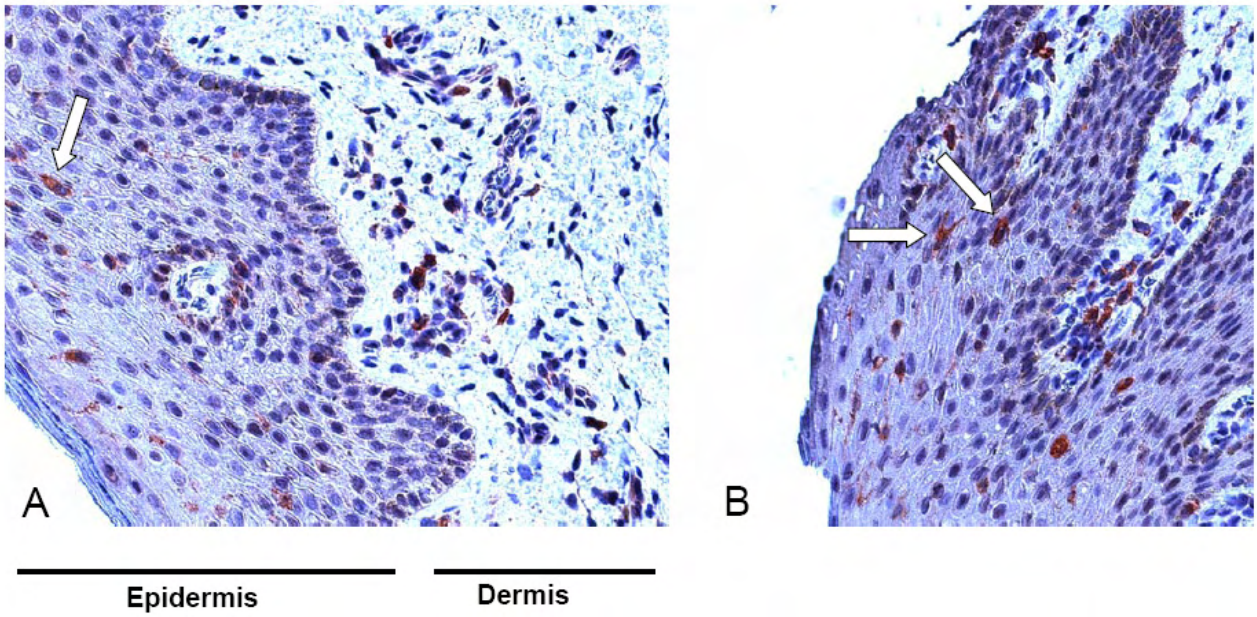

Figure10: ATF3 expression in human pre-pubertal genital skin from a boy with severe hypospadias (A and B), ATF3 expression is observed in both dermal and in epidermal cells. The staining is mostly nuclear, but in certain epidermal cells of different origins ATF3 expression is localized in the cytoplasm (arrows).

