Disorders of Sex Development

Clinical outcomes, (epi)genetic regulation and germ cell cancer

Yvonne G. van der Zwan

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Clinical outcomes, (epi)genetic regulation and germ cell cancer

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“Think and wonder, wonder and think.”

Dr. Seuss

Contents

Chapter 1	General Introduction	9
	Normal gonadal development	9
	Abnormal gonadal development	18
	Germ cell cancer	23
	Disorders of Sex Development and Germ Cell Cancer	25
	Disorders of Sex Development and outcome	27
	Aims and outline of this thesis	29
Chapter 2	Severity of virilization is associated with cosmetic appearance and sexual function in women with Congenital Adrenal Hyperplasia: A Cross – Sectional Study *The Journal of Sexual Medicine 2013; 10(3):866-75*	37
Chapter 3	Long term outcomes in males with Disorders of Sex Development *Journal of Urology 2013; 190(3):1038-42*	55
Chapter 4	Management of Disorders of Sex Development in the Netherlands; Changes over time *Manuscript in preparation*	67
Chapter 5	A 46,XY female DSD patient with bilateral gonadoblastoma, a novel *SRY* missense mutation combined with a *WT1* KTS splice-site mutation. *PLoS One 2012; 7(7):e40858*	81
Chapter 6	*MAP3K1* is a testis cancer susceptibility gene *Submitted*	103
Chapter 7	A novel AMH missense mutation in a patient with persistent Müllerian duct syndrome *Sex Dev. 2012;6(6):279-83*	121
Chapter 8	Role of epigenetics in etiology of Germ Cell Cancer *Int J Dev Biol. 2013;57(2-3-4):299-308*	131

Chapter 9 Seminoma and embryonal carcinoma footprints identified by analysis of integrated genome-wide epigenetic and expression profiles of germ cell cancer cell lines. 153
Submitted

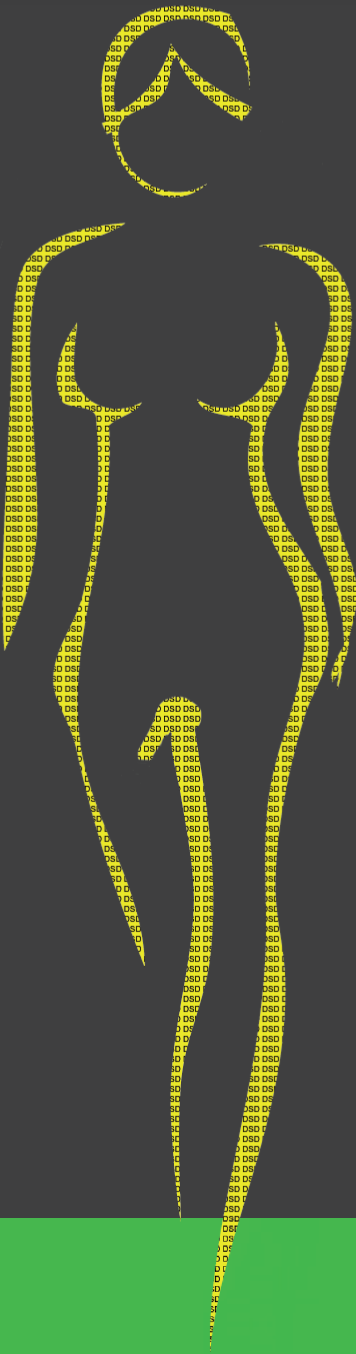
Chapter 10 General discussion 179

Chapter 11 Summary/Samenvatting 207

Chapter 12 Appendices 219
Dankwoord
List of Publications
PhD portfolio

CHAPTER 1

General Introduction



Normal gonadal development

One of the most fundamental aspects of early human development is establishment of sex, which can be defined as the biological qualities that differentiate between male and female. The process of normal sex development is strictly controlled by functionality of a number of genes, both protein encoding- and noncoding-, in which the existing networks can act both on transcription (formation of RNA) and translation (formation of protein) regulation. Sexually dimorphic development of the reproductive system is the result of three sequential processes: chromosomal sex, formation and subsequent differentiation of the bipotential gonad into either testis or ovary (referred to as sex determination), and finally sex-specific development of the reproductive tracts and external genitalia under influence of hormones produced by the gonads (referred to as sex differentiation) [1-3](Figure 1).

During the last decades, relevant genes have been discovered, both in human and mice, involved in the process of both sex determination and differentiation. These discoveries lead to the conclusion that for both formation of female and male in its final form, a cascade of combinatory events has to occur, including both activation and suppression [1, 4]. This is strictly organized, both in time and place. Disturbances in these processes can result in a disruption of the physiological processes, with minor or more significant effects. This is relevant in the context of understanding the molecular pathways during normal development in order to gain insight into various forms of Disorders of Sex Development (DSD). DSD is defined as congenital conditions in which development of chromosomal, gonadal and/or anatomical sex is abnormal. There is ongoing debate about this term, some suggest typical/atypical to be more appropriate, instead of normal/ abnormal and to use the term condition rather than disorder. Some even prefer to maintain the term intersex which

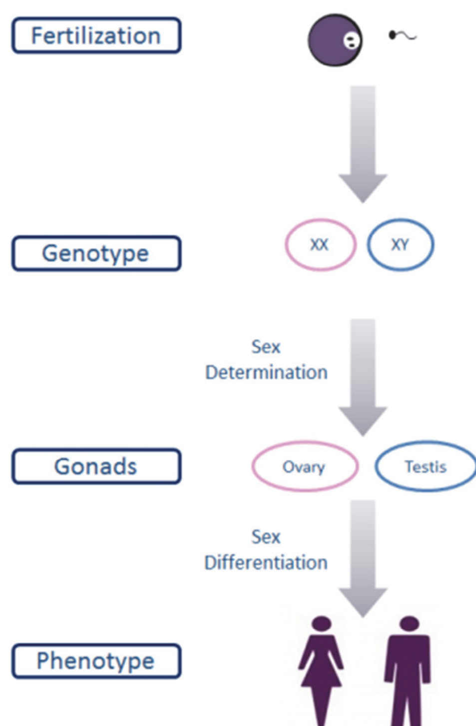


Figure 1 Schematic representation of the sequential steps in the sex development.

purposely has been abandoned [7]. One of the major clinical questions in the context of the development of the gonads in patients with DSD is the risk for malignant transformation of the germ cells. To allow detailed understanding of the possible mechanisms involved, the next paragraphs will provide a more in-depth description of the physiological processes of sex determination and differentiation. Since this thesis focusses on the human, all genes will be mentioned in capital letters unless there is only mouse data available.

Sex Determination

After fertilization the primordial germ cells (PGC), originating from the epiblast, migrate to the genital ridge. PGC are the progenitor cells of gametogenesis in later life. Once they reach the genital ridge, they are under the influence of the sex determination process of the bipotential gonad. This determines germ cell fate towards either the male or female direction and requires a highly regulated process, including multiple pathways.

The bipotential gonad originates from the intermediate mesoderm under influence of various factors like SF1, DMRT1, GATA4, WT1 and FGF9 [8, 9]. At this stage different cell types are present; precursors of the supporting Sertoli/Granulosa cells, precursors of the steroid producing Leydig/Theca cells and PGC that arrive in the bipotential gonad around week 5 human gestational age (GA), now referred to as gonocytes. To keep the gonad in a bipotential state a balanced antagonism of male (Sox9, SRY) and female (RSPO1) opposing signals has been proposed [10]. Figure 2 gives a schematic representation of male and female development and will be discussed in the next paragraphs.

