46,XY DISORDERS OF SEX DEVELOPMENT

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Revised 4/28/2017

ABSTRACT

The 46,XY disorders of sex development (46,XY DSD) are characterized by atypical or female external genitalia, caused by incomplete intrauterine masculinization with or without the presence of Müllerian structures. Male gonads are identified in the majority of 46,XY DSD patients, but in some of them no gonadal tissue is found. Complete absence of virilization results in normal female external genitalia and these patients generally seek medical attention at pubertal age, due to the absence of breast development and/or primary amenorrhea. 46,XY DSD can result either from decreased synthesis of testosterone or DHT or from impairment of androgen action.

A careful clinical evaluation of the neonate is essential because most DSD patients could be recognized in this period and precocious diagnosis allows a better therapeutic approach. Family and prenatal history, complete physical examination and assessment of genital anatomy are the first steps for a correct diagnosis. The diagnostic evaluation of DSD includes hormone measurements (assessment of Leydig and Sertoli cells function), imaging (ultrasonography is always the first and often the most valuable imaging modality in DSD patients' investigation), cytogenetic and molecular studies. Endoscopic and laparoscopic exploitation and/or gonadal biopsy are required in very few cases.

Psychological evaluation is of crucial importance to treat DSD patients. Every couple that has a child with atypical genitalia must be assessed and receive counseling by an experienced psychologist, specialized in gender identity, who must be act as soon as the diagnosis is suspected, and then follow the family periodically, more frequently during the periods before and after genitoplasty.

Parents must be well informed by the physician and psychologist about normal sexual development. A simple, detailed and comprehensive explanation about what to expect regarding integration in social life, sexual activity, need of hormonal and surgical treatment and the possibility or not of fertility according to the sex of rearing, should also be discussed with the parents, before the attainment of final social sex.
Optimal care of DSD patients requires a multidisciplinary team and begins in the newborn period and sometimes in prenatal life. Most of the well-treated DSD patients present a normal quality of life at adulthood.

INTRODUCTION

Male phenotypic development can be viewed as a 2-step process: 1) testis formation from the primitive gonad (sexual determination) and 2) internal and external genitalia differentiation by action of factors secreted by the fetal testis (sexual differentiation.The first step is very complex and involves interplay of several transcription factors and signaling cells (1). Dosage imbalances in genes involved in DSD (deletions or duplication) have been identified as a cause of these disorders (Fig. 1).

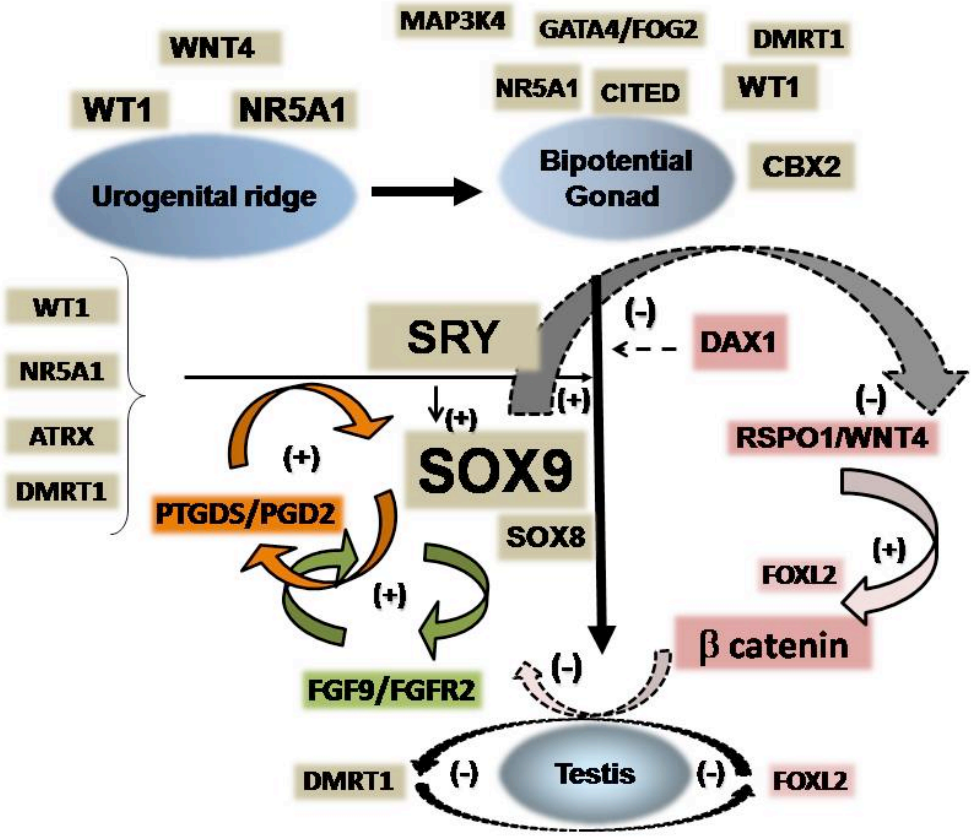

Figure 1 - *Summary of the molecular events in sex determination indicating the genes in which molecular defects can cause gonadal disorders in animal models. Some of these disorders were confirmed in humans.*
NR5A1, Wnt4 and Wt1 are expressed in the urogenital ridge whose development results in formation of the gonads, kidneys and adrenal cortex. Several genes, Wt1, NR5A1, M33 (CBX2 mouse homologue), Lhx9, Lim1, Gata4/Fog2, Dmrt1, Emx2 and Cited are expressed in the bipotential gonad. NR5A1 up-regulates CBX2 expression that is required for upregulation of SRY gene. NR5A1 and Wt1 up-regulate Sry expression in pre-Sertoli cells and Sry initiates male gonad development. Sry strongly up-regulates Sox9 in Sertoli cells. Sox9 up-regulates Fgf9 and Fgf9 maintains Sox9 expression, forming a positive feed-forward loop in XY gonads. The balance between Fgf9 and Rspo1/Wnt4 signals is shifted in favor of Fgf9, establishing the male pathway. If Wnt4/Rspo1 is overexpressed activating the β-catenin pathway, this system blocks Fgf9 and disrupts the feed-forward loop between Sox9 and Fgf9. Pdg2 signaling up-regulates Sox9 and Sox9 activate Ptgds. Sox9 establishes a feed-forward loop with the Pgd2. Sox9 inhibits beta-catenin-mediated Wnt signaling. Overexpression in either Dax1 (locus DSS) or Rspo1/Wnt4 antagonizes testis formation. On the other hand, Dax1 regulates the development of peritubular myoid cells and the formation of testicular cords. Dmrt1 has recently been shown to be required for the maintenance of gonadal sex and to prevent female reprogramming in postnatal testis. CBX2 directly or indirectly represses ovarian development.

The second step, male sex differentiation, is a more straightforward process. Mesonephric (wolffian) and paramesonephric (mullerian) ducts present in both, male and female fetus, originate from the anterolateral epithelium of the urogenital ridge. Anti Müllerian hormone (AMH) secreted by the testicular Sertoli cells acts on its receptor in the Müllerian ducts to cause their regression. Testosterone secreted by the testicular Leydig cells acts on the androgen receptor in the Wolffian ducts to induce the formation of epididymis, deferent ducts and seminal vesicles (Fig. 2) (2).The external genitalia of the fetus derive from mesenchyme cells from the primitive streak. Under androgen stimuli male fetal urethral folds, genital tubercule and genital swellings give rise to corpus spongiosum and primitive urethra, phallus and scrotal swellings respectively. This process is mediated by testosterone and its further reduced dihydrotestosterone (DHT), which acts on the androgen receptor of the prostate and external genitalia leading to its masculinization (3,4) (Fig. 3 and 4).

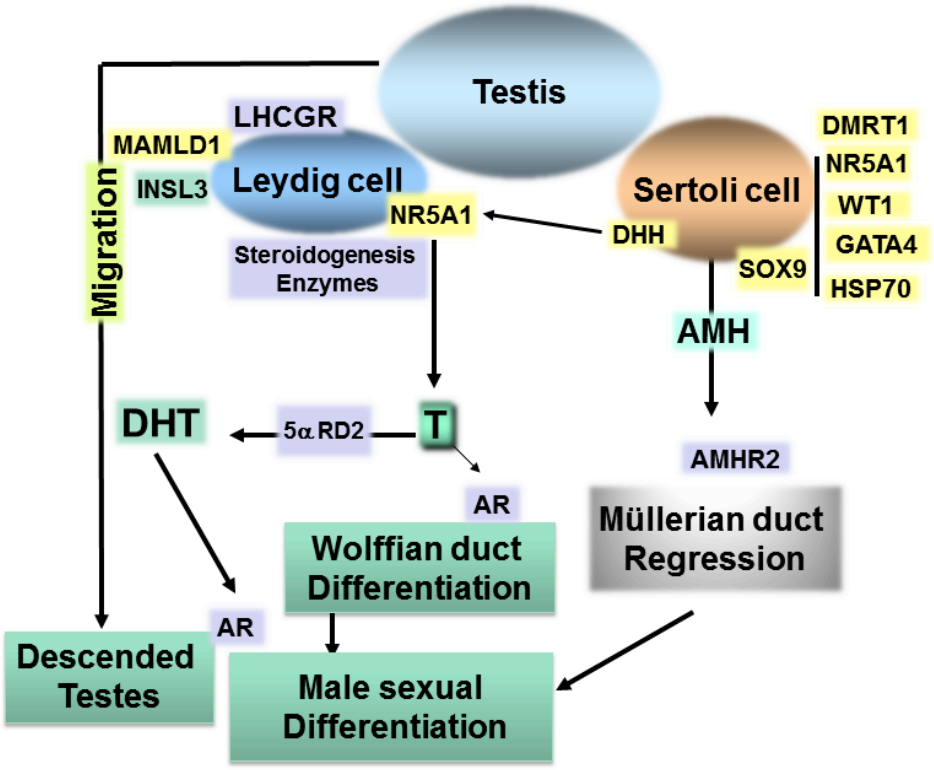

Figure 2 - *Summary of the molecular events in sex differentiation indicating the genes in which molecular defects cause 46,XY DSD in humans. After testis determination, hormones produced by the male gonad induce the differentiation of internal and external genitalia acting on their specific receptor. The regulation of AMH gene requires cooperative interaction between SOX9 and NR5A1, WT1, GATA4 and HSP70 at the AMH promoter. Combined expression of DHH, MAMLD1 and NR5A1 is required for Leydig cell development. NR5A1 regulates gonadal steroidogenesis. The Leydig cells also produce INSL3, which causes the testes to descend to the scrotum.*

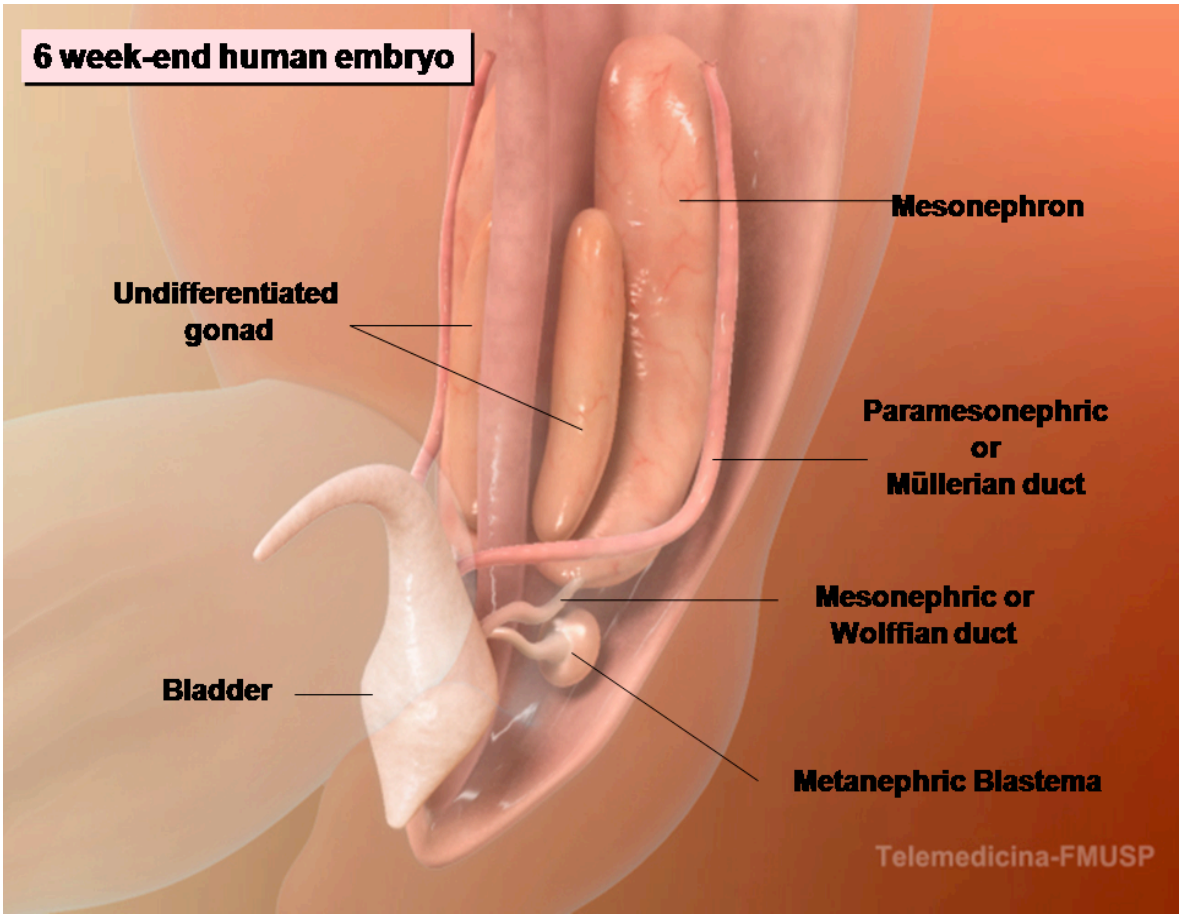

Figure 3 – The development of male internal genitalia in the human embryo. The 6-wk-end embryo is equipped with both male and female genital ducts derived from the mesonephrons

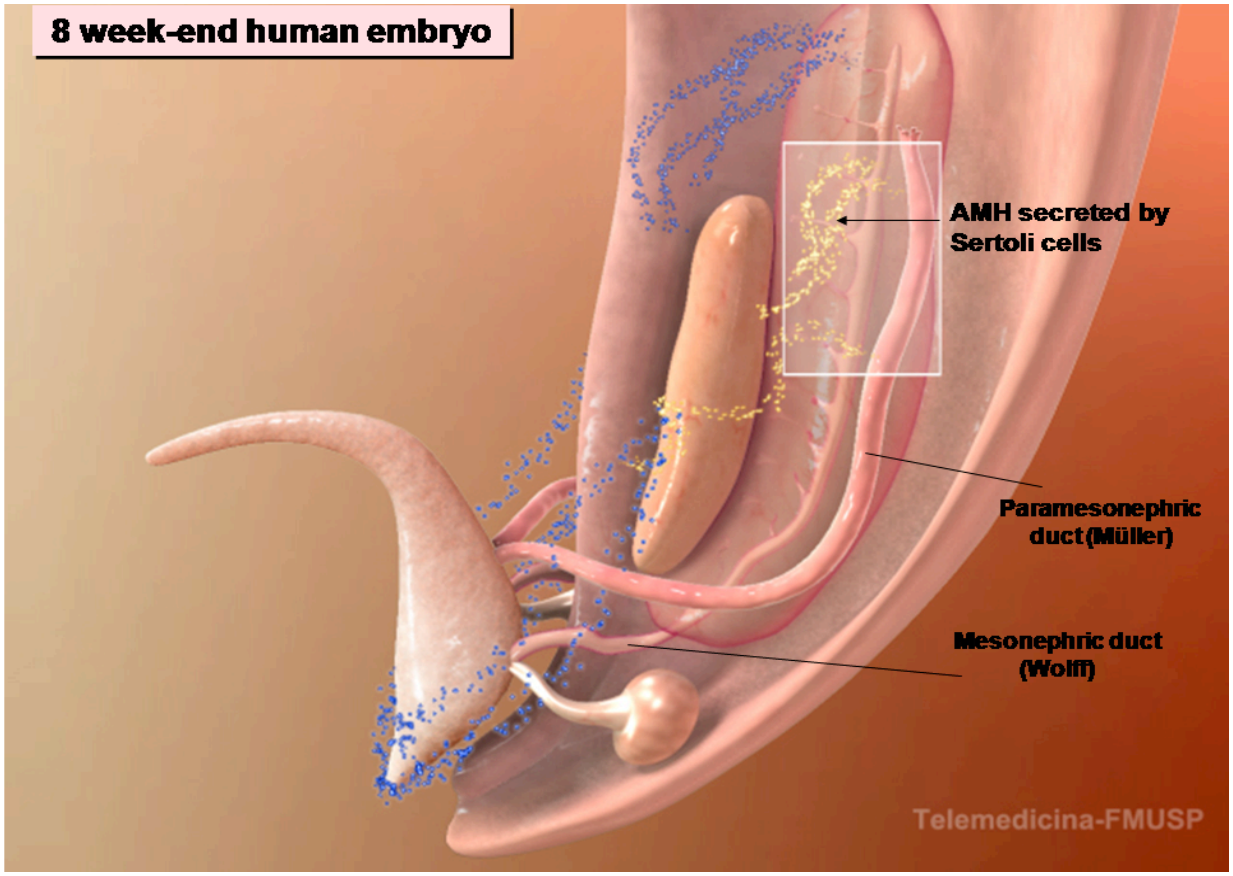

Figure 3 (A) – The development of male internal genitalia in the human embryo. The regression of the Müllerian ducts is mediated by the action of AMH secreted by the fetal Sertoli cells.

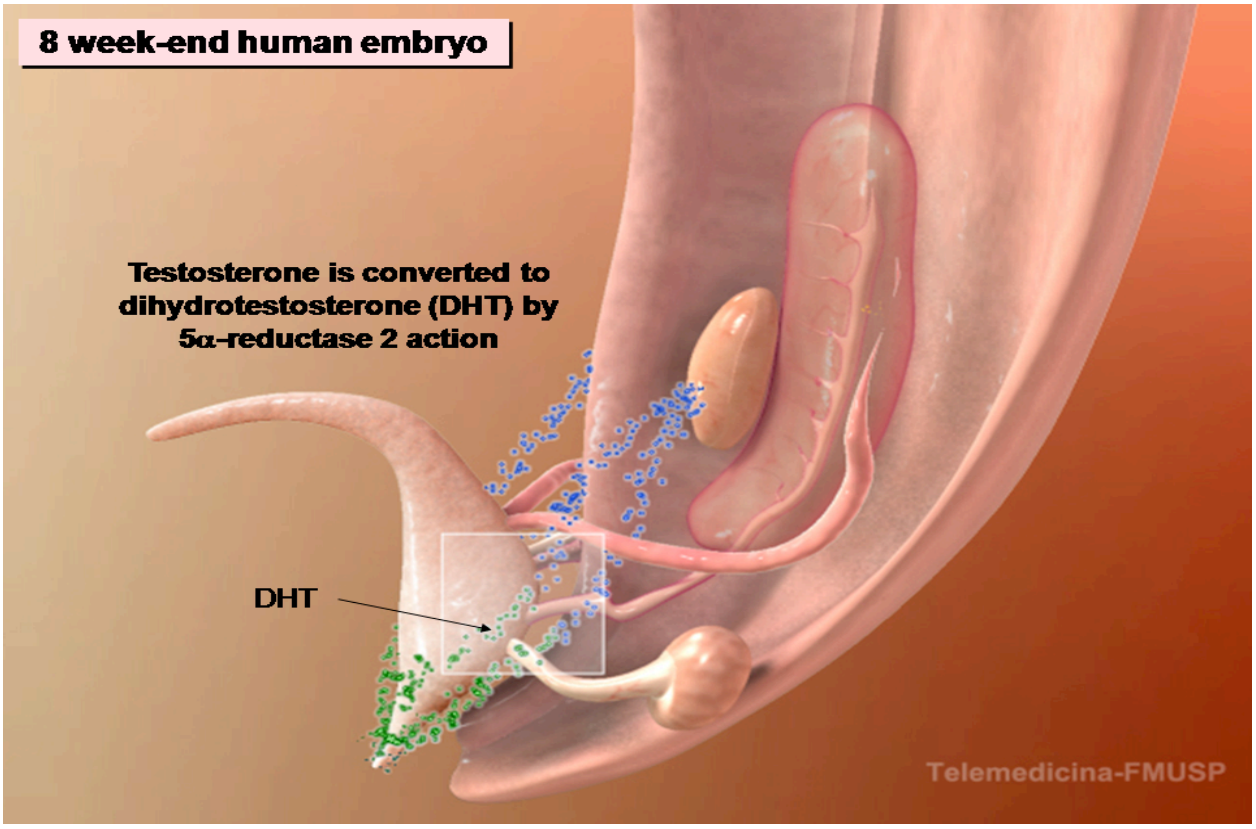

Figure 3 (B) – The development of male internal genitalia in the human embryo. The stabilization and differentiation of the Wolffian ducts are mediated by testosterone synthesized by the fetal Leydig cells. The enzyme 5α-reductase 2 converts testosterone to dihydrotestosterone (DHT). The Wolffian ducts differentiate into epididymis, vas deferens and seminal vesicles. DHT contributes to prostate differentiation.