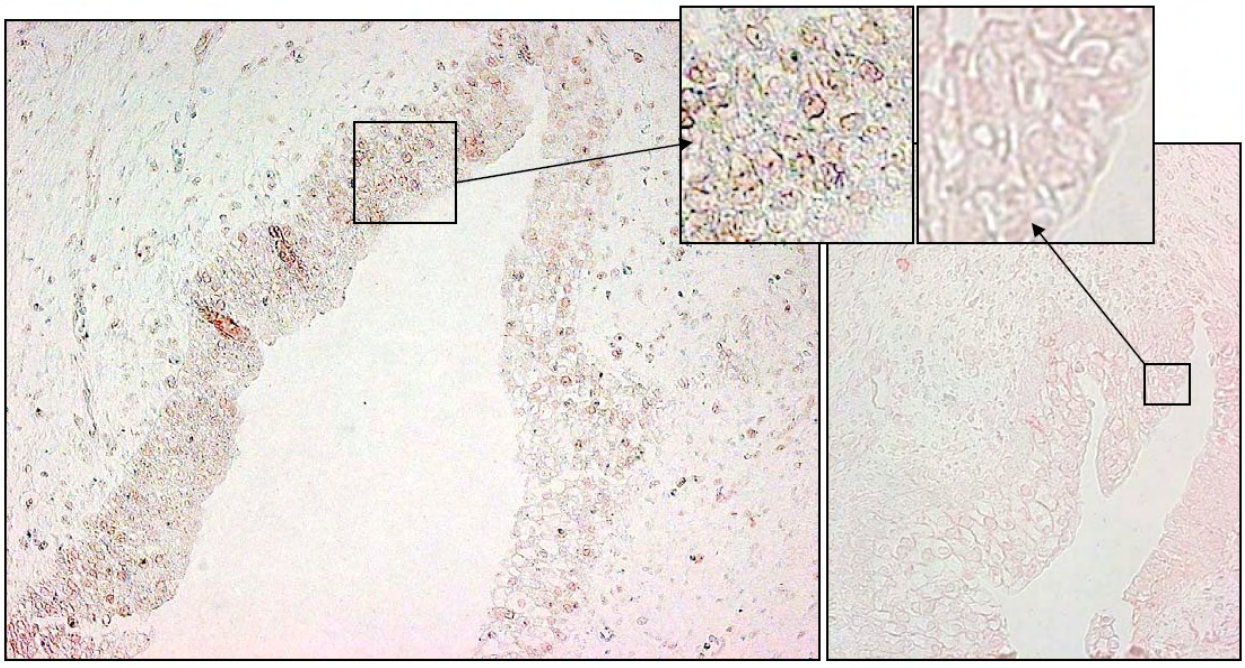

Figure 11: ATF3 expression in posterior urethra from fetal gestation week 15. ATF3 expression is seen in urethral wall, particularly in the epithelium, localizing in the nucleus preferentially located adjacent to the nuclear membrane; no expression was observed before the 15th week. Similar expression pattern is seen in female vagina from 9th-15th gestational weeks. On the right, a similar section of the developing male urethra at 11 weeks shows no ATF3 immuno-staining.

DISCUSSION

The analyses of the selected candidate genes indicated sequence variants that may correlate with the risk to hypospadias. In this section, these findings are discussed.

I. RARE GENE VARIANTS IN FGF8 AND FGFR2 ARE PRESENT IN BOYS WITH HYPOSPADIAS

FGFs, BMPs and their receptors belong to a compact group of conserved "tool kit" genes, which take part in several developmental pathways, including the male urethra. Based on the studies of *Fgf10*, *Fgfr2-IIIb* and *Hoxa13* knock-out mice, we analyzed the *FGF10, FGFR2, FGF8* and *BMP7* sequences in DNA from boys with hypospadias. Gene variants in *FGF8* and *FGFR2* were identified.

The sequence variants on *FGF8* were located around a region coding to the COOH-terminal of the protein. This region is shared by all the reported products of alternative splicing of this gene, which might be of importance to the protein function. However, none of these gene variants alter the predicted protein structure. The sequence variants in *FGFR2* are more spread, and their functional consequences even more difficult to predict. The only non-synonymous variant (p.Met186Thr) does not have any predicted functional consequence. It is possible that these sequence variants might influence splicing or alter the structural folds of mRNA, or be in LD with functional regulatory mutations or polymorphisms. Or maybe, they are of no importance at all.

FGF8 is induced by androgens through an AR mediated mechanism. This regulation acts through its Androgen Response Element sequences in its promoter (Payson RA et al, 1996; Gnanapragasam VJ et al, 2002). Fgfr2-IIIb mediates the closure of the urethral tube through an androgen dependent process. The antagonism of AR leads to down-regulation of Fgfr2-IIIb in mouse genitalia. The presence of an AR binding site in the Fgfr2 promoter also supports an interaction between the two receptors (Petiot A et al, 2005). Since male genital development is an androgen dependent process (Ray R et al, 1998), the gene variants found in the *FGF8* and *FGFR2* genes of boys with hypospadias might interfere with androgen responses during the sexual dimorphic period of the urethral development.

These findings suggest that gene variants in FGF8 and FGFR2 may influence the risk to this hypospadias. The analysis of non coding SNPs and haplotypes of the four genes with, as well as expression studies in human developing urethra are encouraged.