Male sex determination

In the 46,XY constitution, the sex-determining region on the Y-chromosome (SRY) initiates sex determination towards the male pathway [11]. SRY is expressed in the supportive cells starting around week 6 GA. Several transcription factors, like SF1, GATA4 and WT1 (+KTS) have been implicated in SRY expression [8, 12, 13]. Wilm's Tumor 1 (WT1) is an important regulator of early gonadal and kidney development [14]. All known WT1 isoforms share four C-terminal zinc fingers which are necessary for DNA/RNA binding. The two major WT1 isoforms are produced by alternative splicing, resulting in an insertion (+KTS) or exclusion (-KTS) of lysine, threonine and serine between zinc fingers three and four. The –KTS isoform mainly plays a role in transcription and AMH transcriptional activation in Sertoli cells [15]. The +KTS isoform

is involved in RNA processing, and in the mouse plays a role in SRY regulation in vivo [16].

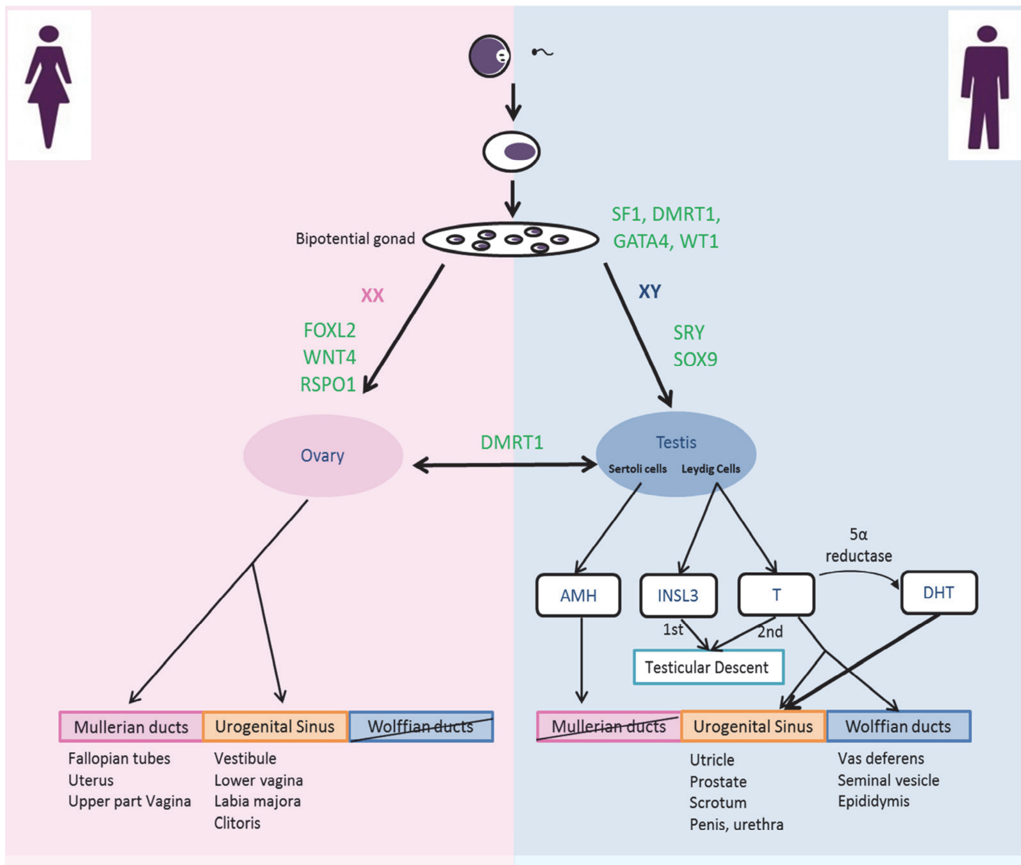


Figure 2 Detailed description of male and female phenotypic development. After fertilization, the PGC migrates to the developing (bipotential) gonad. In case of a XY chromosomal constitution, expression of SRY initiates the process of testicularization via upregulation of SOX9 which in turn activates downstream pathways. Subsequent AMH production by the Sertoli cells results in regression of the Müllerian ducts. Leydig cells form testosterone (T) and INSL3. T stabilizes the Wolffian ducts which form the vas deference, seminal vesicle and epididymis. T together with INSL3 will direct testicular descent, whereas dihydrotestosterone (DHT) together with T directs the formation of the external genitalia. In the XX fetus, the absence of SRY and activation of specific pathways involving factors like WNT4, FOXL2 and RSPO1 results in formation of an ovary. In absence of AMH, the Müllerian ducts develop along the female pathway and in the absence of androgens the formation of female external genitalia takes places.

In addition, signaling molecules involved in receptor tyrosine kinase activity (i.e. MAP3K1 in human and Map3k4 in mice) in the supportive cells are also important for the initial stages of sex determination [17-19]. Transient expression of SRY, specifically in Sertoli cell precursors, activates the downstream target SOX9 [20]. Stabilized high expression of SOX9, due to a feed forward loop involving up-regulation of FGF9 and

stimulation by prostaglandin D2, induces a downstream cascade, eventually resulting in the formation of primitive sex cords and the differentiation of somatic cells into Sertoli cells [21, 22]. During this process the size of the gonad increases due to proliferation of the Sertoli cells and influx of precursors for Leydig cells, peritubular myoid cells and endothelial cells from the mesonephros [23-27]. The presence of germ cells plays no role so far but around the 7th week GA the PGC become enclosed in the further developed sex cords and enter mitotic arrest [21]. A proper supportive micro-environment is essential for the gonocytes to fully maturate. In this respect, epigenetic factors play a role (see below).

Female sex determination

Female sex determination occurs one week later than male sex determination [28]. In the absence of a functional Y-chromosome, the fate of the bipotential gonad is directed to the ovarian lineage. In contrast to testis development, the presence of germ cells (gonocytes) is mandatory for further differentiation of the bipotential gonad into ovaries. In the absence of gonocytes, follicular cells degenerate resulting in non-functional streak gonads [29]. Ovarian follicular granulosa cells surround and nurture oocytes. Ovarian development requires activation of several genes with β-catenin being one of the crucial components driving this process. WNT4, RSPO1 and FOXL2 are factors with a demonstrated function in ovarian determination [30]. Wnt4 collaborates with Rspo1 to stabilize β-catenin that, in turn, acts to limit the expression of the male-specific gene Sox9 [31]. FOXL2 is needed throughout life to maintain the germ cells, granulosa and steroidogenic cells [4, 32-34]. Wnt4/Foxl2 double knockout XX mice show formation of testis-like tubules and spermatogonia in the ovaries, demonstrating that female sex-determining genes, are required to suppress an alternative male fate in the ovary [35]. In addition, FGF9 and WNT4 act as antagonistic signals to regulate mammalian sex determination [36].

The common thought has been that when sex determination to either the female or male pathway was initiated, the course of this pathway would be irreversible. It becomes now clear that there is a delicate balance between both paths and that even throughout life trans-differentiation may occur. As described above, FOXL2 is needed to suppress SOX9. But if FOXL2 function is lost, Sox9 expression is de-repressed in granulosa cells which then trans-differentiate into Sertoli cells and Theca cells into Leydig cells [4]. The other way around, DMRT1 prevents female reprogramming in the postnatal mammalian testis [37].

Germ cell development

A schematic representation of germ cell development is given in figure 3. PGC are the progenitor cells of gametogenesis in later life, first recognized at day E6.5 in mice and 5-6 weeks GA in humans [38]. Pre-PGC begin to express BLIMP1 mRNA and protein, which maintains the pluripotent state [39]. Pluripotency refers to a cell that is capable to self-renew and the potential to differentiate into any of the existing cell lineages. PGC originate from the pluripotent epiblast cells, therefore expressing embryonic pluripotency markers like alkaline phosphatase, OCT3/4, NANOG en c-KIT [40-42]. From the epiblast they migrate along the midline through the hindgut where they leave at its dorsal site moving towards the bipotential gonad. During migration PGC proliferate extensively [43]. The KIT pathway plays an important role in both proliferation as well as the survival of the germ cells [44, 45]. Once they reach the genital ridge (around E9-10 in mice and 6-8 weeks GA in human, then called gonocytes), they are under the influence of the sex determination process of the bipotential gonad, under control of SOX9 and FOXL2, amongst others [34, 46], into either testis or ovary. This determines germ cell fate towards either the male or female direction. While maturating, the gonocytes migrate to the basal lamina, and arrive there around the 13th week GA. Male germ cells continue to proliferate until they differentiate to pre-spermatogonia, which then enter mitotic arrest [43]. During this process they will gradually lose their embryonic markers related to pluripotency and enhance differentiation markers like TSPY and VASA [47, 48]. This process should be completed during the first year postnatal. Germ cells expressing embryonic markers beyond the first year of life are either delayed or blocked in their maturation [49, 50]. The latter are at higher risk for malignant transformation, mostly clinically manifest after puberty. Under influence of testosterone during puberty the testis will fully mature and spermatogenesis will be initiated [51]. In the female embryo, the proliferating germ cells (oogonia) will enter meiosis I arrest and are then referred to as oocytes, they then lost their pluripotency marks [52]. The ovaries will further develop and follicle formation reaches a maximum around 20 weeks GA. At birth there is a pool of a few hundred thousand follicles , gradually decreasing afterwards [53].