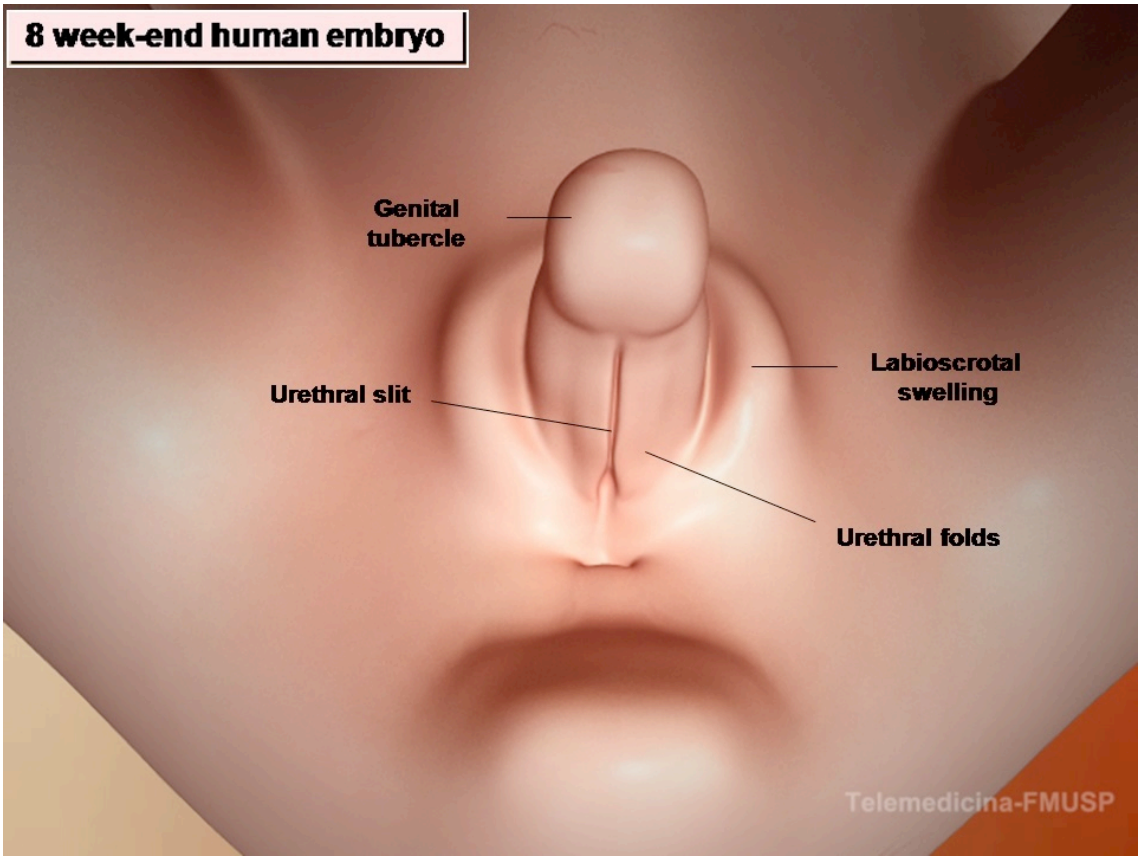

Figure 4 -Development of male external genitalia in the human embryo.
At the 8-wk-end embryo the external genitalia of both sexes are identical and have the capacity to differentiate in both directions: male or female. DHT stimulates growth of the genital tubercle and induces fusion of urethral folds and labioscrotal swellings. It also inhibits growth of the vesicovaginal septum preventing development of the vagina.

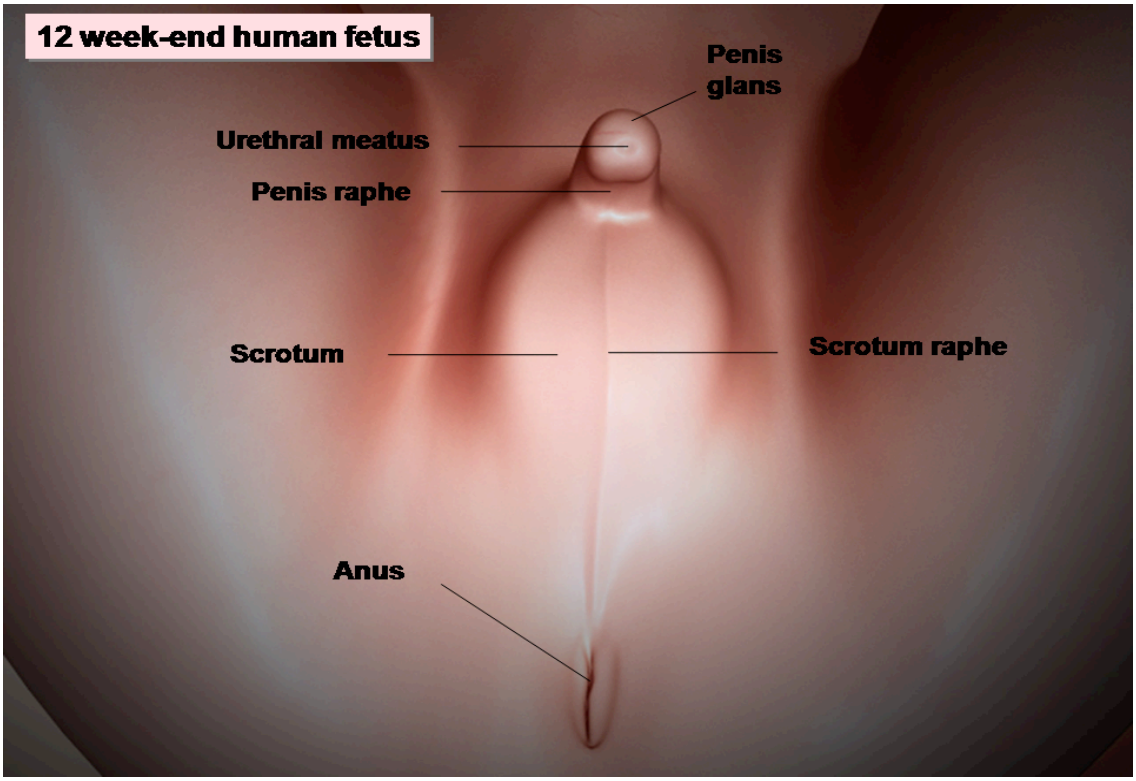

Figure 4 (A) -Development of male external genitalia in the human embryo. At the 12-week-end embryo the male external genitalia is entirely formed.

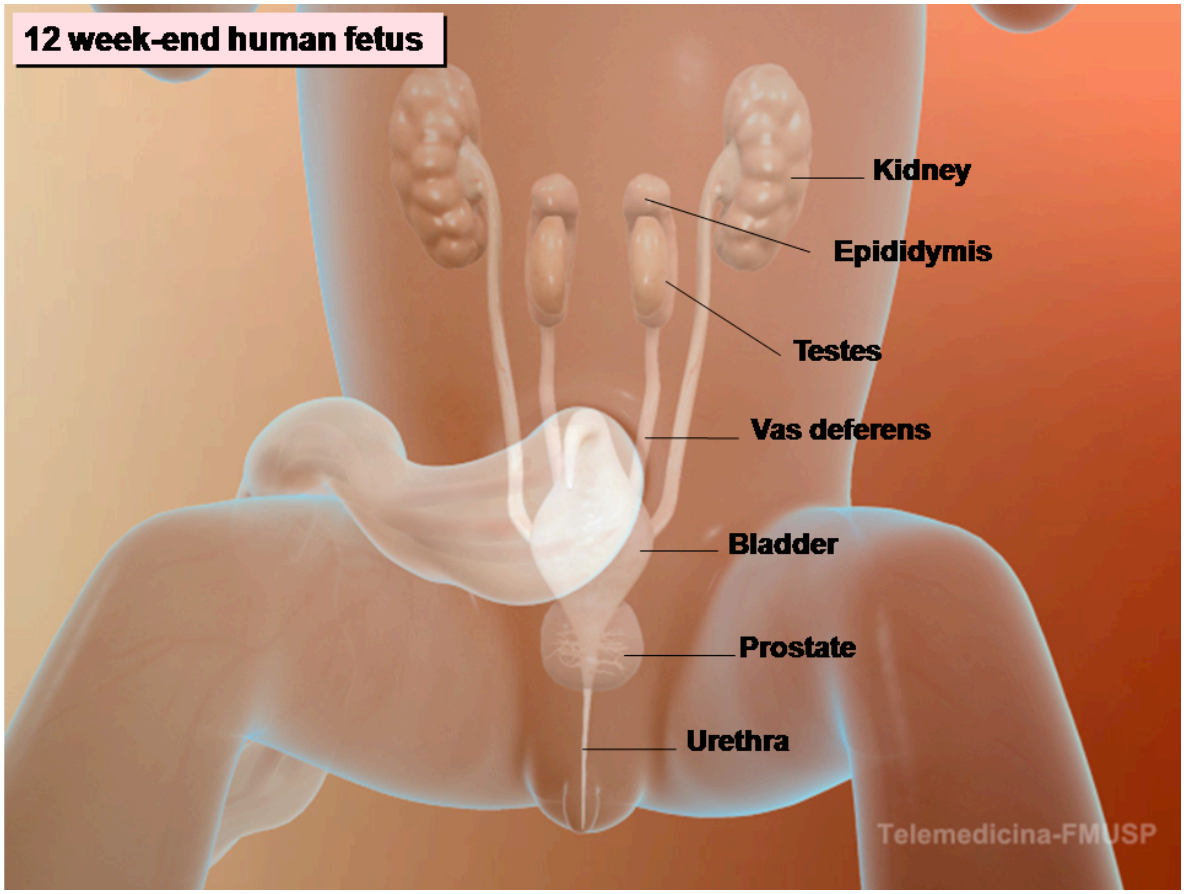

Figure 4 (B) -Development of male internal and external genitalia in the human embryo. At the 12-week-end embryo both internal and external genitalia are completely formed.

The term disorders of sex development (DSD) includes congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical. This nomenclature has been proposed to replace terms such as intersex, pseudohermaphroditism and sex reversal (5,6). These terms, previously used to describe the disorders of sex development, are potentially offensive to the patients and the consensus on the management of intersex disorders recommends a new nomenclature that will be followed in this chapter (5). The proposed changes in terminology aim to integrate upcoming advances in molecular genetics in new DSD classification (7)

The 46,XY disorders of sex development (46,XY DSD) are characterized by atypical or female external genitalia, caused by incomplete intrauterine masculinization with or without the presence of Müllerian structures. Male gonads are identified in the majority of 46,XY DSD patients, but in some of them no gonadal tissue is found. Complete absence of virilization results in normal female external genitalia and these patients generally seek medical attention at pubertal age, due to the absence of breast development and/or primary amenorrhea. 46,XY

DSD can result either from decreased synthesis of testosterone or DHT or from impairment of androgen action (8). Our proposal classification of 46,XY DSD is displayed in Table 1.

Table 1: Classification of 46,XY DSD

46,XY DSD DUE TO ABNORMALITIES OF GONADAL DEVELOPMENT
Gonadal agenesis
Gonadal dysgenesis - complete and partial forms
46,XY DSD due to underexpression of *WT1* gene
Denys-Drash syndrome
46,XY DSD due to the underexpression of steroidogenic factor-1 (NR5A1/SF1)
Dysgenetic 46,XY DSD due to GATA4 and FOG2 underexpression
Dysgenetic 46,XY DSD due to CBX2 underexpression
46,XY DSD due to underexpression of *SRY* gene
Dysgenetic 46,XY DSD associated with campomelic dysplasia (underexpression of the SOX9)
Dysgenetic 46,XY DSD due to FGF9/FGFR2 underexpression
Dysgenetic 46,XY DSD due to disruption in the Hedgehog signaling
I-Desert hedgehog (DHH) gene
II- Hedgehog acetyl-transferase (HHAT) gene
46,XY DSD due to the underexpression of DMRT1 gene
ATR-X syndrome (X-linked α-thalassemia and mental retardation)
Dysgenetic 46,XY DSD due to MAP3K1 underexpression
46,XY DSD due to the overexpression of DAX1 (NR0B1) gene
46,XY DSD due to the overexpression of WNT4 gene
46,XY DSD ASSOCIATED WITH CHOLESTEROL SYNTHESIS DEFECTS
Smith-Lemli-Opitz syndrome
46,XY DSD DUE TO TESTOSTERONE PRODUCTION DEFECTS
Impaired Leydig cell differentiation (*LHCGR* defects)
Complete and partial forms
Enzymatic defects in testosterone synthesis
Defects in adrenal and testicular steroidogenesis
STAR deficiency
P450scc deficiency
3-β-hydroxysteroid dehydrogenase II deficiency
17α-hydroxylase and 17,20 lyase deficiency
P450 oxidoreductase defect (*electron transfer disruption)*
Defects in testicular steroidogenesis
Isolated 17,20-lyase deficiency
Cytochrome b5 defect (*allosteric factor for P450c17 and POR interaction)*
17β-hydroxysteroid dehydrogenase III deficiency
Alternative pathway to DHT
3α- hydroxysteroid dehydrogenase deficiency due to AKR1C2 and AKR1C4 defects
46,XY DSD DUE TO DEFECTS IN TESTOSTERONE METABOLISM
5α-reductase type 2 deficiency

46,XY DSD DUE TO DEFECTS IN ANDROGEN ACTION
Androgen insensitivity syndrome
Complete and partial forms
46,XY DSD DUE TO PERSISTENCE OF MÜLLERIAN DUCTS
Defect in AMH synthesis
Defect in AMH receptor
CONGENITAL NON-GENETIC 46,XY DSD
Maternal intake of endocrine disruptors
Associated with impaired prenatal growth
46,XY OVOTESTICULAR DSD
NON-CLASSIFIED FORMS
Hypospadias
46,XY gender dysphoria

INVESTIGATION OF DSD PATIENTS

Optimal care of patients with disorders of sex development requires a multidisciplinary team and begins in the newborn period. A careful clinical evaluation of the neonate is essential because most DSD patients could be recognized in this period and precocious diagnosis allows a better therapeutic approach. Family and prenatal history, complete physical examination and assessment of genital anatomy are the first steps for a correct diagnosis. The diagnostic evaluation of DSD includes hormone measurements, imaging, cytogenetic and molecular studies. In very few cases, endoscopic and laparoscopic exploitation and/or gonadal biopsy are required (7).

The endocrinological evaluation of 46,XY DSD infants includes assessment of testicular function by basal measurements of LH, FSH, inhibin B, anti-Mullerian hormone (AMH) and steroids.

AMH and inhibin B are useful markers of the Sertoli cells presence and their assessment could help in the diagnosis of testis determination disorders. In boys with bilateral cryptorchidism serum AMH and inhibin B correlate with the presence of testicular tissue and undetectable values are highly suggestive of absence of testicular tissue (9) (10) (11).

In postpubertal patients with testosterone synthesis defects, the diagnosis is made through basal steroid levels. Testosterone levels are low and steroids upstream from the enzymatic blockage are elevated. This pattern can be confirmed by hCG stimulation test, which increases the accumulation of steroids before the enzymatic blockage, with a slight elevation of testosterone. In pre-pubertal individuals, hCG stimulation test is essential for the diagnosis, since basal levels are not altered. There are several hCG stimulation protocols and normative data have to be established for each of them. We established a normal testosterone response 72

and 96 hours after the last of 4 doses of hCG, 50-100 U/kg body weight, given via intramuscular every 4 days in boys with cryptorchidism but an otherwise normal external genitalia: testosterone peak levels reached 391 ± 129 ng/dL and we consider a subnormal response a value <130 ng/dL (equivalent to -2 SD) (12).
Imaging evaluation is indicated in the neonatal period when an atypical genitalia is identified. If apparent female genitalia with clitoral hypertrophy, posterior labial fusion, foreshortened vulva with single opening or inguinal/labial mass is present, imaging study may also be performed. A family history of DSD and later presentations as abnormal puberty or primary amenorrhea, cyclic hematuria in a male, inguinal hernia in a female also require an imaging evaluation.
Ultrasonography is always the first and often the most valuable imaging modality in DSD patients' investigation. Ultrasound shows the presence or absence of Müllerian structures at all ages and can locate the gonads and characterize their echo texture. This exam can also identify associated malformations such as kidney abnormalities (13).
Genitography and cystourethrography can display the type of urethra, the presence of vagina, cervix, and urogenital sinus. Although, the imaging features are non-specific for the cause of DSD, these diagnostic methods are important in gender assignment and specially for surgical planning.

CYTOGENETIC AND MOLECULAR INVESTIGATION

The genetic evaluation includes karyotype, FISH and specific molecular studies to screen for the presence of mutations or gene dosage imbalance. Molecular methodologies have identified already known and also novel causes of DSD, and have led to the adoption of molecular tests into clinical practice for diagnosis and genetic counselling. Among the genetic tests available, most use a candidate-gene approach, while new high-throughput DNA analysis could enable a genetic diagnosis to be made where the aetiology is unknown or differential diagnosis wide. These new high-throughput DNA approach can reduce the need of hormonal and imaging tests to reach the correct diagnosis. Advances in molecular biological techniques for diagnosing DSD is reviewed (14).

aCGH and SNP-array

The association of DSD and syndromic features can be explained by the ubiquous expression of DSD genes or by the contiguous gene syndrome, in which the loss of contiguous genes related and non related to the DSD predispose to the syndromic presentation. aCGH and SNP-array are tools that can detect submicroscopic genome imbalance and copy number variation in the genome as small as 10 KB in apparently normal karyotype patients (15) (16). Pathogenic copy number variations in already known genes related to 46,XY DSD phenotype and novel candidate genes such as *SUPT3H, C2ORF80, KANK1, ADCY2* and *ZEB2* have been

demonstrated by array technics (17) (18). Some authors have proposed that CGH or SNP-array shoud be used as the first genomic test for investigating DSD associated with syndromic features since it is capable to diagnose pathogenic copy number variations in almost 30% of these patients as a single method (17,18). Nevertheless, the attainment of molecular diagnosis is related to a properly established clinical and hormonal diagnosis. Almost all testosterone synthesis defects can be diagnosed by hormonal evaluation.
Careful selection of the genetic test indicated for each condition remains important for good clinical practice.

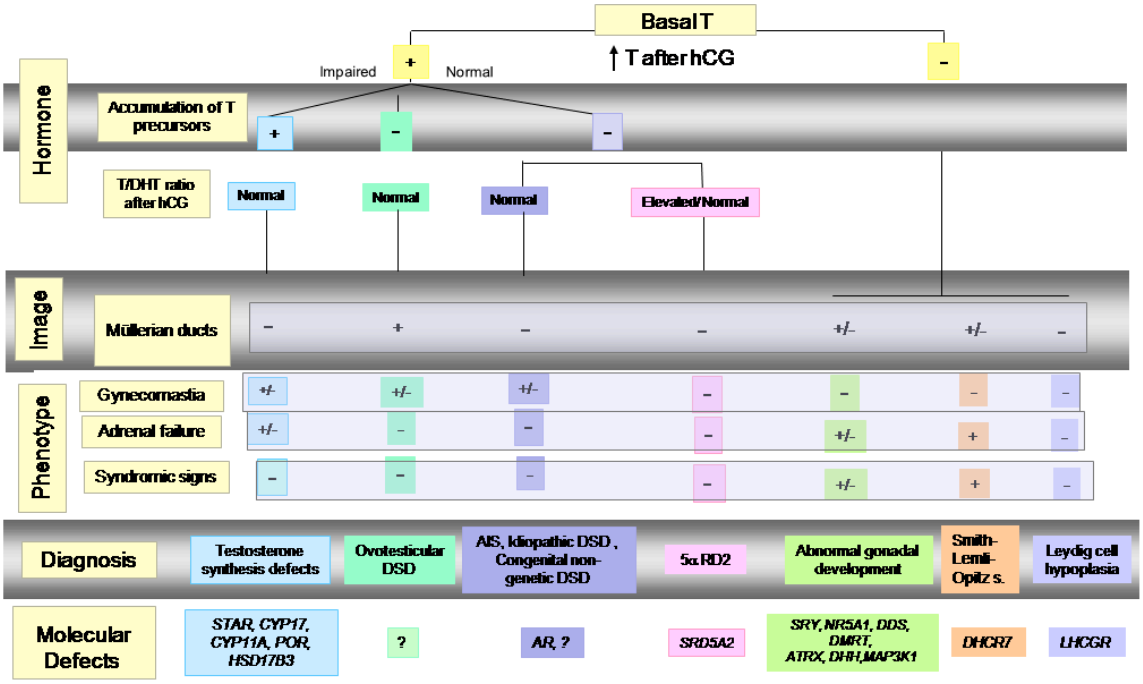

Figure 5 - Algorithm for 46,XY DSD diagnosis

46,XY DSD DUE TO ABNORMALITIES IN GONADAL DEVELOPMENT

In humans, there are several disorders associated with 46,XY gonadal dysgenesis caused by mutations in genes, which are involved in gonadal determination. They will be described according to the period of gene expression in gonadal determination.

