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Mechanisms in Disorders of Sex Development

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Al nonno Michele e alla zia Iole

"I was born twice: first, as a baby girl, on a remarkably smogless Detroit day in January of 1960; and then again, as a teenage boy, in an emergency room near Petoskey, Michigan, in August of 1974."

....

"Sing now, O Muse, of the recessive mutation on my fifth chromosome! Sing how it bloomed two and a half centuries ago on the slopes of Mount Olympus, while the goats bleated and the olives dropped. Sing how it passed down through nine generations, gathering invisibly within the polluted pool of the Stephanides family. And sing how Providence, in the guise of a massacre, sent the gene flying again; how it blew like a seed across the sea to America, where it drifted through our industrial rains until it fell to earth in the fertile soil of my mother's own mid-western womb. Sorry if I get a little Homeric at times. That's genetic, too."

From 'Middlesex'
Jeffrey Eugenides

"...the fact is that not everybody arrives in this world ready to be squeezed into one or the other generally accepted anatomic patterns of what we usually think of as male and female."

From 'Hermaphrodites and the Medical Invention of Sex'
Alice Domurat Dreger

"Sex is just as complicated as humans are"

Vilain E. *et al* Genet Med 2007, 9:65-6

ABSTRACT

"Is it a boy or a girl?" This is usually the first question that parents have when their baby is born. Sometimes it is not possible to give an immediate answer. This is the case when the newborn presents ambiguous external genitalia and an immediate sex assignment is not possible. This situation represents the most typical and dramatic presentation of a disorder of sex development (DSD). Other DSDs can manifest later in life, for example in a girl with primary amenorrhea, who finds out that she has a 46,XY karyotype and will never be fertile. The overall aim of this thesis was to identify mechanisms in DSD, in order to better understand normal and atypical sex development, and furthermore to offer better diagnostics and genetic counselling to patients with DSD and their families.

Congenital adrenal hyperplasia (CAH) due to CYP21A2 deficiency is the single most common cause of ambiguous external genitalia in the newborn. The wide spectrum of clinical manifestations ranges from prenatal virilisation in XX girls and salt-wasting in the neonatal period to precocious pubarche and late-onset hyperandrogenic symptoms during adulthood, depending on the *CYP21A2* genotype. By *in vitro* expression of CYP21A2 we have evaluated the residual enzyme activities of four mutant enzymes that carry novel or rare missense mutations identified in patients with CAH. All mutants had a residual activity below 1%, and are thus associated with severe enzyme deficiency. Therefore, these mutations are predicted to cause classic CAH if found in trans with other severe mutations **(Paper I)**.

Mutations in the androgen receptor (AR) gene cause androgen insensitivity syndrome (AIS). Patients with completely female external genitalia are classified as having complete AIS (CAIS). However, some of these patients have signs of internal male genital differentiation due to missense mutations that show a low degree of residual function. We studied the expression of two isoforms of the AR in two CAIS patients in relation to the development of male internal genital structures. One patient had a mutation (L7fsX33) that affects only the full-length AR-B form of the AR, whereas the other had a nonsense mutation (Q733X) affecting both isoforms, as shown by Western blot analysis of proteins from gonadal and genital skin fibroblasts. No signs of Wolffian duct development were present in any of the patients, indicating that the AR-A form is not sufficient for Wolffian duct maintenance and differentiation **(Paper II)**.

A genome wide investigation by high resolution BAC array CGH (Comparative Genomic Hybridization) was used to identify gene dosage imbalances in 10 patients with female external genitalia due to XY gonadal dysgenesis (GD). We identified and characterised a 637 kb duplication at Xp21 containing *DAX1* in a girl with isolated 46,XY GD **(Paper III)**. We also identified another XY patient with isolated partial GD and ambiguous external genitalia, by MLPA (Multiplex Ligation Probe-dependent Amplification) analysis using a synthetic probe set that we designed to identify gene dosage imbalances for known genes involved in DSD **(Paper IV)**. These reports describe the first duplications on Xp21.2 identified in patients with isolated GD because all previously described XY subjects with Xp21 duplications presented with GD as part of a more complex phenotype, including mental retardation and/or malformations. These data support *DAX1* as a dosage sensitive gene responsible for GD and highlight the importance of considering *DAX1* locus duplications in the evaluation of all cases of 46,XY GD. More recently, we identified an additional family with several members affected with XY GD, where a small *DAX1* duplication is segregating through the female line. These data suggest that *DAX1* duplications might be as common as *SRY* mutations causing 46,XY GD. A terminal 9p deletion, of a region already involved in DSD, was also identified by array-CGH, and confirmed by a MLPA probe set, designed to enable screening of loss of candidate DSD genes at 9p23.4. The identification of submicroscopic deletions at 9p24 is of help to understand the mechanisms that lead to GD in some patients with 9p deletions, and to narrow down the monosomy 9p syndrome candidate region **(Paper V)**. By array-CGH we have also identified two novel chromosomal imbalances that are candidate regions for XY GD: a duplication of 3.7 Mb at chromosome band 12q21.31 and a duplication on chromosome 6 that extends from exon 5 to exon 12 of the *SUPT3H* gene. These regions will be the subjects of further investigations in order to identify new genes involved in gonadal development **(Paper VI)**.

The work of this thesis has led to the establishment of the genetic diagnosis in several patients with DSD, thus allowing not only a better genetic counselling but also, at least in some cases, a better patient management. Furthermore, our genetic diagnostic arsenal has been expanded, as we can offer sequencing of more genes and gene dosage investigations by MLPA.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numerals throughout the text:

I. **Barbaro M**, Baldazzi L, Balsamo A, Lajic S, Robins T, Barp L, Pirazzoli P, Cacciari E, Cicognani A, Wedell A.
Functional studies of two novel and two rare mutations in the 21-hydroxylase gene.
Journal of Molecular Medicine, 2006, 84: 521-528

II. **Barbaro M**, Oscarson M, Almskog I, Hamberg H, Anna Wedell.
Complete Androgen Insensitivity without Wolffian Duct Development - The AR-A Form of the Androgen Receptor is not sufficient for Male Genital Development.
Clinical Endocrinology, 2007, 66: 822-826

III. **Barbaro M** *, Oscarson M *, Schoumans J, Staaf J, Ivarsson SA, Wedell A.
Isolated 46,XY gonadal dysgenesis in two sisters caused by a 637 kb interstitial duplication on Xp21.2 containing the DAX1 (NR0B1) gene.
Journal of Clinical Endocrinology and Metabolism, 2007, 92: 3305-3313

IV. **Barbaro M**, Cicognani A, Balsamo A, Löfgren Å, Baldazzi L, Wedell A, Oscarson M.
Gene dosage imbalances in patients with 46,XY gonadal DSD, detected by an in house designed synthetic probe set for multiplex ligation probe-dependent amplification (MLPA) analysis.
Clinical Genetics. In Press.

V. **Barbaro M**, Balsamo A, Anderlid BM, Myhre AG, Gennari M, Nicoletti A, Pittalis MC, Oscarson M, Wedell A.
Characterization of deletions at 9p affecting the candidate regions for sex reversal and monosomy 9p syndrome by MLPA.
Submitted for publication.

VI. **Barbaro M**, Schoumans J, Ivarsson SA, Staaf J, Elvira Kurvinen, Borg Å, Oscarson M, Wedell A.
Genome wide screening by high-resolution array CGH for submicroscopic chromosome imbalances in patients with 46,XY gonadal dysgenesis.
Submitted for publication.

* Shared authorship

OTHER RELATED PUBLICATIONS

I. **Barbaro M**, Lajic S, Baldazzi L, Balsamo A, Pirazzoli P, Cicognani A, Wedell A, Cacciari E.
Functional Analysis of two Recurrent Amino Acid Substitutions in the *CYP21* Gene from Italian Patients with Congenital Adrenal Hyperplasia.
Journal of Clinical Endocrinology and Metabolism, 2004, 89: 2402-2407

II. Robins T, **Barbaro M**, Lajic S, Wedell A.
Not All Amino Acid Substitutions of the Common Cluster E6 Mutation in *CYP21* Cause Congenital Adrenal Hyperplasia.
Journal of Clinical Endocrinology and Metabolism, 2005, 90: 2148-2153

III. Schoumans J, Wincent J, **Barbaro M**, Djureinovic T, Maguire P, Forsberg L, Staaf J, Thuresson AC, Borg A, Nordgren A, Malm G, Anderlid BM.
Comprehensive mutational analysis of a cohort of Swedish Cornelia de Lange syndrome patients.
European Journal of Human Genetics, 2007, 15: 143-149

IV. Robins T, Bellane-Chantelot C, **Barbaro M**, Cabrol S, Wedell A, Lajic S.
Characterization of Novel Missense Mutations in *CYP21* Causing Congenital Adrenal Hyperplasia.
Journal of Molecular Medicine, 2007, 85: 243-251

V. Beleza-Meireles A, **Barbaro M**, Wedell A, Töhönen V, Nordenskjöld A.
Studies of a co-chaperone of the androgen receptor, *FKBP*52, as candidate for hypospadias.
Reproductive Biology and Endocrinology, 2007, 7: 5-8

VI. Zhang ZF, Ruivenkamp C, Staaf J, Zhu H, **Barbaro M**, Petillo D, Khoo SK, Borg Å, Fan YS, Schoumans J.
Detection of submicroscopic constitutional chromosome aberrations in clinical diagnostics; a validation of the practical performance of different array platforms.
European Journal of Human Genetics. In press.

VII. Soardi FC, **Barbaro M**, Lau IF, Lemos-Marini SHV, Baptista MTM, Guerra-Junior G, Wedell A,Lajic S, de Mello MP. Deleterious effect on the enzyme activity caused by novel *CYP21A2* missense mutations identified in Brazilian and Scandinavian patients with 21-hydroxylase deficiency.
Submitted for publication.

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LIST OF ABBREVIATIONS

17OHP	17-α-hydroxyprogesterone
ACTH	AdrenoCorticoTrophic Hormone
AIS	Androgen Insensitivity Syndrome
AMH	Anti Müllerian Hormone
AR	Androgen Receptor
ARDB	Androgen Receptor gene mutations DataBase
BAC	Bacterial Artificial Chromosome
bp	base pair
CAH	Congenital Adrenal Hyperplasia
CAIS	Complete AIS
CGH	Comparative Genome Hybridisation
CL CAH	Classic CAH
CLAH	Congenital Lipoid Adrenal Hyperplasia
CNV	Copy Number Variant/Variation
CYP11A1	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1 (cholesterol side-chain cleavage enzyme)
CYP17A1	Cytochrome P450, Family 17, Subfamily A, Polypeptide 1 (steroid 17-α-hydroxylase)
CYP21A2	Cytochrome P450, Family 21, Subfamily A, Polypeptide 2 (steroid 21-hydroxylase)
DAX1	DSS Adrenal Hypoplasia congenital critical region on X 1
DHH	Desert Hedgehog Homolog
DHT	Di-Hydro-Testosterone
DMRT	Doublesex and Mab-3 Related Transcription factor
DNA	Deoxyribonucleic acid
DSD	Disorders of Sex Development
DSS	Dosage Sensitive Sex reversal
FISH	Fluorescent *In Situ* Hybridisation
GD	Gonadal Dysgenesis
GT	Genital Tubercule
hCG	Human Chorionic Gonadotrophin
HMG	High Mobility Group
HSD17B3	17ß-hydroxysteroid dehydrogenase type III
HSD3B2	3ß-hydroxysteroid dehydrogenase type II
kb	kilo bases
LH	Luteinising Hormone
LHCGR	Luteinising Hormone/Chorionic Gonadotrophin Receptor
MAGEB	Melanoma Antigen family B
MAIS	Minimal AIS
Mb	Mega bases
MLPA	Multiplex Ligation-dependent Probe Amplification
NR	Nuclear Receptor
nt	nucleotides
OMIM	Online Mendelian Inheritance in Man
PAIS	Partial AIS

PCR	Polymerase Chain Reaction
RSPO1	R Spondin Homolog 1
RT-PCR	Reverse Transcriptase PCR
SF1	Steroidogenic Factor 1
SNP	Single Nucleotide Polymorphism
SOX9	SRY Box 9
SRD5A2	Steroid-5-α-Reductase, polypeptide 2
SRY	Sex Determining Region Y
StAR	Steroidogenic Acute Regulator
SV CAH	Simple Virilising CAH
SW CAH	Salt Wasting CAH
WD	Wolffian Ducts
WNT4	Wingless-Type MMTV Integration Site Family, Member 4
WT1	Wilms Tumor 1

"Is it a boy or a girl?"

This is the question that parents usually have when their baby is born, and that relatives and friends ask them. Sometimes it is not possible to give an answer. This is the case when the newborn presents ambiguous external genitalia and an immediate sex assignment is not possible. This is the most typical and dramatic example of a disorder of sex development (DSD). This situation represents a crisis for the new family and a very difficult clinical situation for the pediatrician. An entire team composed of pediatric endocrinologists, geneticists, surgeons, psychiatrics and psychologists need to collaborate for the best management of this baby. Other DSDs can manifest later in life, for example as a girl with primary amenorrhea, who will find out that she has a 46,XY karyotype and will never be fertile, or as a girl who at puberty suddenly starts to virilise. These are also critical situations where a DSD team needs to be consulted.

The approach to investigate and manage patients with DSD requires an understanding of normal (prenatal and postnatal) sex development, which is therefore briefly described in the following chapter.

SEX DEVELOPMENT

Sex development is a genetically and hormonally controlled process, that in humans starts immediately at fertilisation by the establishment of the chromosomal sex (XX or XY). Sex development is divided in two processes: sex determination and sex differentiation.

Sex determination refers to the formation of the gonad (testis or ovary). Sex determination depends on the sex-chromosome complement of the embryo and is established by multiple genetic and molecular events that direct the development of germ cells, their migration to the urogenital ridge, and the formation of either a testis, in the presence of the Y chromosome (46,XY), or an ovary in the absence of the Y chromosome and the presence of a second X chromosome (46,XX).

Sex differentiation refers to the formation of the genital phenotype (internal and external genitalia) and the future acquisition, at puberty, of the secondary sex characteristics and of the reproductive potential. This process depends on the sex-specific response of tissues to hormones produced by the gonads after they have differentiated in a male or female pattern.

Sex outcome at birth is then the result of a coordinated and sequential series of developmental events controlled by a network of temporally expressed genes and hormones.

GONADS

The gonads derive from the intermediate mesoderm of the embryo. They arise as paired thickenings of the coelomic epithelium at the ventromedial surface of the mesonephros on either side of the dorsal aorta [1]. Gonadal ridges are visible at around 5-6 weeks of gestation. The gonad contains somatic cells and germ cells. The germ cells originate in the wall of the yolk sac and arrive to the gonads by migration through the hindgut [2]. At this stage the gonad is bipotential, there is no difference between female and male. In fact, at this developmental stage all embryos are phenotypically similar regarding sex development (the appearance of the bipotential gonads, the presence of both Müllerian and Wolffian ducts, and the appearance of the bipotential external genitalia) regardless of the chromosome complement.

The earliest sex development step is the differentiation of the bipotential gonad into testes in case of a 46,XY chromosome complement or into ovaries in cases of a 46,XX karyotype.

Four major cell types are present in the indifferent gonads: supporting, steroidogenic, germ lineage and connective cells that will differentiate in the testes as Sertoli cells, Leydig cells, spermatogonia and peritubular myoid cells, respectively [3]. The Sertoli cell is the first cell type that differentiates, triggered by expression of the testis determining factor SRY in the pre-Sertoli cells at 42 days post conception. Sertoli cells produce the Anti Müllerian hormone (AMH, also known as Müllerian inhibiting substance, MIS) and have the function of nurturing the germ cells; the Leydig cells are steroidogenic cells that produce testosterone. Both hormones are therefore needed for normal male foetal development to proceed.

Ovarian differentiation occurs one week later than testis differentiation. Four types of cells are also present in the ovaries: granulosa/follicular cells, steroid-producing cells (theca cells produce androgens, that are converted to oestrogens in the granulosa cells, that also produce progesterone), oocytes and stromal cells. In contrast to the testes, the ovaries are not thought to produce significant amounts of steroids prior to puberty.

INTERNAL DUCTS

The Müllerian ducts are the primordia of female internal genitalia (fallopian tubes, uterus and the upper part of the vagina) whereas the Wolffian ducts give rise to male internal genitalia (the epididymis, vas deferens and seminal vesicles). Both Müllerian and Wolffian ducts develop in all foetuses, regardless of genetic or gonadal sex, during early gestation. In males, the Müllerian ducts regress in response to AMH produced by the Sertoli cells, and the Wolffian ducts differentiate in response to production of testosterone by the Leydig cells between weeks 9 and 13 of gestation [4]. In contrast, the Müllerian ducts develop in females in the absence of AMH, and the Wolffian ducts fail to develop in the absence of testosterone.

EXTERNAL GENITALIA

Like the bipotential gonads, the external genitalia are initially identical in all foetuses, regardless of the genetic or gonadal sex. Initially they consist of the genital tubercule (GT), the urogenital folds and the genital swellings. These undifferentiated structures can develop along either a male or female line. If dihydrotestosterone (DHT) is produced in sufficient amounts from gestational weeks 7–8 until birth, and if the foetus can respond normally to androgens, the bipotential genitalia will develop in a male-typical manner: the GT develops into a penis, the urethral folds fuse creating a tubular penile urethra with the opening located at the tip of the penis, and the genital swellings fuse to form a scrotum.

In the absence of androgenic effects, the GT forms a clitoris, the urethral folds develop into the labia minora, the urethral opening is located in the perineum, and the genital swellings form the labia majora. Finally, in the absence of AMH production, a normal vagina is formed [5]. Although the detailed molecular mechanisms behind the formation of external genitalia are not well characterised it is clear that epithelial-mesenchymal interactions are fundamental for a coordinated and proper differentiation of the external genitalia [6].

GENDER

The final component of a person's sex is their behavioural sex, or gender. Gender is a broad term that encompasses how a person views oneself as a man or woman (gender identity), how that person is viewed by other members of society as masculine or feminine (gender role), and their erotic behaviour.

DISORDERS OF SEX DEVELOPMENT

CLASSIFICATION

In 2006 a new nomenclature system for what previously was called 'intersex' disorders was proposed by a consensus statement on management of intersex disorders organised by the Lawson Wilkins Pediatric Endocrine Society (LWPES) and the European Society for Pediatric Endocrinology (ESPE), involving 50 international experts in the field [7, 8]. The term **disorders of sex development** was proposed, defined as *congenital conditions in which development of chromosomal, gonadal, or anatomical sex it atypical*. Together with this general term a new nomenclature/terminology to classify different forms of DSD was proposed (Table 1). In this thesis the new terminology is used whenever possible.