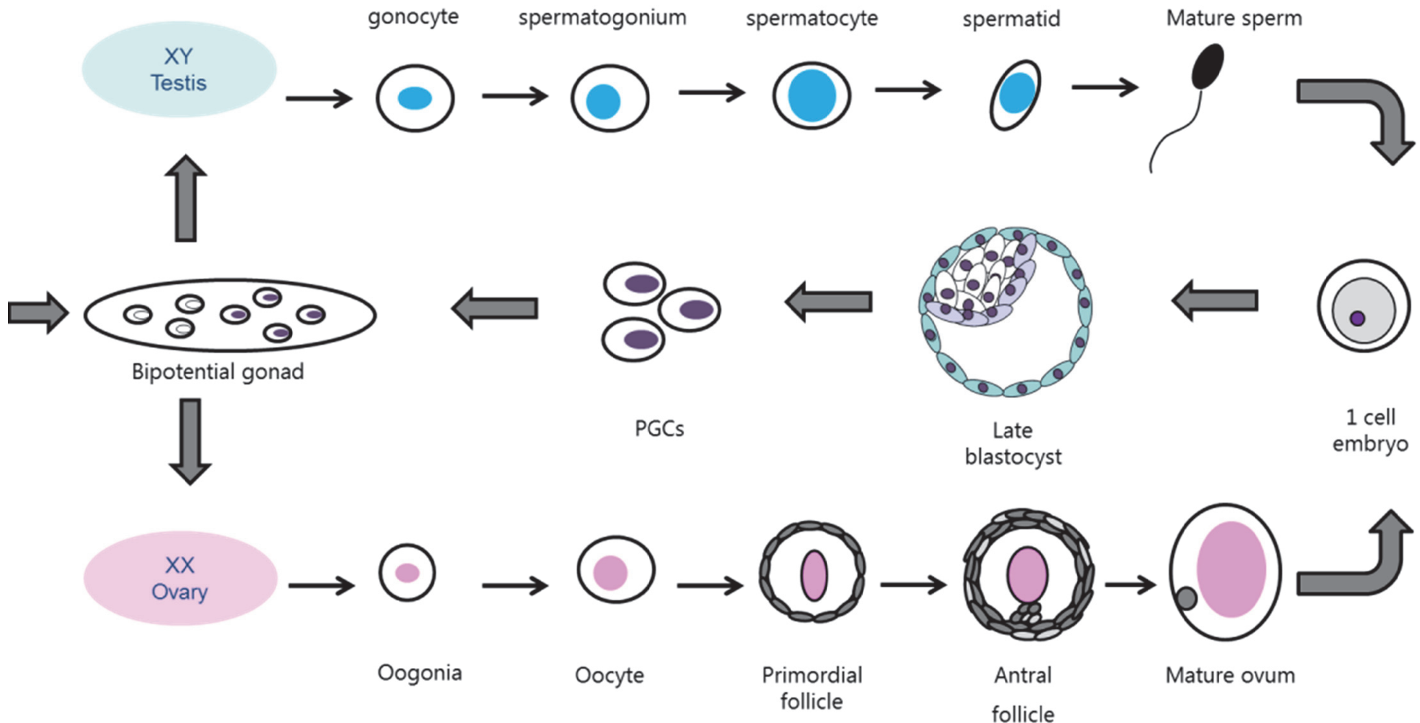


Figure 3 Schematic representation of germ cell development. PGCs undergo extensive epigenetic reprogramming and genomic imprinting is completely erased (white nucleus) before commitment to the female (XX) or male (XY) pathway. A maternal (pink nucleus) or paternal (blue nucleus) imprinting will be established depending on the presence of a testicular or ovarian environment. In case of testis formation, the PGCs/gonocytes will go into mitotic arrest and differentiate to spermatogonia. Full spermatogenesis starts at puberty. When an ovary is formed, the proliferating germ cells (oogonia) will enter meiosis I arrest, and are then referred to as gonocytes. The ovary will further develop and follicle formation takes places. At puberty ovulation starts and together with mature sperm a new life will be initiated starting with a 1 cell embryo. The primordial germ cells arise in the proximal epiblast, first present in the late blastocyst. Which then migrate to the bipotential gonad and erase their genomic imprint to be able to commit to either the female or male pathway.

Epigenetics and germ cell development

In addition to the previously mentioned changes, gene regulation plays an important role. Epigenetics is commonly defined as (possible) inheritable changes affecting gene regulation that are not due to alterations in primary DNA sequence. In fact, epigenetics is related to functionality of the genome. The epigenome is highly dynamic, and can change depending on cell type and developmental stage within a single organism. The epigenetic processes work together to establish and maintain both global as local chromatin states e.g. open or condensed which determines gene expression [54]. Epigenetic modifications are relatively stable in somatic cells. In germ cells, however, the epigenome is reprogrammed on a genome-wide level. By E12.5/10 weeks GA most DNA methylation is lost [55] and *de novo* methylation is initiated in males at E14.5, leading to highly methylated mature gametes. This allows re-establishment of parental imprints in germ cells, the erasure of epi-mutations, and the generation of toti- or multipotent cells [56-58]. Genomic imprinting results in the silencing of one of the parental alleles in a subset of genes, and is different between different tissues and cell types [59].

Sex differentiation

Around the 7th week GA, both Müllerian as well as Wolffian ducts are present in the fetus. Sex differentiation is based on hormone production by the testis and intact hormone action. Testosterone produced by testicular Leydig cells will induce the differentiation of the male reproductive tract, i.e. the Wolffian ducts into epididymis, vas deferens, and seminal vesicles. Anti-Müllerian hormone, produced by the testicular Sertoli cells, is responsible for the regression of the Müllerian ducts [60]. In the absence of AMH the Müllerian ducts will develop into normal female internal organs, i.e. fallopian tubes, uterus and upper part of the vagina [61, 62].Testosterone conversion to DiHydroTestosterone (DHT) occurs due to enzymatic action of 5α reductase. DHT has a higher affinity for the AR receptor and its binding results in a more stable complex [63]. In addition, locally DHT production is much higher compared to testosterone. Therefore, it is the action of DHT that induces virilization of the urogenital sinus and external genitalia [64]. The penile urethra and corpus spongiosum is formed by fusion of the urethral folds, the scrotum is formed by fusion of the labioscrotal swellings, both exists by the end of the first trimester and the glans forms shortly after. Elongation of the penis occurs under influence of androgens in the second and third trimester [65]. The descent of the testis can be subdivided in a transabdominal and an inguinal-scrotal phase. The transabdominal phase takes place under control of INSL3, produced by the Leydig cells. Insl3 null mice have

undescended testis and in cryptorchid patients mutations of this gene, although rare, were found to be causative [66]. The second phase is testosterone dependent.

Female genitalia form in the absence of androgens, being therefore the default pathway. There is no fusion of the urethral folds or labioscrotal swellings and the genital tubercle forms the clitoris and labia majora. The urethral and vaginal openings are separated by the anterior-posterior lengthening of the urogenital sinus. The upper part of the vagina originates from the Müllerian structures and fusion with the lower part of the vagina (originating from the urogenital sinus) is essential to complete vaginal formation.