II. FKBP52 DOES NOT SEEM TO BE INVOLVED IN HYPOSPADIAS IN HUMANS

Androgens, acting via the AR, have an essential role in the development of male genitalia (Rey R et al, 1998; Kim KS et al, 2002). Mutations in the *AR* are an uncommon occurrence in isolated hypospadias (Allera A et al, 1995, Sutherland RW et al, 1996). On the other hand, the complexity of AR regulation opens a highly unexplored area in the research in hypospadias and other undervirilisation phenotypes (Adachi M et al, 2000; Hughes IA, 2000; New MI et al 1999).

Our results indicate that FKBP52, a co-regulator of the AR, which plays a critical role in the murine male genital development (Cheung-Flynn J et al, 2005; Yong W et al, 2006), is expressed in genital skin of prepubertal boys. However, no obvious difference in the FKBP52 expression was observed between hypospadias patients and controls. Furthermore, the analysis of the *FKBP4* sequence did not reveal any mutations or associated polymorphism. In spite of the phenotype of the fkbp52 null mice, and the high homology (89%) between human and mice proteins, with conserved functional domains, our results do not indicate alterations on the human *FKBP4* coding sequence and expression in boys with non syndromic hypospadias.

Differences in the regulation of the androgen pathway in human and mice have previously been described. It has been observed that the disruption of the *Srd5a* in mice does not induce any abnormal reproductive phenotype, while in humans, the presence of less active gene variants in its orthologue, *SRD5A2*, has been associated to hypospadias (Sutherland RW et al), infertility (Elzanaty S et al, 2006) and to various degrees of androgen insensitivity (Wilson JD et al, 1993) in men. Similarly, FKBP52 may be less important for full virilization of the male external genitalia in humans than its orthologue fkbp52 is in mice. The amplification of the androgen activity is required for proper male external genital development in the two species. And it is plausible that this is accomplished in humans mainly by the conversion of testosterone to DHT by SRD5A2; while the FKBP52-mediated AR transactivation plays a more crucial role in mice. Indeed, the fine regulation of gene expression is less well conserved between species then hormones and hormonal receptors sequences (Brigandt I**,** 2003). Another possibility is that mutations in *FKBP4* in humans result in other undermasculinization phenotypes, which have not been the target of our study.

This report indicates that alterations in the sequence and in the expression of the *FKBP4* gene are not a common cause of non-syndromic hypospadias. Furthermore it highlights the risk of extrapolating from an animal model to humans, despite the undeniable usefulness of model organisms. The risk is higher when it concerns the fine regulation of hormonal signaling, which may be species specific.

III. GENE VARIANTS IN *ESR2* INFLUENCE THE RISK TO HYPOSPADIAS

Hypospadias has multifactorial etiology that involves the actions of environmental factors and endocrine-active compounds against a genetic background. The role of estrogen in the development of hypospadias (Crescioli C et al, 2003; Dietrich W et al, 2004; Kim KS et al; 2004; Shapiro E et al, 2006; Yucel S et al, 2003) and the up-regulation of estrogen-responsive genes in hypospadiac tissue (Wang Z et al, 2007), led us to analyze the *ESR* genes in hypospadias. Our studies indicated that sequence variants in the *ESR2* gene affect the risk to hypospadias.

The gene variant c.2681-4 A>G in *ESR2* was found in boys with hypospadias more frequently than in controls. This SNP is located close to the region that codes to the C-terminal fragment domain of the ESR2 receptor, a region involved in the transactivation of the receptor. The C-terminal region seems to impair the binding of ESR2 to 17-beta-estradiol, while the removal of this region increases ESR2's binding ability (Huang J et al, 2005). Furthermore, this gene variant is located close to a splicing site. Therefore, a potential functional implication could be theorized. However, splicing prediction analysis did not indicate any splicing change (data not shown). Moreover, further studies on *ESR2*, by typing tagging SNPs in a larger cohort, did not confirm association to this region of the gene.

With regards to the (CA)n, it is known that intronic microsatellite repeats may lead to altered gene transcription, mRNA splicing, export to cytoplasm, induce heterochromatin- mediated- like gene silencing, or interactions with co-regulators (Li YC et al, 2004). This polymorphism has previously been associated with several conditions, such as with bone mineral density in postmenopausal women, endometrial cancer and systemic blood pressure (Scariano JK et al, 2004; Lau HH et al, 2002; Ogawa S et al, 2000; Setiawan VW et al, 2004; Ogawa S et al, 2000), which substantiates putative functional implications. Moreover, longer prolonged (CA)n variants are associated to lower levels of testosterone in a population-based cohort (Westberg et al, 2001). Lower levels of testosterone could then induce hypospadias in boys with longer (CA)n lengths.