This thesis focuses on patients who at diagnosis presented with unambiguously female external genitalia and a 46,XY karyotype, therefore belonging to the groups of 46,XY DSD with gonadal, androgen synthesis or androgen action defects. XY females with gonadal dysgenesis can be differentiated from XY females with an androgen synthesis or action defect by the presence of a uterus and Fallopian tubes. Completely dysgenetic gonads do not produce AMH, allowing the development of the Müllerian structures. If the gonads are properly formed but there is a testosterone biosynthetic defect, the absence of testosterone leads to female external genital development, WD will not differentiate, however AMH is produced leading to Müllerian structure regression. Other investigations are subsequently required to differentiate between different forms of androgen synthesis defects or action.

Furthermore mutations in the *CYP21A2* gene, causing 21-hydroxylase deficiency which is the most common form 46,XX DSD and the most common defect in a newborn with ambiguous external genitalia, were investigated.

The following paragraphs will therefore in particular focus on the genes important to consider for the evaluation of these types of DSDs.

GENETICS

Tables 2A-2E summarise the genes or the chromosomal regions that have been implicated in DSD, divided according to the DSD classification. Sex development requires the interaction of several factors together with the production of hormones and their consequent signalling action. It is therefore quite obvious that several genes are involved in DSD. Because the formation and differentiation of the gonad is genetically determined, transcription factors and signalling molecules play a very important role. In fact several genes mutated in 46,XY gonadal DSD encode transcription factors. In contrast genes that cause post gonadal 46,XY or 46,XX DSD usually encode enzymes or factors necessary for sex steroid biosynthesis or action.

Table 1. Classification of DSD.

Sex Chromosome DSD	46,XY DSD	46,XX DSD
A. 45,X (Turner syndrome and variants)	**A. Disorders of gonadal (testicular) development** ▪ complete or partial gonadal dysgenesis (SRY, SOX9, SF1,WT1, DAX1 dupl, WNT4 dupl) ▪ gonadal/testis regression ▪ ovotesticular DSD	**A. Disorders of gonadal (ovarian) development** ▪ gonadal dysgenesis ▪ testicular DSD (SRY+, SOX9 dupl, *RSPO1,*) ▪ ovotesticular DSD
B. 47,XXY (Klinefelter syndrome and variants)	**B. Disorders of androgen synthesis and action**	**B. Androgen excess**
	1. Disorders in androgen synthesis ▪ Leydig cell hypoplasia, aplasia (LHCGR defects) ▪ Congenital Lipoid Adreanal Hyperplasia (*STAR*) ▪ Cholesterol side-chain cleavage deficiency (*CYP11A1*) ▪ 17α-hydroxylase/17,20-lyase deficiency (*CYP17A1)* ▪ 3ß-hydroxysteroid dehydrogenase 2 (*HSD3B2)* ▪ 17ß-hydroxysteroid dehydrogenase deficiency (*HSD17B3*) ▪ 5α-reductase 2 deficiency (*SRD5A2)* ▪ P450 oxidoreductase deficiency (*POR)* ▪ Smith-Lemli-Opitz syndrome (*DHCR7*)	**1. Foetal** ▪ 21-hydroxylase deficiency (*CYP21A2)* ▪ 3ß-hydroxysteroid dehydrogenase 2 (*HSD3B2)* ▪ 11ß-hydroxylase deficiency (*CYP11B1)* ▪ P450 oxidoreductase deficiency (*POR)*
	2. Disorders of androgen action ▪ Androgen insensitivity syndrome (AR receptor mutation) ▪ Drugs and environmental modulators	**2. Foetoplacental** ▪ Aromatase deficiency *(CYP19)* ▪ Oxidoreductase deficienecy (*POR)*
		3. Maternal ▪ Maternal virilising tumours (e.g. luteomas) ▪ Androgenic drugs
C. 45,X0/46,XY (Mixed gonadal dysgenesis, ovotesticular DSD)	**C. Other** ▪ Persistent Müllerian duct syndrome (*AMH* and *AMHR*) ▪ Vanishing testis syndrome ▪ Congenital hypogonadotrophic hypogonadism (*DAX1)* ▪ Cryptorchidism (*INSL3, GREAT)* ▪ Isolated hypospadias (*CXorf6)* ▪ Syndromic associations of male genital development (e.g. cloacal anomalies, Robinow, Aarskog, hand-foot-genital, popliteal pterygium)	**C. Other** ▪ Müllerian agenesis / hypoplasia (e.g. MURCS) (*WNT4*) ▪ Vaginal atresis (eg KcKusick-Kaufman) ▪ Uterine abnormalities (e.g. MODY5) ▪ Labial adhesions ▪ Syndromic associations (e.g. cloacal anomalies)
D.46,XX/46,XY (chimeric, ovotesticular DSD)		

Table 2A. Genes known to involved in 46,XY gonadal DSD.

Gene	Cytogenetic band	Protein type	Inheritance	Gonad	Müllerian structures	External genitalia	Other associated features	OMIM no.
SRY	Yp11.3	TF	Y	Dysgenetic testis or ovotestis	+/-	Female or ambiguous		480000
SOX9	17q24-25	TF	AD	Dysgenetic testis or ovotestis	+/-	Female or ambiguous	+ Camptomelic dysplasia	608160
WT1	11p13	TF	AD	Dysgenetic testis	+/-	Female or ambiguous	Wilms' tumor, nephropaties, gonadoblastoma (WAGR, Denys-Drash and Frasier syndromes)	607102
SF1 (NR5A1)	9q33	TF (NR)	AD/AR	Dysgenetic testis	+/-	Female or ambiguous	+/- primary adrenal failure	184757
DHH	12q13.1	Signalling molecule	AD/AR	Dysgenetic testis	+	Female	+/- minifascicular neuropathy (1 case)	605423
ATRX	Xq13.3	Helicase	X	Dysgenetic testis	-	Female, ambiguous, or male	α-Thalassemia, mental retardation	300032
ARX	Xp22.13	TF	X	Dysgenetic testis	-	Ambiguous	+ lissencephaly, epilepsy, temperature instability	300382

TF, transcription factor; NR, nuclear receptor; inheritance: AD, autosomal dominant (or de novo mutation); AR autosomal recessive; Y, Y-linked; X, X-linked. +/- present or absent.

Table 2B. Chromosomal changes known to be involved in 46,XY gonadal DSD.

Candidate gene	Cytogenetic band	Protein	Inheritance	Gonad	Müllerian structures	External genitalia	Other associated features	OMIM no.
DAX1 (NR0B1)	Xp21	TF (NR)	Dupl Xp21	Dysgenetic testis or ovary	+/-	Female or ambiguous		300018
DMRTs	9p24.3	TF	Del 9p24.3 Reduced penetrance	Dysgenetic testis Normal testis	+/-	Female or ambiguous	Mental retardation	602424
WNT4	1p35	Signalling molecule	Dupl 1p35	Dysgenetic testis	+	Ambiguous	Mental retardation	603490

TF, transcription factor; NR, nuclear receptor; inheritance: AD, autosomal dominant (or de novo mutation); AR autosomal recessive; Y, Y-linked; X, X-linked. +/- present or absent.

Table 2C. Genes known to be involved in 46,XY DSD, due to defects in hormones synthesis or action.

Gene	Cytogenetic band	Protein type	Inheritance	Gonad	Müllerian Structures	External genitalia	Other associated features	OMIM no.
LHCGR	2p21	G-protein receptor	AR	Testis, Leydig cell hypoplasia	-	Female, ambiguous, or micropenis		152790
STAR	8p11.2	Shuttle protein in the mitochondrial membrane	AR	Testis	-	Female	Congenital lipoid adrenal hyperplasia, primary adrenal failure	600617
CYP11A1	15q23-24	Enzyme	AR	Testis	-	Female or ambiguous	adrenal failure, pubertal failure	118485
CYP17	10q24.3	Enzyme	AR	Testis	-	Female, ambiguous, or micropenis	CAH, +/- hypertension Hypocortisolism, pubertal failure	202110
HSD3B2	1p13.1	Enzyme	AR	Testis	-	Female or ambiguous	CAH, primary adrenal failure	201810
HSD17B3	9q22	Enzyme	AR	Testis	-	Female or ambiguous	Partial androgenisation at puberty	605573
SRD5A2	2p23	Enzyme	AR	Testis	-	Female, ambiguous or micropenis	Partial androgenisation at puberty	607306
POR	7q11.2	CYP electron donor	AR	Testis	-	Male or ambiguous	Mixed features of 21-hydroxylase deficiency, 17-hydroxylase/17,20-lyase deficiency and aromatase deficiency; +/- Antley Bixler skeletal manifestations	124015
DHCR7	11q12-13	Enzyme	AR	Testis	-	Variable	Smith-Lemli-Opitz syndrome: coarse facies, second-third toe syndactyly, failure to thrive, developmental delay, cardiac and visceral abnormalities	602858
AR	Xq11-12	TF (NR)	X	Testis	-	Female, ambiguous, micropenis or normal male	CAIS, PAIS, MAIS (infertility)	313700

TF, transcription factor; NR, nuclear receptor; inheritance: AD, autosomal dominant (or *de novo* mutation); AR autosomal recessive; Y, Y-linked; X, X-linked. +/- present or absent.

Table 2D. Genes known to involved in 46,XX gonadal DSD with testicular development.

Gene	Cytogenetic band	Protein type	Inheritance	Gonad	Müllerian structures	External genitalia	Other associated features	OMIM no.
SRY	Yp11.3	TF	Translocation	Testis or ovotestis	-	Male or ambiguous	Infertility	480000
SOX9	17q24	TF	Dup17q24	Not investigated	-	Male or ambiguous		608160
RSPO1	1p34.3	Signalling molecules	AR	Testis or ovotestis	-	Male	+ palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin. +/- congenital bilateral corneal opacities, onychodystrophy, and hearing impairment	609595

TF, transcription factor; NR, nuclear receptor; inheritance: AD, autosomal dominant (or *de novo* mutation); AR autosomal recessive; Y, Y-linked; X, X-linked. +/- present or absent.

Table 2E: Genes known to be involved in 46,XX DSD due to androgen excess.

Gene	Cytogenetic band	Protein type	Inheritance	Gonad	Müllerian structures	External genitalia	Other associated features	OMIM no.
HSD3B2	1p13	Enzyme	AR	Ovary	+	Female or ambiguous	CAH, primary adrenal failure, partial androgenisation	201810
CYP21A2	6p21-23	Enzyme	AR	Ovary	+	Female or ambiguous	CAH, +/- adrenal failure	201910
CYP11B1	8q21-22	Enzyme	AR	Ovary	+	Female or ambiguous	CAH, hypertension	202010
POR	7q11.2	CYP electron donor	AR	Ovary	+	Female or ambiguous	Mixed features of 21-hydroxylase deficiency, 17-hydroxylase/17,20-lyase deficiency and aromatase deficiency; Antley Bixler skeletal manifestations	124015
CYP19	15q21	Enzyme	AR	Ovary	+	Ambiguous	Maternal androgenisation during pregnancy, absent breast development at puberty, except in partial cases	107910

TF, transcription factor; NR, nuclear receptor; inheritance: AD, autosomal dominant (or *de novo* mutation); AR autosomal recessive; Y, Y-linked; X, X-linked. +/- present or absent.

GENETICS OF XY GONADAL DEFECTS

The gonads have the very special characteristic to develop as bipotential, with the capacity to differentiate into either an ovary or a testis. Therefore genes that are important for both gonadal ridge formation and gonad determination are candidate genes for XY gonadal DSD. As for other genes that are involved in embryonic development, many genes that control gonad development act in a dosage sensitive manner.

Genes important for human gonadal development have initially been identified studying chromosomal defects in patients with DSD, and studying the process of sex determination in animal models, especially mice. Parallel research has been carried out with candidate genes identified in human patients and then studied in mice models or vice versa. The mice models facilitate studies of embryonic development, identifying individual candidate genes and their spatial and temporal expression, leading to evaluation of functional consequence of excess or absence of a factor. However some differences between mice and humans are also reported, especially regarding sensitivity to gene dosage alterations.

SRY
Sex Determining Region Y

SRY was the first gene identified to be involved in gonadal DSD. It was discovered by analysing Y chromosome translocations in XX males [9]. Subsequently mutations in *SRY* have been shown in patients with XY gonadal dysgenesis (GD) [10]. XX mice transgenic for *Sry* have been shown to develop as males [11], thus confirming *Sry* as the testis determining gene on the Y-chromosome. Both in humans and in mice, the onset of *SRY* expression occurs just before differentiation of the bipotential gonad into a testis, thus defining testis determination [12, 13].

SRY is a single exon gene that encodes a protein with a highly conserved HMG (High Mobility Group) domain that has DNA-binding and DNA-bending functions. SRY is believed to act as a transcription factor. Missense and nonsense mutations in the *SRY* gene are identified along the entire gene, although with a higher frequency in the HMG box [14]. Most mutations are *de novo*, although some familiar cases with apparent normal male carriers have been described [15, 16].

Patients present complete or, more rarely, partial XY GD. Mutations have also been described in some cases with 46,XY ovotesticular DSD [17-19]. Mutations in the *SRY* gene are identified in approximately 10-15% of all cases with 46,XY gonadal DSD.

Although SRY was identified more than 15 years ago, very little is known about its molecular function, and its *in vivo* binding targets have not yet been identified. However, *SOX9* upregulation has been indicated as its ultimate function.

SOX9
Sex-Determining Region Y, Box 9

SOX9 was identified by breakpoint characterisation in patients with chromosome 17 rearrangements presenting campomelic dysplasia (CD) and XY sex reversal due to GD, which is present in 75 % of the XY subjects [20-22]. Several patients carry point mutations in single *SOX9* alleles; these mutations are identified along the entire gene, without correlations between mutations and the severity of the skeletal phenotype or the GD [23]. While patients with CD without XY GD have been described, no patients with GD without CD are reported [24]. Several patients with translocations or deletions that do not disrupt the *SOX9* open reading frame have been described, the phenotype is probably due to disruption of regulatory regions [25-27].

SOX9 also contains a HMG domain that is 70% identical to the SRY-HMG box. This gene is dimorphically expressed in the gonads, where its expression in the testis increases immediately after *SRY* expression [28]. Transgenic expression of *Sox9* in XX mice has been shown to lead to male development indicating that SOX9 plays an important role in testis determination [29]. Interestingly an XX, SRY negative, male individual carrying a mosaic duplication of 17q23-24, including *SOX9* has been described [30].

WT1
Wilms Tumor 1

The *WT1* gene was identified by positional cloning while identifying the gene responsible for Wilms tumour in patients with 11p deletions and the contiguous gene deletion syndrome WAGR (Wilms tumour-Aniridia-Genitourinary anomalies-mental Retardation) [31]. The gene encodes a protein with four zinc fingers and is expressed as four major isoforms, derived from alternative splicing of exon 5 and from the insertion of three amino acids (KTS) encoded by the 3' end of exon 9 [32]. Several other isoforms, using also a non–AUG translation initiation site, have been described [32, 33].

Point mutations have been identified in patients with Denys-Drash syndrome characterised by Wilms tumor, diffuse mesangial nephropathy and different degrees of genital and gonadal development, with hot spots in exon 8-9 that encode the second and third zinc finger [34]. Frasier syndrome is characterised by late onset nephropathy caused by focal segmental glomerular sclerosis, XY GD, gonadoblastoma but no Wilms tumor; in these cases *WT1* mutations affect the splice donor site of exon 9 resulting in imbalance of the +/- KTS isoforms [35-37]. A patient with only genital developmental anomalies without renal defects has also been described [38].

In mice, *Wt1* is expressed in tissues that will develop into kidneys and gonads [39], null mice for *Wt1* show agenesis of the gonads and kidneys. Mice lacking only the –KTS isoform have streak gonads in both sexes indicating that this isoform is necessary for survival of the bipotential gonads. Mice lacking only the +KTS isoform

show complete XY sex reversal due to a dramatic reduction of *Sry* expression, while ovarian development is not affected [40].

SF1 (NR5A1)
Steroidogenic Factor 1

SF1 is a nuclear receptor (NR) that was initially identified as a regulator of steroidogenesis [41, 42]. In mice and humans it is expressed initially in the urogenital ridge and continues in the adrenal, gonads, hypothalamus and pituitary [43] [44]. *Sf1* null mice lack adrenal glands and gonads [45].

Mutations in the human *SF1* have therefore initially been considered in patients presenting with XY GD and adrenal insufficiency. Initially two patients with this phenotype were reported, one with a *de novo* inactivating heterozygous mutation [46] and one homozygous for an inherited partially inactivating mutation [47]. A *SF1* heterozygous mutation has also been reported in a prepubertal XX girl with adrenocortical insufficiency and apparent normal ovarian development [48]. However after the description of a heterozygous mutation in a patient with XY GD but without adrenal failure, several mutations have been identified in patients with varying degrees of GD and normal adrenal function with a frequency apparently similar to *SRY* mutations [49-51].

In this case the mouse phenotype has been important to understand *SF1* function in gonadal development, although it presents some differences from humans. In fact *SF1* haploinsufficiency in humans affects gonadal development while mice heterozygous (+/-) for an *Sf1* deletion present adrenal defects [52].

DHH
Desert Hedgehog Homolog

DHH belongs to the family of hedgehog genes, which produce signalling molecules important in early development. *Dhh* shows specific expression in Schwann and Sertoli cell precursors [53]. Considering the phenotype described for *Dhh* -/- mice by Bitgood *et al.* [54], Umehara *et al.* sequenced the *DHH* gene in a patient with partial XY GD and polyneuropathy and identified a homozygous missense mutation [55]. Homozygous mutations have been reported in three additional patients with isolated 46,XY GD [56]. DHH is the first signalling molecule reported to be mutated in patients with 46,XY gonadal DSD. Feminised XY *Dhh -/-* mice described by Clark *et al.* lacked adult type Leydig cells and displayed numerous undifferentiated fibroblastic cells in the interstitium of the gonads, thus suggesting that DHH triggers Leydig cell differentiation [57, 58].

DAX1 (NR0B1) and Xp21 duplications

Duplications of chromosomal regions containing Xp21 are known to be associated with XY sex reversal; 17 of the 18 patients reported so far [59, 60] carried duplications or translocations that were detectable by conventional karyotyping, and

all patients presented sex reversal as part of a more complex phenotype that included dysmorphic features and/or mental retardation. By comparing patients with different chromosomal rearrangements on Xp, Bardoni *et al.* [61] identified a 160-kb minimal common region denoted dosage sensitive sex reversal that, if duplicated, causes sex reversal. This region contains the *MAGEB* genes and the *DAX1* gene [62], officially named *NR0B1* as it encodes an orphan NR.