Fetal hypothalamic GnRH pulsatile release and the hypothalamic–pituitary–gonadal (HPG) axis are functional by the end of the first trimester. At birth, plasma LH and FSH are low due to high circulating estrogen levels from the mother suppressing the hypothalamus and pituitary. In the first two weeks of life, an increase of LH and FSH is followed by an increase of testosterone [67]. In addition, inhibin B and AMH levels rise during these first two weeks of life with AMH levels continuing to increase beyond the first month of life. This process of 'mini-puberty' is thought to enhance future testicular function by creating a window for final differentiation of Germ, Leydig, and Sertoli cells. Growth of the testes has been noted during this time but not of the penis, likely to be a response to gonadotropin stimulation. The lack of testosterone response may be indicative of relative androgen insensitivity [68]. During childhood the HPG axis is silenced until puberty. Puberty will induce the final maturation steps, inducing secondary sex characteristics as well as achievement of reproductive capacity. In addition, important growth, behavioral, and psychological changes occur at puberty leading to the complete adult phenotype. Leading in this process are testosterone produced by the testis in males and estrogens produced by the ovaries in females.

Abnormal gonadal development

Normal gonadal development is a complex process involving interplay between genetic, epigenetic and environmental factors, no wonder that this sometimes goes wrong. Abnormal development varies (level of testicularization) and is related to the risk to develop germ cell cancer. This ranges from low risk, resulting from minor changes that lead to GCC in males who have apparently normal testes, to higher GCC risk in Testicular Dysgenesis Syndrome (TDS) patients with affected testis development that is not clinically assignable as a DSD, and the highest GCC risk in patients with clinically identifiable DSD. This spectrum is depicted in Figure 4 and in more detail described below.

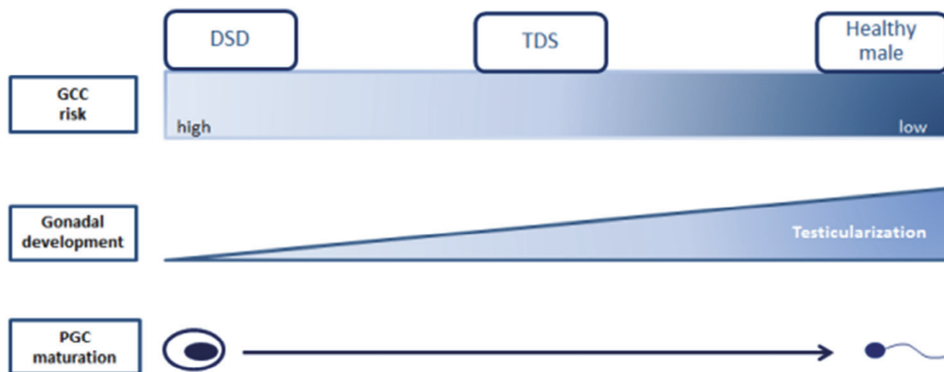


Figure 4 Representation of the phenotypic spectrum, gonadal and germ cell development in relation to GCC risk.

Testicular dysgenesis syndrome and Disorders of Sex Development

TDS syndrome was first proposed in 2001 by Skakkebaek and relates to higher GCC risk and a proposed environmental connection [69]. TDS brings a number of known clinical risk factors related to GCC together i.e. cryptorchidism, hypospadias and infertility [70]. In addition, birth weight, inguinal hernia, twinning and gestational age are factors influencing GCC risk [71]. Aberrant testis development due to influences of anti-androgen and (xeno)estrogens has been proposed as underlying cause for TDS, possibly acting through (epi)genetic mechanisms [72]. This seems to be an effect on the supportive cells rather than a direct effect on the PGC [73]. Exposure to endocrine disrupters was shown to result in demethylation of CpG islands at promoter sites, which in turn has an effect on gene transcription [74]. Animal studies have shown that all TDS symptoms can be induced by fetal exposure to anti-androgenic chemicals [75, 76]. However a direct link with GCC was not found. Taken together, the cause of TDS

and relation with GCC remains to be further elucidated. For DSD however it is known that certain subtypes do have an increased GCC risk, which will be discussed in more detail below.

DSD are defined as congenital conditions in which the development of chromosomal, gonadal or anatomical sex is atypical [77]. Its estimated overall prevalence worldwide is about 1:4500 [78]. In 2006, the Chicago consensus statement on management of intersex disorders resulted in a DSD classification based on karyotype (Table 1) [77]. This classification replaced the different terminologies used before resulting in etiology based definitions which gave new input to research in this field. DSD might be the result of a defect in establishing chromosomal, gonadal and/or phenotypic sex, thus both in sex determination (related to transcriptional regulators) and sex differentiation (related to the production and action of hormones).

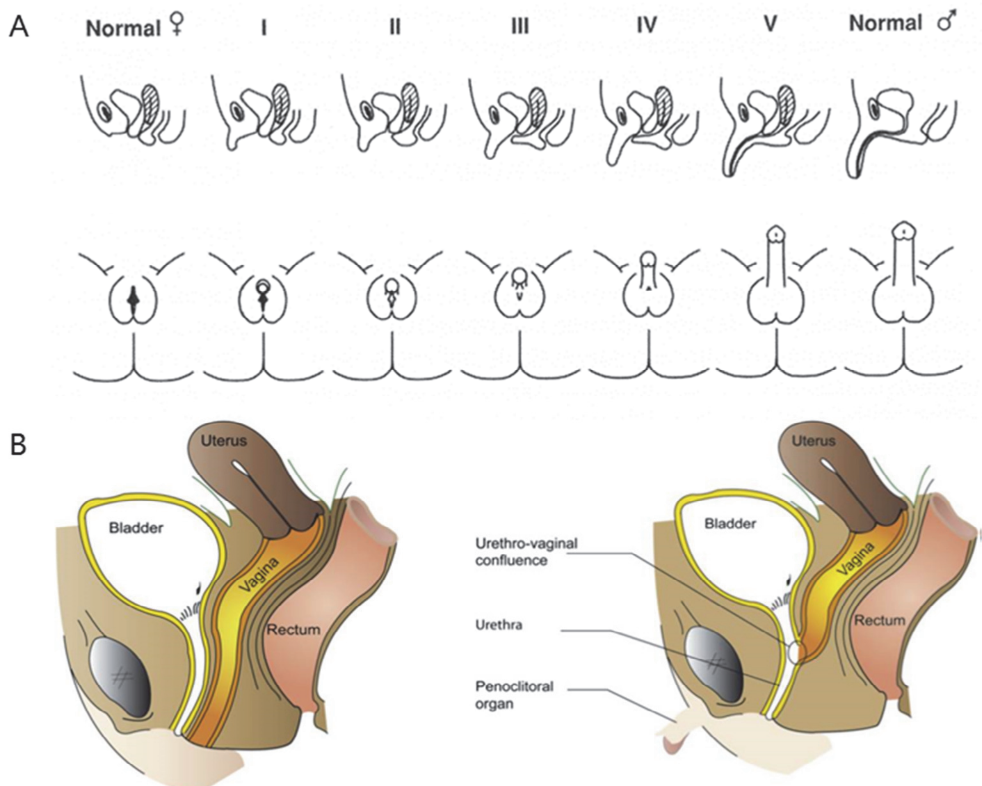


Figure 5 Two ways of defining level of virilization in patients with Congenital Adrenal Hyperplasia (CAH) A. Prader classification. Based on a scoring system where '0' is an unvirilized female and '5' a complete virilized female [5]. **B.** Level of confluence. Left: normal female anatomy; right: representation of the urethral- vaginal confluence which can be absent, low (when the junction is near the perineum) or high (when the junction is near the neck of the bladder) in patients with CAH [6].