Association was also found with promoter gene variants, mapping to the SNP rs2987983, possibly affecting transcription factor binding. Promoter polymorphisms have previously been associated with the risk for several diseases (Setiawan VW et al, 2004; Ogawa S et al, 2000). And this aspect may be of particular relevance in ESR2, a steroid receptor, subjected to a complex regulation by co-regulators and general transcription factors, which modulate ESR2-target gene expression. Moreover, the SNP rs2987983 has previously been associated with prostate cancer risk in a Swedish cohort, corroborating with a possible functional consequence of this variant in the male reproductive tract (Thellenberg-Karlsson C et al, 2006).

The combination of the two risk polymorphisms – the C allele in rs2987983 and longer (CA)n variants - is also over represented in patients. These two polymorphisms are not necessarily inherited together as a haplotype, as demonstrated by a low LD. But when together, their effects are not independent, as indicated by the regression analysis, with the (CA)n polymorphism as the primary risk factor for hypospadias.

Several mechanisms could explain how polymorphisms in *ESR2*, the predominant ESR in the developing male urethra (Dietrich W et al, 2004; Shapiro E et al, 2006), may influence the risk to hypospadias. One of such mechanisms is the interference with the balance between the effects produced by estrogens and androgens, as discussed before. Not only do estrogens modulate the levels of, and the responsiveness to, androgens; but also ESRs and ARs, both ligand-dependent nuclear factors, interact both at DNA and protein level. At the DNA level, these receptors may compete for the same binding sites, or affect protein-DNA interactions. At the protein level, AR & ESR interact with each other through specific domains, affecting their transactivational properties; interactions may also happen by squelching effect, or by competition for common transcriptional factors or co-regulators (Aranda A et al, 2001; Dellovade TL et al, 1995**;** Panet-Raymond V et al, 2000; Toppari J et al, 1996; Zhou Q et al, 2001; Zhu YS et al, 1997).

Sequence variants of the *ESR2* may also directly affect the male genital development (Crescioli C et al 2003; Gooren L, 1989; Westberg L et al, 2001). Sequence variants may interfere with some of the proposed functions of ESR2, such as its anti-proliferative action, regulation of apoptosis and control of antioxidant gene expression (Leung YK et al, 2006). Moreover, since direct interactions of estrogenic chemicals with the ESR2 have been documented (Kuiper GG et al, 1998), ESR2 variants could influence the susceptibility to the deleterious actions of endocrine disrupters, explaining the ethnic differences observed in the response to such agents (Giwercman A et al, 2006).

IV. ATF3, AN HORMONE RESPONSIVE GENE, IS INVOLVED IN HYPOSPADIAS

The evaluation of *ATF3* as a candidate for hypospadias, denotes that sequence variants of this gene, which is expressed in the human developing male urethra, may be involved in the genetic risk for hypospadias in different ways: By the effect of three common, low risk, SNPs in the intron 1 of *ATF3*; or induced by less common variants, possibly with higher effect, such as the missense mutation c.536A>G in the exon 3 and c.817C>T in the 3'UTR. The mechanisms that explain how variants in the sequence of *ATF3* affect the risk to hypospadias are not yet known; but some hypothesis can be suggested.

ATF3 has recently been involved in the hypospadias, both in humans and in animal models (Willingham E & Baskin LS, 2007; Wang Z et al, 2007; Hai T et al, 1999; Liu B el al, 2006). In mice, its orthologue atf3 is up-regulated during sexual differentiation (Liu B et al, 2006) and is estrogen responsive (Liu B et al, 2006).

Furthermore, the expression of ATF3 is up-regulated in human hypospadic tissue, in some reports (Wang Z et al, 2007; Liu B et al, 2005). The potential role of estrogens and ESRs in hypospadias (Crescioli C et al,2003; Shapiro E et al, 2006; Dietrich W et al; 2004; Yucel S et al, 2003; Kim KS et al; 2004), and the estrogen responsiveness of a gene that has been shown up-regulated in hypospadias, motivated the selection of *ATF3* as a candidate gene to hypospadias. This also may explain how variants of this gene may affect urethral development. But other aspects of this gene should also be considered.