Deletions or mutations in *DAX1* in XY subjects cause adrenal hypoplasia congenita [63] and hypogonadotrophic hypogonadism [64]. While many patients with mutations in the *DAX1* gene have been reported, no patient with an isolated *DAX1* duplication has been described previously. *DAX1* is the candidate for sex reversal for many reasons. Its expression during embryonic development in the mouse is compatible with a role both in sex determination and in adrenal and hypothalamic function [65]. XY mice transgenic for *Dax1* show delayed testis development and sex reversal if the transgene is tested against weak alleles of *Sry* [66]. The role of *DAX1* overexpression for sex reversal is also supported by the fact that XY patients with 1p duplications, including *WNT4*, show an abnormal gonadal phenotype (see next paragraph). Furthermore, several functional properties of DAX1 are consistent with its ability to inhibit gonadal development if present at a higher dose. DAX1 can repress transcription by binding to DNA hairpin structures (*e.g.* in the promoter of the *STAR* gene [67]) . Notably, DAX1 has also been reported to inhibit SF1, causing reduction of steroidogenic enzymes and AMH expression. As *SF1* haploinsufficiency causes gonadal deficiency, it has been suggested that DAX1 overexpression might cause GD through inhibition of SF1-mediated transcription. Interestingly, DAX1 has also been reported to interact with and inhibit the transcriptional activity of several other NRs such as the androgen receptor (AR), progesterone receptor (PR), oestrogen receptors (ERα and ERβ), liver receptor homologue-1 (LRH-1) and NUR77 by distinct mechanisms [68, 69]. Furthermore, DAX1 has been shown to bind to RNA in polyribosomes or polyadenylated RNA, and it has been suggested that DAX1 is a shuttling RNA binding protein with a posttranscriptional regulatory role [70]. Thus, DAX1 seems not only to act as a transcriptional repressor of steroidogenesis but also to have a broader functional role during embryonic development and an adult function in the hypothalamic-pituitary-adrenal-gonadal axis [71]. Although the aforementioned data support *DAX1* as the gene responsible for sex reversal, a direct proof in patients has been missing because there have been no reports of single *DAX1* duplications in 46,XY patients with isolated GD.

The *MAGEB* genes belong to the MAGE superfamily that directs the expression of tumour antigens recognised in a human melanoma [72]. While the expression of many *MAGE* genes has been shown in tumours of different origin, the *MAGEB* gene expression is restricted to placenta and testis during normal development. Their functions are however not yet known.

Duplication of 1p35 and *WNT4*

WNT molecules are growth factors responsible for developmental processes. WNT4 was the first identified signalling molecule involved in sex development. XX *Wnt4*

deficient mice lack Müllerian ducts and show masculinisation as Wolffian ducts are present, however no complete female to male sex reversal is shown [73].

Human *WNT4* was cloned in 2001 by Jordan *et al*. [74], while they characterised 1p35 duplications in XY patients with a wide range of DSDs. High overexpression of *WNT4* was demonstrated in gonadal fibroblast of an XY patient presenting XY GD, remnants of both Müllerian and Wolffian structures and ambiguous external genitalia. In cell studies WNT4 was shown to regulate DAX1 expression. In transgenic mice, *Wnt4* interferes with male development by inhibiting steroidogenesis and disrupting testis vasculature, but no male to female sex reversal occurs [75]. The phenotype of humans and mice overexpressing WNT4 is therefore only partially similar. No additional *WNT4* duplications have been reported so far.

Three XX patients have instead been described to be heterozygous for *WNT4* mutations [76-78]. Their common features are primary amenorrhea, high testosterone levels, absent uterus and normal sized ovaries. These results are in concordance with the function of WNT4 as an inhibitor of testosterone synthesis in female gonads.

9p24.3 deletions and *DMRT* genes

The 9p distal region has been extensively investigated to identify genes involved in sex development since the observation of patients with the 9p monosomy syndrome including abnormal sex development. Patients with the 9p monosomy syndrome present with mental retardation, craniofacial dysmorphic features (e.g. trigonocephaly, long philtrum), and delayed motor development [79]; in patients with XY chromosomes genital and/or gonadal disorders are quite frequent. In these patients the external genital phenotype ranges from completely female to male with hypospadias; and the gonadal phenotype ranges from complete GD to ovotestes and to cryptorchid and/or hypoplastic testes. The identification of 9p24 deletions in patients with XY GD but without typical 9p monosomy syndrome features, and the detection of patients with interstitial 9p deletions, has allowed the identification of two distinct regions on 9p, one for sex reversal and one for the 9p monosomy syndrome. The latter has been localised at 9p22.3-p23 [80-82], while the sex reversal region has been progressively narrowed down to the region 9p24.3, extending from the *DMRT* genes to the telomere [83-86]. Within this region the strongest candidate genes for the GD phenotype are the *DMRT* genes that encode proteins with a DM domain. This is a zinc finger-like DNA binding motif that derives its name from the Drosophila *doublesex* (*dsx*) and the Caenorhabditis elegans *mab-3* genes where it was initially identified [87]. Both these genes are involved in downstream pathways of sex determination. There are three *DMRT* genes on 9p24, *DMRT1*, *DMRT3* and *DMRT2* [88, 89]. All deletions reported so far in patients with XY GD include all three genes, except for one case where the deletion is telomeric of the *DMRT* genes in a potential regulatory region [86]. The molecular mechanism that leads to GD is not clear and could either be caused by haploinsufficiency of one or more genes in the deleted region or by unmasking of a recessive mutation on the other chromosome. The haploinsufficiency mechanism is the most likely because, despite several attempts, no mutations in *DMRT1* or *DMRT2* have been identified

[86, 88, 89]. The *DMRT3* gene was identified more recently and no mutation screening has been reported so far. However, it is not clear if haploinsufficiency of one or a combination of the *DMRT* genes is responsible for the phenotype. *Dmrt1-/-* mice present only hypoplastic testis but not sex reversal [90]. These results confirm that the *DMRT1* gene is involved in sex development in mammals and differences in the severity of the phenotype between murine and human abnormal sex development are not surprising. *DMRT2* haploinsufficiency has recently been excluded as the cause of GD because *Dmrt2* knock out mice show embryonic somite patterning defects and no sex development impairments [91]. However, it is still not clear if single *DMRT1* haploinsufficiency leads to GD or if other factors can modulate the phenotype, as 9p24.3 deletions have shown incomplete penetrance in patients.

RSPO1
R-spondin Homolog

RSPO1 is not a candidate gene for 46,XY GD but its identification represents such an important finding that it deserves to be mentioned. *RSPO1* is the first gene that mutated leads to male development in XX subjects in the absence of the *SRY* gene. R-Spondin proteins constitute a family of secreted ligands that, similarly to Wnt proteins, activate ß-catenin/TCF mediated target gene transcription [92].

The gene was identified by linkage analysis in a large family with a syndrome characterised by XX testicular DSD, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin. It was the 90th gene sequenced in a candidate region of 15 Mb [93]. Recently a homozygous mutation has been described in a case with 46,XX ovotesticular DSD, presenting also with palmoplantar keratoderma, congenital bilateral corneal opacities, onychodystrophy, and hearing impairment [94].

Other candidate regions for 46,XY DSD

The identification of a genetic defect in only 20% of 46,XY gonadal DSD patients indicates that more genes are involved in these disorders. Identification of chromosomal rearrangements in XY patients with syndromic features including genital ambiguity or female external genitalia and the description of a large pedigree with several cases of 46,XY or XX DSD patients with no mutations in the already known candidate genes [95], are further indications. Two candidate regions for XY gonadal dysgenesis have been indicated by linkage analysis, one at 5q11.2 [96] and one at Xp11.21-11.23 [97]. Regions suggested by chromosomal rearrangements are at 1p [98], 2q [99] and others. However, when a syndromic XY patient with female or ambiguous genitalia due to a chromosomal defect is described, there is not always an investigation of the gonadal phenotype. Thus it is impossible to distinguish between a developmental defect of the gonad or only of the external genitalia.

Other candidate genes come from mice studies (Table 3), however it is not always possible to translate information from mice to humans. For example *Gata4*$^{ki/ki}$ mice show gonadogenesis defects [100], while heterozygous mutations in human *GATA4*

have been identified in patients with heart defect without gonadal defects. A promising candidate for isolated GD was *LHX9*, as *Lhx9* knock out mice fail to develop gonads and do not show other defects. Nevertheless, no *LHX9* mutations were identified in a large cohort of patients with 46,XY gonadal DSD [101]. Other genes have been shown to cause abnormal gonadal development in mice, these genes are potential candidates for 46,XY DSD, however the mice phenotype is often associated with other defects indicating that these genes should be considered in syndromic cases of DSD.

Table 3. Putative candidate genes for XY GD, based on findings in mouse models.

Gene	Knockout phenotype	Mutant human phenotype (OMIM no.)	Chromosomal location in human
M33 (CBX2)	Impaired development of XY and XX gonads. Male to female sex reversal Gonads present at birth [102]	Not described	17q25.2
EMX2	Regression of XY and XX gonad. Male to female sex reversal [103]	Heterozygous mutations in schizencephaly. No gonadal defects (600035)	10q26.11
LHX9	Gonadal agenesis. Male to female sex reversal [104]	No mutations idenfied in 58 patients with GD [101]	1q31.3
GATA4	Male to female sex reversal [100]	Atrial septal defect. No abnormal gonadal phenotype described (600576)	8p23.1
FOG2	Male to female sex reversal [100]	Heterozygous mutations in some cases with tetralogy of Fallot or with diaphragmatic hernia (603693) Heart defect and GD in a patient with a chromosome translocation disrupting FOG2 [105]	8q23.1
FGF9	Male to female sex reversal [106]	Not described	13q12.11
FGFR2 (receptor of FGF9)	Partial XY sex reversal [107]	Crouzon, Pfeiffer Apert, Jackson-Weiss syndrome (176943)	10q26
IR/IGF1R/IRR	Male to female sex reversal [108]	Not relevant	-
PDGFRα	Defective Leydig cell differentiation [109]	Somatic mutations associated with gastrointestinal tumours (173490)	4q12
POD1 (*TCF21*)	Gonads have expanded steroidogenic cell population [110]	Not described	6q23.2

In conclusion, many different genes have been identified that need to be considered when investigating patients with 46,XY gonadal DSD. Still, a genetic diagnosis is reached in only ≈20 % of the cases, indicating that other genes or factors need to be discovered.

GENETICS OF DISORDERS OF SEX DIFFERENTIATION

Internal and external genital development is regulated by hormone production and their consequent effects. Therefore it is not surprising that defects of genital differentiation can be caused by defects in genes important for hormone biosynthesis and metabolism and their receptors.

Figure 1 shows a schematic representation of adrenal and gonadal steroid biosynthesis and metabolism which is important to know when evaluating DSD of sex differentiation, both in XX and XY subjects. The early steps in the pathways to synthesise sex steroids, mineralocorticoids and glucocorticoids are shared/interconnected. A defect at any step will lead to the decreased or absent production of final products together with the accumulation of intermediate products that will be shunted to the still active pathways. Furthermore, compensatory mechanisms that try to restore mineralocorticoid or cortisol synthesis will lead to excessive production of precursors and biproducts that have undesirable hormonal effects. Depending on which enzyme is defective, androgen biosynthesis can be impaired leading to 46,XY DSD or in excess leading to 46,XX DSD. In absence of negative feedback regulation by cortisol, the pituitary gland continues to stimulate adrenal steroid synthesis by secreting adrenocorticotrophic hormone (ACTH) leading to congenital adrenal hyperplasia (CAH).

In the following paragraphs genetic defects responsible for 46,XY DSD due to androgen biosynthesis defects, 46,XY DSD due to androgen receptor defects and 46,XX DSD due to 21-hydroxylase deficiency will be described.

GENETICS OF 46,XY DSD DUE TO IMPAIRED ANDROGEN SYNTHESIS OR METABOLISM

LHCGR
Luteinising Hormone/Chorionic Gonadotrophin Receptor

LHCGR is a seven transmembrane G-protein coupled receptor, expressed in Leydig cells, that is required for stimulation of testosterone production. The receptor is activated by the binding of the placental chorionic gonadotrophin (hCG) or, in adult life, the pituitary luteinising hormone (LH). XY subjects with inactivating mutations in both alleles present with female external genitalia and absent puberty (sexual infantilism), due to absent testosterone production [111]. Testis biopsies show Leydig cell hypoplasia, due to a Leydig cell maturation defect. Sertoli cells are instead structurally normal and produce AMH, therefore Müllerian structures are absent in these patients. Depending on the degree of inactivation the external genital phenotype can however range from female or ambiguous external genitalia, to hypospadias or cryptorchidism [112].

In XX subjects the phenotype manifests as hypergonadotrophic hypogonadism and primary amenorrhea. Constitutively active mutations instead cause male precocious puberty inherited in an autosomal dominant male-limited pattern [113]. Interestingly *Lhr* -/- mice do not show a defect in prenatal sex development [114, 115]. Mutations in this gene are quite rare.

StAR
Steroidogenic acute regulatory protein

StAR is a shuttle protein that actively transports cholesterol from the outer to the inner side of the mithocondrial membrane where CYP11A1 is located. StAR induces a rapid synthesis of new steroids in steroidogenic cells [116-118].

Mutations in this gene cause congenital lipoid adrenal hyperplasia (CLAH)[119, 120], characterised by greatly diminished or absent synthesis of all adrenal and gonadal steroids. A minimal steroidogenic activity is however still present in absence of StAR activity. The CLAH phenotype is described by a two hit model, an initial step with symptoms due to impaired steroid synthesis (first hit) and a second step with loss of steroidogenic cells due to damage caused by accumulation of cholesterol esters (second hit) [120]. Affected patients present salt wasting as a consequence of impaired synthesis of mineralocorticoids and cortisol, and XY subjects develop female external genitalia because the gonads cannot produce androgens. The two hit model has been confirmed not only by mouse knock out models [121, 122], but also by affected XX females that presented spontaneous puberty [123]. Most patients

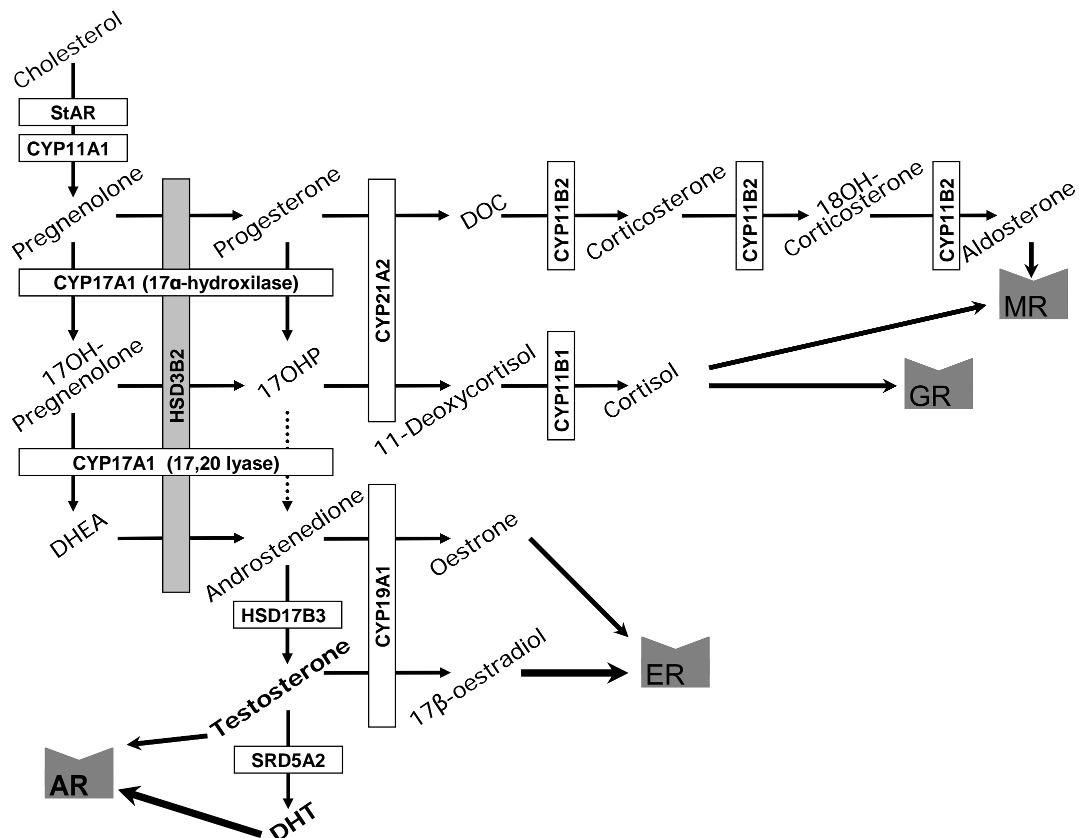

Figure 1. Adrenal and gonadal steroid biosynthesis and target receptors.
StAR, steroidogenic acute regulatory protein; CYP11A1, 20,22 desmolase (cholesterol side-chain cleavage enzyme); CYP17A1, 17α-hydroxylase/17,20-lyase; HSDB3, 3ß-hydroxysteroid dehydrogenase type 2; CYP21A2, 21-hydroxylase; CYP11B1, 11ß-hydroxylase; CYP11B2, aldosterone synthase; HSD17B3, 17ß- hydroxysteroid dehydrogenase type 3; CYP19A1, aromatase; SRD5A2, 5α-reductase type 2; MR, mineralocorticoid receptor, GR, glucocorticoid receptor; ER, oestrogen receptor; AR, androgen receptor; DOC, 11-deoxycorticosterone; 17OHP, 17 hydroxyprogesterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone.

present adrenal hyperplasia, however at least one patient has been described with small adrenals [124]. Adrenal insufficiency usually manifests in the neonatal period or within the first year [125], however a mild form of CLAH has been recently described with an onset at 2-4 years and normal male external genitalia in two XY brothers [126]. It is important to know that StAR does not regulate steroidogenesis in the placenta, therefore mutations in this gene do not affect progesterone synthesis, which is required in pregnancy.

CYP11A1
Cholesterol side-chain cleavage enzyme

CYP11A1 (cholesterol side chain cleavage enzyme, P450scc or cholesterol 20,22 desmolase) is the enzyme responsible for the initial and rate-limiting step in the synthetic pathway of all steroid hormones in steroidogenic tissues (adrenal cortex, gonads, and placenta). It converts cholesterol to pregnenolone in the mitochondria and CYP11A1 is therefore necessary for the production of glucocorticoids, mineralocorticoids and sex steroids in prenatal and postnatal life [127].

Initially this was the candidate gene for CLAH, but no mutations could be identified in *CYP11A1* [128], and instead *STAR* mutations were identified. Because of the absence of *CYP11A1* mutations and because CYP11A1 is required for placental progesterone synthesis, deficiency of CYP11A1 was earlier considered to be incompatible with life [129]. During normal human pregnancy, progesterone is initially produced by the maternal corpus luteum, but after 6-8 weeks the placental trophoblasts which are of foetal origin take over. Thus CYP11A1 deficiency would compromise gestation.