<u>*46,XX DSD*</u>

The most common cause of 46,XX DSD is Congenital Adrenal Hyperplasia (CAH), a group of autosomal recessive disorders resulting from the deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. About 90-95% of individuals with CAH have a mutation in the *CYP21A2* gene, encoding the 21–hydroxylase enzyme [79]. There are two types of CAH; the classical, and a non-classical form or late onset CAH. Genital ambiguity at birth is present only in the classical form, which can be subdivided in simple virilizing CAH (SV-CAH) when only a defect in cortisol biosynthesis is present, and Salt Wasting CAH (SW-CAH) when the patient also show a concurrent defect in aldosterone biosynthesis. Intra- uterine androgen exposure (before week 12 GA) can lead to a variable degree of labioscrotal fusion and clitoral enlargement, after week 12 GA androgen exposure causes isolated clitoromegaly. Level of virilization is described by Prader stage or level of confluence as depicted in figure 5. Patients with the non-classical form and untreated or insufficiently treated patients with the classical form have manifestations of androgen excess such as hirsutism, menstrual dysfunction, and acne [80].

46,XX Gonadal dysgenesis is characterized by abnormal ovarian determination subdivided into three categories [77, 81, 82] :

1. Testicular DSD: complete sex reversal i.e. XX male, usually caused by a translocation of SRY to the X-chromosome.
2. Ovotesticular DSD: this term defines the presence of both ovarian and testicular tissue in the same individual. Ovarian tissue should contain stroma and normal appearing follicles, whereas seminiferous tubules must be present in the testicular tissue.
3. Gonadal dysgenesis: all other forms of 46,XX gonadal dysgenesis

Additional causes of 46,XX DSD (Table 1) are extremely rare and not further described in this thesis.

Sex chromosome DSD	46,XY-DSD	46,XX-DSD
47,XXY (Klinefelter syndrome and variants)	**Disorders of gonadal (testicular) development**	**Disorders of gonadal (ovarian) development**
45,X (Turner syndrome and variants)	1. Complete or partial gonadal dysgenesis	1. Gonadal dysgenesis
45,X/46,XY (mixed gonadal dysgenesis)	2. Ovotesticular DSD	2. Ovotesticular DSD
46,XX/46,XY (chimerism)	3. Gonadal regression	3. Testicular DSD
	Disorders in androgen synthesis or action	**Androgen excess**
	1. Disorders of androgen synthesis	1. Fetal
	a. LH receptor mutations	a. 3 β-hydroxysteroid dehydrogenase 2
	b. Smith-Lemli-Opitz syndrome	b. 21-hydroxylase
	c. Steroidogenic acute regulatory protein mutations	c. P450 oxidoreductase
	d. Cholesterol side-chain cleavage	d. 11 β-hydroxylase
	e. 3 β-hydroxysteroid dehydrogenase 2	e. Glucocorticoid receptor mutations
	f. 17 β-hydroxysteroid dehydrogenase	
	g. 5 α-reductase 2	2. Fetoplacental
		a. Aromatase deficiency
	2. Disorders of androgen action	b. Oxidoreductase deficiency
	a. Androgen insensitivity syndrome	
	b. Drugs and environmental modulators	3. Maternal
		a. Maternal virilizing tumours
		b. androgenic drugs
	Other	**Other**
	1. Syndromic associations of male genital development (e.g. cloacal anomalies, Robinow, Aarskog)	1. Syndromic associations (e.g. cloacal anomalies)
	2. Persistent Müllerian duct syndrome	2. Müllerian agenesis/hypoplasia
	3. Vanishing testis syndrome	3. Uterine abnormalities
	4. Isolated hypospadias	4. Vaginal atresia
	5. Congenital hypogonadotropic hypogonadism	5. Labial adhesions
	6. Cryptorchidism	
	7. Environmental influences	

Table 1 DSD classification [74]

46,XY DSD

Undervirilization is characterized by the presence of an ambiguous/female phenotype in 46,XY individuals with bilateral testis, in whom testosterone production and/or action is inadequate. Most common in this group is androgen insensitivity syndrome. In addition there is a large group in which no definite diagnoses can be made referred to as 'undefined 46,XY DSD' or severe hypospadias. Complete androgen insensitivity syndrome (CAIS) is characterized by female external genitalia, a short, blind ending vagina, the absence of Wolffian duct derived structures, breast development and the absence of pubic and axillary hair. Patients with CAIS present with inguinal hernia in the first years of life or at puberty when menarche is not initiated. Testosterone and LH levels are elevated at the time of puberty. Partial androgen insensitivity syndrome (PAIS), characterized by a partial action of testosterone, could lead to a range of phenotypes, from mostly female, to ambiguous or predominantly male [83, 84].

46,XY Gonadal Dysgenesis (GD) is characterized by abnormal testis determination, divided in three forms:

1. Complete/Partial GD: complete/partial lack of testicular development. The complete forms have bilateral streak gonads, well developed Müllerian structures, absent Wolffian structures and female phenotype, whereas the

partial form have bilateral dysgenetic testis and a mix of Müllerian and Wolffian structures, ambiguity of the external genitalia is variable.

2. Ovotesticular DSD: described above
3. Gonadal regression: also referred to as vanishing testis, during surgery only the presence of a fibrovascular nodule with associated macrophages and dystrophic calcification are present. Residual testicular tubules are found in less than 10% of the cases [85].

Testosterone produced by testicular Leydig cells will induce the differentiation of the male reproductive tract, i.e. the Wolffian ducts into epididymis, vas deferens, and seminal vesicles. Testosterone synthesis disorders are caused by an enzyme defect in the biosynthesis of testosterone/dihydrotestosterone [64]. Mutations in the genes that codify the enzymes acting in the steps from cholesterol to DHT have been identified in affected patients. Patients with 46,XY DSD secondary to defects in androgen production show a variable phenotype, strongly depending of the specific mutated gene. Often, these conditions are detected at birth due to the ambiguous genitalia [86]. Anti-Müllerian hormone (AMH), produced by the testicular Sertoli cells, is responsible for the regression of the Müllerian ducts. In the absence of AMH the Müllerian ducts will develop into normal female internal organs [62]. Persistent Müllerian Duct Syndrome (PMDS) is characterized by the presence of a uterus, fallopian tubes and the upper part of the vagina in phenotypic normal male patients, and is usually discovered at surgery for cryptorchidism or inguinal hernias.

Chromosomal DSD

This group comprises of all patients with an aberrant chromosomal pattern, including Turner and Klinefelter syndromes who are not the subject of this thesis. The mosaic gonadal dysgenesis patients however are, and usually present with mixed gonadal dysgenesis. The usual gonadal pattern is a streak gonad on one side and a dysgenetic or normal appearing testis on the other side of the abdomen. Müllerian and Wolffian duct development usually correlates with the character of the ipsilateral gonad. Then there is a rare form of chromosomal DSD, 46,XX/ 46,XY karyotype, which is thought to present a chimera. Affected patients present with ambiguous genitalia, the degree of which is related to the dominance of 46,XX of 46,XY cells. In addition, ovotesticular DSD could occur [87].

The next paragraph will give an introduction on GCC, after which GCC will be discussed in the context of DSD.

Germ cell cancer

Germ cell cancers arise from the germ cell lineage and are subdivided in five separate groups based on their clinical and patho-biological characteristic [88]. Type II GCCs (further referred to as GCC) are related to TDS and DSD and therefore discussed here.

Although the overall incidence is low, GCCs are the most common malignancy in Caucasian adolescents and young adults and their incidence is still rising [89, 90]. In addition, there are populations (TDS, DSD) that have an increased risk, and specific forms of DSD have a high risk to develop GCC [49]. The incidence differs between European countries (highest incidences in Switzerland and Denmark) and worldwide (less frequent in Asian and African populations)[91]. Of interest is that the incidence rates among immigrants to Sweden showed similar rates to the men in their country of origin, whereas this changed to the Swedish risk in the second generation, indicating that in utero environmental exposures contribute to the GCC risk [92]. These findings were more pronounced for seminoma compared to non-seminoma [92].