Gonadal determination and differentiation

The intermediary mesoderm is the primary embrionic tissue at gastrulation that gives rise to the urogenital ridge. This, in turns, is going to derive the primitive gonad from a condensation of the medioventral region of the urogenital ridge. The primitive gonad separates from the adrenal primordium at about 5 weeks, but

remains bipotential until the 6th week after conception. Mammalin sex determination is a complex process, which involves a large number of genes acting in networks. Several genes have been involved in the development of the urogenital ridge, including *Emx2, Lim1, Lhx9, WT1, Gata-4/Fog2, Nr5a1/NR5A1.* Although mutations or knockout models of these genes produce abnormal gonads in mice, not all these genes have been implicated in gonadal-dysgenesis syndromes in humans. To date, *Emx2* null mice have absent kidneys, ureters, gonads and genital tracts and have developmental abnormalities of the brain (19). In humans, mutations in *EMX2* have been found in patients with schizencephaly (a rare condition in which a person is born with clefts in the brain that are filled with liquor) but no gonadal phenotype have been described. In mice lacking Lhx9 function, germ cells migrate normally, but somatic cells of the genital ridge fail to proliferate and gonads fail to form (20). No human mutation in *LHX9* has been identified (21). *WT1, NR5A1* and *DAX* are well known genes that are critical for the formation of the urogenital ridge in humans. The products of the Wilms' tumor-suppressor gene (*WT1*) are essential for both gonadal and renal formation (22) whereas steroidogenic factor 1 (NR5A1) protein is essential for gonadal and adrenal formation (23,24). DAX 1 is also essential for gonadal and adrenal differentiation and when duplicated results in adrenal hypoplasia congenital and hypogonadotropic hypogonadism (25).

After the formation of the bipotential gonad, by the 6th week after concepcion, in 46, XY individuals, the expression of the testis-determining gene *Sry,* which is transcriptionally regulated by the expression of WT1 (26) and its co-factor zinc finger protein FOG2 (27) and chromobox protein homolog 2 (CBX2) (28) triggers the gonadal masculinizing fate process (29). In the mammalian male embryo, the first molecular signal of sex determination is the expression of *Sry* within a subpopulation of somatic cells of the indifferent genital ridge. The transient expression of Sry drives the initial differentiation of pre-Sertoli cells that would otherwise follow a female pathway becoming granulosa cells. Once Sry expression begins, it initiates the cascade of gene interactions and cellular events that direct to the formation of a testis from the indifferential fetal gonad. So, pre-Sertoli cells proliferate, polarize and aggregate around the germ cells to define the testes cords. Migration of cells into the gonad from the mesonephros or the coelomic epithelium is subsequently induced by signals emanating from the pre-Sertoli cells. Peritubular myoid cells surround the testes cords and cooperate with pre-Sertoli cells to deposit the basal lamina and further define the testis cords. Signalling molecules produced by the pre-Sertoli cells promote the differentiation of somatic cells, found outside the cords, into fetal Leydig cells, thus ultimately allowing the production of testosterone. Endotelial cells are associated to form the coelomic vessel, which promotes efficient export of testosterone into plasma.

The gene *Sox9* is up-regulated immediately after *Sry* expression and is involved in the initiation and maintenance of Sertoli cell differentiation during the early phases of testis differentiation (30). The mechanism by which NR5A1 and SRY increase endogenous SOX9 expression was clearly demonstrated in human embryonal carcinoma cell line NT2/D1 (31).

Extracellular signaling pathways (*Fgf9* and Igf1r/Irr/Ir) play a significant role in *Sox9* expression. A model has been suggested in that the fate of the bipotential gonad is controlled by mutually antagonistic signals between *Fgf9* and *Wnt4/Rspo1*. In this model *Sox9* up-regulates *Fgf9-Fgfr2* and *Fgf9* maintains *Sox9* expression, forming a positive feed-forward loop in XY gonads. The balance between *Fgf9* and *Wnt4/Rspo1* signals is shifted in favor of *Fgf9*, establishing the male pathway. In addition, *Sry* inhibits β-catenin-mediated Wnt signaling (32). In the absence of this feed-forward loop between *Sox9* and *Fgf9*, *Wnt4/Rspo1,* the activated β-catenin pathway, blocks *Fgf9* and promotes the ovarian fate (33,34). Furthermore, SOX9 directly binds to the promoter of the *Ptgds* gene which encodes prostaglandin D synthase that mediates the production of PGD2 (35) which, in turn, promotes nuclear translocation of SOX9, facilitating Sertoli cell differentiation (36).

Antagonism between *Dmrt1* and *Foxl2* comprises another step for sex-determining decision. *Dmrt1* has been described as essential to maintain mammalian testis determination, preventing female reprogramming in the postnatal mammalian testis (37). *MAP3K1* has been described to be important to the balance between *SOX9/FGF9* to *WNT/beta-catenin* signaling in functional studies (38,39). However, the role of MAP3K1 in human sex-determination remains unknown as downstream effectors of MAP3K1 in the human developing testis have not been identified, as reviewed by Bashamboo and McElreavey (40).

Abnormalities in the expression (underexpression or overexpression or timing of expression) of genes involved in the cascade of testis determination can cause anomalies of gonadal development and consequently, 46,XY DSD. The absence, regression or the presence of dysgenetic testes results in abnormal development of the genital ducts and/or external genitalia in these patients.

46,XY DSD DUE TO ABNORMALITIES OF GONADAL DEVELOPMENT

Gonadal Agenesis

Total absence of gonadal tissue confirmed by laparoscopy has rarely been described in XY subjects with female external and internal genitalia indicating the absence of testicular determination (41). Mendonca et al described a pair of siblings, one XY and the other XX, born to a consanguineous marriage, with normal female external and internal genitalia associated to gonadal agenesis (42). Mutations in *NR5A1 and LHX9* were latter ruled out in these siblings (43). The origin of this disorder remains to be determined, but a defect in another gene

essential for bipotential gonad development is the most likely cause of this disorder.

46,XY DSD due to Gonadal Dysgenesis

Complete and partial 46,XY gonadal dysgenesis

46,XY gonadal dysgenesis consists of a variety of clinical conditions in which the development of the fetal gonad is abnormal and encompasses both a complete and a partial form. The complete form of gonadal dysgenesis was first described by Swyer et al. (44) and is characterized by female external and internal genitalia, lack of secondary sexual characteristics, normal or tall stature without somatic stigmata of Turner syndrome, eunuchoid habitus and the presence of bilateral dysgenetic gonads in XY subjects. Mild clitoromegaly is present in some cases.

The partial form of this syndrome is characterized by variable degrees of impaired testicular development and of testicular function. These patients present a spectrum of atypical genitalia with or without Müllerian structures. Similar phenotypes can also result from a 45,X/46,XY karyotype.

Serum gonadotropin levels are elevated in both the complete and partial forms, mainly FSH levels, which predominate over LH serum levels. Testosterone levels are at prepubertal range in the complete form. Meanwhile, in the partial form, it can range from prepubertal levels to normal adult male levels.

The clinical condition named embryonic testicular regression syndrome (ETRS) has been considered part of the clinical spectrum of partial 46,XY gonadal dysgenesis (45). In this syndrome, most of the patients present atypical genitalia or micropenis associated with complete regression of testicular tissue in one or both sides. The variable degree of masculinization of the internal and external genitalia is a consequence of the time of testicular function prior to its loss. The dysgenetic testes showed disorganized seminiferous tubules and ovarian stroma with occasional primitive sex cords without germ cells (46). Familial cases have been reported with variable degrees of sexual ambiguity, but the nature of the underlying defect is still unknown (45).

An interesting study describes a remarkable family pedigree across four generations with multiple affected family members. Phenotypic, with variable degrees of gonadal dysgenesis. The phenotypic mode of inheritance was strongly suggestive of X-linkage (47). In this report, a fertile woman had a 46,XY karyotype in peripheral lymphocytes, mosaicism in cultured skin fibroblasts (80% 46,XY and 20% 45,X) and a predominantly 46,XY karyotype in the ovary (93% 46,XY and 6% 45,X). She gave birth to a 46,XY daughter with complete gonadal dysgenesis. The range of phenotypes observed in this unique family suggests a new mechanism, which predisposes to chromosomal mosaicism (47).

Regarding the genetic etiology, 46,XY gonadal dysgenesis is heterogeneous and can results from defects of any gene involved in the process of gonadal formation.

Mutations in *NR5A1, MAP3K1 and SRY* are the most frequent molecular cause of 46,XY gonadal dysgenesis. The following review will focus on the main genes causing gonadal dysgenesis in humans, presenting as an isolated or syndromic phenotype. The genes are described in the text accordingly their expression time during gonadal development.

46,XY DSD due to underexpression of *WT1* gene

The Wilms' tumor suppressor gene (*WT1*) encodes a zinc-finger transcription factor involved in the development of the kidneys and gonads and their subsequent normal function. *WT1* gene is located on 11p13 and mutations in this gene impair gonadal and urinary tract development. Three disorders are associated with *WT1* mutations: *WAGR syndrome, Denys-Drash syndrome* and *Frasier syndrome*.
WAGR syndrome: is characterized by Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation. The genitourinary anomalies are renal agenesis or horseshoe kidney, urethral atresia, hypospadias, cryptorchidism and more rarely atypical genitalia (48). Heterozygous deletions of *WT1 and contiguous* gene are the cause of this syndrome (49). Deletions of *PAX6* gene are related to the presence of aniridia in these patients. Severe obesity is present in some subjects with the WAGR syndrome and the acronym WAGRO has been suggested for this (50). The existence of a gene in the 11p14-p12 regions responsible for obesity is proposed. A 46,XY patient with WAGR syndrome and female external and internal genitalia with an interstitial deletion of approximately 10 Mb encompassing *WT1* and *PAX6* was described (51). This report demonstrated an overlap of clinical and molecular features in WAGR, Frasier and Denys-Drash syndromes that confirms these conditions as a spectrum of disease due to *WT1* alterations.
Denys-Drash syndrome
is characterized by dysgenetic 46,XY DSD associated with early-onset renal failure (diffuse mesangial sclerosis) and Wilms´ tumor development in the first decade of life (52). Müllerian ducts differentiation varies according to the Sertoli cells function. The molecular defect of this syndrome is the presence of heterozygous missense mutations in the zinc finger encoding exons (DNA-binding domain) of *WT1* gene (53). Gonadal development is impaired to variable degrees, resulting in a spectrum of 46,XY DSD (54). Frasier syndrome: is characterized by a female to atypical external genitalia phenotype in 46,XY patients, streak gonads and high risk of gonadoblastoma development and renal failure in the second decade of life. We described a patient presenting an unusual DDS nephropathy progression, which reinforces that patients carrying WT1 mutations should have the renal function carefully monitored due to the possibility of late-onset

nephropathy (55) (56). However, the nephrotic syndrome may be evident early in life (57).

The *WT1* gene contains 10 exons, of which exons 1–6 encode a proline/glutamine-rich transcriptional-regulation region and exons 7–10 encode the four zinc fingers of the DNA-binding domain. There are four major species of RNA with conserved relative amounts, different binding specificities, and different subnuclear localizations, generated by two alternative splicing regions (58). Splicing at the first site results in either inclusion or exclusion of exon 5. The second alternative splicing site is in the 3' end of exon 9 and allows the inclusion or exclusion of three amino acids lysine, threonine and serine (KTS) between the third and fourth zinc fingers, resulting in either KTS-positive or negative isoforms. Isoforms that only differ by the presence or absence of the KTS amino acids have different affinities for DNA and, therefore, possibly different regulatory functions (59). The c.1432+4C>T mutation leads to a change in splicing resulting in deficiency of the usually more abundant KTS positive isoforms and reversal of the normal KTS positive to negative ratio, indicating that a precise balance between WT1 isoforms is necessary for normal WT1 function (56). Constitutional heterozygous mutations of the *WT1* gene, almost all located at intron 9, are found in patients with Frasier syndrome, leading to a change in splicing that results in reversal of the normal KTS positive/negative ratio from 2:1 to 1:2 (52) (60). Frasier syndrome is usually associated with the c.1432+4C>T mutation (61), although exonic mutations also cause Frasier syndrome (62). We reported a patient presenting an overlapping of some typical characteristics of Frasier syndrome (end-stage renal failure in the second decade, gonadoblastoma and the c.1432+4C>T mutation, but with the gonadal and external genitalia development usually found in Denys-Drash syndrome (56).

The report of atypical external genitalia (62), the presence of Wilms' tumor (63), and the description of exonic mutations in the DNA binding domain of *WT1* gene (62) in patients with Frasier syndrome indicate an overlap of clinical and molecular features in Denys Drash and Frasier syndromes.

46,XY DSD due to the underexpression of steroidogenic factor-1 (NR5A1/SF1)

NR5A1 was originally identified as a master-regulator of steroidogenic enzymes in the early 1990s following the Keith L. Parker and Kenichirou Morohashi inspiring work (64,65) (66). NR5A1 has since been shown to control many aspects of adrenal and gonadal function (67) (23,24) (68). NR5A1, together with several signaling molecules are also involved in adrenal stem cell maintenance, proliferation and differentiation inducing adrenal zonation, probably acting in the progenitor cells (69). Homozygous 46,XY null mice (−/−) have adrenal agenesis, complete testicular dysgenesis, persistent Müllerian structures, partial

hypogonadotropic hypogonadism, and other features such as late-onset obesity (70). Therefore, it was clear demonstrated that NR5A1 is an essential factor in sexual and adrenal differentiation and a key regulator of adrenal and gonadal steroidogenesis and also of the hypothalamic-pituitary-gonadal axis.

The first reported human case of *NR5A1* mutation, the heterozygous p.G35E, was a 46,XY patient who presented female external genitalia and Müllerian duct derivatives, indicating the absence of male gonadal development, associated with adrenal insufficiency. This patient presented with salt-losing adrenal failure in early infancy and was thought to have a high block in steroidogenesis (e.g. in CYP11A1, STAR) affecting both adrenal and testicular functions. However, the identification of a streak-like gonad and Müllerian structures was consistent with testicular dysgenesis, thereby, a disruption of a common developmental regulator such as NR5A1 was hypothesized. The patient was found to have a *de novo* heterozygous p.G35E change in the P-box of *NR5A1* which is important in dictating DNA binding specificity through its interaction with DNA response elements in the regulatory regions of target genes (71).

The second report of *NR5A1* defects in humans was described by Biason-Lauber and Schoenle, in a 14 month-old 46,XX girl who had presented primary adrenal insufficiency and seizures (72). She had a *de novo* heterozygous *NR5A1* change resulting in the p.R255L mutation into the proximal part of the ligand-like binding domain of the protein. The mutant NR5A1 protein was transcriptionally inactive, without a dominant negative effect. The ovaries were detected by MRI scan and Inhibin A levels was normal for her age, suggesting that NR5A1 change had not disrupted ovarian function.

The third report of *NR5A1* defects in humans was found in an infant with a similar phenotype of the first case: primary adrenal failure and 46,XY DSD. However, this child had inherited the homozygous p.R92Q alteration in a recessive manner (73). The change lies within the A-box of NR5A1, which interferes with monomeric DNA binding stability, but *in vitro* functional activity was in the order of 30–40% of the wild type (73) (74) (75). Carrier parents showed normal adrenal function suggesting that the loss of both alleles is required for the phenotype development when disrupted protein keeps this level of functional activity. In addition, another family has been reported with a homozygous missense mutation (p.D293N) in the LBD of NR5A1 (76). This change also showed partial loss-of-function (50%) in gene transcription assays.

In 2004, we reported the fourth *NR5A1* mutation in humans which brought two novel variables to NR5A1 phenotype: it was the first frameshift mutation and it appeared in a 34 year old 46,XY DSD female with normal adrenal function (77). Another interesting aspect in this patient was the absence of gonadal tissue at laparoscopy. Since she had atypical genitalia and absence of Müllerian derivatives we assumed that testicular tissue regressed completely late in fetal life.

NR5A1 changes associated with 46,XY DSD are usually frameshift, nonsense or missense changes that affect DNA-binding and gene transcription (74). Most of the point mutations identified in *NR5A1* are located in the DNA-binding domain of the protein. The p.L437Q mutation, the first located in the ligand-binding region, was identified in a patient with a mild phenotype, a penoscrotal hypospadias; this protein retained partial function in several NR5A1-expressing cell lines and its location points to the existence of a ligand for NR5A1, considered an orphan receptor so far (75). NR5A1 is bound to sphingosine (SPH) and lyso-sphingomyelin (lysoSM) under basal conditions (78,79). Progressive androgen production and virilization in adolescence has been observed in several XY patients with *NR5A1* mutations, in contrast to the severe undervirilized external genitalia found in most patients (79-81). The almost normal testosterone levels after hCG stimulation or at pubertal age suggest that *NR5A1* action might be less implicated in pubertal steroidogenesis than during fetal life.

In contrast, fetal Sertoli cell function seems to be preserved in the most patients with heterozygous *NR5A1* mutations based on the common observation of absent Müllerian derivatives and primitive seminiferous tubules on histology. The reviewed data of seventy-two 46,XY DSD patients with NR5A1 mutations reported in the literature, for whom information on presence or absence of Müllerian derivatives was available, suggested that Müllerian derivatives are present in about 24% of the cases (82-85). However, persistently elevated FSH levels after puberty found in all patients studied suggest an impairment of Sertoli cells function in post pubertal age (82).

More than 90 different *NR5A1* variants, distributed across the full length of the protein, have been described and the majority is nonsynonymous mutations (73,79,81,86,87). Most of these mutations are located in the DNA binding domain and are in heterozygous state or compound heterozygous state with the p.Gly146Ala (rs1110061) variant. A clear correlation between the location of a mutation, its *in vitro* functional performance and the associated phenotype is not observed. Indeed, family members bearing the same *NR5A1* mutation may present with diferent phenotypes (88).

The contribution of other genetic modifiers has been suggested to explain phenotypic variability. Exome sequencing analyses of DSD patients have identified pathogenic variants or variants of uncertain significance in several genes involved in sexual development (29,89). In a 46,XY patient with atypical external genitalia, palpable inguinal gonads, absent uterus in pelvic ultrasonography and poor testosterone response to hCG stimulation, Mazen and colleagues identified, by exome sequencing, the previously described p.Arg313Cys *NR5A1* mutation in compound heterozygous state with a p.Gln237Arg *MAP3K1* variant (90). This *NR5A1* mutation was previously reported in association with mild hypospadias

(91), and a possible digenic inheritance was proposed to explain the phenotypic heterogeneity (90).

In several cohort studies, NR5A1 changes have been reported in approximately 10–15% of the individuals with gonadal dysgenesis (67,74,79). Although many of the heterozygous changes are *de novo*, about one-third of these changes have been shown to be inherited from the mother in a sex-limited dominant manner (74). These women are at potential risk of primary ovarian insufficiency but while fertile they can pass NR5A1 heterozygous changes to their children. This mode of transmission can mimic X-linked inheritance (74). The features in different affected family members can be variable.

A novel role of *NR5A1* in human reproductive function was described by Bashamboo and co-workers (92). They investigated whether changes in *NR5A1* could be found in a cohort of 315 men with normal external genitalia and non-obstructive male factor infertility where the underlying cause was unknown (92). Analysis of *NR5A1* in this cohort identified heterozygous changes in seven individuals; all of them were located within the hinge region of the NR5A1 protein. The men who harbored *NR5A1* changes had more severe forms of infertility (azoospermia, severe oligozoospermia) and in several cases low testosterone and elevated gonadotropins were found. A serial decrease in sperm count was found in one-studied men raising the possibility that heterozygous changes in NR5A1 might be transmitted to offspring, especially if fatherhood occurs in young adulthood rather than later in life (93)As progressive gonadal dysgenesis is likely, gonadal function should be monitored in adolescence and adulthood, and early sperm cryopreservation considered in male patients, if possible. In conclusion, this study shows that changes in NR5A1 may be found in a small subset of phenotypically normal men with non- obstructive male factor infertility where the cause is currently unknown. These individuals may be at risk of low testosterone in adult life and may represent part of the adult testicular dysgenesis syndrome (93) (94) (95).

A novel heterozygous missense mutation (p.V355M) in *NR5A1* was identified in one boy with a micropenis and testicular regression syndrome *(96). NR5A1* mutations have also been identified in familial and sporadic forms of 46,XX primary ovarian insufficiency (POI) not associated with adrenal failure (76) (97). Most of these women harbored heterozygous alterations in *NR5A1* and had been identified in families with history of 46,XY DSD and 46,XX POI. Heterozygous *NR5A1* changes were also found in two girls with sporadic forms of POI (76). In one large kindred a partial loss-of-function NR5A1 change (p.D293N) was inherited in an autosomal recessive manner. These 46,XX women with p.D293N NR5A1 mutation presented with either primary or secondary amenorrhea and with a variable age of features onset. The detection of NR5A1 alterations in 46,XX ovarian failure shows that *NR5A1* is also a key factor in ovarian development and function in humans. Thus, some 46,XX women with *NR5A1* mutations have normal ovarian function

and can transmit the mutation in a sex-limited dominant pattern. Therefore, the inheritance patterns associated with *NR5A1* changes can be autosomal dominant, autosomal recessive or sex-limited dominant.

NR5A1 defects can be found in association with a wide range of human reproductive phenotypes such as 46,XY and 46,XX disorders of sex development (DSD) associated or not with primary adrenal insufficiency, male infertility, primary ovarian insufficiency.and finally testicular or ovariotesticular 46,XX DSD (79) (Table 2).

Table 2- Spectrum of phenotypes caused by NR5A1 defects

Karyotype	Phenotypes	Number of described patients	Reference
46,XY	DSD and adrenal failure	2	(71,73)
	DSD without adrenal failure	69	(75,79,81,86,87,94,98-101)
	Male infertility	10	(92,101)
	Ovotesticular DSD and genitopatelar syndrome*	1	(102)
46,XX	Adrenal failure	2	(72,103)
	Female infertility	14	(76,79,98)
	(Ovo) testicular DSD without adrenal failure	11	(79,104-106)

Dysgenetic 46,XY DSD due to GATA4 and FOG2 underexpression

Gata4 (GATA-binding factor 4 gene) cooperatively interacts with several proteins to regulate the expression of genes involved in testis determination and differentiation as *SRY, SOX9, NR5A1, AMH, DMRT1, STAR, CYP19A1*, and others (107).
In humans, *GATA4* mutations were first described in patients with congenital heart defects without genital abnormalities (108). However, genitourinary anomalies, as hypospadias and cryptorchidism, were described in 46,XY patients with deletion of 8p23.1 region in which GATA4 is located (109).