To date, six patients with *CYP11A1* mutations have been identified worldwide [130-134]. They present with adrenal insufficiency neonatally or later in childhood and XY subjects present female external genitalia or clitoromegaly. In contrast to CLAH, the adrenal glands in these patients are of normal size or even absent. A two hit model of the disease has been proposed for these patients too, although with a different mechanism than in patients with *STAR* mutations. The accumulation of cholesterol most probably occurs inside the mitochondria and this may induce apoptosis in the steroidogenic cells. The cell damage would lead to earlier cell loss that could explain the absence of adrenal enlargement (compared to StAR patients) and even a possible absence of the adrenals at birth in these patients [133]. There is not a clear genotype-phenotype correlation, with null mutations present in patients born pre-term and at-term. Furthermore, several patients show additional clinical problems such as absent corpus callosum, tethered spinal cord, central hypothyroidism and short stature [133, 134]. No links between these conditions and CYP11A1 deficiency have been established yet. However, to note is that the *Cyp11a1* null mice are reported not only to show XY sex reversal and lethal adrenal insufficiency, which can be rescued with corticosteroid treatment, but also to manifest growth retardation, muscle atrophy, lethargy and anorexia [135].

CYP11A1 is an important candidate gene not only in cases presenting with sex reversal and adrenal insufficiency at birth, but also in patients with isolated 46,XY sex reversal when no mutations can be identified in the more obvious candidate genes (i.e. *AR* and *SRD5A2*), as partially inactivating *CYP11A1* mutations can lead to adrenal insufficiency that only presents later in life.

CYP17A1
Steroid 17-a-hydroxylase

CYP17A1 can catalyse two different enzymatic reactions: the 17α-hydroxylation of pregnenolone and progesterone, and the 17,20-lysis of 17α-hydroxypregnenolone and, with less efficiency, of 17-α-hydroxyprogesterone (17OHP) [136], and it is the qualitative regulator of steroidogenesis [137]. Defects of this enzyme lead to partial or complete deficiency of cortisol and sex steroids, and accumulation of the mineralocorticoid precursors 11-deoxyxcorticosterone (11-DOC) and corticosterone. These two metabolites have weak but significant mineralocorticoid activity preventing an adrenal crisis, however their accumulation leads to an excess of mineralocortocoids that may cause severe hypokalaemic hypertension. The deficit of sex steroids causes 46,XY DSD presenting as undervirilisation in male newborns, with the phenotype of external genitalia ranging from ambiguous to completely female, and absent pubertal development with primary amenorrhea in 46,XX individuals. Some mutations affect only the 17,20-lyase activity, leading therefore to deficit of sex steroids, without evidence of cortisol deficiency or mineralocorticoid excess [138, 139]. Often patients come to attention late in life because of absent pubertal development (sexual infantilism) due to hypergonadotropic hypogonadism, in both sexes [140].

HSD3B2
3β-hydroxysteroid dehydrogenase type II

HSD3B2 is predominantly expressed in the adrenal gland, ovary, and testis where it converts Δ5-3β-hydroxysteroids (pregnenolone, 17- pregnenolone and DHEA) into Δ4-3β-hydroxysteroids (progesterone, 17OHP and androstenedione). Type I is expressed in placenta and peripheral tissues.

Defects of HSD3B2 affect all three steroidogenic pathways. Patients present a quite wide clinical spectrum with or without salt wasting, that correlates with the genetic defect. Males can present normal or ambiguous genitalia, in most cases presenting as perineoscrotal hypospadias and a bifid scrotum, but also normal male genitalia, without correlation with the salt wasting symptoms. Some cases of both sexes show isolated premature pubarche. Females present normal or mildly virilised genitalia (clitoromegaly) [141]. These cases can be misdiagnosed as 21-hydroxylase deficiency [141, 142]. HSD3B2 is however a rare form of CAH.

HSD17B3
17-β-hydroxysteroid dehydrogenase type III

HSD17B3 is specifically expressed in the testes where it converts androstenedione to testosterone; four other isoenzymes with different expression and encoded by separate genes have been identified [143, 144].

HSD17B3 deficiency represents a rare autosomal recessive cause of 46,XY DSD, although a higher frequency is reported in Brazil. External genitalia at birth are usually female, but cases with genital ambiguity have also been identified [145, 146]. Patients present WD development and Müllerian structures [147]. Most patients are diagnosed at puberty when phenotypical females show severe virilisation and a 46,XY karyotype is revealed. It is hypothetised that pubertal testosterone is synthesised by one or more of the other HSD17B enzymes. Homozygously affected XX subjects are asymptomatic [148].

46,XY DSD patients with HSD17B3 defects are hard to distinguish, prepubertally, from patients with mutations in the AR or SRD5A2 genes, making genetic diagnostics very valuable for a correct diagnosis [147].

SRD5A2
Steroid-5-a-Reductase type 2

SRD5A2 converts testosterone to the more potent androgen dihydrotestosterone (DHT). Although both androgens act through the same androgen receptor (AR), DHT is required for external genital development. In fact SRD5A2 is expressed in the urogenital sinus, GT and genital swellings [149]. In most cases SRD5A2 deficiency results in ambiguous external genitalia in 46,XY subjects. Wolffian duct differentiation occurs normally and patients have epididymides, vas deferens and seminal vesicles. Furthermore spermatogenesis is normal if the testes are descended. At puberty spontaneous virilisation (i.e. growth of the phallus, increased muscle mass and deepening of the voice) can occur. In the past patients were often raised as females, however gender identity changes have been reported after puberty. Thus management of subjects diagnosed as having 5α-reductase-2 deficiency should be evaluated carefully. Affected 46,XY individuals have normal to elevated plasma testosterone levels with decreased DHT levels and elevated testosterone/DHT ratios. Mutations have been identified in all exons without a clear genotype-phenotype correlation [150].

GENETICS OF 46,XY DSD DUE TO ANDROGEN RECEPTOR DEFECTS

The actions of testosterone and DHT are mediated by the AR, a transcription factor that belongs to the superfamily of NRs. Like other NRs, the AR contains an N-terminal segment involved in transactivation, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD)[151]. The AR is normally present in two forms, AR-A and AR-B, in a wide variety of foetal and adult tissues [152, 153]. The AR-A form represents a truncated form of the receptor that is thought to arise

by initiation of translation at methionine 188, thus lacking 187 amino acids in the N-terminal part compared to the full-length AR-B form [154].

Mutations in the *AR* gene, located at Xq12, are responsible for the androgen insensitivity syndrome (AIS), the most common cause of 46,XY DSD. AIS manifests according to the severity of the AR defect [155]. The CAIS phenotype is that of a normal female, despite the presence of a 46,XY karyotype, and the presence of normal testes that produce testosterone. Internal genitalia (both WD and Müllerian structures) are absent, and there is a blind ending, short vagina. Recent information has revealed that the group of patients with apparent CAIS can be further subdivided into two distinct groups [156]. In true CAIS resulting from null mutations in the *AR* gene, there is virtually no effect of testosterone and a complete lack of male differentiation regarding both external and internal genitalia. Another group of patients is indistinguishable from CAIS patients by inspection of external genitalia, but they have well-developed WD structures internally (epididymis and/or vasa deferentia). The term severe AIS was proposed for this phenotype. Partial AIS (PAIS), characterised by partial androgen activity, leads to the formation of ambiguous external genitalia at birth, with a phenotype that can range from clitoromegaly to isolated hypospadias. This is a typical case of DSD when gender assignment is not immediately possible. AR mutations have also been described in males with isolated infertility; this phenotype is called minimal AIS (MAIS).

A large number of mutations in the *AR* gene have been identified in AIS patients, including obvious null mutations (deletions, frameshift, nonsense and splice site mutations), as well as missense mutations causing single amino acid substitutions. To date, more than 300 different mutations in the *AR* gene have been reported to be associated with AIS (see the androgen receptor gene mutations database (ARDB) [157] at www.androgendb.mcgill.ca/). The type of mutation differs along the length of the gene; in particular, nearly all mutations in exon 1 cause CAIS and they are nonsense mutations or frameshift mutations, due to insertions/deletions, that cause the introduction of premature termination codons. Indeed, all null mutations have been found in CAIS phenotypes except in a case of mosaicisms [158]. Missense mutations have instead been shown to be responsible for both CAIS and less severe AIS as they can result in partially functioning receptors, as assessed by transactivation assays following expression of mutant protein in cultured cells. In some cases the same missense mutation has been reported in PAIS and CAIS phenotypes and a variable expressivity is even observed in affected individuals within the same family [159].

The incomplete genotype-phenotype correlation represents a problem for a reliable genetic counselling to families of affected patients. Several genetic and environmental factors can be considered to explain the phenotype heterogeneity such as mosaicism, variable androgen production and availability during the embryonic stage and differences in AR coregulator activity and expression [155, 160].

46,XX DSD DUE TO 21-HYDROXYLASE DEFICIENCY (i.e. FOETAL ANDROGEN EXCESS)

CYP21A2 converts progesterone to 11-deoxycorticosterone and 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol. A defect leads to decreased or absent aldosterone and cortisol synthesis and excessive synthesis of androgens from the accumulated precursors. The clinical manifestations include a wide spectrum of symptoms, and the disease has traditionally been divided into three groups according to severity. The most severe form is salt wasting (SW) CAH, which includes life-threatening neonatal salt loss due to mineralocorticoid deficiency together with prenatal virilisation of external genitalia in females due to excess of androgen production. The slightly less severe simple virilising (SV) form is characterised by prenatal genital virilisation of external genitalia in females and early postnatal virilisation together with rapid somatic growth with early epiphyseal fusion in both sexes, due to excessive production of adrenal androgens. Salt loss is not manifested in these patients. SW and SV forms are together referred to as classic CAH (CL CAH). The mildest form is non-classical (NC) CAH which is characterised by late-onset hyperandrogenic symptoms such as precocious pubarche in children, acne, hirsutism, and menstrual irregularities in young women. The severity of hyperandrogenic symptoms is variable in these patients and some, especially males, may remain asymptomatic [161].

Steroid 21-hydroxylase deficiency is an autosomal recessive disorder responsible for more than 90–95% of all cases of CAH. CL CAH is the most common cause of ambiguous external genitalia in newborns. The incidence of 21-hydroxylase deficiency in Sweden is 1/10 000, figures from other populations vary somewhat [161, 162].

The 21-hydroxylase locus has a complicated structure where the active gene *CYP21A2* and the inactive pseudogene *CYP21A1P* are adjacent to and alternating with the *C4B* and *C4A* genes encoding the fourth component of complement [163]. One *C4* gene and one *CYP21* gene are part of a repeated module (RCCX) located in the HLA class III region on chromosome 6p21.3 [163]. The genetic defects that account for the majority of all cases of 21-hydroxylase deficiency are deletions and various gene conversion-type mutations in which *CYP21A2* has partially been converted to pseudogene-like sequences. These gene conversion events give rise to *CYP21A2* genes that carry one or multiple mutations normally present in the pseudogene or a chimeric *CYP21A1P/CYP21A2* gene [164, 165]. There is generally a good relationship between genotype and phenotype where the clinical manifestations of CAH reflect the less severely mutated *CYP21A2* allele [166, 167] (Figure 2). More rarely, mutations arise independently of the pseudogene, and these unique mutations are thus found in a single family or one specific population [168-172] [173, 174]. Especially for rare missense mutations, where large groups of patients are not available for clinical investigation, functional investigations of mutant enzymes have been helpful in classifying mutations according to severity. Mutants with activities ranging from less than 1% to over 70% of normal when assayed in a standardised cell culture system have been reported, and the activities have generally been in agreement with disease severity (Figure 2) [173, 175-178].

All reported disease-causing *CYP21A2* alleles together with, when known, associated clinical phenotypes and residual *in vitro* activities are listed in the Home Page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee at http://www.cypalleles.ki.se/cyp21.htm.

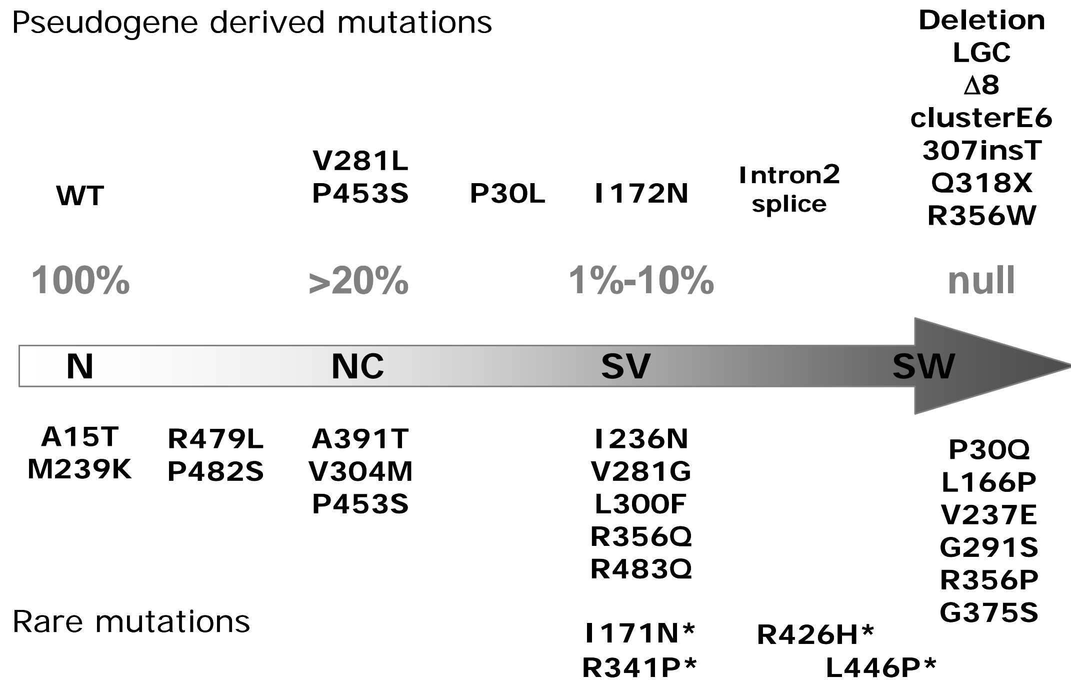

Figure 2. Genotype/phenotype correlations in CAH due to CYP21A2 deficiency. Common pseudogene derived mutations and rare mutations with associated clinical phenotypes and *in vitro* enzyme activities. WT, wild type; LGC, large gene conversion; Δ8, deletion of 8 bp in exon 3; SW, salt wasting; SV, simple virilising; NC, non classic. * Mutations studied in this thesis.

PATIENT INVESTIGATION AND MANAGEMENT

DSDs consist of a heterogenous group of conditions caused by different genetic or environmental factors that interfere with normal sex determination or sex differentiation. The modes of presentation of DSDs are also diverse, and patients can come to attention either at birth and in the neonatal period or later at puberty when sex development does not proceed as expected. A newborn with DSD comes to attention because of ambiguous genitalia, apparent male genitalia without testes, female genitalia with an inguinal/labial mass, hypospadias, a discordance between the genital appearance and a prenatal karyotype, or a family history of DSD (e.g. CAIS, CAH). Presentations of DSDs later in childhood or at puberty include signs of virilisation in a female at puberty, delayed or incomplete puberty, primary amenorrhea, gynecomastia and male infertility.

Different forms of DSDs can initially have a similar clinical presentation, therefore a series of analyses need to be performed to establish a diagnosis. Even if the choice of the tests depends on the finding at the clinical examination of each single patient and on the laboratory resources present in each center, it is possible to delineate some general lines. The first and fundamental investigations that can be useful to make a working diagnosis are:

- An thorough clinical investigation

- Karyotype analysis
  The karyotype is required to discriminate between a sex chromosome DSD, XY DSD or XX DSD. It is important to analyse a sufficient number of cells to be able to detect a mosaicism or chimerism.

- Imaging (ultrasound)
  Imaging gives information about the internal genital structures, it may lead to the location of the gonads, and may identify additional abnormalities (e.g. renal or adrenal). In an XY female, identification of Müllerian structures indicates a gonadal defect, while the absence of a uterus indicates normal testis development but absent testosterone synthesis or action.

- Biochemical investigations: measurement of serum 17OHP, testosterone, DHT, cortisol, DHEAS, androstenedione, gonadotrophins, AMH or inhibin-B, and electrolytes.

Decision making algorithms are also available [179-181]. Possible further investigations include:

- hGC stimulation test
  This test is performed to determine the presence of a testis and whether functional Leydig cells are present and capable of producing testosterone. The evaluation of steroid production can show a defect in androgen biosynthesis (for example a block of testosterone production from androstenedione in case

of 17ß-HSD deficiency or a defect in the conversion of testosterone to DHT in case of SRD5A2 deficiency).

- ACTH stimulation test
  This test can be performed if a defect involving the adrenal is suspected. Different steroid ratios can be used to identify the type of enzymatic defect.

- Laparoscopy
  This is used to identify and locate gonads and to determine the anatomy of the internal genitalia. At laparoscopy, biopsy of the gonads can be taken for further analysis.

- Gonadal biopsy
  Gonadal biopsy in cases of XY DSD is very important, and several analyses can be done. A histological investigation can evaluate the gonadal tissue morphology to distinguish for example between GD and ovotesticular DSD. It can also evaluate the presence of gonadoblastoma, which is important as some DSD conditions confer an increased tumor risk.

- Gonadal karyotyping
  A karyotype analysis on gonadal fibroblasts can be performed when GD is suspected, to reveal a local mosaicism not detected by the initial analysis on blood cells. FISH analysis using X and Y specific probes can also be used, on imprints of fresh gonadal tissue or after culture of fibroblasts. The detection of a gonadal X0/XY mosaicism will lead to a diagnosis of mixed GD.

- Urinary steroid analysis by gas chromatography mass spectrometry.

DNA analyses are used to finally confirm the diagnosis. Genetic analysis is important not only to confirm a diagnosis and offer a better genetic counselling, but may also have direct implications for e.g. choice of therapy, sex assignment and evaluation of tumor risk. Tumour risk seems to vary between different forms of DSDs. In cases at high risk of gonadoblastoma (e.g. 46,XY GD), gonadectomy needs to be performed at an early age and/or soon after diagnosis. In other cases, gonadectomy can probably be performed after puberty (e.g. CAIS), or perhaps gonads can just be monitored. For some types of DSD significant information about tumour risk is still lacking [182, 183]. Many of the patients also remain undiagnosed at the molecular level. More long-term clinical follow-up studies of DSD patients with verified molecular diagnoses need to be performed, to enable prognostic evaluations to be made for patients belonging to each subgroup.

AIMS

The overall aim of this thesis was to identify mechanisms of DSD, in order to better understand normal and atypical sex development, and furthermore to offer better diagnostics and genetic counselling to patients with DSD and their families.

The specific aims of my studies were

- To study functional consequences of novel missense mutations identified in patients with CAH due to 21-hydroxylase deficiency.

- To improve the diagnostics for XY female patients by setting up
    - PCR and sequencing to identify additional mutations in candidate genes, not yet routinely analysed in the Clinical Genetics laboratory
    - RT-PCR from fibroblasts enabling genetic evaluation at the mRNA level
    - Western blotting analysis to
        - evaluate AR expression in genital skin fibroblasts from patients who show an AIS phenotype but for whom no mutations in the AR gene have been identified
        - evaluate expression of the different AR isoforms in skin and gonadal fibroblasts of patients with CAIS due to different AR mutations

- To perform array comparative genome hybridisation (array CGH) in patients with XY gonadal dysgenesis in order to identify submicroscopic chromosome imbalances. Genes located in deleted or duplicated regions would be novel candidates for genes involved in gonadal development.