The precursor lesion of GCC is carcinoma *in situ* (CIS) in the testis, also known as Intratubular Germ Cell Neoplasia Unclassified (IGCNU) and Testicular Intratubular Neoplasia (TIN) and GonadoBlastoma (GB) in the undifferentiated/dysgenetic gonad/ovary (related to DSD)[93-95]. Both are derived from an embryonic germ cell, blocked in their maturation in an early stage of development and are known to progress to an invasive GCC when left untreated [94, 96]. GCC arise from PGC or gonocytes and are subdivided into seminomas/dysgerminomas and non-seminomas [97]. Non-seminomas can be further categorized into embryonal carcinoma, which can differentiate into somatic lineages and extra – embryonic tissues (teratoma vs yolk sac tumor and choriocarcinoma respectively) (see also Figure 6) [97]. An important diagnostic marker for GCC detection is OCT3/4, a pluripotency factor, which is positive in CIS, seminomas en embryonal carcinomas [98]. To differentiate between seminomas and embryonal carcinomas, SOX17 and SOX2 proved to be informative. SOX17 is positive in CIS/GB and seminomas, as well as normal spermatogonia, whereas SOX2 is positive in embryonal carcinoma [99]. Together, this set of markers will allow diagnosis of these GCCs. PGC have the intrinsic capacity for pluri/totipotency, reflected in GCC, in which even the germ line can be formed in non–seminomatous tumors [96].

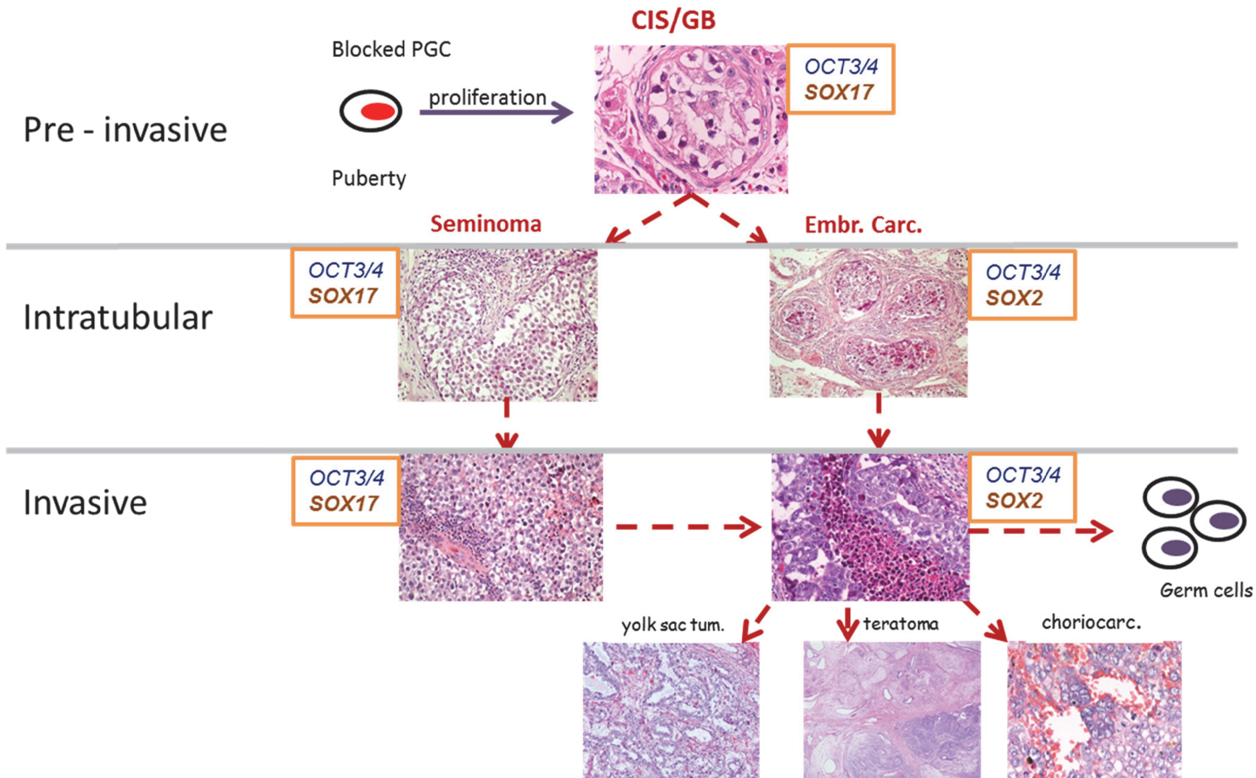


Figure 6 Pathogenesis of GCC and related diagnostic markers. GCCs arise from an embryonic germ cell blocked in its maturation (OCT3/4 positive) and is supposed to start proliferating during puberty under influence of androgens. The precursor lesion is CIS in a testis environment and GB in a dysgenetic/streak environment, both will progress to an invasive GCC (via an intratubular stage) when left untreated. GCC are subdivided into seminomas and non-seminomas who can be distinguished based on the presence of either SOX17 or SOX2. Non-seminoma can be further categorized in embryonal carcinoma, which can differentiate into somatic lineages and extra – embryonic tissues, even the germ line can be formed.

Ten year survival of patients with a GCC is high, approximately 95%, due to effective treatment strategies like surgery, irradiation and/or chemotherapy [100]. Seminomas and non-seminomas differ from other solid cancers in their overall responsiveness to DNA damaging agents, including irradiation and cisplatin-based chemotherapy, a chemotherapeutic agent which binds to DNA, inducing crosslinks, which ultimately triggers apoptosis [101]. Seminomas are usually sensitive to chemotherapy with cisplatin, whereas the response of non-seminomas differs according to their histology [102, 103]. Since GCC arise in early adulthood, the impact of the long term side effects of systemic therapy is high. These side effects include heart- and vascular disease, infertility, metabolic syndrome, cognitive impairment and secondary malignancies [104].
It is therefore important to identify risk factors that will allow early diagnosis, thereby preventing the necessity of systemic therapy.

Disorders of Sex Development and Germ Cell Cancer

DSD form an intriguing model to study the impact of intrinsic and environmental factors on normal and abnormal gonadal development. Indeed the diagnosis of 46,XY and chromosomal DSD is a risk factor for the development of GCC, with higher risk associated with an earlier block in differentiation. Other risk factors include anatomical position of the gonad, the presence of Y-chromosomal material (GBY region), genetic and epigenetic anomalies (see Figure 7) [49, 105]. This supports the theory that developmental disturbances of the micro-environment could result in inadequate maturation of the germ cells. This may result in a fetal epigenetic profile which, upon hormone stimulation during puberty, leads to an aberrant induction of transcription and proliferation, ultimately leading to GCC later in life [106]. It has been shown that seminomas are more often found in abdominal testes compared to scrotal testes [107]. This might also explain the occurrence of dysgerminomas in the ovary and dysgenetic gonads, which are both located in the abdomen. Indeed, seminoma and dysgerminomas are similar in morphology and gene expression profile, and therefore might have the same epigenetic profiles [108-110].