The p.Gly221Arg *GATA4* mutation was identified in five members of a French family, three 46,XY DSD patients, two of them with cardiac anomalies and in their two apparently unaffected mothers (110).
The role of *FOG2* in human testis development was corroborated by the identification of a balanced translocation t(8;10) (q23.1;q21.1) in a patient with partial gonadal dysgenesis and congenital heart abnormalities (111). Bashamboo et al. identified independent missense mutations in FOG2, by using exome sequencing, in two patients with 46,XY gonadal dysgenesis. One patient carried the non-synonymous p.S402R, heterozygous mutation. The second patient carried the inherited homozygous p.M544I mutation and the *de novo* heterozygous p.R260Q mutation. The p.M544I variant by itself has little effect on the biological activity of FOG2 protein in transactivation of the gonadal promoters, but it shows reduced binding with GATA4. In the *in vitro* assays, a combination of both the p.R260Q and the p.M544I variants altered the biological activity of the FOG2 protein on specific downstream targets, as well as obliterated its interaction with GATA4. In the patient, the two mutations together may result in an imbalance of the delicate equilibrium between antagonistic male and female pathways leading ultimately to gonadal dysgenesis (112).

Dysgenetic 46,XY DSD due to CBX2 underexpression

CBX2 (Chromobox homolog 2 gene) defects in *SRY*-positive mice cause male-to-female sex reversal with small or absent ovaries suggesting that CBX2 acts repressing ovarian development in XY gonads (113) (114).
A girl, with a 46,XY karyotype performed during prenatal life, was born with a completely normal female phenotype, including uterus and histologically normal ovaries. The gonads were evaluated at 4.5 years of age and at this time she had high serum FSH levels. Direct sequencing of the *CBX2* gene revealed the presence of the heterozygous variants c.C293T and the c.G1370C, both in exon 5 leading to p.P98L (inherited from the father) and p.R443P (inherited from the mother) mutations in the CBX2 protein (115).

46,XY DSD due to underexpression of *SRY* gene

Most of the authors reported mutations in *SRY* gene in less than 20% of the patients with complete 46,XY gonadal dysgenesis (116-118). In the partial form, the frequency of *SRY* mutation is even lower than in the complete form. To date, most of the *SRY* mutations are located in the HMG box, showing the critical role of this domain and are predominantly *de novo* mutations. However, some cases of fertile fathers and their XY affected children, sharing the same altered *SRY* sequence, have been reported (116,119). In few of these cases, the father's

somatic mosaicism for the normal and mutant *SRY* gene have been proven (120). The variable penetrance of *SRY* mutations in familial cases have been described in SRY mutant proteins with relatively well preserved *in vitro* activity (121).

Dysgenetic 46,XY DSD associated with campomelic dysplasia (underexpression of the SOX9)

SRY-related HMG-box gene 9 (*SOX9*) is a transcription factor involved in chondrogenesis and sex determination. *SOX9* gene*,* located on human chromosome 17, is a highly conserved HMG family member and it is also implicated in the sex-determining pathway (122) (123). In all affected subjects, *SOX9* mutation was identified in heterozygous state indicating that this disorder is due to haploinsufficiency of *SOX9* gene (122). This syndrome is characterized by severe skeletal malformations (campomelic dysplasia) associated to dysgenetic 46,XY DSD in three-quarters of the affected 46,XY patients. The external genitalia vary from that of normal males with cryptorchidism through atypical to female and internal genitalia can include vagina, uterus and fallopian tubes (124).
Patients with campomelic dysplasia and 46,XY gonadal dysgenesis with intact *SOX9* were reported. In one patient a microdeletion of ~380 kb upstream of *SOX9* was identified (125). In the other patient an apparently balanced chromosome translocation with breakpoints scattered ~1.3 Mb downstream of *SOX9* was found (126).

Dysgenetic 46,XY DSD due to FGF9/FGFR2 underexpression

The importance of Fgf9/Fgfr2 signaling pathway in mouse testis determination is well known (127-129). In the developing testis occurs a positive feedback loop among Fgf9/Fgfr2/Sox9; Fgf9 is upregulated by Sox9 and signals through Fgfr2 maintain Sox9 expression (33) (130) (127) and this loop represses Wnt4 (131). Mice homozygous for a null mutation in *Fgf9* or *Fgfr2* exhibit male-to-female sex reversal, with all testis-specific cellular events being disrupted, including cell proliferation, mesonephric cell migration, Sertoli cell differentiation, and testis cord formation (132) (127) (129). However, in human sex development the role of *FGF9* and *FGFR2* remains unclear. No *FGF9* mutations were identified in 46,XY GD patients (133). Human *FGFR2* mutations have been related with some syndromes as lacrimoauriculodentodigital (LADD) characterized by tear tract, ear, teeth and digit abnormalities (134) and craniosynostosis syndromes including Crouzon, Pfeiffer, Apert and Antley-Bixler syndromes (135) (136), (137). *FGFR2* mutations can lead to loss (LAAD syndrome) or gain (craniosynostosis syndromes) of function in these disorders (138) (139). No gonadal defects were described in patients with LADD or craniosynostosis syndromes.

A single 46,XY patient with gonadal dysgenesis and craniosynostosis was described by Bagheri-Fam et al (140). This patient had abnormalities found in different craniosynostosis syndromes (short stature, brachycephaly, proptosis, downslanting palpebral fissures, low-set dorsally rotated ears, reduced extension at the elbows but absence of hand and feet anomalies) and a specific syndromic diagnosis was not established. She also presented female external genitalia, primary amenorrhea and gonadal dysgenesis with dysgerminoma. DNA sequencing revealed a cysteine-to-serine substitution at position 342 in the FGFR2c isoform (Cys342Ser). Cys342 substitutions by Ser or other amino acids (Arg/Phe/Trp/Tyr) occur frequently in the craniosynostosis syndromes Crouzon and Pfeiffer but these patients do not present gonadal abnormalities. Mutation in the 2c isoform of FGFR2 is in agreement with knockout data showing that FGFR2c is the critical isoform during sex determination in the mouse. Taken together, these data suggest that the FGFR2c c.1025G>C (p.Cys342Ser) mutation contributed to 46,XY DSD in the sex-reversed patient. The authors proposed that this heterozygous mutation leads to gain of function in the skull, but to loss of function in the developing gonads and that she might harbor a unique set of modifier genes, which exacerbate this testicular phenotype (140). Expressivity of the XY gonadal sex reversal phenotype in Fgfr2 knockout mice was greatly dependent on the genetic background (127).

Dysgenetic 46,XY DSD due to disruption in the Hedgehog signaling
I-Desert hedgehog (DHH) gene

It is a member of the hedgehog family of signaling proteins, is located in chromosome 12-q13.1 and is one of the genes involved in the testis-determining pathway (141). Dhh seems to be necessary for Nr5a1 up-regulation in Leydig cells in mouse (142). To date, six homozygous mutations have been described in DHH gene in 46,XY patients conferring phenotypes ranging from partial to complete gonadal dysgenesis, associated or not with polyneuropathy. The first one, the homozygous missense mutation (p.M1T) is located at the initiation codon of exon 1 and was found in a 46,XY patient with partial gonadal dysgenesis associated with polyneuropathy (143). Two other mutations, one the p.L162P located at exon 2 and the other the p.L363CfsX4 located in exon 3 were identified in three patients with complete gonadal dysgenesis without polyneuropathy; two of them harbored gonadal tumors (bilateral gonadoblastoma and dysgerminoma, respectively) (144). Later, the c.1086delG mutation was identified in heterozygous state in two patients with partial gonadal dysgenesis (145). In addition, two novel homozygous mutations were described in two patients with complete 46,XY gonadal dysgenesis without clinically overt polyneuropathy (146). In both sisters, clinical neurological examination revealed signs of a glove and stocking like polyneuropathy. The first

defect the c.271_273delGAC resulted in deletion of one amino acid (p.D90del) and the second one, a duplication c.57_60dupAGCC resulted in a premature termination of DHH protein (146). The p.R124Q mutation was identified by exome sequencing in two sisters of a consanguineous family with 46, XY gonadal dysgenesis and testicular seminoma (147).

II- Hedgehog acetyl-transferase (HHAT) gene

The HHAT protein is a member of the MBOAT family of membrane-bound acyl-transferases which catalyzes amino-terminal palmitoylation of Hh proteins. The novel mutation (p.G287V) in the Hedgehog acetyl-transferase gene (HHAT) was found in a syndromic 46,XY DSD patient with complete gonadal dysgenesis and skeletal malformation by exome sequencing. This mutation disrupted the ability of HHAT protein to palmitoylate Hh proteins including DHH and SHH (148). In mice, the absence of *Hhat* in the XY gonad did not affect testis-determination, but impaired fetal Leydig cells and testis cords development (148). The phenotype of the girl carrying the homozygous p.G287V mutation is a rare combination of gonadal dysgenesis and chondrodysplasia. Moreover, a *de novo* dominant mutation in the MBOAT domain of *HHAT* was reported in association with intellectual disability and apparently normal testis development (149).

46,XY DSD due to the underexpression of DMRT1 gene

Raymond et al identified both DNA-binding Motif (DM) domain genes expressed in testis *(DMRT1* and *DMRT2)* located in chromosome 9p24.3, a region associated with gonadal dysgenesis and 46,XY DSD (150) (151) (152). The human 9p monossomy syndrome is characterized by variable degrees of 46,XY DSD, from female genitalia to male external genitalia with cryptorchidism associated to agonadism, streak gonads or hypoplastic testes and internal genitalia disclosing normal Müllerian or Wolffian ducts, mental retardation and craniofacial abnormalities (153). Gonadal function varies from insufficient to near normal testicular production. It is inferred that haploinsufficiency of *DMRT1* and *DMRT2* primarily impairs the formation of the undiferentiated gonad, leading to various degrees of testis or ovary formation defects (153).

Genomic–wide copy number variation screening revealed that *DMRT1* deletions were associated with isolated 46,XY gonadal dysgenesis in addition to inactivation mutations (133,151).

In vitro studies to analyze the fuctional activity of the DMRT1 p.R111G mutation identified by exome sequencing in a patient with 46,XY complete gonadal dysgenesis, indicated that this protein had reduced DNA affinity and altered sequence specificity. This mutant DMRT1, when mixed with the wild-type protein bounded as a tetramer complex to an *in vitro* Sox9 DMRT1-binding site, differently

of the wild-type DMRT1 that usually bound as a trimer. This suggests that a combination of haploinsufficiency and a dominant disruption of the normal DMRT1 target binding site is the cause of the abnormal process of testis-determination seen in this patient (154).
Matson et al. (2011) have shown in mouse that *Dmrt1* and *Foxl2* create another regulatory network necessary for maintenance of the testis during adulthood. Loss of *Dmrt1* in mouse Sertoli cells induces the reprogramming of those into granulosa cells, due to *Foxl2* upregulation. Consequently, theca cells are formed, estrogens is produced and germ cells appear feminized (37).

ATR-X syndrome (X-linked α-thalassemia and mental retardation)

ATR-X syndrome results from mutations in the gene that encodes for X-linked helicase-2, implicating *ATR-X* in the development of the human testis (155). Genital anomalies leading to a female sex of rearing were reported in several affected 46,XY patients with ATR-X syndrome (156).
ATR-X syndrome is characterized by severe mental retardation, alpha thalassemia and a range of genital abnormalities in 80% of cases (155). In addition to these definitive phenotypes, patients also present with typical facial anomalies comprising a carp-like mouth and a small triangular nose, skeletal deformities and a range of lung, kidney and digestive problems. A variety of phenotypically overlapping conditions (Carpenter-Waziri syndrome, Holmes-Gang syndrome, Jubert-Marsidi syndrome, Smith-Fineman-Myers syndrome, Chudley-Lowry syndrome and X-linked mental retardation with spastic paraplegia without thalassemia) have also been associated with *ATRX* mutations. *ATRX* lies on the X chromosome (Xq13) and the disease has been confined to males; in female carriers of an *ATRX* mutation, the X-inactivating pattern is skewed against the.X carrying the mutant allele.
Urogenital abnormalities associated to mutations in human *ATRX* range from undescended testes to testicular dysgenesis with female or atypical genitalia. Duplication of Xq12.2-Xq21.31 that encompasses ATRX along with other genes has been described in a male patient with bilateral criptorquidism and severe mental retardation. The patient entered spontaneous puberty by the age of 12 and developed bilateral gynecomastia (157). There are two major functional domains in ATRX protein: 1- the ATRX-DNMT3-DNMT3L (ADD) domain at the N-terminus and 2- the helicase/ATPase domain at the C-terminal half of the protein, both acting as chromatin remodeling. Mutations in the ADD domain have been related to severe psychomotor impairment associated to urogenital abnormalities. On the other hand, mutations in the C-terminus region have been related with mild psychomotor impairment without severe urogenital abnormalities (158) (159).

Although all cases of severe genital abnormality reported in ATRX syndrome have been associated with severe mental retardation, this is not true for alpha-thalassemia. The role of *ATRX* in the sexual development cascade is poorly understood and it is suggested that it could be involved in the development of the Leydig cells (160).

Dysgenetic 46,XY DSD due to MAP3K1 underexpression

MAPK signaling pathway role in mammalian sex-determination is still poorly understood. In mice, it has been shown that the Map3k4 gene is essential for testicular determination, since the lack of activity of this protein leads to failure of testicular cord development and disorganization of gonadal tissue in formation (161). In mice, the reduction of the Gadd45/Map3k4/p38 pathway activity is associated with a reduction in the Sry expression in the XY mice gonad at sex-determination causing sex-reversal in these animals (162).
Studies with knockin animals for the *Map3k1* gene demonstrated a lower repercussion in the testicular tissue which present a reduction in the Leydig cells number (163,164). However, in patients with 46, XY gonadal dysgenesis, different non-synonymous allelic variants were identified in the *MAP3K1* gene. The first mutation described was identified for mapping by linkage analysis of an autosomal sex-determining gene locus at the long arm of chromosome 5 in two families with 46,XY DSD, including patients with complete and partial gonadal dysgenesis. The splice-acceptor mutation c.634-8T>A in the *MAP3K1* disrupted RNA splicing and segregated with the phenotype in the first family. Mutations in the *MAP3K1* were also demonstrated in the second family (p.Gly616Arg) and in two of 11 sporadic 46,XY DSD patients (p.Leu189Pro, p.Leu189Arg) studied (38)(39). Subsequently, the two novel mutations p.Pro153Leu and c.2180- 2A>G in the *MAP3K1* were identified in non-syndromic patients with 46,XY gonadal dysgenesis. Functional studies of mutated MAP3K1 proteins identified change in phosphorylation targets in subsequent steps of the cascade of MAP3K1, p38 and ERK1/2 and enhanced the binding of the Ras homolog gene family, member A (RHOA) to the MAP3K1 complex (39). In normal male gonadal development, the binding of MAP3K1 to the RHOA protein promotes a normal phosphorylation of p38 and ERK1/2, and a blockade of the β-catenin pathway is determined by MAP3K4. In the female development, hyperphosphorylation of p38 and ERK1/2 occurs and the presence of p38 and ERK1/2 hyperphosphorylated determine the activation of the β-catenin pathway, that result in a block of the positive feedback pathway of SOX9 and the testicular development (39).
Cohorts of patients with 46,XY DSD studied by targeted gene panel has found several new potentially deleterious variants and uncertain significance variants in the *MAP3K1* (165) (166). Although, the findings strongly indicate the participation

of the *MAP3K1* mutations in the etiology of testicular development abnormalities a better understanding of the mechanisms of MAPK pathway in the gene regulatory networks of the human testicular determination process is still necessary (40) (90).

46,XY DSD due to the overexpression of DAX1 (NR0B1) gene

Male patients with female or atypical external and internal genitalia due to partial duplications of Xp in the presence of an intact *SRY* gene have been described (167). These patients present with dysgenetic or absent gonads associated or not with mental retardation, cleft palate and dysmorphic face. Bardoni et al identified in these patients, a common 160-kb region of Xp2 containing *DAX1* gene named dosage sensitive sex (DSS) locus which, when duplicated, resulted in 46,XY DSD (167).
The large duplications of Xp21 reported prior to array-CGH and MLPA techniques were identified by conventional karyotyping. Patients carried large genomic rearrangements involving several genes. In these patients, the presence of XY gonadal dysgenesis was part of a more complex phenotype which also included dysmorphic features and/or mental retardation (168).
Interestingly, in all cases with isolated 46,XY gonadal dysgenesis, the *IL1RAPL1* gene, located immediately telomeric to the duplication containing *NR0B1*, is not disrupted. Deletions or mutations of this gene have been identified in patients with mental retardation (169). Disruption of this gene could explain the mental retardation previously described in patients with larger Xp21 duplications (170).
Several patients with isolated 46,XY gonadal dysgenesis due to duplications of Xp21 have been described. The first report identified a 637 kb tandem duplication on Xp21.2 that in addition to *DAX1* includes the four *MAGEB* genes in two sisters with isolated 46,XY gonadal dysgenesis and gonadoblastomas (171). The second case exhibited a duplication with approximately 800 kb in size and, in addition to *DAX1,* contains the four MAGEB, Cxorf21 and GK genes. The healthy mother was a carrier of the duplication (172).
Smyk et *al.* described a 21-years-old 46,XY patient manifesting primary amenorrhea, a small immature uterus, gonadal dysgenesis and absence of adrenal insufficiency with a submicroscopic deletion (257 kb) upstream of *DAX1*. The authors hypothesized that loss of regulatory sequences may have resulted in up-regulation of *DAX1* expression, consistent with phenotypic consequences of *DAX1* duplication (173).
By using array-CGH and MLPA techniques, additional *NR0B1* locus duplications have been identified in patients with isolated 46,XY gonadal dysgenesis (17) (174) (172).
Barbaro et al identified a relatively small NR0B1 locus duplication responsible for isolated complete 46,XY gonadal dysgenesis in a large English family (172). The

duplication extends from the *MAGEB* genes to part of the *MAP3K7IP3* gene, including *NR0B1*, *CXorf21*, and *GK* genes. Unfortunately, the authors were unable to set up the rearrangement mechanism and distinguish between a nonallelic homologous recombination or a nonhomologous end joining mechanism.

Therefore, until now, there is not a direct proof that an isolated *DAX1* duplication is sufficient to cause 46,XY gonadal dysgenesis in humans, suggesting that other contiguous genes located in the DSS locus, should be involved in dosage-sensitive 46,XY DSD.
X-inactivation patterns in fertile female carriers of each of the three small *NR0B1* locus duplications were analyzed (172). They established that female carriers of macroscopic Xp21 duplications are healthy and fertile due to the preferential inactivating of the duplicated chromosome and thereby protecting them from increased *NR0B1* expression (172).

46,XY DSD due to the overexpression of WNT4 gene

The *Wnt4* (wingless-type mouse mammary tumor virus integration site member 4) gene belongs to a family that consists of structurally related genes that encode cysteine-rich secreted glycoproteins that act as extracellular signaling factors (175).
Overexpression of the *WNT4 and RSPO1* may be a cause of 46,XY DSD. A 46,XY newborn infant, with multiple congenital anomalies including bilateral cleft lips and palate, intrauterine growth retardation, microcephaly, tetralogy of Fallot, atypical external and internal genitalia, and undescended gonads consisted of rete testes and rudimentary seminiferous tubules, who carried a duplication of 1p31-p35, including both *WNT4* and *RSPO1* gene, was reported (176). *In vitro* functional studies showed that Wnt4 up-regulates *Dax1* in Sertoli cells, suggesting that *Dax1* overexpression was the cause of 46,XY DSD in this infant (177).

46,XY DSD ASSOCIATED WITH CHOLESTEROL SYNTHESIS

Smith-Lemli-Opitz Syndrome (SLOS)

This syndrome*,* caused by a deficiency of 7-dehydrocholesterol reductase, is the first true metabolic syndrome leading to multiple congenital malformations (178,179). This disorder is caused by mutations in the sterol delta-7-reductase (*DHCR7*) gene, which maps to 11q12-q13. Typical facial appearance is characterized by short nose with anteverted nostrils, blepharoptosis, microcephaly, photosensitivity, mental retardation, syndactyly of toes 2 and 3, hypotonia and genital ambiguity. Adrenal insufficiency maybe be present or evolve with time. Atypical external genitalia is a frequent feature of males (71%) and ranges from

hypospadias to female external genitalia despite normal 46,XY karyotype and *SRY* sequences. Müllerian derivative ducts can also be present (180) (181) (182). The etiology of masculinization failure in the SLOS remains unclear. However, the description of patients with SLOS who present with hyponatremia, hyperkalemia, and decreased aldosterone-to-renin ratio suggest that the lack of substrate to produce adrenal and testicular steroids is the cause of adrenal insufficiency and atypical genitalia (183), although, a revision of HPA axis in these patients showed normal HPA axis function (184).

Affected children present elevations of 7-dehydrocholesterol (7DHC) in plasma or tissues. 7DHC is best assayed using Gas Chromatography/Mass Spectroscopy (GC/MS). Considering the relative high frequency of Smith-Lemli-Opitz syndrome, approximately 1 in 20,000 to 60,000 births, we suggest that at least cholesterol levels should be routinely measured in patients with 46,XY DSD. However, although frequently low, plasma cholesterol levels can be within normal limits in affected patients.

DHCR7 mutation analysis can confirm a diagnosis of SLOS. The human *DHCR7* gene is localized on chromosome 11q13 and contains nine exons encoding a 425 amino-acid protein (111). More than 130 different mutations of *DHCR7* have been identified and the great majority of them are located at the exons 6 to 9 (185) (186). However, the genotype-phenotype correlation in SLOS is relatively poor (187).