- To develop new diagnostic tools based on my new findings.

SUBJECTS AND METHODS

PATIENTS

The work of this thesis has focused on some specific groups of DSD patients.

- 46,XX DSD due to CYP21A2 deficiency
  In particular novel or rare mutations have been the subject of study. Functional studies have been performed to evaluate residual activities of mutant enzymes to better understand the genotype-phenotype relationships. With this knowledge a better management of the patients and a more accurate genetic counselling to the patients and members of their family can be offered.

- 46,XY DSD patients
  Genetic and molecular studies were performed for 46,XY patients who presented unambiguously female external genitalia (i.e. XY female patients).

The type of samples used in the studies were

- Genomic DNA
  from blood samples, EBV-immortalised cell lines, gonadal and genital skin fibroblasts.

- Total RNA
  from EBV-immortalised cell lines, gonadal and (genital) skin fibroblasts.

- Proteins
  from EBV-immortalised cell lines, gonadal and (genital) skin fibroblasts.

- Metaphase nuclei from EBV-immortalised cell lines.

After we developed some new diagnostic tools, a group of patients, initially referred to the Molecular Genetic laboratory of the Pediatric Unit of the S.Orsola-Malpighi Hospital-University of Bologna, Italy, were included. The 10 Italian patients formed a more heterogeneous group of 46,XY patients with abnormal gonadal development without a genetic diagnosis; the external genitalia ranged from completely female to ambiguous. *SRY* gene mutations had been excluded in all cases.

Sample availability was different for each patient and this affected the genetic and functional investigations that could be performed. Informed consent was collected in each case.

CONTROLS

Blood samples were collected anonymously from five fertile males and five healthy post pubertal females. Lymphocytes were isolated and EBV-immortalised. DNA was extracted by a standard phenol/clorophorm extraction protocol.
Control DNA samples were also obtained from Karolinska University Hospital blood donors.

CYP21A2 FUNCTIONAL STUDIES (Paper I)

To evaluate the functional consequences of novel or rare CYP21A2 missense mutations identified in patients with CAH, functional studies have been performed. A schematic representation of the experimental design is presented in Figure 3.

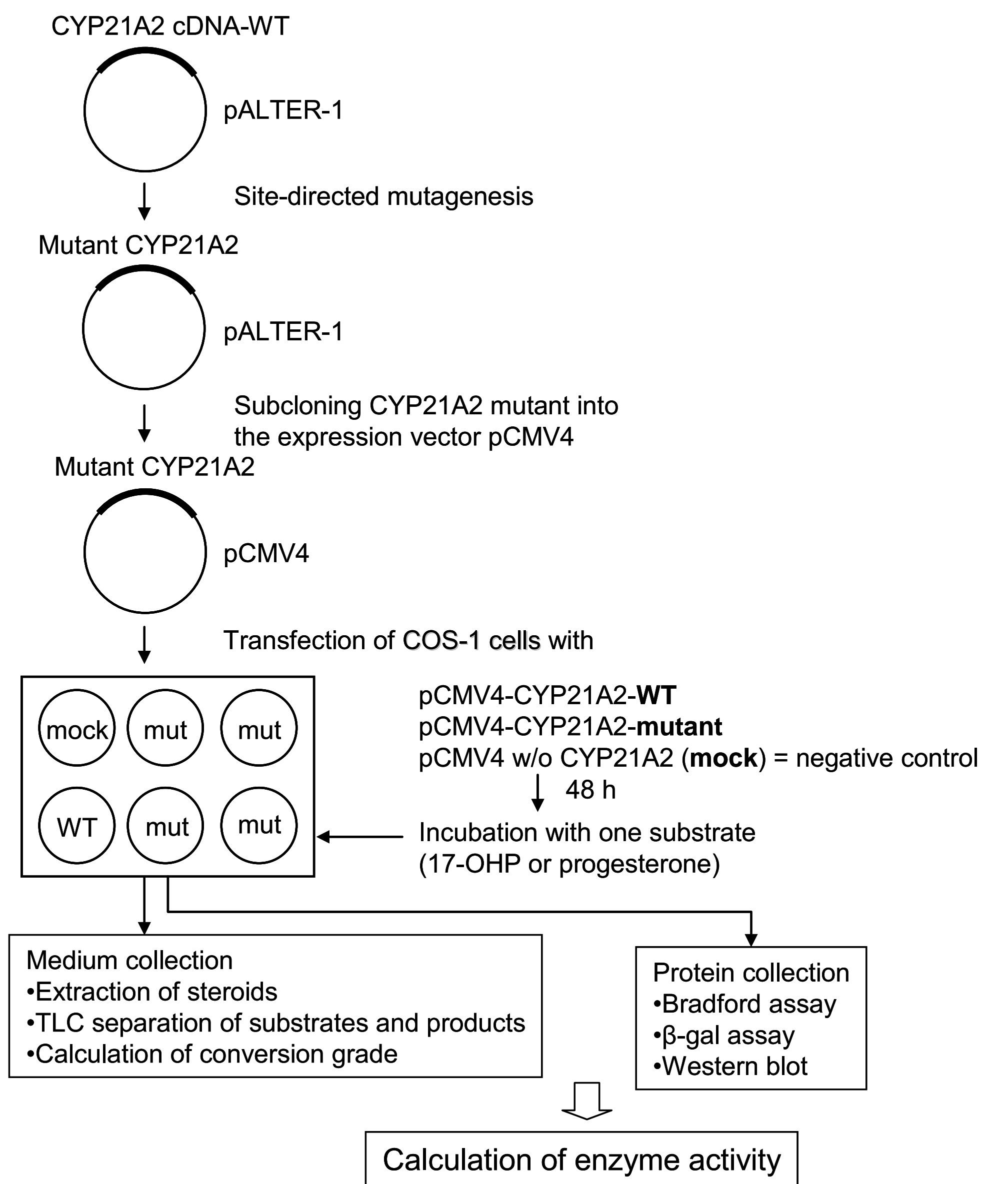

Figure 3. Strategy for CYP21A2 *in vitro* functional studies.
WT, wild-type; TLC, thin layer chromatography; ß-gal, ß-galactosidase.

Mutagenesis and preparation of expression vectors

The missense mutations of interest were introduced in the pALTER-CYP21 vector [184] using the Altered site II *in vitro* mutagenesis system (Promega) (Figure 4). Two phosphorylated primers were used for each mutagenesis reaction; one primer to introduce the specific point mutation and one primer to introduce a change to restore the functionality of the mutated ampicillin resistance gene. After primer annealing to the denatured pALTER vector, mutant strands were synthesised by a T4 DNA polymerase and ligated by a T4 DNA ligase. A mutant strand of the pALTER vector, containing the specific mutation and resistance to ampicillin was thus produced *in vitro*. The strands were then purified by isopropanol precipitation and replicated by transforming by electroporation the BMH17-18 mut S strain of *E. coli*. This strain is defective of the mismatch repair system so the mismatches, due to the introduction of the mutations, are not repaired; selective growth of the bacteria in ampicillin leads to isolation of colonies that contain only the mutated pALTER-CYP21. Plasmids were then purified from single colonies and the cDNA insert was sequenced to ensure the introduction of only the specific mutation and to exclude the presence of additional variations.

In general, the entire mutagenised cDNA was subcloned into the expression vector pCMV4-CYP21 [176], using the restriction enzymes *Bgl*II and *Kpn*I. However, for pALTER-CYP21-I171N the vectors were digested with *Bgl*II/*Pml*I, because another mutation had accidentally been introduced in the insert and needed to be eliminated. After subcloning, the insert was sequenced one more time.

Expression of CYP21A2 in COS-1 cells

The COS-1 cell expression system was chosen to study CYP21A2 enzyme activity. This mammalian system ensures a proper post-transcriptional machinery necessary for the correct processing and folding of the CYP21A2 protein. Furthermore COS-1 cells do not produce steroids that could affect the interpretation of the results. The COS-1 cell line is derived from monkey kidney fibroblasts. It originates from the CV1 cell line that has been transformed with an origin defective SV40 virus that has integrated in the genome. The cells constitutively express at high levels the SV40 large T antigen [185]. This antigen can bind to any SV40 origin present in a plasmid and initiate replication of the plasmid. The expression vector pCMV4 contains an SV40 origin and is therefore replicated in high copy number when transfected to the COS-1 cells [186].

Wild-type pCMV4-CYP21, mutant pCMV4-CYP21 constructs and native pCMV4 without CYP21A2 cDNA (mock), used as negative control, were co-transfected with β-galactosidase vector pCH110 (Pharmacia) in COS-1 cells using the multi component lipid-based transfection reagent Fugene (Roche). After at least 48 hours from transfection enzyme activity assays were started.

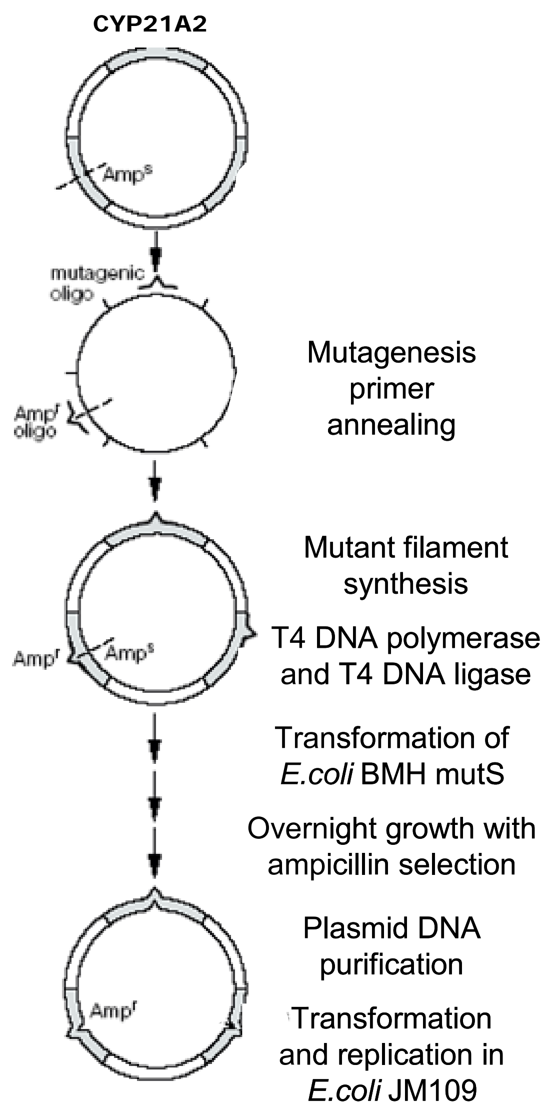

Figure 4. pALTER mutagenesis. Modified from the technical manual provided by Promega. Amp^s, ampicillin sensitive; Amp^r, ampicillin resistant.

Enzyme activity assay

^{3}H-labelled substrate, 17OHP or progesterone, was added to the medium together with unlabelled steroids and the co-factor NADPH. After incubation under non-saturated conditions the medium was collected, steroids were extracted and separated by thin-layer chromatography. Radioactivity corresponding to products and substrates was subsequently measured by liquid scintillation spectrophotometry.

After cell collection and sonication, protein extracts were collected. Total protein content was measured using a protein assay based on the Bradford method [187]. ß-gal activity was measured by incubation of the protein extract with a substrate that when converted by ß-galactisidase generates a yellow colored product, the intensity of the color was measured by a photometer. The ratio of ß-galactosidase

activity/total protein content was measured in each experiment to verify an equal transfection efficiency.

Apparent specific activity was calculated for each mutant as pmol substrate conversion / mg of total protein / minute of incubation. Background signal measured in the cells transfected with the mock was subtracted. Enzyme activities were expressed as a percentage of conversion, considering the apparent specific activity of the wild-type CYP21A2 as 100%.

To verify that the different forms of the CYP21A2 protein were equally expressed in the transfected cells, Western blotting was also performed (see Western blotting section).

DNA SEQUENCING

Direct DNA sequencing is the most specific and accurate method to identify single base substitutions (point mutations) or small insertions or deletions in a specific gene. Candidate genes for XY DSD (*SF1*, *WT1*, *DHH* and *CYP11A1*), not yet routinely analysed at the Clinical Genetics laboratory, were sequenced to identify possible disease-causing mutations.

PCR primers to enable amplification and sequencing of all exons and at least 20 nt of the intronic sequence at the intron-exon boundaries were designed with the Primer3 software available online [188]. PCR fragments were purified, initially, by a silica membrane column (Qiagen) and, afterward, by incubation with the ExoSap-IT enzyme mix (USB Europe GmbH). Sequencing was performed using the Big Dye terminator v3.1 kit (Applied Biosystems) that is based on a modified version of the Sanger method [189]. Dideoxynucleotides (ddNPTs), labelled with four different fluorophores corresponding to the four different bases (A, C, G, T), are used as chain terminators. Primers used for the PCR reaction were also used for sequencing. When possible, both strands were sequenced. Fragments were size separated and fluorescence was measured using an ABI 3730 XL capillary sequencer (Applied Biosystems). Electropherograms were analysed by visual inspection and with the SeqScape version 2.5 software.

RT-PCR (REVERSE TRANSCRIPTASE-PCR)

RT-PCR is a method that allows the amplification of RNA. This technique can be used to verify the expression of a gene in a specific tissue or cell line. After the total RNA or the mRNA has been extracted from the tissue of interest, it is converted into single stranded complementary DNA (cDNA) by a reverse transcriptase enzyme. The DNA synthesis can be initiated by hexamer primers that randomly bind to the RNA molecules or by poly(T) primers that bind to the poly(A) tails of the mRNAs. The cDNA is then used as a target for a PCR reaction. Primer pairs are designed strategically in order to differentiate between amplification from genomic DNA and

cDNA molecules. Primers can be designed to bind to sequences within different exons or at the junction between two exons.

RT-PCR was used to verify the expression of the *AR* gene in genital skin or gonadal fibroblasts from patients with DSD with a suspected androgen receptor defect. Total RNA was converted to cDNA using the random primer method and primers were targeting different exons **(Paper II).**

RT-PCR also offers the advantage to enable the sequencing of mRNA or verify the correct splicing of a gene. The limitation of RT-PCR is represented by the availability of the right tissue expressing the gene of interest. Furthermore, the expression information is limited to the RNA and does not necessarily correlate with protein expression.

WESTERN BLOTTING

Western blotting is a technique used to verify the presence of a specific protein in a tissue or cell extract. Proteins from a cell extract are first size separated by electrophoresis, blotted onto a nitrocellulose membrane, detected by an antibody and visualised. The visualisation system we chose consists of using HRP-conjugated secondary antibodies, adding a chemiluminescent substrate (ECL) and detecting the signal by an image analyser (Image Reader LAS-1000; Fujifilm).

To study CYP21A2 expression in transfected cells, protein extracts were concentrated by trichloroacetic-acid-precipitation and directly resolved in loading buffer. However, as cell harvesting by trypsination and the following procedures before protein precipitation may lead to degradation of proteins, proteins were also collected directly from plated cells 48 h after transfection, using a lysis buffer and direct loading on the gel [190]. Two different primary antibodies were used: polyclonal antibodies raised in rabbit against a synthetic peptide derived from human CYP21 [191] and antibodies present in serum from a patient with Addison's disease having natural antibodies against CYP21.

To study the AR in patient fibroblasts, adherent cells were collected at confluence in 100 mM Na phosphate buffer (pH 7.4) containing a protease inhibitor cocktail (Roche). Four different antibodies that recognise different epitopes of the AR were used (Table 4).

Table 4. AR antibodies used for Western blotting analysis.

Antibody	Recognised epitope	Source	Company
AR-441	aa 299-315 of human AR	mouse	Santa Cruz Biotechnology
AR-N20	N-terminus of human AR	rabbit	Santa Cruz Biotechnology
AR-C19	C-terminal of human AR	rabbit	Santa Cruz Biotechnology
F39.4.1	Human AR	mouse	BioGenex

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

MLPA is a technique that allows the detection of copy number changes of several nucleic acid target sequences in one reaction. The method has been described for the first time by Shouten *et al.* in 2002 [192] and represent a powerful technique that makes it easier and faster to study single gene or single exon deletions or duplications. These types of copy number change, of a size too small to be identified by cytogenetic techniques and too large to be detected by PCR and sequencing, were previously very difficult to study. The available techniques such as FISH and Southern blotting were quite laborious and not amenable to a high throughput format, leading to an underestimation of the frequency of deletions or duplications of such intermediate size.

The few steps necessary for MLPA analysis are schematically represented in Figure 5. Two sequence-tagged oligonucleotides (Figure 6) are adjacently hybridised to a specific target sequence and ligated together by a DNA ligase to create a probe. The amount of ligated probes is proportional to the copy number of the target sequence. Each oligonucleotide pair (or half probe pair) used in one MLPA reaction is designed to generate a fragment (probe) of a unique size but with the same end sequences as the others (tagged sequences). Thus all probes can be simultaneously amplified by a fluorescently labelled universal primer pair and PCR products are size separated by capillary electrophoresis and quantified. A sample is analysed by comparing its peak profile to the corresponding peak profile obtained from a control sample. The relative peak area of a PCR product reflects the relative amount (copy number) of the target sequence in the sample.

Initially the two oligonucleotides were one synthetic and one M13 derived. This gives the advantage to analyse simultaneously up to 45 target sequences. Probes of unique sizes, with target sequences of 50-70 nt but the final size ranging from 130 to 480 nt can be generated thanks to the insertion of a stuffer fragment in the M13 derived probe. The PCR fragments differ between each other in a step wise fashion of 6-9 nt in length. Completely synthetic probe sets can be also used, in this case the probes are of shorter length, ranging from 84 to 140 nt, with a stepwise difference in length of 3-4 nt. The advantage is that the probes are produced without the laborious work of creating M13 phage vectors. The disadvantage is represented by the lower number of probes that can be simultaneously included in the probe set. The limitation is caused by the difficulty of producing long length oligonucleotides without partially synthesised contaminants.

One possibility to add more probes in a completely synthetic probe set is the development of two color MLPA [193], where another probe set is added, with oligonucleotides with different tag sequences that will be amplified by a second universal primer pair, labelled by a different fluorophore. This approach was used to design the DSD-MLPA probe set presented in **Paper IV**.