ATF3 is a key immediate early gene induced by gonadotropin-releasing hormone (GnRH) (Xie J et al, 2005). GnRH is required for synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), essential signals in the establishment of primary and secondary sexual characteristics. Interestingly, LH and FSH response to GnRH is higher in boys with hypospadias than those in prepubertal control subjects (Nonomura K et al, 1984) and genetic variants of the LH and its receptor have been associated to disorders of the male reproductive tract (Pals-Rylaarsdam R et al, 2005; Kaleva M et al, 2005; Themmen AP et al, 1996). Furthermore, ATF3 expression is up-regulated by the AR's activation by both androgens and estrogens in prostate cancer cell lines. And the treatment of these cells with an anti-androgen decreases ATF3 levels and partially abrogated induction by androgen (Pelzer AE et al, 2006). These evidences are of interest, due to the importance of androgens in male development (Klonish T et al, 2004).

Furthermore, ATF3 responds to a given signal in epithelial cells via the TGF-beta pathway (Valcourt U et al, 2005; Kang Y et al, 2003). Interestingly, genes involved in the TGF-beta pathway are up-regulated during several stages of murine urethral tube development (Willingham E & Baskin LS, 2007), where it facilitates epithelial and mesenchymal differentiation (Valcourt U et al, 2005; Kang Y et al, 2003; Massague J et al, 2005). Furthermore, it has been suggested that *ATF3* may lie at the centre of interactions between the TGF-beta signaling pathway, and steroid-hormone receptors. These aspects provide further elucidation for the involvement of ATF3 in hypospadias.

In other contexts, ATF3 has been characterized as a cellular *adaptive response* gene: The expression of ATF3 protein is normally at a steady state in quiescent cells; but can be rapidly induced to a high level in response to multiple stress signals, such as ischemia, inflammation, nutrient limitation, wounding and toxicity (Hai T et al, 1999; Hartman MG, 2001; Pan YX et al, 2007). ATF3 has also been related to cell fate and to p38 and p53 related pro-apoptotic events (Fan F et al, 2002; Lu D et al, 2007; Yan C et al, 2006). Furthermore, the ATF3deltaZip2 isoform sensitizes cells to apoptotic cell death in response to tumor necrosis factor-alpha (Hua B et al, 2006). The role of ATF3 in apoptosis and adaptive responses may shed light into the molecular basis of its involvement in hypospadias.

Indeed, the normal male genital tubercle development depends on hormonal action, epithelial-mesenchymal interactions and a balance between cell proliferation and apoptosis (Morgan EA, et al, 2003). ATF3 seem to be involved in all these aspects. We hypothesize that the inappropriate regulation of ATF3, by variants in its gene sequence, or by any other mechanism, such as lower nutrient availability (Pan YX et al, 2007)–a link to low birth weight? - might halter the seam fusion process of the developing urethra and result in a shorter urethra with a proximal opening.

CONCLUDING REMARKS

Hypospadias is one of the most common inborn errors of development with complex and still elusive etiology. Genes and environmental factors are believed to influence the risk to this disorder of the male genital development. Currently, hypospadias is repaired surgically, which constitutes one of the most common surgeries performed in neonates.

The molecular mechanisms involved in the development of external genitalia during fetal life seem to depend on a complex balance between early morphogenetic cell-cell interactions; and between sex steroid hormones and related pathways. These balances can be disturbed by the exposure to environmental endocrine disruptors. But due to know ethnical differences in the response to such exposures, genetic factors may play an important role.

The projects that were included in this thesis evidenced the involvement of sequence variants in *FGFR2*, *FGF8*, *ESR2* and *ATF3*, four hormonal responsive genes, in hypospadias. These reports do not only increased our understanding about the complex etiology of this inborn error of development, but also highlight the importance of hormonal cascades in genital development. Further functional analysis of the implicated genes, the analysis of other endocrine related genes and pathways, and gene-environment studies are strongly encouraged.