Currently, most SLOS patients are treated with cholesterol supplementation that can be achieved by including high cholesterol foods and/or suspensions of pharmaceutical grade cholesterol. Data suggests that early intervention may be of benefit to SLOS patients (188). Observational studies report improved growth and muscle tone and strength, increased socialization, decreased irritability and aggression in SLOS patients treated with cholesterol supplementation. However, in a group of SLOS patients' treatment with a high cholesterol diet did not improve developmental scores (111).(189).

Treatment with sinvastatin, an HMG-CoA reductase inhibitor, aiming to block the cholesterol synthesis pathway avoiding the formation of large amounts of 7DHC/8DHC, and in this manner limiting exposure to potentially toxic metabolites in SLOS patients has been proposed. Simvastatin can also cross the blood–brain barrier and may provide a means to treat the biochemical defect present in the CNS of SLOS patients (190). A major effect of statins therapy is the transcriptional upregulation of genes controlled by the transcriptional factor SREBP, as DHCR7. Thus, if any residual activity is present in the mutant DHCR7, its upregulation could increase intracellular cholesterol synthesis. Simvastatin use in SLOS patients resulted in a paradoxical increase in serum and cerebral spinal fluid cholesterol levels (190). Randomized controlled-placebo trial were performer with simvastatin

in SLOS showing significant reduction in plasmatic 7DHC associated with improvement in irritability symptoms (191). Determination of residual DHCR7 enzymatic activity may be helpful in selecting SLOS patients being considered for a beneficial response of statins (186). Recently, promising gene therapy using an adeno-associated virus vector carrying a functional copy of the DHCR7 gene was administered by intrathecal injection in mouse model with improvement of cholesterol levels in the central nervous system (192).

Table 4. Phenotype of 46,XY subjects with Smith-Lemli-Optiz syndrome	
Inheritance	Autosomal recessive
External genitalia	Micropenis and/or hypospadias, hypoplasic or bifid scrotum; female
Müllerian duct derivatives	May be present
Wolffian duct derivatives	Absent to male
Testes	Scrotum, inguinal or intraabdominal region
Clinical features	Facial and bone abnormalities. Heart and pulmonary defects. Renal agenesis. Mental retardation, Seizures, hypotonia, syndactyly of second and third toes.
Puberty	Apparently normal
Hormonal diagnosis	Low cholesterol, elevated 7-dehydrocholesterol. Decreased aldosterone-to-renin ratio
Gender role	Male
DHCR7 gene location	11q12-q13
Molecular defect	Mutations in *DHCR7* gene
Treatment	Dietary cholesterol supplies accompanied by ursodeoxycholic acid, and statins
Outcome	Severe mental retardation

46,XY DSD DUE TO TESTOSTERONE PRODUCTION DEFECTS

46,XY DSD due to Impaired Leydig Cell Differentiation (Complete and Partial Forms)

Inactivating mutations of human LHCG receptor (*LHCGR*) have been described in 46,XY individuals with a rare form of disorder of sex development, termed Leydig cell hypoplasia. These inactivating mutations in the LHCGR prevent LH and hCG signal transduction and thus testosterone production both pre- and postnatally in genetic males (193).

Both hCG and LH act by stimulating a common transmembrane receptor, the LHCGR (194) (195). *LHCGR* is a member of G protein-coupled receptors, which were characterized by the canonical serpentine region, composed of seven transmembrane helices interconnected by three extracellular and three intracellular loops (196) (197). The large amino-terminal extracellular domain, rich in leucine-

repeats, mediates the high affinity binding of pituitary LH or placental human chorionic gonadotropin (hCG) (197).

LHCGR activates the Gs protein, which determines an increase in intracellular cAMP and a subsequent stimulation of steroidogenesis in gonadal cells such as testicular Leydig cells, ovarian theca cells and differentiated granulosa cells (194) (195). A secondary mechanism of LHCGR stimulation is through $G_{q/11}$ protein activation and the inositol phosphate signaling pathway (197).

The *LHCGR* gene is located on the short arm of chromosome 2 (2p21). It spans nearly 80 kb and has been thought to be composed of 11 exons and 10 introns. Exon 11 of the *LHCGR* gene encodes the entire serpentine domain as well as the carboxy-terminal portion of the hinge region (NCBI GeneID 3973; http://www.ncbi.nlm.nih.gov). The amino-terminal portion of the hinge region is encoded by exon 10 and the signal peptide and remaining portion of the extracellular domain are encoded by exons 1-9 (196) (193). A novel primate-specific exon (termed exon 6A) was identified within intron 6 of the *LHCGR* gene. This exon is not used by the wild-type full-length receptor. It displays composite characteristics of an internal/terminal exon and possesses stop codons triggering nonsense-mediated mRNA decay in *LHCGR.* When exon 6A is utilized, it results in a truncated LHCGR protein (198).

In 1976, Berthezene et al. (199) described the first patient with Leydig cell hypoplasia and subsequently several cases have been reported (200) (201) (202). The clinical features are heterogeneous and result of a failure of intrauterine and pubertal virilization. A review of the literature allowed to delineate the characteristics of 46,XY DSD due to the complete form of Leydig cell hypoplasia as: 1) female external genitalia leading to female sex assignment 2) no development of sexual characteristics at puberty, 3) undescended testes slightly smaller than normal with relatively preserved seminiferous tubules and absence of mature Leydig cells, 4) presence of rudimentary epididymis and vas deferens and absence of uterus and fallopian tubes, 5) low testosterone levels despite elevated gonadotropin levels, with elevated LH levels predominant over FSH levels, 6) testicular unresponsiveness to hCG stimulation, and 7) no abnormal step up in testosterone biosynthesis precursors (193) (203) (204).

Several different mutations in the LHCGR gene were reported in patients with Leydig cell hypoplasia in both sexes (193).

Table 5. Phenotype of 46,XY subjects with the complete form of Leydig cell hypoplasia	
Inheritance	Autosomal recessive
External genitalia	Female, occasionally mild clitoromegaly or labial fusion
Müllerian derivatives	Absent
Wolfian ducts derivatives	Absent or vestigial
Testes	Inguinal or intra-abdominal, slightly subnormal size
Puberty	Absence of spontaneous virilization or feminization
Hormonal diagnosis	Elevated serum LH, normal or slightly elevated FSH and very low testosterone levels with normal levels of testosterone precursors
Gender role	Female
LHCGR gene location	2p21
Molecular defect	Mutations in *LHCGR* gene (complete inactivation) and in the internal exon 6A *LHCGR* (increase of nonfunctional isoform); defects in *LHCGR* were not identified in several families
Treatment	Estrogen replacement at pubertal age, bilateral orchiectomy and vaginal dilation
Outcome	Female gender role and behavior, infertility

In contrast to the homogenous phenotype of the complete form of Leydig cell hypoplasia, the partial form features a broad spectrum, ranging from incomplete male sexual differentiation characterized by micropenis and/or hypospadias to hypergonadotropic hypogonadism without ambiguity of the male external genitalia (194) (205) (195) (206) (207) (208). Testes are cryptorchidic or in the scrotum and during puberty, partial virilization occurs and testicular size is normal or only slightly reduced, while penile growth is significantly impaired. Spontaneous gynecomastia does not occur. Before puberty, the testosterone response to the hCG test is subnormal without accumulation of testosterone precursors. After puberty, LH levels are elevated as a result of insufficient negative feedback of gonadal steroid hormones on the anterior pituitary and testosterone levels are intermediate between those of children and normal males.

Several mutations in the *LHCGR* gene have also been identified in patients with the partial form of Leydig cell hypoplasia. Latronico et al (194) first reported a homozygous mutation in the LHCGR (p.Ser616Tyr) in a boy with micropenis. Subsequently, other milder mutations were identified in further patients with the partial form of Leydig cell hypoplasia (195) (205) (206). *In vitro* studies showed that cells transfected with LHCGR gene containing these mutations had an impaired hCG-stimulated cAMP production (205) (206).

Leydig cell hypoplasia was found to be a genetic heterogenous disorder since Zenteno *et al.* (209) ruled out, by segregation analysis of a known polymorphism in exon 11 of the LHCG receptor gene, molecular defects in the LHCG receptor as being responsible for Leydig cell hypoplasia in three siblings with 46,XY DSD. Most inactivating mutations of the *LHCGR* are missense mutations that result in a single amino acid substitution in the LHCGR. In addition, mutations causing amino acid deletions, amino acid insertions, splice acceptor mutation or premature truncations of the receptor have also been reported. These mutations are usually located in the coding sequence, resulting in impairment of either LH/CG binding or signal transduction (197).

Although it is well known that hCG and LH act by stimulating a common receptor, a differential action of them in the LHCGR has been suggested. The identification of a deletion of exon 10 of the *LHCGR* in a patient with normal male genitalia at birth, but no pubertal development indicated that the mutant LHCGR was responsive to fetal hCG, but resistant to pituitary LH. The binding affinity of hCG for LHCGR was normal *in vitro* analysis, suggesting that exon 10 is necessary for LH, but not for hCG action (210).

The identification and characterization of a novel, primate-specific bona fide exon (exon 6A) within the *LHCGR* determined a new regulatory element within the genomic organization of this receptor and a new potential mechanism of this disorder. Kossack *et al* analyzing the exon 6A in 16 patients with 46,XY DSD due to Leydig cells hypoplasia without molecular diagnosis, detected mutations

(p.A557C or p.G558C) in three patients. Functional studies revealed a dramatic increase in expression of the mutated internal exon 6A transcripts, resulting in the generation of predominantly nonfunctional isoforms of the LHCGR, thereby preventing its proper expression and functioning (198).

A new compound heterozygous mutation of the *LHCGR*, constituted by a previously described missense mutation (p.Cys13Arg) and a large deletion of the paternal chromosome 2 was identified by array-Comparative Genomic Hybridization (array-CGH) in a 46,XY infant with sexual ambiguity and low hCG-stimulated testosterone levels associated with high LH and FSH levels (211).

In addition, causative mutations in *LHCGR* were absent in around 50% of the patients strongly suspected to have Leydig cell hypoplasia. These findings supported the idea that other genes must be implicated in the molecular basis of this disorder.

We observed that 46,XX sisters of the patients with 46,XY DSD due to Leydig cell hypoplasia, carrying the same homozygous mutation in the *LHCGR*, have primary or secondary amenorrhea, spontaneous breast development, infertility, normal or enlarged cystic ovaries with elevated LH and LH/FSH ratio, normal estradiol and progesterone levels for early to mid-follicular phase, but not for luteal phase levels, confirming lack of ovulation (193,207). Our findings were subsequently confirmed by other authors who studied 46,XX sisters of 46,XY DSD patients with Leydig cell hypoplasia (212) (213) (214).

Subsequently, a novel homozygous missense mutation, p.N400S, has been identified by whole genome sequencing in two sisters with empty follicle syndrome (215).

Table 6. Phenotype of 46,XY subjects with partial Leydig cells hypoplasia	
Inheritance	Autosomal recessive
External genitalia	Atypical to male
Müllerian derivatives	Absent
Wolfian ducts derivatives	Rudimentary to male
Testes	Scrotum, labial folds or inguinal regions, normal or only slightly subnormal size
Puberty	Partial virilization without gynecomastia, discrepancy between reduced penis size and normal testicular growth
Hormonal diagnosis	Elevated serum LH levels, normal or slightly elevated FSH and low T levels with normal levels of T precursors in relation to T
Gender role	Male
LHCGR gene location	2p21
Molecular defect	Mutations which confer partial inactivation of *LHCGR*
Treatment	Repair of the hypospadias, testosterone replacement at pubertal age
Outcome	Male gender role and behavior, possible fertility under treatment

46,XY DSD DUE TO ENZYMATIC DEFECTS IN TESTOSTERONE SYNTHESIS

Six enzymatic defects that alter the normal synthesis of testosterone have been described to date (Figure 6). Three of them are associated with defects in cortisol synthesis leading to congenital adrenal hyperplasia. All of them present an autosomal recessive mode of inheritance and genetic counseling is mandatory, since the chance of recurring synthesis defects among siblings is 25%.

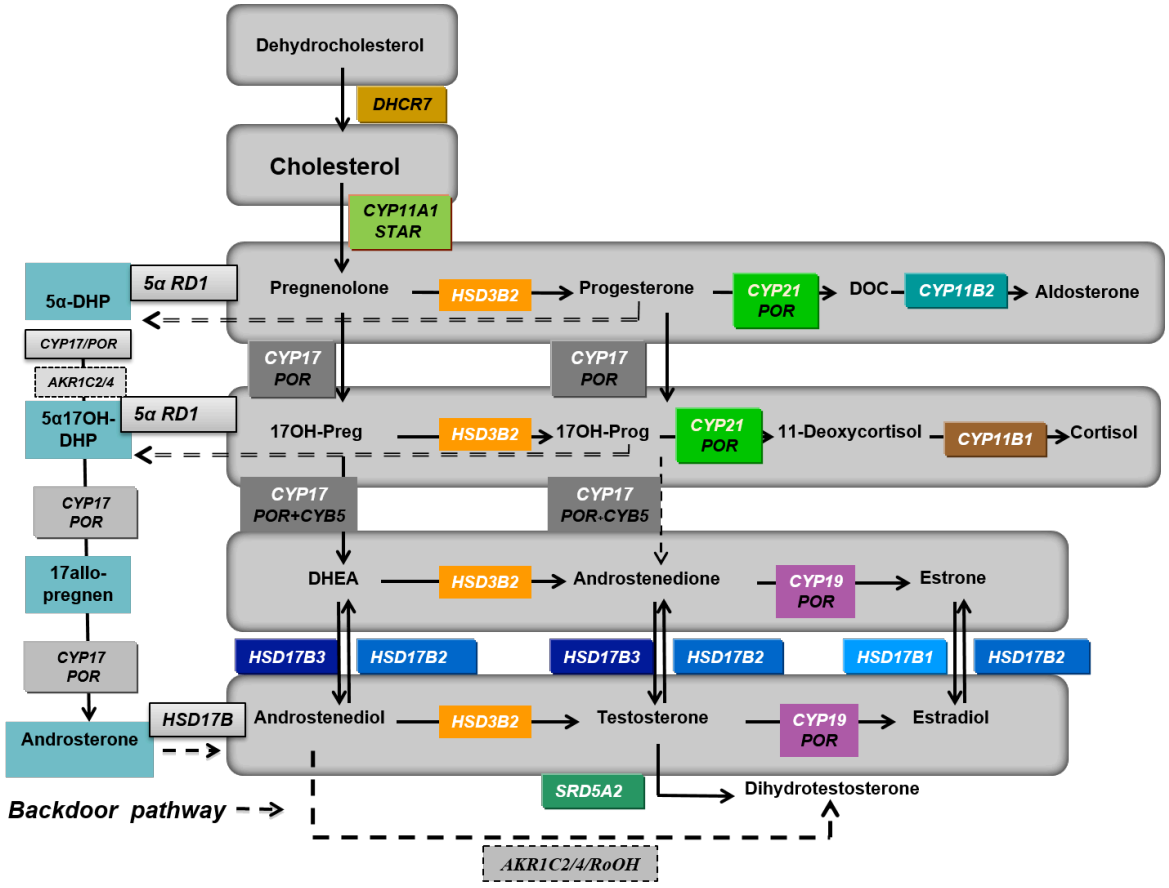

Figure 6- Ordinary steroidogenesis and alternative pathway to DHT synthesis.

Defects in Adrenal and Testicular Steroidogenesis

Adrenal hyperplasia syndromes are examples of hypoadrenocorticism or mixed hypo- and hyper corticoadrenal steroid secretion. Synthesis of cortisol or both cortisol and aldosterone are impaired. When cortisol production is impaired there is a compensatory increase in ACTH secretion. If mineralocorticoid production is impeded, there is a compensatory increase in renin-angiotensin production. These compensatory mechanisms may return cortisol or aldosterone production to normal or near normal levels, but at the expense of excessive production of precursors that can cause undesirable hormonal effects.

Deficiency of the acute steroidogenesis regulatory protein (StAR)

The earliest step in the conversion of cholesterol to hormonal steroids is hydroxylation at carbon 20, with subsequent cleavage of the 20-22 side chain to form pregnenolone. In steroidogenic tissues, such as adrenal cortex, testis, ovary, and placenta, the initial and rate-limiting step in the pathway leading from cholesterol to steroid hormones is the cleavage of the side chain of cholesterol to yield pregnenolone. This reaction, known as cholesterol side-chain cleavage, is catalyzed by a specific cytochrome P450 called P450scc or P45011A and by the steroidogenic acute regulatory (StAR) protein, a mitochondrial phosphoprotein (216).

It is the most severe form of congenital adrenal hyperplasia (217). Lipoid adrenal hyperplasia is rare in Europe and America but it is thought to be the second most common form of adrenal hyperplasia in Japan. Affected subjects are phenotypic females irrespective of gonadal sex or sometimes have slightly virilized external genitalia with or without cryptorchidism, underdeveloped internal male organs and an enlarged adrenal cortex, engorged with cholesterol and cholesterol esters (218). Adrenal steroidogenesis deficiency leads to salt wasting, hyponatremia, hyperkalemia, hypovolemia, acidosis, and death in infancy, although patients can survive to adulthood with appropriate mineralocorticoid- and glucocorticoid-replacement therapy (219).

Hormonal diagnosis is based on high ACTH and renin levels and the presence of low levels of all glucocorticoids, mineralocorticoids and androgens.

The disease was firstly attributed to P450scc deficiency, but most of the cases studied through molecular analysis showed an intact *P45011A* gene and its RNA (220). Since StAR is also required for the conversion of cholesterol to pregnenolone, molecular studies were performed in *StAR* gene and mutations were found in most of the affected patients (221). Congenital lipoid adrenal hyperplasia (LCAH) in most Palestinian cases is caused by a founder c.201_202delCT mutation causing premature termination of the StAR protein (222). Histopathological findings of excised XY gonads included accumulation of fat in Leydig cells since 1 yr of age, positive placental alkaline phosphatase and

octamer binding transcription factor (OCT4) staining indicating a neoplastic potential (222).

A two-hit model has been proposed by Bose *et al.* (221) as the pathophysiological explanation for LCAH. In response to a stimulus (*e.g.* ACTH), the normal steroidogenic cell recruits cholesterol from endogenous synthesis, stored lipid droplets or low-density lipoprotein-receptor mediated endocytosis. Subsequently StAR promotes the cholesterol transport from the outer to the inner mitochondrial membrane in which cholesterol is further processed to pregnenolone. In cells with mutant StAR (first hit), there is no rapid steroid synthesis, but still some StAR-independent cholesterol flows into the mitochondria, resulting in a low level of steroidogenesis. Due to increased steroidogenic stimuli in response to inadequately low steroid levels, additional cholesterol accumulates. Massive cholesterol storage and resulting biochemical reactions eventually destroy all steroidogenic capacity (second hit) (221). This two-hit model has been confirmed by clinical studies (223) (224) as well as StAR knockout mice research (225).

The human *STAR* gene is localized on chromosome 8p11.2 and consists of seven exons (226). It is translated as a 285-amino acid protein including a mitochondrial target sequence (N terminal 62 amino acids), which guides StAR to the outer mitochondrial membrane and a cholesterol binding site, which is located at the C-terminal region. *In vitro* studies revealed that StAR protein lacking the N terminal targeting sequence (N-62 StAR) can still stimulate steroidogenesis in transfected COS-1 cells, whereas mutations in the C-terminal region lead to severely diminished or absent function (227) (228) (229). Most of the *STAR* gene mutations associated with LCAH are located in the C-terminal coding region between exon 5 and 7 StAR related lipid transfer (START) domain (230). Mild phenotype of lipoid CAH was a recognized disorder caused by StAR mutations that retain partial activity (231). Affected males can present with adrenal insufficiency resembling to autoimmune Addison disease with micropenis or normal development with hypergonadotropic hypogonadism (231) (232). More than 40 StAR mutations causing classic lipoid CAH have been described (221,230) (233) (234), but very few partial loss-of-function mutations have been reported (231) (232) (233). Therefore, there is a broad clinical spectrum of StAR mutations, however, the StAR activities in vitro correlate well with clinical phenotypes (235,236).

Three 46,XY patients with the homozygous p.R188C STAR mutation causing primary adrenocortical insufficiency without atypical genitalia were reported (237).

Table 7. Phenotype of 46,XY subjects with StAR deficiency	
Inheritance	Autosomal recessive
External genitalia	Female Micropenis (mild form)
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Absent -> hypoplastic
Testes	Small size
Clinical Features	Early adrenal insufficiency; no pubertal development; hypergonadotropic hypogonadism
Hormonal diagnosis	Elevated ACTH and rennin levels; low levels of all glucocorticoids, mineralocorticoids and androgens
Gender role	Female Male (mild form)
STAR gene location	8p11.2
Molecular defect	Inactivating mutation in *STAR*
Treatment	Early gluco- and mineralocorticoid replacement; estrogen replacement at pubertal age
Outcome	Infertile, female or male gender role and behavior

Deficiency of P450scc

It has been thought that *CYP11A* mutations are incompatible with human term gestation, because P450scc is needed for placental biosynthesis of progesterone, which is essential to maintain pregnancy. In rodents and some other animals, the mother's corpus luteum of pregnancy produces progesterone throughout gestation, consequently, *Cyp11a1* knockout mice reach term without difficulty [185]. However, in humans, pregnancy is characterized by a second-trimester "luteo-placental shift" wherein the mother's corpus luteum involutes and placental progesterone biosynthesis takes over. Thus this statement would predict that mutations in P450scc would be incompatible with term gestation [186].