The synthetic probe sets used in **Papers III, IV, V and VI** have been designed according to the recommendations described by *Stern et al.* [194]. Briefly, the target oligonucleotide sequences were designed to have a GC content of 40-60% when possible, a Tm >65°C, a G or a C at the junction between the target and the

universal primer sequence, and the ligation site was never between GG, GC or CC. When possible the two adjacent oligonucleotides were designed to have similar length and properties. The oligonucleotides' GC content, length and Tm were obtained from the RawProbe program (available from the MRC Holland web site at www.mlpa.com). The uniqueness of the selected target sequence was verified by performing a BLAST analysis to identify possible similar sequences or pseudogenes, using in the query the mRNA sequence of the target gene or a fragment of at least 500 bp for intergenic sequences. If similar sequences were detected and it was not possible to change the target region, the oligonucleotides were designed to have the ligation site between nucleotides that discriminate between one sequence and the other, as the ligase enzyme is sensitive to mismatches. The presence of SNPs in the target was also excluded. Once designed, oligonucleotide and probe sequences were analysed with the BLAT function [195] in the UCSC genome browser (http://genome.ucsc.edu/ [196]) to find possible cross hybridisations in the genome.

In all probe sets at least 3 control probes were included. In addition to the *PCLN1* and_*CLDN16* control probe located on 3q28 and 3q26 reported by Stern *et al.* [194], other control probes were designed in coding regions of genes located on different chromosomes: *ALB* on 4q13.3, *RB1* on 13q14.2, *RELN2* on 4q13.3, *PITX2* on 4q25, *PAX6* on 11p13, *ATP2C1* on 3q22.1.

We noticed that the PCR product with the smallest size, which is the first one to be separated during the electrophoresis, has a high variability. To overcome this problem we included a "pilot" probe pair in each probe set that will result in the smallest product and will act as a filter/shield for the other products that will be quantified.

MLPA reactions were carried out using the in house designed probe sets and the reagents contained in the EK1 kit (MRC Holland), according to the manufacturer's protocol. Fragments were size separated using a ABI 3100 genetic analyser (Applied Biosystems). Peak traces were visualised and analysed using the GeneMapper v3.7 software (Applied Biosystems), data were exported and further analysed using Excel (Microsoft). For each sample the peak areas corresponding to each probe were first normalised to the average of the peak areas of the control probes (block normalisation). Ratio values were then calculated between the normalised probe peak areas in all samples and the corresponding average value in the control samples. The sample run was considered acceptable if the ratio for the internal control probes was between 0.8 and 1.2. Threshold values for deletion and duplication were set at 0.75 and 1.25, respectively.

In conclusion, MLPA analysis offers the possibility to simultaneously screen for copy number variations of several specific target sequences. It is also rapid, cheap, sensitive and reproducible. As the probes used are small, it can detect deletions or duplications of small regions (i.e. single exons). It can also be used for breakpoint fine mapping at a higher resolution compared to FISH. The disadvantages are the limited number of probes that can be included in a mix and that high quality DNA is required to obtain reliable results.

Synthetic oligonucleotides

Genomic DNA

Hybridisation

Target A

Target B

Target C

Ligation

Target A

Target B

Target C

Multiplex PCR

Target A

Target B

Target C

Separation of amplification products by electrophoresis

Fragment analysis with peak area calculation

Figure 5. Principles of Multiplex Ligation-dependent Probe Amplification (MLPA).

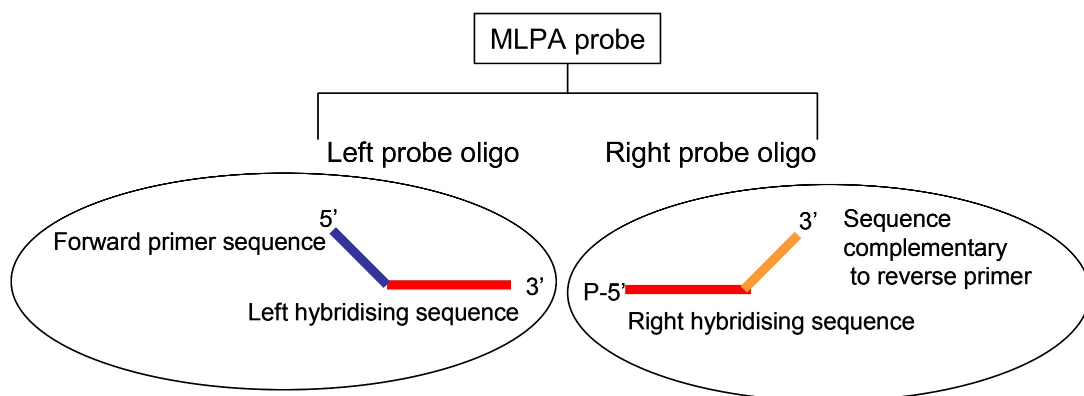

Figure 6. MLPA probe. Terminology of probe components. P, 5'-phosphoritation.

FLUORESCENT *IN SITU* HYBRIDISATION (FISH)

FISH allows the localisation of a specific DNA sequence (probe) in individual chromosomes or cells. Chromosomes or cells are fixed onto a glass slide. Fluorescently labelled probes are hybridised to the slides and visualised by a fluorescent microscope. Several types of FISH probe (BAC, PAC or cosmid clones or PCR products) can be used and several investigations can be performed (detection of deletions, duplications, chromosome rearrangements, mapping of translocation breakpoints). However the resolution is not as high as with MLPA. In **Paper III, IV, and VI**, FISH has been used to establish the localisation of the duplicated genomic material identified by array-CGH. Probes were labelled by random priming and different fluorophores were used in order to perform co-hybridisation experiments. BAC clones were used to localise the region of interest and cosmids or centromere specific probes were used to recognise specific chromosomes.

ARRAY COMPARATIVE GENOMIC HYBRIDISATION (ARRAY CGH)

CGH is a technique that was developed to detect genomic copy number variation using a genome wide approach. Sample DNA and a reference DNA are fluorescently labelled and co-hybridised to a glass slide that contains several target sequences. Initially the target was represented by normal metaphase chromosomes, giving a resolution of >3Mb. In array CGH the target sequences are mapped DNA sequences (cDNA or BAC clones) or oligonucleotides arrayed onto the glass slide. After hybridisation, the slide is scanned to quantify the fluorescent signal of each target on the array. After proper analysis, gains and losses are identified by ratio values. Sequences that are present in the same number of copies in the sample and the reference have a ratio value of 1, (1:1=1; 2:2=1), a deletion or a duplication in the sample will give a value <1 or >1, respectively. The resolution of the method depends on the number of targets, the distribution of the targets in the genome and the type of target.

The target sequences in the array CGH slides used in **Paper III and VI** (from Swegene DNA Microarray Resource Centre, Lund University, Sweden) were tailored BAC clones to enable a genome wide screening with a theoretical resolution of 100 kb [197]. However, during the analysis a smoothing transformation over three clones was performed, this function slightly decreases the resolution. As genomic reference DNA we used a commercially available pool of DNA from 10 normal males (Promega, Madison, WI, USA). The same batch was used for all experiments. Sample DNA and reference DNA were labelled with Cy5 and Cy3 fluorophores and dye swap experiments were carried out to reduce/eliminate the labelling bias effect. Identification of individual spots on scanned arrays was performed with Gene Pix Pro 6.0 (Axon Instruments), and the quantified data matrix was loaded into Bio Array Software Environment BASE [198]. Background-corrected Cy3 and Cy5 intensities were calculated using the median-feature and median-local background intensities provided in the quantified data matrix. Spots with bad morphology were excluded, pin based Lowess normalisation was applied [199] and to reduce noise, data from the two duplicates were merged. Within arrays, intensity ratios for individual probes

were calculated as background corrected intensity of the patient sample divided by background corrected intensity of the reference sample. To identify gains and losses throughout the genome, the two BASE implemented automatic breakpoint identification tools CGH plotter [200] and GLAD [201] were applied after smoothing with a sliding window over three clones. The threshold for gains and losses was set to $\log_2$(ratio) of ± 0.25.

The array CGH technique offers the advantage of a high resolution genome wide analysis that can identify submicroscopic deletions or duplications. However, as for every screening approach, the results need to be confirmed by an alternative technique, such as FISH, MLPA or quantitative PCR. The choice of the technique(s) depends on the type and size of the abnormality and the type of sample available. A disadvantage of the array CGH analysis is that it does not give information about possible chromosomal rearrangements or the configuration of a rearrangement, for example a duplication can be interstitial (with the duplicated segments with the same or opposite orientation) or due to a translocation to another chromosome. Thus, complementary techniques need to be used for further investigation.

RESULTS AND DISCUSSION

FUNCTIONAL STUDIES OF TWO NOVEL AND TWO RARE MUTATIONS IN THE *CYP21A2* GENE (Paper I)

By sequence analysis of the *CYP21A2* gene we have identified two novel (I171N and L446P) and two rare (R341P and R426H) mutations in seven Italian patients with CAH. We performed functional studies to determine the degree of impairment caused by each mutation on CYP21A2 activity and consequently to verify that the encountered mutations were disease causing.

In CAH due to 21-hydroxylase deficiency there is in general a good genotype-phenotype relationship, where the disease expression depends on the less severe of the two alleles present in the patients. Mutants with *in vitro* activities ranging from below 1% to over 70% in a standardised cell culture system have been reported and the activities have generally been in agreement with clinical disease severity [173, 202]. Especially for rare and novel mutations, where no large group of patient are available for clinical investigation, functional studies of mutant enzyme are thus helpful, and in some cases the only way to classify mutations according to severity.

I171N

The I171N mutation was found in a female patient with a NC phenotype usually associated with the V281L mutation that she presented on her other allele. This makes it impossible to classify her new mutation according to severity based on clinical findings. By functional studies, we determined that the I171N mutant had 0.7% and 0.6% residual activity towards 17OHP and progesterone, respectively. It also seemed less stable than the wild-type protein as it was affected by trypsination before Western blotting. The highly reduced enzyme activity we found is concordant with the results obtained for I171N and I172N forms of the protein produced in a yeast system [203]. Thus I171N, as the similar known pseudogene derived mutation I172N, is associated with a CL CAH phenotype.

R341P

The R341P mutation was found in four unrelated patients that all had SV CAH phenotypes characterised by precocious pubarche, accelerated growth, and advanced bone age in the male patients and by clitoromegaly in the female. The mutations present in the other alleles were the Intron 2 splice and the I172N mutations in the males and the female, respectively. Intron 2 splice is in most cases associated with SW CAH, whereas I172N is the most common mutation causing SV CAH. The R341P mutation had previously been reported in homo- or hemizygous form in a brother and sister with NC CAH [204]. However, the girl presented with precocious pubarche before 2 years of age, thus clearly indicating a more severe phenotype than what is typical of NC CAH. In our functional studies, the R341P protein maintained a minimal enzyme activity of 0.7% toward both substrates. The

mutation is therefore associated with a CL CAH phenotype. The remaining residual activity seems to be enough to produce sufficient amounts of aldosterone to prevent salt wasting, at least under non-stressed conditions.

R426H

The R426H mutation is caused by a typical mutation of CG dinucleotides that changes the codon from CGC to CAC. The substitution was found in a severely prenatally virilised female patient that carried the null mutation Q318X in the other allele. The same alteration was also reported in hemizygous form in three Austrian sisters that had a severe classic form of CAH, with Prader stage IV virilisation but apparently no salt-wasting [205]. Arg 426 is very important for 21-hydroxylase function as it is one of the four residues needed for heme propionate coordination. This amino acid is conserved in CYP21 of other species and is also present in all mammalian CYPs that have been crystallised, while a histidine is in this position in all microbial crystallised CYPs, except in class II bacterial CYP102 (P450BM3). Arginine to histidine substitutions are generally considered conservative, but a change in such a key functional position is still likely to have a drastic effect on enzyme function. In our non-saturated system, the R426H mutation caused a drastic functional impairment, but a very low activity remained for both substrates, 0.5% and 0.4% of normal activity towards 17OHP and progesterone, respectively. This mutation therefore belongs to the CL CAH phenotype group. These results have also been confirmed by functional studies performed by another group that also studied the R426C substitution, which seems to have a more severe impact completely abolishing enzyme activity [178].

L446P

The novel L446P mutation was identified in a male patient with CL CAH with latent salt wasting. The rare R356Q mutation that has previously been associated with SV CAH [184] was present on the other allele. Leu 446 is a highly conserved residue at one end of one of the two helices that sandwich the heme. A proline residue is likely to cause the disruption of this helix structure that is essential for enzyme activity. In fact, in the functional experiments the mutant showed almost null activity, 0.5% for 17OHP and 0.0% for progesterone. Consequently this mutation is associated with a CL CAH phenotype.

In conclusion, all four rare CYP21A2 mutations analysed in this study result in drastically impaired enzyme function, which is associated with severe CL CAH. For some of them, sufficient amounts of aldosterone may be produced *in vivo* to prevent salt-wasting, at least under non-stressed conditions. The distinction between SW and SV forms of CAH is not absolute, however, and the risk of salt-wasting should not be neglected in patients carrying any of these mutations in their mildest allele. With the introduction of neonatal screening programs for CAH and the possibility of prenatal diagnosis and treatment, many patients are diagnosed before symptoms and signs are fully developed. In addition, a clinical classification is not always clear-cut as the disease severity reflects the mildest of the two alleles present in each

patient. Thus, *CYP21A2* genotyping and functional characterisation of each disease-causing mutation has relevance both for treatment of the patients and for genetic counselling to the family. Furthermore, as the crystal structure of CYP21A2 is not yet available, *in vitro* functional studies of mutations, together with clinical data and homology modelling [206] can provide important information to better understand clinically relevant functional and structural relationships in the CYP21A2 protein.

DIAGNOSTICS IN 46,XY FEMALE PATIENTS

During the last ten years, forty-six 46,XY female patients have been referred to our unit for genetic diagnostics. (Only patients with unambiguously female external genitalia are discussed here.) Initial genetic investigations were carried out at the Clinical Genetics laboratory at the Karolinska University Hospital. Analyses included karyotyping and DNA sequencing of one the following genes: *AR, SRY* and *CYP17,* depending on the patient's clinical presentation. Typical findings indicating a clinical diagnosis of CAIS were absence of internal genitalia and high testosterone levels, whereas the presence of female internal genitalia and high FSH levels lead to a working diagnosis of XY GD. When no genetic diagnosis could be made by the routine analyses, the cases were subjected to the analyses described in the present study. An overview of the patients, together with our genetic findings, is presented in Table 5. My new findings are indicated in bold.

Table 5. Summary of 46 investigated XY female patients: clinical diagnosis and genetic defects.

Clinical Diagnosis	Number of patients	Number of patients (N) and genetic defects
CAIS	24	(21) *AR* mutation **(1) absent expression of the AR protein, although no identification of mutation in the AR gene** (2) still unexplained
XY GD (one XYY)	20 (2 pairs of siblings)	(2) *SRY* mutations (3) mixed GD (1 47,XYY in blood cells and 45,X0/47,XYY in gonads; 1 X0/XY mosaicism in blood cells, 1 gonadal X0/XY mosaicism) **(2 siblings) *DAX1* locus duplications** **(1) terminal 9p deletion** **(1) *WT1* mutation** (11) still unexplained
XY female with adrenal failure	1 (+ 1 XX sibling with adrenal insufficiency)	**(1) CYP11A1 mutations**
XY female with hypocortisolism	1	(1) *CYP17* mutations

Some general conclusions regarding XY female patients can be made:

- CAIS and XY GD accounted for 52% and 43% of the cases, respectively.
- Mutations in the *AR* gene were found in 88% of the patients with clinical signs of CAIS.
- In one of the three CAIS patients without identified *AR* mutations, genital skin fibroblasts were available. This enabled confirmation of the diagnosis by analysis of AR protein expression.
- Rare enzyme deficiencies affecting both gonadal and adrenal steroid synthesis accounted for around 4% of the cases.
- An initially missed X0/XY mosaicism was found in 15% of the cases referred for XY GD.
- We could establish a genetic diagnosis in 45% of the cases referred for XY GD.

The specific findings are discussed in more detail in the following sections.

Complete Androgen Insensitivity Syndrome and the Androgen Receptor

In the patients referred to us with a suspected CAIS diagnosis, mutations in the *AR* gene have been identified in 88% (21/24) of the cases. Most of the patients were identified in late puberty because of primary amenorrhea, while at least two were identified during childhood because of bilateral inguinal hernia. Interestingly, one of the patients identified at puberty reported to have been operated for bilateral hernia in childhood. These data stress that AIS should be considered in every young girl that is operated for hard mass inguinal hernia, and that karyotype investigation should be routinely performed in these patients.

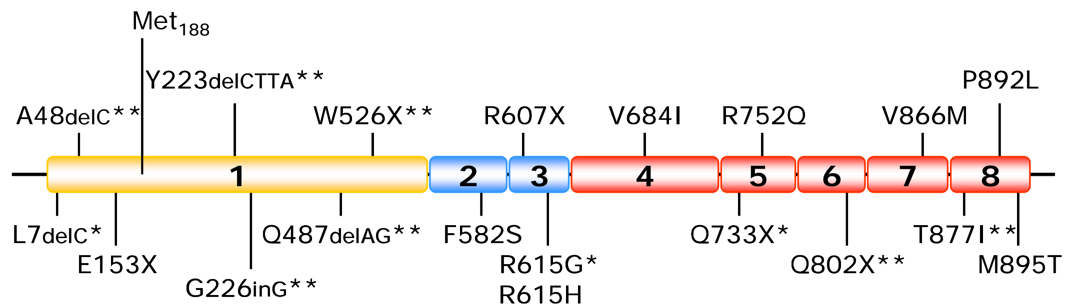

Figure 7. AR mutations identified in our CAIS patients.
* novel mutations and ** not yet reported in the literature.

A summary of the 19 different mutations identified in our CAIS girls is presented in Figure 7. Twelve mutations were novel and are marked by an asterisk; a second asterisk is added if they are not yet reported in the literature or in the androgen receptor gene mutations database (ARDB). The type and distribution of the mutations is in concordance with the data reported in literature. To date, more than 300 different mutations in the *AR* gene have been reported to be associated with AIS. Only 54 of these are located in exon 1 even though it encodes more than half of the AR protein. The type of mutation differs along the length of the gene; in

particular, nearly all mutations in exon 1 cause CAIS and they are nonsense mutations or frameshift mutations, due to insertions/deletions, that cause the introduction of premature termination codons. It is therefore not surprising that more than one third of the mutations identified in our CAIS patients lie in exon 1 and are all missense or frameshift mutations. The apparent higher frequency of mutations in exon 1 we found, compared to the ARDB is due to the fact that we analysed only CAIS patients, while the data from the database include also other forms of AIS. While nonsense and frameshift mutations are identified only in CAIS patients, missense mutations have been shown to be responsible for both CAIS and less severe AIS forms as they can result in partially functioning receptors. In some cases the same missense mutation has been reported in PAIS and CAIS phenotypes and a variable expressivity is also observed in affected individuals within the same family [159, 207]. Most of the mutations reported in the ARDB are individual or familial mutations, however a few recurrent mutations are reported. The mutations identified in our patients are spread along the entire gene and are both missense and nonsense mutations. Nine of the mutations we have identified are described also by other groups, to note 6 (2/3) occur at CpG dinucleotides (Table 6). The R615H and V866M mutations have both been identified in two patients. Both mutations have already been reported in several other patients and thus most likely represent hot spot mutations. Furthermore, these two missense mutations, as well as others, have been reported not only associated with a CAIS phenotype but also to PAIS and even to a MAIS phenotype. Interestingly, in some cases with PAIS the variable expressivity was explained by somatic mosaicism.