The overall aim of this thesis was to gain an increased understanding of the pathogenesis of hypospadias. An additional benefit would be to shed light on key mechanisms in sex differentiation. Despite no immediate clinical application of the presented results, the importance of increased information to affected families must not be under rated. Furthermore, although surgery may remain the therapy of choice for hypospadias, a better knowledge of the hormonal and molecular mechanisms of GT development may be the basis for preventive strategies reducing the incidence of this common malformation.

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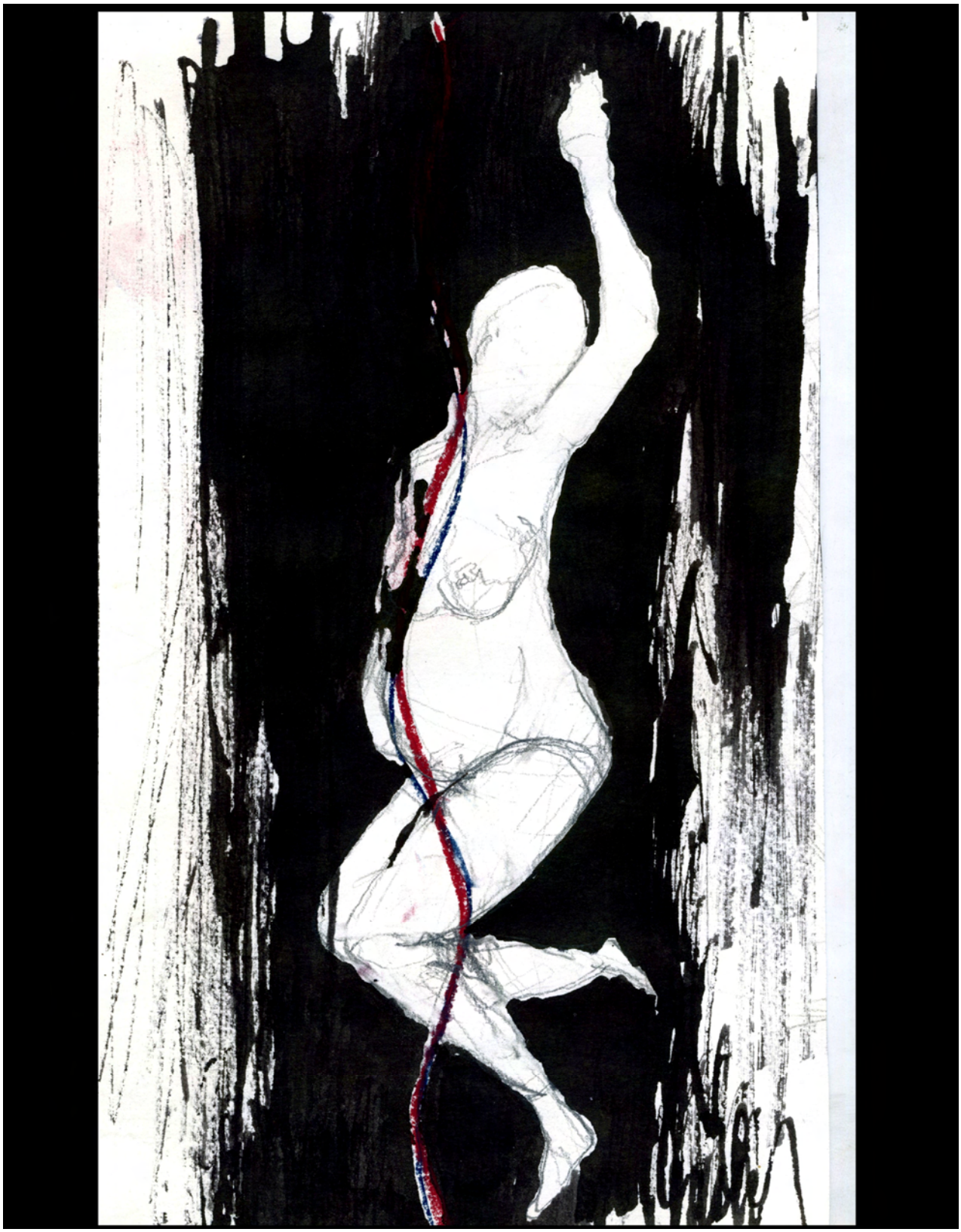
Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa.
Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis

Let’s also acknowledge the curious, creative and insubordinate Human nature, which is what keeps the world moving...



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