Nevertheless, a number of patients with *CYP11A1* mutations have now been described [187-191], including late-onset non-classical forms secondary to mutations that retain partial enzyme activity [191-194]. Clinically, these patients are indistinguishable from those with lipoid CAH, but none of them present enlarged adrenals that characterize lipoid CAH. Once the majority of these patients have born prematurely following unsuppressible labor, it appears that the maternal corpus luteum may simply survive longer in these pregnancies, but this hypothesis remains unproven [186].

Analyzing infants with adrenal failure and disorder of sexual differentiation compound heterozygous mutations in CYP 11A1 have been identified, recognizing that this disorder may be more frequent than originally thought. The phenotypic spectrum of P450scc deficiency ranges from severe loss-of-function mutations associated with prematurity, complete underandrogenization, and severe early-onset adrenal failure, to partial deficiencies found in children born at term with mild masculinization and later-onset adrenal failure. [191].

3β-Hydroxysteroid Dehydrogenase type II Deficiency

3β-HSD converts 3β-hydroxy Δ^5 steroids to 3-keto Δ^4 steroids and is essential for the biosynthesis of mineralocorticoids, glucocorticoids and sex steroids Two forms of the enzyme have been described in man: the type I enzyme which is expressed in placenta and peripheral tissues such as the liver and skin, and type II that is the major form expressed in the adrenals and gonads (238). The two forms are very closely related in structure and substrate specificity, though the type I enzyme has higher substrate affinities and a 5-fold greater enzymatic activity than type II (239). Male patients with 3β-HSD type II deficiency present with atypical external genitalia, characterized by micropenis, proximal hypospadias, bifid scrotum and a blind vaginal pouch associated or not with salt loss (240). Gynecomastia is common at pubertal stage.

Serum levels of Δ-5 steroids such as pregnenolone, 17OHpregnenolone (17OHPreg), DHEA, DHEAS are elevated and basal levels of 17OHPreg and 17OHPreg/17OHP ratio are the best markers of this deficiency in both prepubertal and postpubertal stage. Δ-4 steroids are slightly increased due to the peripheral action of 3β-HSD type I enzyme but the ratio of Δ-5/Δ-4 steroids is elevated. Cortisol secretion is reduced but the response to exogenous ACTH stimulation varies from decreased (more severe deficiency) to normal. At adult age, affected males can reach normal or almost normal levels of testosterone due to the peripheral conversion of elevated Δ-5 steroids by 3β-HSD type I enzyme and also due to testicular stimulation by the high LH levels (241).

The human genome encodes two functional 3βHSD genes on chromosome 1p13.1. The HSD3B2 gene is expressed in adrenal and gonads and consists of four exons coding for a 372 aminoacid protein (242). To date, around 40 mutations in HSD3B2 gene have been described. Most of them are base substitutions, and they are located especially at the N-terminal region of the protein. The amino acids A10, A82, P222 and T259 could be considered as mutational hotspots since different mutations were reported in these *HSD3B2* positions.

Mutations abolishing 3β-HSD type II activity lead to congenital adrenal hyperplasia (CAH) with severe salt-loss (216) (239) (243) (244). Mutations that reduce, but do not abolish type II activity lead to CAH with mild or no salt-loss, which in males is associated with 46,XY DSD due to the reduction in androgen synthesis (245,246).

Male subjects with 46,XY DSD due 3β-HSD type II deficiency without salt loss showed clinical features in common with the deficiencies of 17β-HSD3 and 5α-reductase 2.
Most of the patients were raised as males and kept the male social sex at puberty. In one Brazilian family, two cousins with 46,XY DSD due to 3β-HSD type II deficiency were reared as females; one of them was underwent orchiectomy in childhood and kept the female social sex; the other did not undergo orchiectomy at childhood and changed to male social sex at puberty (241).

Table 8. Phenotype of 46,XY subjects with 3β-HSD type II deficiency	
Inheritance	Autosomal recessive
External genitalia	Atypical (proximal hypospadias, bifid scrotum, urogenital sinus)
Müllerian derivatives	Absent
Wolfian duct derivatives	Normal
Testes	Well developed; generally topic
Clinical features	Adrenal insufficiency or not in infancy; virilization at puberty with or without gynecomastia
Hormonal diagnosis	Elevated basal and ACTH-stimulated 17OHPreg and 17OHPreg/17OHP ratio
Gender role	Male; female to male
HSD3B2 gene location	1p13.1
Molecular defect	Inactivating mutations in *HSD3B2*
Treatment	Glucocorticoid replacement along with mineralocorticoids in salt-losing form; at puberty variable necessity for testosterone replacement
Outcome	Variable spermatogenesis; fertility possible by *in vitro* fertilization

Combined 17-Hydroxylase and C-17-20 lyase deficiency

CYP17 is a steroidogenic enzyme that has dual functions: hydroxylation and lyase and is located in the fasciculata and reticularis zone of the adrenal cortex and gonadal tissues. The first activity results in hydroxylation of pregnenolone and progesterone at the C(17) position to generate 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, while the second enzyme activity cleaves the C(17)-C(20) bond of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to form dehydroepiandrosterone and androstenedione, respectively. The modulation of these two activities occurs through cytochrome b5, necessary for lyase activity (247).

Deficiency of adrenal 17-hydroxylation activity was first demonstrated by Biglieri et al. (248). The phenotype of 17-hydroxylase deficiency in most of the male patients described is a female-like or slightly virilized external genitalia with blind vaginal pouch, cryptorchidism and high blood pressure, usually associated with hypokalemia. New in 1970, reported the first affected patient with atypical genitalia which was assigned to the male sex (249).

At puberty, patients usually present sparse axillary and pubic hair. Male internal genitalia are hypoplastic and gynecomastia can appear at puberty. Most of the male patients were reared as females and sought treatment due to primary amenorrhea or lack of breast development. Genetic female patients may also be affected and present normal development of internal and external genitalia at birth and hypergonadotropic hypogonadism and amenorrhea at post pubertal age; enlarged ovaries at adult age and infarction from twisting can occur (250) (251). These patients do not present signs of glucocorticoid insufficiency, due to the elevated levels of corticosterone, which has a glucocorticoid effect. The phenotype is similar to 46,XX or 46,XY complete gonadal dysgenesis and the presence of systemic hypertension and absence of pubic hair in post pubertal patients suggests the diagnosis of 17-hydroxylase deficiency (252).

Serum levels of progesterone, corticosterone, and 18-OH-corticosterone are elevated, while aldosterone, 17-OH-progesterone, cortisol, androgens and estrogens are decreased. Martin et al, performed a clinical, hormonal, and molecular study of 11 patients from 6 Brazilian families with the combined 17-alpha-hydroxylase/17,20-lyase deficiency phenotype (253). All patients had elevated basal serum levels of progesterone and suppressed plasma renin activity. The authors concluded that basal progesterone measurement is a useful marker of P450c17 deficiency and suggest that its use should reduce the misdiagnosis of this deficiency in patients presenting with male DSD, primary or secondary amenorrhea, and mineralocorticoid excess syndrome.

Excessive production of deoxycorticosterone and corticosterone results in systemic hypertension, suppression of renin levels and inhibition of aldosterone synthesis. The *CYP17A1* gene, which encodes the enzymes 17-hydroxylase and 17-20 lyase,

is a member of a gene family within the P450 supergene family and is mapped at 10q24.3 (254). Several mutations in the *CYP17A1* gene have been identified in patients with both 17-hydroxylase and 17,20 lyase deficiencies (250) (251) (253) (255). Four homozygote mutations, p.A302P, p.K327del, p.E331del and p.R416H, were identified by direct sequencing of the *CYP17A1* gene. Both P450c17 activities were abolished in all the mutant proteins but the mutant proteins were normally expressed, suggesting that the loss of enzymatic activity is not due to defects of synthesis, stability, or localization of P450c17 proteins (255).

Glucocorticoid replacement for hypertension management, gonadectomy and estrogen replacement at puberty for patients reared in the female social sex are indicated. In male patients, androgen replacement is usually necessary since they present very low levels of testosterone. These patients are very sensitive to glucocorticoids and low doses of dexamethasone (0.125-0.5 mg at night) are sufficient to control blood pressure. In some patients, however, estrogens might aggravate hypertension. The control of blood pressure can be initially achieved by salt restriction although mineralocorticoid antagonists might be necessary (255).

Table 9.- Phenotype of 46,XY subjects with 17a-hydroxylase and 17,20-lyase deficiency	
Inheritance	Autosomal recessive
External genitalia	Female like --> atypical
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Hypoplastic --> normal
Testes	Intra-abdominal or inguinal
Clinical features	Low renin hypertension; absent or slight virilization at puberty; gynecomastia
Hormonal diagnosis	Elevated progesterone, DOC, corticosterone; low plasma renin activity low cortisol not stimulated by ACTH
Gender role	Female in most patients
CYP17 gene location	10q24.3
Molecular defect	Mutations in *CYP17A1* gene
Treatment	Repair of sexual ambiguity; glucocorticoid and estrogen or testosterone replacement according to social sex
Outcome	Female behavior, infertility

Cytochrome P450 reductase (POR) deficiency (electron transfer disruption)

The apparent combined P450C17 and P450C21 deficiency is a rare variant of congenital adrenal hyperplasia, first reported by Peterson et al in 1985 (256). Affected girls and boys are born with atypical genitalia, indicating intrauterine androgen excess in females and androgen deficiency in males. Boys and girls can also present with skeletal malformations, which in some cases resemble a pattern seen in patients with Antley-Bixler syndrome. Findings of biochemical investigations of urinary steroid excretion in affected patients have shown accumulation of steroid metabolites, indicating impaired C17 and C21 hydroxylation, suggesting concurrent partial deficiencies of the 2 steroidogenic enzymes, P450C17 and P450C21. However, sequencing of the genes encoding these enzymes showed no mutations, suggesting a defect in a cofactor that interacts with both enzymes. POR is a flavoprotein that donates electrons to all microsomal P450 enzymes, including the steroidogenic enzymes P450c17, P450c21 and P450aro (218). Shephard et al. (1989) isolated and sequenced cDNA clones that encode the rat and human NADPH-dependent cytochrome P-450 reductase and located the human gene at 7q11.2 (257).

The underlying molecular basis of congenital adrenal hyperplasia with apparent combined P450C17 and P450C21 deficiency was defined in 3 patients, who were compound heterozygotes for mutations in POR (258) (259). Antley-Bixler syndrome is characterized by craniosynostosis, severe midface hypoplasia, proptosis, choanal atresia/stenosis, frontal bossing, dysplastic ears, depressed nasal bridge, radiohumeral synostosis, long bone fractures, femoral bowing, phalangeal malformation (arachno-/campto-/clinodactilyly, brachytelephalangia, rocker bottom feet) and urogenital abnormalities (260). The occurrence of genital abnormalities in patients with Antley-Bixler syndrome, especially females was reported in 2000 (261). In a recent large survey of patients with Antley-Bixler syndrome, it was demonstrated that individuals with an Antley-Bixler-like phenotype and normal steroidogenesis have *FGFR2* mutations, whereas those with atypical genitalia and altered steroidogenesis have POR deficiency (262). The skeletal malformations observed in many, but not all patients with POR deficiency, are thought to be due to disruption of enzymes involved in sterol synthesis, 14α-lanosterol demethylase (CYP51A1) and squalene epoxidase, and disruption of retinoic acid metabolism catalyzed by CYP26 isoenzymes that depend on electron transfer from POR (263).

Pubertal presentation in females with congenital POR deficiency were described. Incomplete pubertal development and large ovarian cysts prone to spontaneous rupture were the predominant findings in females. The ovarian cysts may be driven not only by high gonadotropins but possibly also by impaired CYP51A1-mediated production of meiosis-activating sterols due to mutant POR. In the two boys

evaluated, pubertal development was more mildly affected, with some spontaneous progression. These findings may suggest that testicular steroidogenesis may be less dependent on POR than adrenal and ovarian steroidogenesis (264).

Table 10 - Phenotype of 46,XY patients with POR deficiency	
Inheritance	Autosomal recessive
External genitália	Atypical
Müllerian duct derivatives	Normally developed
Wolfian duct derivatives	Normally developed
Testes	Well developed, frequent cryptorchidism
Hormonal diagnosis	Low T and cortisol and elevated basal ACTH, Prog and 17OHP
POR gene location	7q11.2
Molecular defect	Inactivating mutation of *POR* gene
Puberty	Spontaneous pubertal development in males
Gender role	Male
Treatment	Repair of sexual ambiguity; glucocorticoid replacement and estrogen or testosterone replacement according to social sex
Outcome	Puberty development, fertility?

Defects in Testicular Steroidogenesis

Three defects in testosterone synthesis that are not associated with adrenal insufficiency have been described: CYP17A1 deficiency, cytochrome B5 deficiency and 17-β-HSD3 deficiency

CYP17A1 (17,20 lyase activity) Deficiency

Human male sexual differentiation requires production of fetal testicular testosterone, whose biosynthesis requires steroid 17,20-lyase activity. The existence of true isolated 17,20-lyase deficiency has been questioned because 17-α-hydroxylase and 17,20-lyase activities are catalyzed by a single enzyme and because combined deficiencies of both activities were found in functional studies of the mutation found in a patient thought to have had isolated 17,20-lyase deficiency (265). Later, clear molecular evidence of the existence of isolated 17,20 desmolase deficiency was demonstrated (247,266) (251) (267).

The patients present atypical genitalia with micropenis, proximal hypospadias and cryptorchidism. Gynecomastia Tanner stage V can occur at puberty (267).

Elevated serum levels of 17-OHP and 17-OHPreg, with low levels of androstenedione, dehydroepiandrosterone and testosterone are found. The hCG stimulation test results in a slight stimulation in androstenedione and testosterone secretion with an accumulation of 17-OHP and 17-OHPreg.

The *CYP17A1* gene of two Brazilian 46,XY DSD patients with clinical and hormonal findings indicative of isolated 17,20-lyase deficiency, since they produce cortisol normally, were studied. Both were homozygous for substitution mutations in *CYP17A1* (267). When expressed in COS-1 cells, the mutants retained 17α-hydroxylase activity and had minimal 17,20-lyase activity. Both mutations alter the electrostatic charge distribution in the redox-partner binding site, so that the electron transfer for the 17,20-lyase reaction is selectively lost (267).

Table 11. Phenotype of 46,XY subjects with 17,20 lyase deficiency

Inheritance	Autosomal recessive
External genitalia	Atypical (proximal hypospadias, bifid scrotum, urogenital sinus)
Müllerian derivatives	Absent
Wolfian ducts derivatives	Hypoplastic --> normal
Testes	At inguinal region, small size
Clinical features	Gynecomastia variable; poor virilization at puberty

Hormonal diagnosis	Elevated 17OHP and 17OHP/A ratio after hCG stimulation and decreased DHEA, A and T levels;
Gender role	Male or female
CYP17 gene location	10q24.3
Molecular defect	Mutations in the redox partner binding site of CYP17A1 enzyme
Treatment	Repair of hypospadias and gynecomastia; testosterone replacement at pubertal age
Outcome	Male or female behavior

Cytochrome B5 deficiency (allosteric factor for P450c17 and POR interaction)
In 1994, Hegesh et al described a 46,XY DSD patient with type IV hereditary methaemoglobinemia (268). The patient had a 16-bp deletion in the cytochrome b5 mRNA leading to a new in-frame termination codon and a truncated protein. The etiology of 46,XY DSD in this patient was attributed to the cytochrome b5 defect since cytocrome b5, acts as an allosteric factor, promoting the interaction of. P450c17 and POR favoring 17,20 lyase reaction (247).

Two homozygous mutations in CYB5 in 46,XY DSD patients with elevated methaemoglobinemia levels but without clinical phenotype of methaemoglobinemia were reported (269) (270).

46,XY DSD due to 17β-HSD 3 Deficiency

This disorder consists in a defect in the last phase of steroidogenesis, when androstenedione is converted to testosterone and estrone to estradiol. This disorder was described by Saez and his colleagues (271,272) and is the most common disorder of androgen synthesis, reported from several parts of the world (273) (274).
There are 5 steroid 17β-HSD enzymes that catalyze this reaction (275) and 46,XY DSD results from mutations in the gene encoding the 17β-HSD3 isoenzyme (275,276). Patients present female-like or atypical genitalia at birth, with the presence of a blind vaginal pouch, intra-abdominal or inguinal testes and epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts. Most affected males are raised as females (277) (278) (279), but some have less severe defects in virilization and are raised as males (275). Virilization in subjects with 17β-HSD3 deficiency occurs at the time of expected puberty. This late virilization is usually a consequence of the presence of testosterone in the circulation as a result of the conversion of androstenedione to testosterone by some other 17β-HSD isoenzyme (presumably 17β-HSD 5) in extra-gonadal tissue and, occasionally, of the secretion of testosterone by the testes when levels of LH are elevated in subjects with some residual 17β-HSD3 function (275). However, the discrepancy between the failure of intrauterine masculinization and the virilization that occurs at the time of expected puberty is poorly understood. A limited capacity to convert androstenedione into testosterone in the fetal extragonadal tissues may explain the impairment of virilization of the external genitalia in the newborn. Bilateral orchiectomy resulted in a clear reduction of androstenedione levels indicating that the main origin of this androgen is the testis (275) (278). 46,XY DSD phenotype is sufficiently variable in 17β-HSD3 deficiency to cause problems in accurate diagnosis, particularly in distinguishing it from partial androgen insensitivity syndrome (PAIS) (280) (277).

Laboratory diagnosis is based on elevated serum levels of androstenedione and estrone and low levels of testosterone and estradiol resulting in elevated androstenedione/testosterone and estrone/ estradiol ratios or low (or low testosterone/androstenedione and estradiol/estrone ratios) indicating impairment in the conversion of 17-keto into 17-hydroxysteroids. Testosterone/Androstenedione ratio of 0.4±0.2 was found in prepubertal patients with 17β-HSD3 deficiency after hCG stimulation. Based on these data, a T/A ratio below <0.8 is suggestive of 17β-HSD3 deficiency (273). At the time of expected puberty, serum LH and testosterone levels rise in all affected males and testosterone levels may reach the normal adult male range (281) (279).

Pitfalls in the hormonal diagnosis of 17β-HSD3 deficiency had been reported in the literature. Two of the fourteen cases of 17β-HSD3 deficiency reported from the UK database had a T/A ratio > 0.8 (277). Both patients were from a consanguineous pedigree, with two affected sisters (both assigned in the female social sex) and one nephew. The former patient had atypical genitalia with proximal hypospadias and was assigned as male. The hCG test was performed at 2 years and 2 months of age, respectively, resulting in a T/A ratio of 3.4 and 1.5. Two other patients with atypical genitalia, who were also assigned in the female social sex, were evaluated at 5 months and 9.2 year of age, respectively (282). After the hCG stimulation test, there was a clear elevation of serum testosterone (measured by HPLC tandem mass spectrometry) with a small increase of the androstenedione levels resulting in a high T/A ratio (2.47 and 2.27 respectively). Sequencing of the *HSD17B3* gene identified deleterious molecular defects in both alleles in both patients. The possible explanation for the normal T/A ratio in these 4 children is the individual and temporal variability in the *HSD17B* isoenzymes activity (282).

The disorder is due to homozygous or compound heterozygous mutations in the *HSD17B3* gene which encodes the 17β-HSD3 isoenzyme. Up to now, almost 37 mutations in the *HSD17B3* gene have been reported. These include missense, nonsense, exonic deletion, duplication, intronic splice site and amplification mutations (283) (275) (279). Although mutations have been described throughout the *HSD17B3*, a mutation cluster region was identified in the exon 9. The 17β-HSD3 activity was completely eliminated in the majority of the *HSD17B3* mutations (277). Outside exon 9, the most frequent site of mutation in *HSD17B3 gene* is the R80 in exon 3, which primarily disrupts the binding of the NADPH cofactor to the protein. The p.R80Q mutation has been found in Palestinian, Brazilian and Turkish families (284).

Most patients are raised as girls during childhood. Change to male gender role behavior at puberty has been frequently described in individuals with this disorder who were reared as females (285) (281) (279) (286) including members of a large consanguineous family in the Gaza strip (287). In a revision of all adult patients

with 46,XY DSD due to 17β-HSD3 deficiency reared as female and not castrated during childhood reported until now, we found that 30 of them (61%) kept the female social sex and 19 of them (39%) changed to male social sex (279).
A higher risk of tumor development (28%) has been reported in 46,XY DSD patients due to 17β-HSD3 deficiency (6). However, this high frequency was based on the gonadal tissue analysis of only 7 patients with 17β-HSD3 deficiency (5). Considering the histological analysis of testicular tissue stained with hematoxylin-eosin from all the 40 reported cases 46,XY patients with,17β-HSD3 deficiency the prevalence of germ cell tumor is actually 5.0% (277) (279) (288,289) (290). Therefore, the evidence to support the statement not to encourage patients to assume male gender role due to the risk of gonadal malignancy, is not defendable and the maintenance of the testes in patients with male social sex is safe when the testes can be positioned into the scrotum (266) (279) (291).