Table 6. Comparison of phenotypes associated with already reported AR mutations.

Mutation	Our patient phenotype	Phenotype in other patients	Reference
E153X	CAIS [208]	CAIS	[209]
F582S	CAIS	PAIS	[210]
R607X (CpG)	CAIS [211]	CAIS	[212]
R615H (CpG)	CAIS (2)	9 CAIS (2 siblings) 1 PAIS 1 MAIS	[156, 159, 212-218]
V684I	CAIS	CAIS	[219]
R752Q (CpG)	CAIS	6 CAIS	[118, 215, 220-223]
V866M (CpG)	CAIS (2)	8 CAIS 3 PAIS (1 somatic mosaicism) 1 MAIS	[156, 160, 216, 218, 224-228]
P892L (CpG)	CAIS	4 CAIS	[160, 229]
M895T (CpG)	CAIS [230]	1 PAIS (somatic mosaicism)	[231]

Absent AR protein expression despite no identification of mutations in the *AR* gene (unpublished data)

In three patients (approximately 12%) with a clinical diagnosis of CAIS no mutations were identified in the AR gene. For one patient genital skin fibroblasts were available and further molecular studies could be done. RT-PCR and Western blotting analysis were performed to verify AR expression.

RT-PCR using a forward primer located in exon 1 and a reverse primer in the last exon of the gene produced a band of the expected full length size. However, although the AR gene was normally expressed at the RNA level, no AR protein was detected by Western blotting carried out using antibodies against different epitopes of the AR protein (Figure 8). This indicates that the phenotype is caused by absence of AR, however the mechanism that leads to the absent expression is not yet clear. We do not know if AR is translated and immediately degraded or if translation occurs at all. Further studies are required to better understand the molecular defect in this patient.

These results stress the importance of investigating AR expression in patients with clinical signs of CAIS in whom no mutations in the AR coding region are identified. This would allow to discriminate between patients with undetected mutations in the AR gene and patients with other defects, for instance a co-factor defect. Defects of AR expression at the mRNA or protein level have already been described in PAIS patients indicating that mechanisms affecting AR transcription and translation can occur, however they have not yet been understood in molecular detail [232]. The absence of an AR cofactor has also been shown in a patient with a CAIS phenotype, however the protein has never been identified [233].

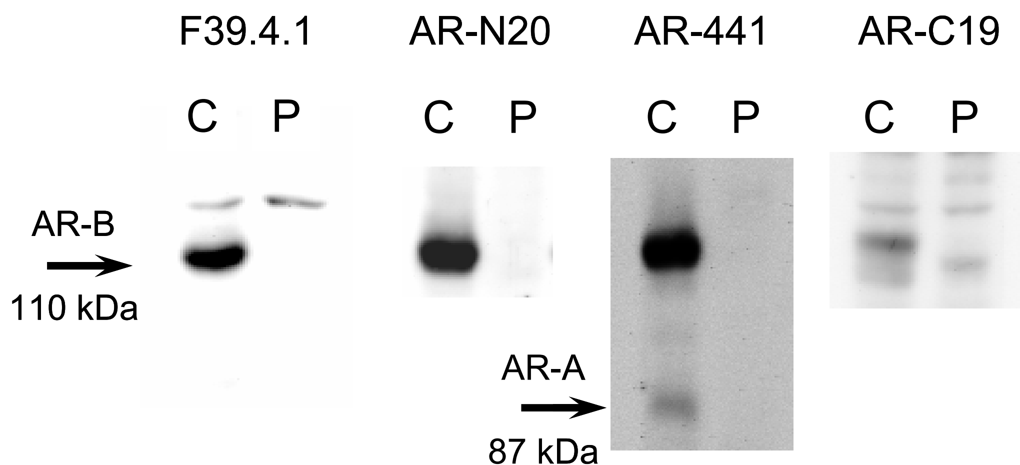

Figure 8. Western blot analysis. Different antibodies against the AR receptor were used to detect AR expression in proteins extracted from genital skin fibroblasts of a CAIS patient (P) with no mutations in the *AR* gene and from foreskin fibroblasts from a normal male (C).

CAIS without Wolffian duct development: the AR-A form of the AR is not sufficient for male genital development (Paper II)

The AR is clearly essential for the differentiation of male external and internal genitalia. As for many other nuclear receptors, it is present in two isoforms in a wide variety of foetal and adult tissues [152, 153]. There is a full length form B and an N-terminal truncated form A that arises by initiation of translation at Metionine 188, however for this latter isoform the function has not yet been identified. After the publication of Hannema's studies [156] with the distinction between severe AIS (female external genitalia but male internal genitalia) and complete AIS, two CAIS patients were referred to us for genetic evaluation. In one patient the L7fsX33 mutation, that affects only the full length form of the AR, was identified, while the other patient carried the nonsense mutation Q733X that affects both isoforms. This gave us the opportunity to investigate whether the truncated form of the AR can promote some degree of male internal genital development. Both patients were gonadectomised and the presence of Wolffian duct structures was specifically looked for during surgery and at subsequent histological analysis of the removed gonads and adjacent tissue. No signs of androgen stimulated Wolffian duct structures could be identified. By RT-PCR and Western blotting using antibodies that recognise different AR epitopes we could show that the gonadal fibroblasts carrying the L7fsX33 were not expressing the AR-B isoform, but were still able to express the truncated AR-A isoform, while the fibroblasts with the Q733X mutation did not express any AR protein. These data suggest that the AR-A isoform of the receptor is incapable of promoting development of male internal genitalia in the human foetus *in vivo*. Although at least 14 mutations that can lead to expression of only the AR-A form have been described in CAIS patients, this is the first time that the presence or absence of male internal genitalia, together with the differential expression of the AR isoforms, has been studied in these patients.

Mixed gonadal dysgenesis (unpublished data)

Three patients with a final diagnosis of mixed GD were identified. One was initially found to have a 47,XYY karyotype in peripheral blood. The other two were referred to us for molecular investigation of 46,XY gonadal dysgenesis. In all three cases, karyotyping had been performed in other laboratories.

In the 47,XYY girl and in one of the 46,XY patients, gonadal biopsies had been taken and fibroblast cell lines had been established. Karyotyping of these revealed mosaicism with the presence of a 45,X0 cell line that had not been detected in blood. From the third patient, we performed QF-PCR (quantitative fluorescent PCR) on peripheral blood DNA, using a kit for prenatal screening of aneuploidy of chromosomes 13, 18, 21, X and Y. The results indicated the presence of both X and Y chromosomes but in amounts suggesting mosaicism. This was confirmed by FISH analysis using X and Y specific probes on the blood sample, which revealed a previously undetected X0/XY mosaicism (39% X0).

These results clearly indicate that X0/XY mosaicism should always be looked for when a 46,XY karyotype is identified in a female patient. If only blood is available,

FISH analysis of the X and Y chromosomes enables analysis of several hundred cells and is thus a sensitive assay for detecting mosaicism in this tissue. If gonadectomy is performed or gonad biopsies are taken, karyotyping should be done from this tissue, as mosaicism may be confined to the gonads. As this investigation is not routinely done, the frequency of X0/XY mosaicism is probably underestimated in these patients.

CANDIDATE GENE APPROACH TO IDENTIFY DISEASE CAUSING MUTATIONS IN PATIENTS WITH 46,XY GONADAL DSD (unpublished data)

When a patient with 46,XY DSD affecting the gonads is identified, the first gene to be analysed is *SRY*. However, mutations in this gene are identified in only 10-15% of the cases. In our material, mutations in *SRY* were identified in only two of the 20 (10%) patients with 46,XY GD analysed. The first mutation was a novel missense mutation (A66G) within the conserved HMG box. Ala66 belongs to the N-terminal bipartite nuclear localisation sequence of SRY [234]. The second mutation was a nonsense mutation (Q93X) already reported in another case [235].

In 11 patients without a genetic diagnosis and for whom DNA was available, other candidate genes involved in GD were considered for further genetic investigation by PCR and sequencing (*SF1*, *WT1* and *DHH)*. Surprisingly, no *SF1* mutations were identified, although several mutations in the *SF1* gene have recently been reported in the literature with an apparent higher frequency than *SRY* mutations, in patients with XY GD without adrenal insufficiency [51, 236]. No mutations in the *DHH* gene were identified.

A novel missense mutation (M342K) in the *WT1* gene was found in a patient with GD. The patient was initially identified because of delayed puberty. Gonadal tissue investigation after gonadectomy revealed a gonadoblastoma. No signs of impaired renal function, which is typically seen in patients with gonadal dysgenesis caused by missense mutations in *WT1,* were present. The M342K mutation has not previously been described but a missense mutation affecting the same residue, M342R, has been reported in an XY female child with renal failure [37]. Renal function investigations are recommended for this patient as she is at risk of developing renal failure or a kidney tumour. Unfortunately, parental samples are not available and evaluation of the inheritance of the mutation is not possible.

In another XY female patient we identified a synonymous mutation (G51G), the triplet of codon 51 changes from GGC to GGT. Synonymous mutations do not change the protein sequence and for that reason they have generally been considered as neutral changes without effects on gene function or phenotype. Some synonymous mutations can however affect normal mRNA splicing or miRNA binding, and have been described as causative mutations in several diseases [237]. Two more mechanisms by which synonymous mutations can affect normal protein expression and function have recently been described. In one case a synonymous SNP changes

the secondary mRNA structure leading to reduced protein translation and activity [238]. In another the substitution of a frequent GGC codon to the more rare synonymous GGT codon affects the timing of translation leading to changes in protein folding and function [239, 240]. These two newly described mutation mechanisms can represent special rare cases or be more frequent but underestimated due to the difficulty to study mRNA secondary structures and protein folding compared to studying mRNA splicing. Whether the synonymous mutation we have identified in our patient is responsible for the phenotype remains to be evaluated. This change is not a common SNP and does not seem to create an obvious splice site. However, before proceeding with any further studies, parental samples should be analysed, to indicate a potential role in the phenotype.

CANDIDATE GENE APPROACH TO IDENTIFY DISEASE CAUSING MUTATIONS IN PATIENTS WITH 46,XY DSD DUE TO DISORDERS IN ANDROGEN SYNTHESIS (unpublished data)

CYP17 mutations

On the basis of very low cortisol levels in an 46,XY girl with absent pubertal development, CYP17A1 deficiency was suspected. By sequencing of the *CYP17A1* gene the patient was shown to be homozygous for the nonsense mutation R358X, thus explaining the complete 17-α-hydroxylase/17,20-lyase deficiency.

CYP11A1 mutations

In a 46,XY female patient with adrenal insufficiency, who had a 46,XX sister also affected with adrenal insufficiency, *SF1* and *STAR* had been the first candidate genes investigated, but no mutations were identified. Subsequently, sequencing of the *CYP11A1* gene lead to the establishment of the genetic diagnosis. The two siblings were homozygous for the missense mutation A359V. Both parents were heterozygous carriers. This mutation has already been described by Al Kandari *et al.* [133] in a patient with female genitalia, a 46,XY karyotype, and adrenal insufficiency that severely manifested at 1y and 9 m of age. The mutation occurs at a CpG dinucleotide suggesting a possible mutational hot spot. The patient described by Al Kandari also presented agenesis of the corpus callosum. Further clinical investigation of our affected sisters is ongoing.

GENE DOSAGE ALTERATIONS IN 46, XY GONADAL DSD (Papers III, IV, V and VI)

Gonadal development has been shown to be sensitive to gene dosage and genes involved in sex development are located on several chromosomes. Therefore, genome wide array-CGH represents an attractive screening tool to identify submicroscopic imbalances that could be responsible for gonadal DSD. We have analysed 10 unrelated patients with 46,XY gonadal DSD by high resolution tiling BAC arrays **(Paper VI)**.

In two patients with isolated 46,XY GD, alterations affecting already known candidate regions for XY DSD were identified, a duplication on Xp21.2 **(Paper III)** and a deletion on 9p24, that were further investigated. These data suggested that dosage imbalances affecting known genes or regions were more frequent than we expected. Therefore, we developed two MLPA probe sets, one to investigate dosage imbalances affecting known genes involved in DSD **(Paper IV)** and one to investigate in detail the candidate region for sex reversal on the short arm of chromosome 9 **(Paper V)**.

Isolated 46,XY GD in two sisters caused by a 637 kb interstitial duplication on Xp21.2 containing the *DAX1* (*NR0B1*) gene (Paper III)

The Xp21.2 duplication was confirmed and further characterised by MLPA, FISH, PCR and sequencing. This revealed a 637 kb tandem duplication that in addition to *DAX1* includes the four *MAGEB* genes, the hypothetical gene *CXorf21*, *GK*, and part of the *MAP3K7IP3* gene, with the breakpoints located in intron 1 of the *MAGEB2* gene and in intron 7 of the *MAP3K7IP3* gene. The duplication was also present in the affected sister of the patient analysed by array-CGH and the mother was shown to be a healthy carrier of the duplication. The duplication contains the 160 kb minimal common region for dosage sensitive sex reversal [61], that is shared by all patients with XY sex reversal and Xp duplications. This region contains *DAX1* and the *MAGEB* genes [62]. *DAX1* is the candidate gene responsible for sex reversal due to several of its characteristics (expression pattern, functional properties, transgenic mouse data). No patients with duplications including only *DAX1* have been reported previously and a role for the *MAGEB* genes that have unknown functions and specific testis expression cannot be excluded. In the two sisters the *MAGEB2* regulatory region is not duplicated and therefore the extra copy is probably not transcribed, however the transcription of the extra copies of the other three *MAGEB* genes might be functional resulting in over expression of these genes.

The duplication described in this study is the first Xp21 duplication containing *DAX1* identified in patients with isolated GD. All previously described XY subjects with Xp21 duplications presented GD as part of a more complex phenotype, including mental retardation and/or malformations. Most probably it is also the smallest duplication identified so far, as all previously reported patients were identified by conventional karyotyping, apart from a patient with a duplication of less than 1 Mb identified by Southern blot [61].

These data support *DAX1* duplication as the genetic cause of GD, even if a role for the *MAGEB* genes cannot be completely excluded. Furthermore, we show the importance of using methods that can detect submicroscopic *DAX1* locus duplication in the evaluation of patients with isolated 46,XY GD and not only when GD is part of a more complex phenotype. As will be described, we subsequently identified additional XY GD patients with small *DAX1* duplications, verifying their pathogenetic importance.

Gene dosage imbalances in patients with 46,XY gonadal DSD, detected by an in house designed synthetic probe set for MLPA analysis (Paper IV).

The detection by array CGH of two submicroscopic imbalances affecting already known regions involved in XY gonadal DSD made us suspect that dosage imbalances of genes or regions already known to be involved in DSD are more frequent than previously thought. The underestimation could be the consequence of the fact that single gene deletions or duplications for genes involved in DSD are not routinely investigated.

The recently developed MLPA technique that allows investigation of copy number variations of several target sequences in one reaction, is an easy, reliable and less laborious way than before, to detect deletions or duplications of genes involved in DSD. We therefore designed an MLPA synthetic probe set to use as a screening method to investigate patients with different forms of DSD. For genes already known to act in a dosage manner, such as *SF1*, *WT1*, *SOX9*, *WNT4* and *DHH*, two probes were designed. For *DAX1* only one probe was designed, and instead a probe for the *MAGEB1* gene was included. Furthermore probes for the *LHCGR*, *SRY*, *RSPO-1*, *SRD5A2*, *STAR* and *CYP11A1* genes were designed. We initially used this probe set to analyse a group of patients with 46,XY gonadal DSD. The analysis led to the identification of two duplications, one containing the *SRD5A2* gene, the other containing the *DAX1* and *MAGEB* genes.

The first duplication has a minimal size of approximately 170 kb and a maximal size of 360 kb, and it contains the entire *SRD5A2* gene and part of the *XDH* gene. The strategy of series of probe sets used to narrow down the duplication breakpoints is represented in Figure 9. Considering the enzymatic function of the gene products and that the alteration is paternally inherited, we excluded this alteration as the cause of the GD phenotype in the patient. Nevertheless the presence of this duplication, that represents a rare normal variant, should be taken into account when the *SRD5A2* and *XDH* gene are analysed by PCR and sequencing.

The duplication of the *DAX1* locus extends for 800 kb and in addition to *DAX1* contains the *MAGEB* genes, the hypothetical gene *CXorf21* and *GK.* No genes were disrupted at the breakpoint as the telomeric breakpoint is located 193 kb upstream of the *MAGEB* genes and the centromeric breakpoint is 76 kb downstream of the *GK* gene. The duplication was identified in a patient with isolated partial GD and ambiguous external genitalia, and was inherited from the healthy mother. A phenotype with partial GD has been reported in three other unrelated patients with Xp21 duplications, indicating a variable affect of the *DAX1* locus when present in a double dose. A positional effect that affects *DAX1* expression and the modulation of DAX1 action by its interaction with several co-factors should be considered as potential mechanisms for a variable phenotype expressivity.

The identification of an additional *DAX1* locus duplication in a patient with 46,XY GD without other dysmorphic features and/or mental retardation, clearly indicates that *DAX1* duplications should be investigated in all patients with 46,XY GD, complete or partial, independently of the manifestation of other symptoms.

The fact that the mothers were healthy carriers indicates that *DAX1* locus duplications can be transmitted and spread through the female line in the family, and *DAX1* locus duplications, of a size undetectable by conventional karyotyping, could be more frequent than previously thought.