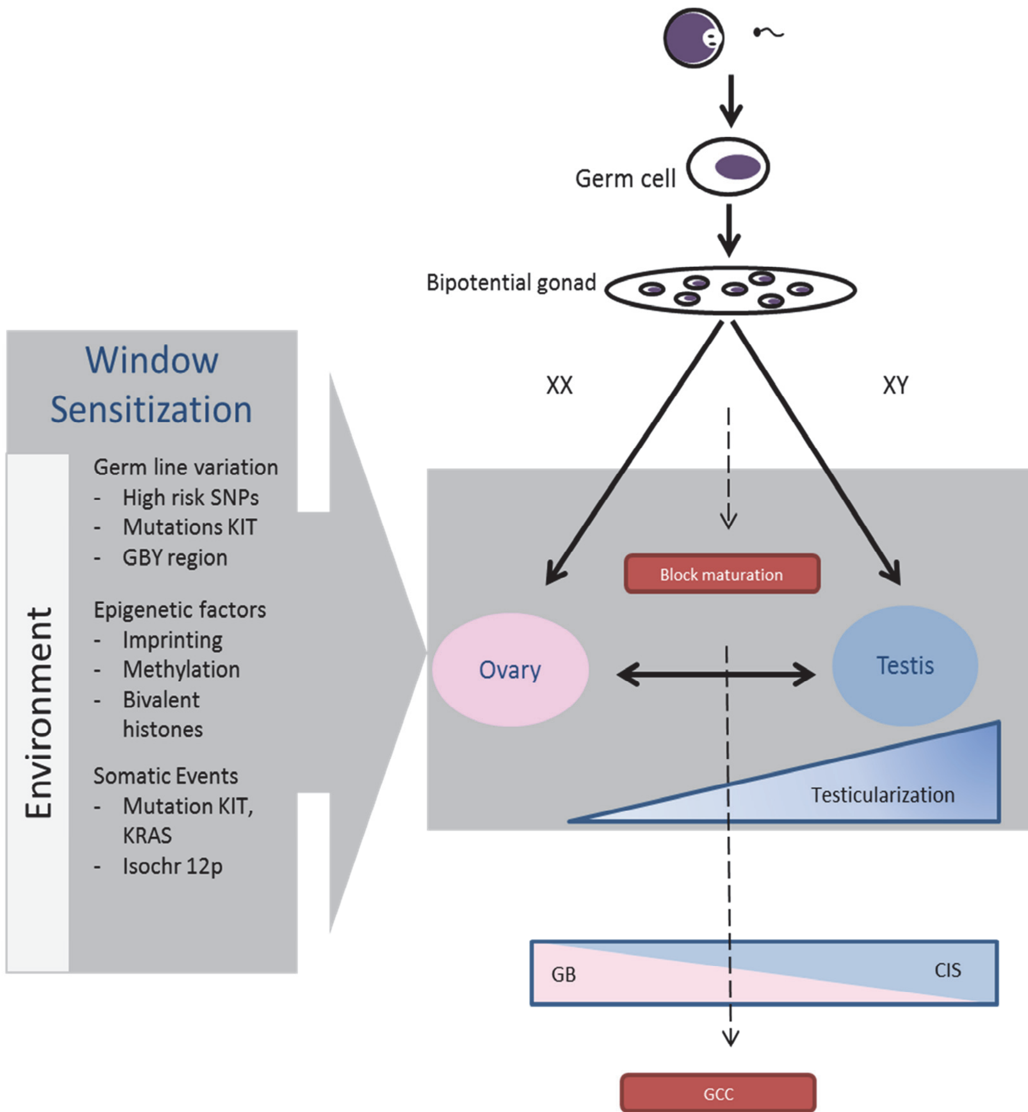


Figure 7 Window of sensitization. During gonadal development, a specific window (grey area) exists in which the germ cells and micro-environment are susceptible for genetic and epigenetic parameters. In addition, there is evidence that the environment has influences on both genetic and epigenetic level during the same window.

Prolonged expression of OCT3/4 and increased expression of TSPY are thought to play a role in the survival and proliferation of CIS and GB cells. TSPY expression is only sometimes expressed at a higher level in seminomas. In addition, TSPY was found to be a repressor for androgen signaling due to entrapping of the cytosolic androgen receptor, even in the presence of androgens. Androgen treatment stimulated cell proliferation and TSPY expression was found to be reduced in more malignant GCC [111]. These results underline the theory that the androgen-estrogen balance is important in the etiology of GCC.

Important to notice is that in patients with cryptorchidism or DSD, delayed maturation of germ cells can occur. These germ cells express OCT3/4 after the age of one, therefore this could lead to overdiagnosis of CIS. To distinguish germ cells delayed versus blocked in their maturation, the ligand of the aforementioned tyrosine kinase receptor c-KIT (i.e. SCF) was found to be informative. SCF is found to show a positive staining in CIS/GB cells while absent in germ cells showing maturation delay [112].

Disorders of Sex Development and outcome

In addition to GCC it is important to study endocrine, surgical and psychosexual outcomes in a systematic way to evaluate the management of patients with DSD and to develop evidence based guidelines for future management. It is widely recognized that care for DSD patients should be directed by a multidisciplinary team to allow all different aspects to be included. Most long term outcome data is available for CAH. The excessive amount of androgens circulating in the fetus probably also mould brain development in a masculine direction, leading to virilization of personality and cognitive, psychosocial, and psychosexual functioning [113, 114]. Female patients with moderate or severe genital virilization may undergo feminizing surgery, e.g. clitoroplasty, vaginoplasty, and labioplasty, with the aim to create female appearing external genitalia, and to enable sexual intercourse. Genital surgery is controversial since loss of sensitivity resulting in diminished sexual functioning, has been reported [115-120]. However, available data are conflicting possibly because of confounding factors. The underlying chromosomal constitution of infants with markedly ambiguous genitalia may be 46,XY, 46,XX or a mosaic pattern. Improved reconstructive techniques and observations of gender dysphoria and a wish for a gender role change in patients with 46,XY hypovirilization raised as girls [121, 122] resulted in more male sex assignments in the last decades, particularly in patients with less severe hypovirilization [77, 123-125]. The aim of masculizing surgery in DSD patients is to improve cosmesis and function of the external genitalia, to enable sexual intercourse

and to avoid stigmatization. It is therefore important to assess the functional and sexual outcome of these patients. There have been a limited number of outcome studies in male DSD patients, including those with a undefined 46,XY DSD [123, 126-130], but in general studies with combined data on urological- and in-depth psychological examination in relation to surgical history are scarce. It is thus important that systematic long-term outcome data will be generated for all categories of DSD patients to be able to develop guidelines for future management.

Aims and outline of this thesis

DSD is important to study from a number of perspectives. Since DSD occurs due to disruption in the earliest stages of sex determination or sex differentiation, it will give insight into the processes involved in gonadal development.

To improve medical care for patients with DSD, outcome studies are highly relevant. These studies need to address surgical, cosmetic and psychosexual outcomes. Therefore in **Chapter 2** and **3** we performed a cross-sectional study to determine these outcomes in CAH women and DSD males respectively. In addition, care of DSD patients had changed over the last decades due to insights in molecular biological mechanisms underlying these disorders, screening for CAH, advanced surgical techniques and GCC risk awareness. Therefore we performed a study, presented in **Chapter 4**, to determine distribution of the various DSD diagnoses and the age of first presentation. The overarching aim was to see if we could determine shifts in management regarding age at gonadectomy, number of surgeries, and to estimate the incidence of GCC and its precursors.

Embryonic development is strictly controlled by functionality of genes in which the existing networks can act both on transcription and translation regulation. When a Y chromosome is present, these patients might have an increased risk to develop GCC. These cancers are unique because of a number of characteristics. In spite of their clinical presentation, i.e. predominantly after puberty, they arise from primordial germ cells/gonocytes that have failed appropriate maturation to either pre-spermatogonia or oogonia. In 15% of the patients with 46,XY DSD a mutation in *SRY* is found and therefore routinely screened in patients with compete GD. In **Chapter 5** we describe a novel *SRY* mutation in a patient with complete GD and bilateral GB. Late onset progressive kidney failure triggered the analysis of *WT1*, and indeed a pathogenic *WT1* intron 9 splice-site mutation was found, known to be causative for Frasier Syndrome (being a variant of 46,XY DSD), in addition to the *SRY* mutation.

Interaction with the micro-environment is essential for normal maturation and it is therefore thought that the deregulation of supportive cells, thereby not properly nourishing the germ cell could be the underlying cause. **Chapter 6** describes *MAP3K1* as a GCC susceptibility gene, aberrant expression could influence malignant transformation through a direct effect on the germ cell or indirect via the environment/Sertoli cells.

Functional Sertoli cells produce AMH which causes regression of the Müllerian ducts; **Chapter 7** describes a patient with PMDS caused by a novel mutation, and biopsy of both gonads showed irregular distribution of germ cells but no signs of abnormal maturation.