Table 12- Phenotype of 46,XY patients with 17β-HSD 3 deficiency	
Inheritance	Autosomal recessive
External genitalia	Atypical, frequently female-like at birth
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Normally developed
Testes	Well developed, frequent cryptorchidism
Hormonal diagnosis	Low T and elevated basal and hCG-stimulated A and A/T ratio
HSD17B3 gene location	9q22
Molecular defect	Inactivating mutation of *HSD17B3*
Puberty	Virilization at puberty; variable gynecomastia
Gender role	Most patients keep the female social sex; some change to male social sex
Treatment	Repair of sexual ambiguity; estrogen or testosterone replacement according to social sex
Outcome	Male or female behavior; in males fertility possible by *in vitro* fertilization

Alternative Pathway To DHT Synthesis

46,XY DSD due to 3α-hydroxysteroid dehydrogenase deficiency (AKR1C2 and AKR1C4 defects)

Molecular analysis of the patients initially described, in 1972, as having 46,XY DSD due to isolated 17,20-lyase deficiency failed to find mutations in *CYP17A1* (265). The hormonal data were inconsistent with other enzymatic deficiencies, then the alternative or backdoor pathway was considered to explain the etiology of the DSD in these patients. The backdoor pathway was firstly described in marsupials and is remarkable for having both reductive and oxidative 3α-HSD steps: the reductive reaction converts 17-OH-dihydroprogesterone (17OH-DHP) to 17OH-allopregnanolone (17OH-Allo), and the oxidative reaction converts androstanediol to DHT (266,292) (293) (Figure 7). Therefore, synthesis of dihydrotestosterone (DHT) occurs without the intermediacy of DHEA, androstenedione or testosterone (292). All the human genes participating in the backdoor pathway have not been identified, however it has been thought that the reductive 3α-HSD activity can be catalyzed by an aldo-keto reductase called AKR1C2) (294) and possibly by other enzymes as the oxidative 3α-HSD activity by 17β-HSD6, also called as RoDH (295) and possibly by AKR1C4 (296).

The initially reported cases with isolated 17,20 lyase deficiency from 1972 (265) were found to carry mutations in two aldo-keto reductases, AKR1C2 and AKR1C4 which catalyze 3α-hydroxysteroid dehydrogenase activity. The two affected 46,XY females were compound heterozygotes for AKR1C2 mutations, the p.I79V/H90Q and p.I79V/N300T. However the mutant AKR1C2 enzymes retained 22-82% of wild-type activity *in vitro* analysis suggesting that another gene was probably involved. Analysis of AKR1C cDNA found that AKR1C4 was spliced incorrectly and gene sequencing displayed an intronic mutation 106 bases upstream from exon 2 that caused this exon to be skipped. So, in this family, a digenetic inheritance was found to impair testicular synthesis of DHT during prenatal life (297).

AKR1C2 is abundantly expressed in the fetal testis, but minimally expressed in the adult testis; on the other hand, the AKR1C4 was found in fetal and adult testes at lower levels. Therefore, it appears that both AKR1C2 and AKR1C4 participate in the backdoor pathway to DHT in the fetal testis, and that molecular defects in these genes appear to cause incomplete male genital development. However, the relative roles of these two AKR1C enzymes remain unclear and testosterone levels at adult age are not available in these patients.

The finding described above, which substantially advanced our understanding of the mechanisms by which male sexual differentiation occurs, illustrates the importance of detailed studies of rare patients who appear to have 17,20 lyase deficiency (247).

46,XY DSD DUE TO DEFECTS IN TESTOSTERONE METABOLISM

5α-Reductase Type 2 Deficiency

An autosomal recessive disorder of sex development (DSD) in males termed pseudovaginal perineoscrotal hypospadias was described in 1961 by Nowakowski and Lenz (298). The main features of this syndrome was that affected males presented with female external genitalia but bilateral testes and male urogenital tracts in which the ejaculatory ducts terminate in a blind-ending vagina. This phenotype was in accordance with what would be expected for steroid 5α-reductase 2 deficiency (299). The clinical syndrome of 5α-reductase type 2 (5α-RD2) deficiency was first described, clinically and biochemically in 1974, in studies of 24 affected subjects from the Dominican Republic and in two siblings from Dallas, Texas USA (300) (301). The gene *SRD5A2* codifies 5α-RD2, it contains 5 exons and 4 introns and is located in chromosome 2p23 and mutations in this gene cause 46,XY DSD (301).

Affected individuals have variable external genitalia ranging from almost normal female external genitalia to microphallus associated with various degrees of hypospadias (302) (303).

Normal internal reproductive structures include seminal vesicles, vasa deferentia, epididymides and ejaculatory ducts, but prostate hypoplasia is common in these patients. No mullerian structures are present and the testes are usually located in the inguinal region. At puberty, deepening of the voice, development of muscle mass and virilization of external genitalia occur. Gynecomastia is only rarely observed in males with 5α-reductase type 2 deficiency and this is an important feature to differentiate from partial androgen insensitivity syndrome. Facial and body hair is decreased in comparison with unaffected males and male pattern baldness does not occur in 5α-reductase type 2 deficiency (302) (304,305).

The phenotype of 46,XY DSD due to 5α-reductase type 2 deficiency in the newborn, overlaps with other forms of 46,XY DSD such as partial androgen insensitivity and testosterone synthesis defects. At puberty or in young adult men, the basal hormonal evaluation demonstrates normal male serum testosterone levels, low or low normal dihydrotestosterone levels, and elevated or normal serum testosterone to dihydrotestosterone ratio (306) (307). In prepubertal children it is necessary to increase serum testosterone levels with hCG stimulation or after exogenous testosterone enanthate injection to analyse T/DHT ratio (303) (308) (309). In newborn the ratio of serum testosterone to dihydrotestosterone may be normal, because expression of the 5α-reductase type 1 enzyme can occasionally be higher than average (310) (308). Elevated 5β/5α urinary metabolites ratio is also an accurate method but not largely available to diagnose 5α-reductase 2, even at prepubertal age or in orchiectomized adult patients (311) (310). Genetic analysis of *SRD5A2* gene is recommended to define the diagnosis of 5α-reductase

2 deficiency before sex assignment in 46,XY DSD newborns with atypical genitalia (312) (313) (314). Mutations in the *SRD5A2* are inherited in an autosomal recessive pattern, and homozygous defects are more frequent than compound heterozygous states (301). One case of uniparental disomy is reported in which a patient with 5α-reductase type 2 deficiency was found to have inherited the paternal allele in homozygous state (315). Variability in phenotypic expression depends on the type of mutation and its effects on enzymatic activity (316). It is interesting that individuals carrying the same mutation may have different phenotypes, suggesting that other factors in addition to 5α-reductase type 2 enzyme activity contribute to the phenotype (317) (318). To date, around 90 different mutations have been described in the *SRD5A2* gene causing 5α-reductase type 2 deficiency (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff, Wales, UK: SRD5A2 gene: http://www.hgmd.cf.ac.uk). The majority of SRD5A2 defects are missense mutations (319). Most males with 5α-reductase type 2 deficiency are raised in the female social sex, but many such individuals not subjected to orchiectomy in childhood undergo change to male social sex at puberty or in adulthood. There are three large cohorts comprasing 136 affected individuals which a similar predominance of the female sex of rearing but differ in the percentual of change to male social sex (303) (306) (313) . In the cohorts from Sao Paulo, SP (Brazil) and Dallas, Texas (USA), the prevalence of sex change to male social sex was around 50% (303) (306). In the other cohort, from France, the percentage of sex change was 12% (313). These differences were possibly due to differences in the age of diagnosis: in the Sao Paulo cohort the patients were diagnosed at an average age of 16 years old whereas in the French study the average age at diagnosis was 7.6 years. In the cohort from Dallas in whom the age at diagnosis was older in many subjects, the incidence of change in social sex was similar to that in Sao Paulo (303) (306). Correlation between the type of mutation and change to male social sex in adulthood was not established.

Regarding long-term follow up, the majority of these subjects were satisfied with the long-term results of their treatment including the appearance of the external genitalia and sexual activity, although a small penile length made sexual intercourse difficult for some of them (320). Most of the adult males patients get married, and those reared as male report a more satisfactory quality of life (QoL) than the female social sex patients (321,322). In female subjects, most of them describe a satisfactory sexual life, but none are married or have adopted children. In the males from Sao Paulo cohort, three patients adopted children and two cases had biological children after successful *in vitro* fertilization (FIV). For FIV procedure, the patient's sperm cells were used resulting in twin siblings in one family and in a single pregnancy in the other (302).

The management in subjects with female social sex includes a careful psychological evaluation to define gender identity (323). Subsequent management

is similar to that in women with others forms of 46,XY DSD. Treatment must simulate a normal puberty pattern and low to normal estrogen doses, taking in account the height, should be administered at the age of expected puberty (10 – 12 years old). After complete breast development, adult estrogen doses are maintained continuously. Progesterone replacement is not necessary because these patients do not have a uterus (324) (325). Feminizing genitoplasty is necessary to provide an adequate vaginal opening, a functional vaginal introitus, fully separation between urethral and vaginal orifice and phallic erectile tissue remotion. Vaginal dilatation with acrylic molds to promote vaginal length is proposed when the patients decide to initiate sexual activity (326) (327). Orchiectomy is recommended for all female patients and laparoscopy procedure is the best technique to perform it.Testosterone replacement is not usually necessary in male patients because most retain testicular function at the time of puberty. However, since the degree of virilization is usually unsatisfactory, a limited course with intramuscular testosterone or transdermal dihydrotestorone may be used for better virilization (328). Dihydrotestosterone replacement provides some advantages such as more activity than testosterone and lack of promotion of bone maturation or of development of gynecomastia since this steroid is not aromatized to estrogen (309) (303). Maximum penile length is obtained after 6 months of high dose testosterone therapy (e.g., 500 mg of testosterone cypionate per week) (302). The therapeutic penile response does not result in normal penile length in all subjects, even when initiated during childhood, and the final penile lenght is below 2 SD in all patients (320). Surgical treatment consists of orthophaloplasty, scrotumplasty, resection of the vaginal pouch and proximal and distal urethroplasthy. Correction of hypospadias is indicated in the first 2 years of life (320).

Table 13. Phenotype of 46,XY subjects with 5α-reductase 2 deficiency	
Inheritance	Autosomal recessive
External genitalia	Atypical, small phallus, perineal hypospadias, bifid scrotum, blind vaginal pouch
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Normal
Testes	Normal size at inguinal or intra abdominal region
Puberty	Virilization at puberty, absence of gynecomastia
Hormonal diagnosis	Increased T/DHT ratio in basal and hCG-stimulation conditions in pospubertal patients and after hCG-stimulation in pre-pubertal subjects. Elevated 5β/5α C_{21} and C_{19} steroids in urine in all ages
SRD5A2 gene location	2 p23
Molecular defect	Mutations in *5RD5A2*
Gender role	Female → male in 50% of the cases
Treatment	High doses of T and/or DHT for 6 months to increase penis size
Outcome	Maximum penis size in males after treatment is below 2 SD; fertility is possible by *in vitro* fertilization

46,XY DSD DUE TO DEFECTS IN ANDROGEN ACTION

Androgen Insensitivity Syndrome

Androgen insensitivity syndrome (AIS) is the most frequent cause of atypical genitalia in individuals with 46,XY karyotype. The undervirilization can be complete (female external genitalia) or incomplete virilization with a spectrum of atypical genitalia. AIS is caused by mutations in the androgen receptor gene (AR), resulting in resistance to the physiologic activity of the androgens. AR is located on the long arm of the X chromosome at Xq11-12 and the pattern of inheritance is X-linked , but *de novo* mutations are found in up to 30% of the cases (329). Differing degrees of resistance lead to 3 three phenotypes: a complete form with female normal-appearing external genitalia, a partial form with a wide range of virilization of the external genitalia, and a mild form with only oligospermia, infertility and/or micropenis (330).

The AR gene is encoded by eight exons and coding a protein about 920 amino acids. Like other members of the nuclear receptor superfamily, the AR is composed of three major functional domains: the N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD (331). The main difference between the *AR* and other steroid receptors is the presence of a longer NTD. The exon 1 encode for the NTD, while exons 2 and 3 encode for the DBD and exons 4-8 encode for the LDB. In the presence of androgens, the AR recruits multiples epigenetic coregulators (332). This co-regulators can be co-activators or co-repressors and acting upon AR influencing DNA binding, nuclear translocation, chromatin remodeling, AR stability and bridging AR with transcriptional machinery (333). AR coding region has two polymorphic trinucleotid repeat regions, located at exon 1, the CAG and GGC repeats (334). The number of these repeats can cause human diseases. In general, longer CAG repeats are related with impairement of AR transactivaction and shorter CAG repeats with enhanced transactivaction (335). A high number of CAG repeats (>38) is the molecular cause of Spinal and Bulbar Muscular Atrophy (Kennedy's disease) (336). This disease is characterized by severe muscular atrophy and a mild AIS phnothype, including gynecomastia. Shorter CAG repeats are related with increased risk for prostate cancer (337).

AIS results from mutations in the AR gene and there are more than 500 mutations in the *AR* gene reported in AIS patients (www.androgendb.mcgill.ca/). Most of them are point mutations leading to amino acids substitutions in the protein structure. However, small insertions and deletions, splicing mutations, point mutations leding to a premature stop codon and complete deletions were describe, most of them related to complete AIS (338). A recurrent germline mutation in two

unrelated patients with complete androgen insensitivity syndrome (CAIS) generating an upstream open reading frame in the 5' untranslated region (5'-UTR) of the *AR* gene (339) and a deep intronic pseudoexon-activing mutation were described (340). Some AIS patients have been described with an unaltered coding region of the AR gene including the intron-exon boundaries supporting the concept that in a subset of AIS patients, particulary those with partial form, molecular alterations outside the coding region of the AR gene must be presumed. This group was been named as AIS type 2 (341) (342). However, a specific role of certain coregulators in the pathophysiology of AIS is not established yet and the contribution of AR-associated coregulators in AIS remains poorly understood (343).

Knowledge about the molecular mechanism of androgen action and how the range and type of mutations distributed throughout the AR gene affect phenotype is important to clinician to establish a correct diagnosis and management of this disease. Despite the advances in molecular diagnosis, mutations are identified in 28-50% of PAIS and 90-95% of CAIS (329) (338) (335).

Complete Androgen Insensitivity Syndrome

Prenatal diagnosis of CAIS can be suspected based on the discordance between 46,XY karyotype on prenatal fetal sex determination and female genitalia at prenatal ultrasound. At birth, there is the presence of a typical female external genitalia. At prepubertal age, an inguinal hernia in a girl can indicate the presence of testes in 2.4% (344). At puberty, CAIS patients presenting with complete breast development and primary amenorrhea. Pubic hair and axilar hair are sparse in most of them. Mullerian ducts are generally absent in CAIS patients but there are some reports referring the presence of this derivatives in these patients (345).

Whereas the clinical picture of CAIS is homogeneous, the phenotype of partial androgen insensitivity syndrome (PAIS) is quite variable and similar to other causes of 46,XY DSD (280) (277). Patients with PAIS have atypical genitalia, ranging from predominantly female genitalia with mild clitoromegaly to predominantly male genitalia with micropenis and hypospadias. The development of gynecomastia at puberty is common and this feature is important to diferential diagnosis (346). Hormonal diagnosis, after the age of puberty, is performed by the demonstration of normal or elevated serum testosterone levels and slightly elevated LH levels. FSH levels can be slightly elevated, especially in cases with cryptorchidism. Testosterone precursors are not elevated in relation to testosterone levels (347).

Patients with CAIS are raised as girls and have a female gender identity and role behavior (285) (348) (347). Estrogen replacement is recommended to induce puberty if bilateral gonadectomy has been performed. The risk of gonadal tumours

in CAIS patients has been estimated from 0% to 22% (349) (350) (351). It is consensus that gonadectomy should be performed because of the increased risk of testicular tumors after puberty. In order to define the better age to indicate the bilateral gonadectomy is important to consider that the decline or delay of gonadectomy is a common situation in these patients for reasons such as fear of surgery, to avoid estrogen replacement and expectations for future fertility (290) (352). Due to a probably low risk of gonadal tumour in these patients, an increasing number of adult women with CAIS prefer to retain their gonads indefinitely (353) (354).

Table 14. Phenotype of 46,XY subjects with complete androgen insensitivity syndrome	
Inheritance	X-linked recessive
External genitalia	Female
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Absent or vestigial
Testes	Inguinal or intraabdominal, slightly subnormal size
Puberty	Complete breast development
Hormonal diagnosis	High or normal serum LH and T levels, normal or slightly elevated FSH levels
Gender role	Female
AR gene location	Xq11-12
Molecular defect	Mutations in androgen receptor gene
Treatment	Psychological follow-up Replacement with estrogens after gonadectomy. Vaginal dilation
Outcome	Female gender role and behavior, infertility

Partial Androgen Insensitivity Syndrome (PAIS)

Patients with PAIS have a broad spectrum of impairment in virilization. The external genitalia ranged from predominantly female with clitoromegaly and labial fusion to predominantly male with micropenis and hypospadias. Testes are in the inguinal canal or labioscrotal folds or, less frequently, intraabdominal. At puberty is observed undervirilization and gynecomastia (347). It is estimated that subjects with PAIS had mean final height intermediate between mean normal male and female and decreased bone mineral density in the lumbar spine (355). These findings suggest an important role for androgens in normal growth and bone density.

Serum LH levels are in the upper normal range or slightly elevated and testosterone levels are normal or also slightly elevated. Testosterone precursors are not increased in relation to testosterone whereas testosterone/DHT ratio may be slightly higher than in the normal population. A definitive diagnosis of PAIS is established by the identification of mutation in the *AR* gene but mutations are not found in more than 40% of the patients with PAIS.

The sex of rearing is female in half of the cases. The social sex change is not common in PAIS and most of the patients with PAIS who were raised as females or males maintained their original social sex after postpubertal age (323). This is interesting because in these patients, the gender identity is in line with sex of rearing (356).

Estrogen replacement is necessary for female patients to induce adequate puberty and to be maintained. For male patients, androgen supplementation, either to induce puberty or to enhance virilization post-puberty is commonly required. High doses of intramuscular testosterone preparations or topical DHT can be tried for 6 months (357).

Gonadectomy is mandatory for all female patients and male patients need to have the gonads accessible, preferably in the scrotum.

Table 15. Phenotype of 46,XY subjects with partial androgen insensitivity syndrome	
Inheritance	X-linked recessive
External genitalia	Broad spectrum from female with mild clitoromegaly to male with micropenis and/or hypospadias
Müllerian duct derivatives	Absent
Wolfian duct derivatives	Broad spectrum from absent or male
Testes	Eutopic, inguinal or intraabdominal, normal or slightly subnormal size
Puberty	Gynecomastia
Hormonal diagnosis	High or normal serum LH and T levels, normal or slightly elevated FSH levels
Gender role	Female or male
AR gene location	Xq11-12
Molecular defect	Mutations in *AR* gene
Treatment	Females: surgical feminization, gonadectomy, replacement with estrogens at the time of puberty, vaginal dilation (if necessary) Males: hypospadias repair, bifid scrotum; high doses of T or DHT to increase penis size
Outcome	Infertile, female or male gender role

46,XY DSD DUE TO PERSISTENT MÜLLERIAN DUCT

Defect in AMH Synthesis in AMH Receptor

The development of female internal genitalia in a male individual is due to the incapacity of Sertoli cells to synthesize or secrete anti-mullerian hormone (AMH) or to alterations in the hormone receptor. Persistent Müllerian duct syndrome (PMDS) phenotype can be produced by a mutation in the gene encoding anti-Müllerian hormone or by a mutation in the AMH receptor. These two forms result in the same phenotype and are referred to as type I and type II, respectively (358).

AMH is a 145,000 MW glycoprotein homodimer produced by Sertoli cells not only during the period when it is responsible for regression of the Müllerian ducts but also in late pregnancy, after birth, and even, albeit at a much reduced rate, in adulthood (9,358-360).

AMH is a small gene containing 5 exons, located in chromosome19p.13.3 (361) and its protein product acts through its specific receptor type 2 (AMHR2) a serine/threonine kinase, member of the family of type II receptors for TGF-β-related proteins (362).

Affected patients present a male phenotype, usually along with bilateral cryptorchidism and inguinal hernia (363). Leydig cell function is preserved, but azoospermia is common due to the malformation of *ductus deferens* or agenesis of epididymis. When the hernia is surgically corrected, the presence of a uterus, fallopian tubes and superior part of the vagina can be verified.

PMDS is a heterogeneous disorder that is inherited in a sex-limited autosomal recessive manner. Mutations in *AMH* gene or *AMH* receptor 2 gene in similar proportions are the cause of approximately 85% of the cases of PMDS (364,365). In the remaining cases the cause of the persistent Mullerian duct syndrome is unknown (9).

Normally, AMH levels are measurable during childhood and decrease at puberty. Patients with AMH gene defects have low AMH levels since birth whereas patients with mutations in AMH receptor gene have elevated AMH levels (366).

Treatment is directed toward an attempt to assure fertility in males. Early orchiopexy, proximal salpingectomy (preserving the epididymis), and a complete hysterectomy with dissection of the vas deferens from the lateral walls of the uterus are indicated (367,368).