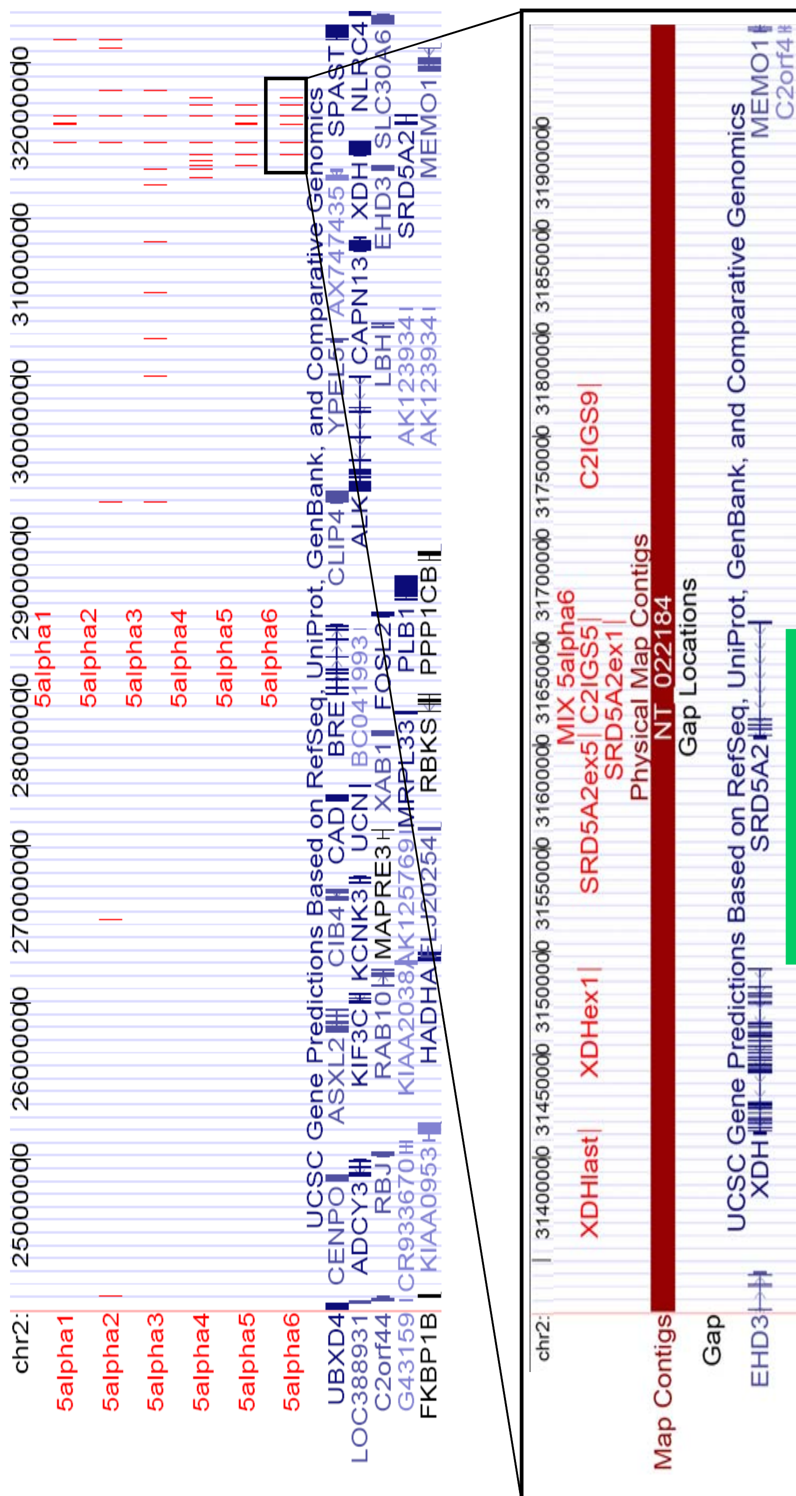

Figure 9. Schematic representation of the MLPA strategy used to identify the breakpoints of the duplication initially detected by SRD5A2ex2 MLPA probe. Representation from the UCSCgenome browser of the *SRD5A2* locus on 2p23.1. Probes included in the differ probe sets are indicated by red vertical lines. The thick green horizontal line indicates the minimal duplicated region.

9p deletions in 46, XY DSD and monosomy 9p syndrome (Paper V)

To confirm the 9p terminal deletion identified by array-CGH, we decided to develop an MLPA probe set to use for screening of all further patients with 46,XY GD. Although the 9p24.3 region has been extensively investigated, the gene(s) and the mechanisms responsible for GD have not yet been identified. Thus the identification and fine mapping of submicroscopic terminal or interstitial deletions on 9p24.3 would help the definition of the minimal sex reversal region and could lead to the identification of gene(s) responsible for 46,XY gonadal DSD. The target genes selected for the MLPA analysis were: the three *DMRT* genes, with *DMRT1* historically being the strongest candidate gene, the three more telomeric genes *FOXD4*, *DOCK8* and *ANKDR15*, and the three centromeric genes *SMARCA2, UHRF2* and *MPDZ* outside the candidate sex reversal region, with *MPDZ* located within the 9p-monosomy syndrome critical region. In addition we designed probes for the *DMRTB1* and *DMRTA3* genes located on 1p32.3. These genes were considered to be of interest not only because of their high expression in the adult testis but also because duplications including the 1p31-32.3 region have been reported in some cases of 46,XY DSD and we wanted to screen for possible imbalances of these genes. Unfortunately, the MLPA analysis detected deletions only in the patient already identified by array-CGH and in a patient with a 46,XY del(9)(p23) karyotype. However the high molecular resolution of the MLPA analysis together with the data available in the literature made it possible to make new considerations/observations on the sex reversal and the monosomy 9p candidate regions.

The identification, by array-CGH, of the terminal deletion that does not include *DMRT1* in a patient with isolated 46,XY GD gives rise to many hypotheses and considerations both in favour for and against *DMRT1* haploinsufficiency as the cause of GD. Because *DMRT1* and its regulatory region are not deleted, DMRT1 should still be normally expressed in the patient, suggesting that the gonadal phenotype could be caused by haploinsufficiency of one of the more telomeric genes. However, very little information is available for the expression and function of the genes in the *DMRT1* telomeric region, making it impossible to indicate another candidate gene. Only the *ANKRD15* gene can be excluded as a candidate because the copy number of this gene seems to be highly polymorphic. Another possibility is that the deletion positioned the *DMRT1* gene in proximity of the telomere and that this could negatively affect the expression of *DMRT1* even if its regulatory region is intact.

The patient with the 46,XYdel(9)(p23) karyotype presented not only with female external genitalia due to GD but also typical signs of monosomy 9p syndrome. We therefore decided to better characterise the deletion using MLPA analysis, and to further narrow down the 3.5 Mb monosomy 9p candidate region interval [82]. We thought we had narrowed down the minimal overlapping region to a 1.3 Mb interval, however no genes were present in this region. After careful evaluation of the literature and comparison of the molecular characterisation of several patients, we realised that at least two patients with monosomy 9p syndrome without loss of the latest defined candidate region had been described [241, 242]. In fact, different types of rearrangement (terminal deletions, interstitial deletions, unbalanced translocations, complex rearrangements), have been described in patients with the

monosomy 9p syndrome, that could differentially affect gene expression, for example by haploinsufficiency and/or positional effects. To define a reliable minimal common region responsible for a phenotype it is generally better to use patients with simple interstitial deletions and compare several patients at the same time. For the monosomy 9p syndrome, information from patients with terminal 9p deletions without the monosomy 9p syndrome can also be taken into account. With the data available at the moment the region would extend for 8 Mb, from D9S286 to D9S285.

MLPA analysis of all patients with 46,XY gonadal DSD and even patients with 9p-monosomy syndrome would lead to the identification and characterisation of more patients with rearrangements on 9p24. Detailed genetic characterisation together with careful morphological examination of more patients is needed to identify the molecular mechanisms that lead to GD and to typical features of monosomy 9p.

The understanding of the molecular mechanisms that lead to GD is made more difficult by the incomplete penetrance of the phenotype. In fact, while new patients with 9p24 deletions and 46,XY GD are identified [243-247], at the same time also patients with 9p24 deletions and normal male external genitalia are described [248]. The study of the gonadal tissue of these patients could be a way to identify the candidate gene(s) for gonadal dysgenesis. It would, for example, make it possible to verify and quantify the expression of candidate genes, or to evaluate the presence of methylation mechanisms or to verify other hypotheses. This requires the collection of a sufficient number of patients and their gonadal material, which at the moment is almost impossible due to the rarity of such patients and that 9p deletions are not yet routinely investigated in 46,XY DSD patients. The investigations of additional murine knock-outs for one or more *DMRT* genes as well as for other genes in 9p24.3 would also lead to a better understanding of the pathological mechanisms causing the impaired gonadal development.

Novel candidate regions for gonadal dysgenesis (PaperVI)

By array CGH several copy number variations (CNVs) were identified in the other eight patients analysed. It tourned out to be more difficult to distinguish benign CNVs from pathogenic CNVs in patients with DSD than in patients with other disorders with a dominant inheritance because duplications or deletions of genes involved in gonadal development have been shown to cause DSD in a sex chromosome limited way. Therefore, if a CNV identified in control studies is listed in the public database of genomic variants, this has to be interpreted with caution especially if it is identified in a small number of subjects. After excluding benign CNVs, three potential causative CNVs were confirmed by MLPA and further evaluated.

A duplication on chromosome 6 that extends from exon 5 to exon 12 of the *SUPT3H* gene is shared by two sisters with 46,XY GD. The duplication is maternally inherited. Because gene dosage can affect sex development in a sex chromosome limited manner, this alteration is still potentially involved in the patient phenotype. SUPT3H

(suppressor of Ty 3 homolog isoform 1) is the human homologue of the yeast (*Saccharomyces cerevisiae*) transcription factor spt3 [249]. While several studies on the yeast spt3 protein have been performed to understand its role in transcription, very little is known about the human *SUPT3H* gene. Several gene isoforms are described in the UCSC genome browser (Built 36, May 2006) and in the Ensembl database (release 48, Dec 2007). Interestingly, in the latter database a novel protein transcript (ENST00000371455) that seems to correspond to the duplicated region is described. According to expression array data, human *SUPT3H* is expressed in the testis as well as in a range of other tissues, with no clear tissue dominating. The mouse homologue is also expressed in the testis. However, no obvious connection between SUPT3H function and sex development can be deduced. At the same time it cannot be excluded as a candidate gene for DSD as many transcription factors have shown a dosage action in sex development.

A duplication at chromosome band 12q21.31 was identified in the patient who also carries the novel missense mutation M342K in the *WT1* gene, which could be the cause of her GD. This duplication is quite large and it is worth to report, independently of its involvement in the patient phenotype. It is not reported in the public database of genomic variants. For most of the genes present in this region very little information is available, the only gene that seems to be expressed in the testis, according to the UCSC genome browser, is the *LIN7A* gene. Further investigations of genes present in this region are necessary to select or exclude genes from being candidates for DSD.

Although further investigations are needed, these two duplications constitute two candidate regions for GD. Analysis of a sufficient number of controls with known sex chromosome complement is necessary to exclude the possibility that they represent very rare normal variants. Accurate expression data and determination of protein function could also indicate a possible role in gonad development. Knock out and transgenic mouse models for the genes with testis expression would also be of great interest. Another possible way to evaluate the significance of the duplications identified in this study is to analyse several patients with 46,XY GD to evaluate if some more patients carry the same alterations. However patients with 46,XY GD are rare and the genetic alterations considered to be most frequent, *SRY* mutations, are identified in only 10-15% of the cases. A similar or even lower frequency should be expected for new genetic alterations causing GD.

We can conclude that array CGH is a valuable method to analyse patients with 46,XY GD, that can lead to the identification of genomic imbalances at loci with genes involved in sex development and the detection of novel CNVs. These are new candidate regions for genes important in gonadal development.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Patients with DSD present a very challenging situation both clinically and genetically. A correct diagnosis has several implications including choice of sex assignment, treatment, evaluation of gonadal malignancy risk and possibility of a more specific genetic counselling to the family. Thus the understanding of genetic and functional mechanisms that cause DSD can have an immediate translational consequence. Furthermore, understanding mechanisms of DSD gives us the opportunity to better understand normal human sex development.

Functional studies, candidate gene sequencing and genome wide screening techniques all represent valuable tools to provide more information about mechanisms of DSD.

With the *in vitro* studies of *CYP21A2* mutations we have been able to confirm the genetic diagnosis in patients with CAH, in particular the new and rare mutations identified have all been associated to a severe phenotype. For one mutation (I171N) this could not be predicted by the clinical manifestation of disease in the patient since she carried a milder mutation on the other allele. Furthermore the rare R426H mutation has recently been detected in two additional unrelated patients with CAH. One patient had a deletion on the other allele and presented a SV phenotype, in concordance with our results. The other patient showed a NC phenotype typically associated with the V281L mutation that she carried on the second allele. Knowing the association of R426H to a SV phenotype has allowed a more accurate genetic counselling to this patient and to advise her about the risk to transmit a severe allele for CAH, although her phenotype was mild. This situation explains how functional studies for novel or rare mutations have important translational consequences.

Studying the expression of the two isoforms of the AR protein in two patients with CAIS, we could determine that the truncated isoform of the AR is not sufficient for Wolffian duct development *in vivo*. We could also confirm a clinical diagnosis of CAIS in a patient where no mutations in the coding region of the AR gene were identified. This finding illustrates that when a CAIS diagnosis is not genetically confirmed, AR expression should be investigated.

By a candidate gene approach to study patients with 46,XY DSD (i.e. XY female), mutations in the *AR, SRY* and *CYP17A1* gene have been identified in the clinical setting. Investigation of more candidate genes have revealed mutations in the *CYP11A1* gene in a patient with gonadal and adrenal insufficiency and a *WT1* mutation in a patient with XY GD and no symptoms of abnormal kidney function. Quite surprisingly we did not identify any *SF1* mutations. However due to the relatively high frequency of such alterations recently reported in the literature, this

gene has been added to the list of genes analysed in the Clinical Genetics laboratory.

Genome wide high resolution array-CGH and MLPA analysis using in house designed synthetic probe sets have proved to be valuable methods for the identification and characterisation of submicroscopic genome imbalances in patients with 46,XY GD. We identified two independent *DAX1* locus duplications, a 9p deletion and two novel candidate regions for GD. These techniques should be considered as a complementary diagnostic approach to sequencing candidate genes, for the genetic investigation of patients with 46,XY gonadal disorders.

In particular the MLPA analysis with the DSD-probe set is easily implemented by the genetic laboratory. MLPA analysis is quite cheap, easy and does not require the acquisition of new equipment. For genetic investigation of patients with 46,XY GD we consider it more convenient, after sequencing the *SRY* gene, to use an MLPA screening approach before proceeding to sequencing the other candidate genes. Furthermore the DSD-MLPA kit can also be used to analyse patients with 46,XX DSD, as it contains probes for *SOX9*, *SRY* and *RSPO1*. Array CGH, although more expensive, offers the advantage to screen for genomic imbalances in an unbiased way, potentially leading to the identification of new candidate regions. Depending on the resolution and the type of platform used for the analysis it can be used as an alternative or complement to the MLPA investigation. This is a choice that depends on the resources available in each single genetic and research laboratory.

The identification of two independent families with *DAX1* locus duplications stresses the importance of using methods that can detect submicroscopic duplications of the region surrounding *DAX1* in the evaluation of patients with isolated 46,XY GD (complete or partial) and not only when GD is part of a complex dysmorphic phenotype. Such events are most probably more frequent, but have escaped detection due to the methods that have been used so far and the selection of the patients investigated. The lack of phenotype in the carrier mother also illustrates that such duplications can be spread through the female line in the family. In fact we have recently found another *DAX1* locus duplication in two 46,XY sisters with GD and an apparent X-linked inheritance of the defect (Figure 10). This suggests that *DAX1* duplications might be as common as *SRY* mutations, which have hitherto been considered as the single most common cause of XY GD. Further investigation of this family is ongoing.

It would be interesting to identify an Xp21.2 duplication involving only the *DAX1* or the *MAGEB* genes to finally prove that single *DAX1* duplications cause GD, or to discover a functional role of the *MAGEB* genes. As patients with XY GD undergo preventive gonadectomy, it could be useful to collect the gonadal samples enabling the measurement of *DAX1* and *MAGEB* expression.

The identification of the two novel candidate regions for GD prompts further investigations. The duplication at chromosome 12 is difficult to interpret because it was found in a patient with a novel *WT1* missense mutation. The interstitial duplication of *SUPT3H* instead represents an interesting potential mutation. A series

of initial investigations can be taken. The analysis of several controls and verification of the expression pattern of this gene in different human tissues, with particular attention to sex organs, is an obvious start. Afterwards, functional studies and construction of animal models should be taken into consideration.

Gene dosage imbalance mechanisms have been shown not only to affect XY gonadal development but also XX gonadal development. It would be interesting to use array CGH also to analyse patients with XX GD and patients with premature ovarian failure, considering this phenotype as a partial or late onset XX GD, when no mutations in candidate genes have been identified.

In conclusion, the work of this thesis has led to the establishment of the genetic diagnosis in several patients with DSD, thus allowing not only a better genetic counselling but also, at least in some cases, a better patient management. Furthermore, our genetic diagnostic arsenal has been expanded, as we can offer sequencing of more genes and gene dosage investigations by MLPA.

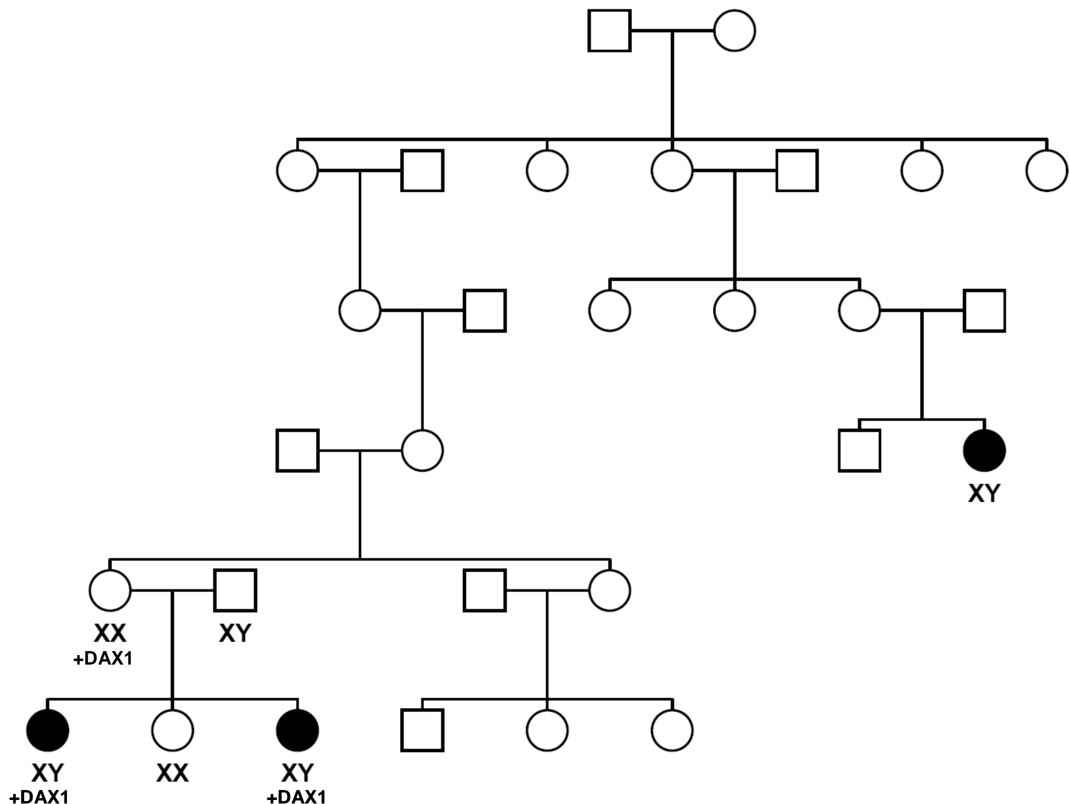

Figure 10. Pedigree of a recently identified family with two sisters with XY GD due to a *DAX1* locus duplication (+DAX1), inherited from the mother. The affected relative could be a carrier of the same mutation spread through the female line. Investigations are ongoing.

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