CONGENITAL NON-GENETIC 46,XY DSD

Maternal Intake of Endocrine Disruptors

The use of synthetic progesterone or its analogs during the gestational period has been implicated in the etiology of 46,XY DSD (369). Some hypothesis have been proposed to explain the effect of progesterone in the development of male external genitalia, such as reduction of testosterone synthesis by the fetal testes or a decrease in the conversion of testosterone to DHT due to competition with

progesterone (also a substrate for 5α-reductase 2 action). The effect of estrogen use during gestation in the etiology of 46,XY DSD has not been confirmed to date (370). Recently, a study in Japanese subjects supports the hypothesis that homozygosis for the specific estrogen receptor alpha 'AGATA' haplotype may increase the susceptibility to the development of male genital abnormalities in response to estrogenic effects of environmental endocrine disruptors (371).
Environmental chemicals that display anti-androgenic activity via multiple mechanisms of action have been identified. They are: pesticides, fungicides, insecticides, plasticizers and herbicides. They can work as androgen receptor antagonists like pesticides, or they can decrease mRNA expression of key steroidogenic enzymes and also the peptide hormone insl3 from the foetal Leydig cells, like plasticizers and fungicides (372).
Daily exposure to residues of a fungicide (vinclozolin), either alone or in association with a phytoestrogen genistein (present in soy products), induce hypospadias in 41% of mice, supporting the idea that exposure to environmental endocrine disruptors during gestation could contribute to the development of hypospadias (373).
Supporting the idea that exposure to a mixture of chemicals can produce greater incidences of genital malformations, Rider *et al* examined the effects of exposure to a mixture of two chemicals that act as androgen receptor antagonists. They observed that the exposure to vinclozolin (fungicide) alone resulted in a 10% incidence of hypospadias and no vaginal pouch development in male rats, whereas procymidone, another fungicide exposure failed to generate malformations. However, the combined exposure resulted in a 96% incidence of hypospadias and 54% incidence of vaginal pouch in treated animals. Similar results were observed in phthalate (plasticizer) mixture studies (372).
Given that severe alterations of sexual differentiation can be produced in animal laboratory studies, the question arises of what would be expected in exposed humans given that humans are exposed to mixtures of compounds in their environment.

Congenital non-Genetic 46,XY DSD Associated to Impaired Prenatal Growth

Despite the multiple genetic causes of 46,XY DSD, around 30-40% of cases remain without diagnosis. Currently, there is a frequent, non-genetic variant of 46,XY DSD characterized by reduced prenatal growth and lack of evidence for any associated malformation or endocrinopathy (374) (375). Using the model of monozygotic twins, hypospadias has now been linked to low birth weight (374). We have identified a pair of 46,XY monozygotic twins (identical for 13 informative DNA loci) born at term after an uneventful pregnancy sustained by one placenta who were discordant for genital development (perineal hypospadias *versus* normal male

genitalia) and postnatal growth (low birth weight versus normal birth weight). No evidence for uniparental dissomy was found (376). The most plausible cause of incomplete male differentiation associated with early-onset growth failure is a post-zygotic, micro-environmental factor since different DNA methylation patterns associated with silencing of genes important for sex differentiation has been shown (377).
Additionally, three cohorts of undetermined 46,XY DSD report around 30% of cases as associated with low birth weight, indicating that adverse events in early pregnancy are frequent causes of congenital non-genetic 46,XY DSD (378) (379) (380).

46,XY OVOTESTICULAR DSD

There are rare descriptions of 46,XY DSD patients with well characterized ovarian tissue with primordial follicles and testicular tissue, a condition that histologically characterized 46,XY ovotesticular DSD. The diferential diagnosis of 46,XY ovotesticular DSD with partial 46,XY gonadal dysgenesis should be performed considering that in the latter condition there are descriptions of dysgenetic testes with disorganized seminiferous tubules and ovarian stroma with occasional primordial follicles in the first years of life (46). To our knowledge there are no descriptions of an adult patient with 46,XY ovotesticular DSD with functioning ovarian tissue, as occurs in all 46,XX ovotesticular DSD. Therefore the diagnosis of 46,XY ovotesticular DSD is debatable.

NON-CLASSIFIED FORMS

Hypospadias

Hypospadias is one of the most frequent genital malformation in the male newborn and 40% of the cases are associated with other defects of the urogenital system. Hypospadias results from an abnormal penile and urethral development that appears to be a consequence of various mechanisms including genetic and environmental factors. It is usually a sporadic phenomenon, but familial cases can be observed, with several affected members.
The presence of hypospadias indicates an *intrauterus* interference in the correct genetic programme of the complex tissue interactions and hormonal action through enzymatic activities or transduction signals. MAMLD1 (mastermind-like domain containing 1) has been reported to be a causative gene for hypospadias (381). It appears to play a supportive role in testosterone production around the critical period for sex development. To date, microdeletions involving *MAMLD1* and nonsense and frameshift mutations in the gene have been found in 46, XY DSD patients, suggesting that *MAMLD1* mutations cause 46,XY DSD primarily because

of compromised fetal testosterone production, however, its role in the molecular network involved in fetal testosterone production is not known so far (382).
The activating transcription factor 3 (ATF3) expression was evidenced in the developing male urethra. Apparently ATF3 variants may influence the risk of hypospadias (383).
By definition, hypospadias is a form of 46,XY DSD and although most of the patients present fertility and masculinization at puberty, their testicular function should be assessed to rule out causes such as defects in testosterone synthesis and action, which require hormonal treatment and genetic counseling in addition to surgical treatment.

GONADAL TUMOR DEVELOPMENT IN 46,XY DSD PATIENTS

Specific variants of DSD (especially in patients with gonadal dysgenesis and hypovirilization) have a significant risk factor for type II germ cell tumors. A high risk of gonadoblastoma is found when sex determination is disrupted in an early stage of Sertoli cell differentiation (due to abnormalities in *SRY, WT1, SOX9*). Early Sertoli cell development is also disturbed in patients with 45X/46,XY mosaicism.
The presence of the well-defined Y chromosome region, known as the gonadoblastoma Y *locus* (GBY), is a prerequisite for malignant transformation. Among the genes located on GBY region the testis-specific protein Y (TSPY) seems to be the most significant candidate gene for tumor-promoting process (288). The presence of undifferentiated gonadal tissue containing germ cells, that abundantly express TSPY and OCT4 has also been identified as a gonadal differentiation pattern bearing a high risk for the development of gonadoblastoma (288).
Careful histological analysis of gonadal tissue of DSD patients revealed that undifferentiated gonadal tissue (UGT) of DSD is the most likely precursor stage of gonadoblastomas.
The risk for germ cell tumors is increased in patients with undescended testes, including all other 46,XY DSD syndrome.
Neoplastic transformation of germ cells in dysgenetic gonads (gonadoblastomas and/or an invasive germ cell tumor) occurs in 20-30% of 46,XY DSD patients and is associated with the presence of Y chromosome or part of it (288).
Spontaneous breast development suggests the presence of an estrogen-secreting tumor (gonadoblastomas). Bilateral gonadectomy should be performed in 46,XY patients before pubertal age to avoid degeneration of dysgenetic tissue, unless the gonad is functional and easily accessible to palpation and imaging studies, which should be performed yearly. A gonadal biopsy showing the presence of undifferentiated gonadal tissue or testicular tissue with OCT4-positive cells on the basal lamina suggests a high risk for germ cell tumors whereas testicular tissue

displaying maturation delay of germ cells and stroma ovarian tissue can be safely be left *in situ* (288). The risk for germ cell tumors is increased in patients with undescended testes, including all other 46,XY DSD syndromes (288).
Although data are limited, in the androgen insensitivity syndrome the risk seems to be markedly higher in the partial form than in the complete form and tumor prevalence in AIS is markedly increased after puberty. On the other hand, series reporting other causes of 46,XY undervirilized patients and gonadal tumors are too small and do not allow any conclusion.
The use of a uniform classification system of the various forms of DSD will hopefully shed light on the actual risk for malignant transformation of germ cells in the different DSD subgroups, which might result in a more conservative approach of gonadectomy in some patients. The benefits may include physiological induction of puberty and even fertility.

FERTILITY IN PATIENTS WITH 46,XY DSD

Infertility is almost always present in 46,XY DSD patients due to impaired spermatogenesis secondary to gonadal dysgenesis, testosterone deficiency or action, cryptorchidism or retrograde ejaculation, frequently found in patients with perineal hypospadias. Currently, *in vitro* fertilization techniques have enabled 46,XY DSD patients to produce offspring (384) (307). Successful pregnancy and delivery following in vitro fertilization using donor oocytes and embryo transfer in a patient with complete 46,XY gonadal dysgenesis was reported (385).

46,XY GENDER IDENTITY DISORDERS

Male To Female Transsexualism (Transgender Woman)

Male to female transsexualism is characterized by the wish to live as member of the female sex with conviction and consistently and progressively efforts to achieve such state. 46,XY gender identity disorders are more frequent among the male sex, although it also occurs in the female sex. Its first manifestations usually start during childhood. Its etiology remains unknown, although some hormonal alterations during *intrauterus* life and familial factors before and after birth cannot be ruled out (386).
Term used to name men and women who live a significant incongruence between their gender identity and their inborn physical phenotype has undergone changes over time. The term “transssexualism” was coined by Hirschfeld in 1923 and is still used by the International Classification of Diseases – version 10 (ICD-10). The American Psychiatric Association, in its 4th edition, adopted “gender identity disorder” to define persons who are not satisfied with their natal gender

(Association, American Psychiatric. "Diagnostic and statistical manual of mental disorders (2000).

Finally, the current classification system of the American Psychiatric Association (DSM-5) replaced the term “gender identity disorder” with “gender dysphoria” and the upcoming version of International Classification of Diseases – version 11 (ICD-11) has proposed using the term “gender incongruence” (387).

In this chapter we will use the current term coined by DSM-5, the “gender dysphoria”. To refer to male to female gender-dysphoric persons we will use the term transgender woman (American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition - DSM-5; 2013). Therefore, the term “transgender woman” refers to all 46, XY individuals with normal male phenotype who wishes to live and be accepted as a female

Higher prevalence of addictions and suicidal thoughts or suicide attempt than those observed in the general population, revealed the need for early care of these patients by health professional**s.** Among transgender woman, total mortality was 51% higher than in the general population, mainly from increased mortality rates due to suicide, acquired immunodeficiency syndrome, cardiovascular disease, drug abuse, and unknown cause (388). Based on these data, supervised gender-affirming treatment for gender dysphoric persons is absolutely essential because they are at increased risk of committing suicide and self-harm (389).

Management of adult transgender woman

As proposed by the Harry Benjamin International Gender Dysphoria Association, now known as World Professional Association for Transgender Health (WPATH), the process of gender-affirming treatment should be given by a multi and interdisciplinary team, in which the endocrinologist has a key role.

The interdisciplinary team should consist of a psychologist, a psychiatrist, an endocrinologist, and a surgeon, at least. It would be ideal that they all participate in an integrated and consistent way across all the steps of the treatment (390).

The mental health professionals (psychologist and psychiatrist) make a distinction between gender dysphoria and conditions with similar features (e.g. body dimorphic disorder and body identity integrity disorder), decide whether the individuals fulfills ICD-10 and DSM-5 criteria, recommend the appropriate treatment and follow them before, during and after gender-affirming treatment. The endocrinologist will inform about the possibilities and limitations of all kinds of treatment, initiate and monitor the cross-sex hormonal treatment and participate in the indication of gender-affirming surgery. At the final step, the surgeon performed surgical procedures of the treatment (390).

***Diagnostic Assessment and Mental Health Care*.**

Psychological evaluation of persons with gender dysphoria should consider the evolution of the individual as whole, using psychological assessment

instruments, such as: freely structured interviews and patterned psychological assessment instruments. For the structured interview, we use a specific questionnaire developed by our mental health professionals that covers childhood, adolescence and adulthood aspects.

During the psychotherapeutic follow up, besides offering an ideal condition for elaborating conflicts and issues regarding gender identity, other variables should take into account, such as individual general state of mental health, ability and manner of conflict resolution, quality of interpersonal relationships, ability to deal with frustrations and limitations, particularly regarding to surgery's esthetic and functional results idealization. It is recommended that the relatives and/or spouses were invited for interviews to clear up them upon the offered treatment.

***Hormonal therapy for adult* transgender woman**

Endocrinologists have the responsibility to confirm that persons fulfill criteria for hormonal treatment and clarify the consequences, risks and benefits of the treatment.

The hormone therapy must follow well-defined criteria. The person with gender dysphoria have to: 1) demonstrate knowledge and understanding of the expected and side effects of cross-sex hormone use; 2) complete a real life experience in the gender identity for at least three months, or psychotherapy for a period determined by the mental health professional to consolidate gender identity; and 3) be likely to take hormones in a responsible manner (390).

The two major goals of hormonal therapy are:1) to replace endogenous sex hormone levels and, thus, induce the appearance of sexual characteristics compatible with male gender identity; 2) to reduce endogenous sex hormone levels and, thereby, the secondary male sexual characteristics and 3) to establish the ideal hormones dosage which allows physiological hormone serum levels compatible with male gender identity by adopting the principles of hormone replacement treatment of hypogonadal patients (391) (390).

Hormone therapy provides a strong relief from suffering caused by the incongruence of the phenotype with the gender identity.

In our clinical practice, we observe that the majority of transgender women consume very high doses of female sex hormones, guided by their wish to obtain rapid development of breasts and control of facial hair growth. However, high doses of hormones are not necessary to achieve the desired effects, and are frequently associated with undesirable side effects.

The chosen hormone to induce female secondary sexual characteristics in this group is the estrogens. A large number of pharmaceutical estrogen preparations, including oral, injectable, transdermal and intravaginal forms associated or not with progesterone are available. Due to the higher cost of the transdermal preparations, oral route are the most widely used. Nevertheless, the

transdermal route is recommended for transgender women over 40 years of age due to the lower association of 17β-estradiol replacement with thromboembolic events (392).

Anti-androgens are used as adjuvants to estrogen, especially in the reduction of male sexual characteristics and the suppression of testosterone to levels compatible with the female sex. Cyproterone acetate blocks testosterone binding to its receptor, and in a dose of 50-100 mg/day associated with estrogen can maintain testosterone in female levels in transgender women (393).

At the time, most of patients followed in our clinic makes use of conjugated equine estrogens at a dose of 0.625-1.25 mg/day associated with 50 mg/day of cyproterone acetate for an average period of 11 years. At clinical examination we observed satisfactory breast development, decrease of spontaneous erections, thinning of facial and body hair (especially after association with cyproterone acetate), body fat redistribution, enlargement of the areola and nipple and reduction of testicular volume (391).

Testosterone levels remained at pre or intra-pubertal female range (< 14-99 ng/dL) in all patients; LH levels were pre-pubertal (<0.6-0.7 U/L) in 72% of the cases, and the FSH levels were suppressed (<1.0 U/L) in 40% of cases. Therefore, daily use of oral conjugated estrogens at low doses in association with cyproterone acetate is effective in suppressing the hypothalamic-pituitary-testicular axis in transgender women (391).

Venous thromboembolism may be a serious complication related to estrogen therapy in this group of patients, particularly during the first year of treatment,when the incidence of this event is 2-6% falling to 0.4% in the second year, significantly higher when compared to the overall young population (0.005 to 0.01%/year).This high incidence of thromboembolic events in transgender women seems to be more associated with ethinyl estradiol than natural oral or transdermal estrogens (392). All patients on estrogen therapy have a mild prolactin levels increase. However, a small percentage of these subjects have galactorrhea. In our cohort, two patients had macroprolactinoma, which totally regressed with dopamine agonist treatment. Both of them had previously used high doses of estrogen (394). Endocrinologist should half yearly monitoring weight, blood pressure, breast enlargement, body hair involution, body fat redistribution, and testicular size. The laboratory evaluation should include measurement of LH, FSH, testosterone, estradiol, prolactin, liver enzymes, complete blood count, coagulation factors, and lipid profile. Bone densitometry and breast ultrasound should be performed yearly.

After surgery in patients over 50 years old, the dosage of PSA should be conducted yearly (391).

The current key issues include avoiding supraphisiological doses of estrogen and the use of ethinyl estradiol. The preference should be given to

conjugated estrogens or transdermal natural estrogen, especially in patients over 40 years of age (395).

Hormone therapy provides a strong relief from suffering caused by the incongruence of the phenotype with the male gender identity (391).

MANAGEMENT OF PATIENTS WITH 46,XY DSD

It is important to stress that the treatment of 46,XY DSD patients requires an appropriately trained multi-disciplinary team. Early diagnosis is important for good outcome of the patients and should start with a careful examination of the newborn's genitalia at birth (14,357) (396) (397).

Psychological Evaluation

It is of crucial importance to treat DSD patients. Every couple that has a child with atypical genitalia must be assessed and receive counseling by an experienced psychologist, specialized in gender identity, who must be act as soon as the diagnosis is suspected, and then follow the family periodically, more frequently during the periods before and after genitoplasty, (398,399).

Parents must be well informed by the physician and psychologist about normal sexual development. A simple, detailed and comprehensive explanation about what to expect regarding integration in social life, sexual activity, need of hormonal and surgical treatment and the possibility or not of fertility according to the sex of rearing, should also be discussed with the parents, before the attainment of final social sex.

The determination of social sex must take into account the etiological diagnosis, penis size, ethnic traditions, sexual identity and the acceptance of the assigned social sex by the parents. In case parents and health care providers disagree over the sex of rearing, the parents' choice must be respected. The affected child and his/her family must be followed throughout life to ascertain the patient's adjustment to his/her social sex.

Hormonal Therapy

Female social sex: The purpose of the hormonal therapy is the development of female sexual characteristics and menses in the patients with uterus. The treatment must simulate normal puberty, by introducing low doses of estrogen at 9-11 years to avoid excessive bone maturation in short children. Estrogen therapy should be initiated at a low dose (one sixth to one quarter of the adult dose) and increased gradually at intervals of 6 months. Doses can then be adjusted to the response (Tanner stage, bone age), with the aim of completing feminization gradually over a period of 2–3 yr. In tall 46,XY females, adult estrogen dosage is recommended to avoid high final stature. The initial dosage of conjugate estrogens (0.07 to 0.15 mg/day orally) or oral or topic 17β-estradiol (0.5 mg daily) is kept as the patient presents progressive breast development. If breast development is not

progressive, the estrogen dose is doubled. Low-dose transdermal hormone therapy is also a viable alternative estrogen replacement, offering lipid protection and preservation of bone mass. After breast development is complete, the estrogen dose is maintained at (0.625 mg/day of conjugate estrogen) or 1 mg twice a day of oral or topic 17β-estradiol) continuously and medroxyprogesterone acetate (5 to 10 mg/day) or micronized progesterone 50 mg/day, from the 1st to the 12th day of the month), is added to induce menses. In patients without uterus only estrogen is indicated. The dilation of the blind vaginal pouch with acrylic molds (327) or surgical neovagina promote development of a vagina adequate for sexual intercourse after 6-10 months of treatment when patients desire to initiate sexual activity (400).

Male social sex: Testosterone replacement is started between 10 and 11 yrs, simulating normal puberty according to the child's psychological evaluation and height. Intramuscular depot injections of testosterone esters are commonly used; another option is oral testosterone undecanoate and transdermal preparations (401). The initial dose of depot injections of testosterone esters is 25 to 50 mg/month administered IM. The maintenance dose in an adult patient is 200 to 250 mg every 2 weeks or 1000 mg each 3 months. In male patients with androgen insensitivity, higher doses of testosterone esters (250-500 mg twice a week) are used to increase penis size and male secondary characteristics. Maximum penis enlargement is obtained after 6 months of high doses and after that, the normal dosage is re-instituted 273) (311). The use of topic DHT gel is also useful to increase penis size with the advantage of not causing gynecomastia and promoting faster increase of penis size as it is 50 times more active than testosterone. Considering that DHT is not aromatized, one would expect it to have no effect on bone maturation, allowing the use of higher doses than testosterone and consequently attaining a higher degree of virilization.

SURGICAL TREATMENT

The aim of surgical treatment is to allow development of adequate external genitalia and remove internal structures that are inappropriate for the social sex. Patients must undergo surgical treatment preferably before 2 years of age, which is the time when the child becomes aware of his/her genitals and social sex. Only skilled surgeons with specific training in the surgery of DSD should perform these procedures (5) (402).

Laparoscopy is the ideal method of surgical treatment of the internal genital organs in patients with 46,XY DSD (403). In these patients, the indications for laparoscopy are the removal of normal gonads and ductal structures that are contrary to the assigned gender and the removal of dysgenetic gonads, which are nonfunctional and present potential for malignancy. In addition to being a minimally invasive surgery, one of the main advantages of this method is the lack of scars.

Feminizing genitoplasty should provide an adequate vaginal opening into the perineum, create a normal-looking vaginal introitus, fully separate the urethral from the vaginal orifice, remove phallic erectile tissue preserving glandular enervation and blood supply, and prevent urinary tract complications (326). The most reasonable procedure to perform clitoroplasty is based on the concept of maintaining the clitoral glans and sensory input, which facilitates orgasm. The use of an adequate size of tissue flap is mandatory in Y-V vaginoplasty, to avoid introital stenosis. Failure to interpose an adequate flap will result in persistent introital stenosis, requiring later revision. Vaginal dilation with acrylic molds in patients with introitus stenosis showed to be a good treatment choice when these patients wished to start sexual intercourse, resulting in good outcomes (327). In our experience, the single-stage feminizing genitoplasty consisting of clitoroplasty with the preservation of dorsal nerves and vessels and ventral mucosa, vulvoplasty and Y-V perineal flap, followed by vaginal dilation with acrylic molds, allowed good cosmetic and functional results (326).

For those raised as males, surgery consists in orthophaloplasty, scrotumplasty with resection of vaginal pouch, proximal and distal urethroplasty and orchidopexy when necessary. Surgeries were performed in 2 or 3 steps in the patients with perineal hypospadias. The most frequent complication is urethral fistula in the penoscrotal angle and urethral stenosis that can occur several years after surgery. The results of surgical correction are good, from both the aesthetical and functional points of view in our series as well as in others (5) (6) (404) (405) (320).

Most of our patients present satisfactory sexual performance as long as they present a penis size of at least 6 cm. New approaches, such as the use of donor-grafting tissue to elongate the urethra and penis may help these patients in the future.

Acknowlegdment: The authors would like to thank the posgraduated students Nathalia Lisboa Gomes, Rafael Loch Batista and Jose Antonio D Faria Junior for their help in the update of this chapter.

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