Since mutations are rarely found in GCC, epigenetic deregulation, possibly caused by environmental factors, could be one of the underlying mechanisms, described in more detail in **Chapter 8**. Therefore, in **Chapter 9**, we compared the epigenetic profiles of TCam-2 and NCCIT (representative for seminoma and non-seminoma, EC respectively) related to expression to determine differences between SE and NS and possibly linking environmental factors with genetic aberrations.

A detailed insight into the mechanisms that cause GCC in patients with DSD, combined with clinical characteristics will lead to better risk stratification and in the future allows individualized screening and guidelines whether or not to perform prophylactic gonadectomy.

Together, the overarching aim of this thesis is to improve the care for patients with DSD with respect to guidelines for multidisciplinary management based on a better insight of the biology of normal and abnormal gonadal development combined with knowledge from long term outcome studies which will be discussed in **Chapter 10.**

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CHAPTER 2

Severity of virilization is associated with cosmetic appearance and sexual function in women with Congenital Adrenal Hyperplasia: A Cross – Sectional Study

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Abstract

Introduction:

Women with the classical form of Congenital Adrenal Hyperplasia (CAH) are born with different degrees of virilization of the external genitalia. Feminizing surgery is often performed in childhood to change the appearance of the genitalia and to enable penile-vaginal intercourse later in life. There are suggestions that this affect sexual functioning.

Aims:

To study the anatomical, surgical, cosmetic and psychosexual outcomes in women with CAH.

Methods:

Forty women with CAH, aged over 15 years, from two referral centers for management of Disorders of Sex Development in the Netherlands were included. Physical and functional status was assessed by a gynaecological interview and examination. Sexual functioning was assessed with the FSFI and FSDS-R scales and compared to a reference group.

Results:

36 of the 40 women had undergone feminizing surgery; 25 (69%) underwent more than one operation. Re–surgery was performed in seven of the 13 (54%) women who had had a single stage-procedure. Anatomical assessment showed reasonable outcomes. Multiple linear regression showed that only level of confluence had a significant effect on cosmetic outcome, the impact depending on the number of surgeries performed. Cosmetic evaluations did not differ between the women and the gynaecologists. Only twenty women had experience of intercourse. Eight women reported dyspareunia; seven reported urinary incontinence. The women's perceived sexual functioning was less satisfactory than in the reference group, and they reported more sexual distress.

Conclusion:

The level of confluence was the major determinant for cosmetic outcome; the impact depended on the number of surgeries performed. Fifty four procent of the women required re-surgery after a single-stage procedure in childhood. Anatomical assessment showed reasonable outcomes. The women evaluated their sexual functioning and functional outcome less favourable than the reference group and they experienced less often sexual intercourse.

Introduction

Congenital Adrenal Hyperplasia (CAH) is a group of autosomal recessive disorders resulting from the deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. About 90-95% of individuals with CAH have a mutation in the *CYP21A2* gene, encoding the 21–hydroxylase enzyme [1].

There are two types of CAH; the classical, and a non-classical form or late onset CAH. Genital ambiguity at birth is present only in the classical form, which can be subdivided in simple virilizing CAH (SV-CAH) when only a defect in cortisol biosynthesis is present, and salt wasting CAH (SW-CAH) when the patient also show a concurrent defect in aldosterone biosynthesis. Patients with the non-classical form and untreated or insufficiently treated patients with the classical form have manifestations of androgen excess such as hirsutism, menstrual dysfunction, and acne [2]. The excessive amount of androgens circulating in the fetus probably also mould brain development in a masculine direction, leading to virilization of personality and cognitive, psychosocial, and psychosexual functioning [3, 4].

Female patients with moderate or severe genital virilization may undergo feminizing surgery, e.g. clitoroplasty, vaginoplasty, and labioplasty, with the aim to create female appearing external genitalia, and to enable sexual intercourse.

Genital surgery is controversial since loss of sensitivity resulting in diminished sexual functioning, has been reported [5-12]. However, available data are conflicting possibly because of confounding factors.

Aims:

To study the long term anatomical, surgical, cosmetic, and psychosexual outcomes of a large cohort of women with CAH.

Methods

Study Design:

Cross-sectional study

Patients:

Eighty nine patients with CAH (>15 yrs of age) were invited to participate in the study between 2007 and 2009. Participating centers were ErasmusMC Rotterdam (n=53) and Radboud University Nijmegen MC, Nijmegen (n=36), the Netherlands. The study was approved by the Medical Ethics Committees of both centres. Participants signed a

written consent. Participants were free to refuse parts of the gynaecological examination or the psychological assessment.

Procedure:

The study consisted of two parts. Data on genital virilization at birth and genital surgery were collected retrospectively from medical files. Patients were invited to take part in a follow-up study consisting of a standardized gynaecological examination, psychosocial assessment on sexual functioning, and an interview.

Outcome measures:

Surgery and level of confluence:

A description of the urogenital sinus (UGS) was available in the medical records [13]. Therefore the degree of virilization was classified by the level of confluence of the vagina into the UGS at birth. Three levels were distinguished: low, i.e the junction of the vagina and urethra is near the perineum (n=14); high, i.e. the junction is near the neck of the bladder (n=8); and no confluence, i.e. clitoral hypertrophy only (n=5). From 13 patients we had no data on level of virilization.

Gynaecological examination:

The standardized gynaecological examination consisted of visual inspection (size of clitoris, labia majora and labia minora; pigmentation; meatus externus urethrae, hair growth, labial scarring, perineal length), speculum examination (assessment of vagina, internal hair growth, granulation tissue, epithelial atrophy, presence of a cervix) and pelvic examination (accessibility of the vagina by number of fingers, vagina length, and width (measured by Hegar), strictures, pelvic floor tone, vaginal discharge).

The three gynaecologists who performed the gynaecological examination had not been involved previously in the care of these patients.

The gynaecologist, and the patient herself independently rated the general appearance and the appearance of different parts of the vulva (i.e. clitoris, labia majora and minora) on a 1 to 10 scale (1=extremely poor, 10=excellent, <6 was considered insufficient). The cosmetic outcome score per patient was calculated as the mean of the gynaecologists' scores for the different parts of the genitalia.

Psychosexual Assessment:

Psychosexual functioning was assessed by the Dutch versions of the Female Sexual Function Index (FSFI) [14, 15] and the Female Sexual Distress Scale - Revised (FSDS-R) [15, 16].

The FSFI assesses sexual functioning by six key dimensions of female sexual function in the preceding four weeks: desire, subjective arousal, lubrication, orgasm,

global emotional/relational satisfaction and pain. The FSDS-R assesses perceived stress with respect to sexuality. The combined results of the FSFI and FSDS-R suggest the diagnosis of one or more sexual dysfunction(s) according to the Diagnostic and Statistical Manual of Mental Disorders (Version four, text revision)[17]. A FSFI score < 26.55 combined with a FSDS-R score > 11 implies the existence of at least one sexual dysfunction according to the DSM-IV-TR [17].

Since questions in the FSFI and FSDS-R relate mainly to the 4-week period before completing the surveys, and some items can only be filled out when having a partner (i.e. satisfaction domain of the FSFI), or when having intercourse (i.e. pain domain of the FSFI), a 'valid' total FSFI score could only be calculated for a minority of the women. Therefore we did not interpret zero responses ('no sexual activity in last 4 weeks') as extreme degrees of dysfunction, but excluded these women from further analyses [18, 19].

Patients' psychosexual scores were compared to data of a Dutch reference group of 108 healthy women with mean age 27.1 years (SD 9.4) [15]. These authors had made up a reference group of women who were all engaged in heterosexual partner relationships, and had reported they had no sexual dysfunctions. By making use of these inclusion criteria, the reference group is probably not representative for the Dutch female population. Analysis showed that the reference group is similar to our study group with respect to age (p= 0.43) and educational level (p=0.67). On the FSFI and FSDS-R the reference group did not report problems in psychosexual functioning.

The psychological interview inquired about the age sexual developmental milestones had been passed, such as age at first engagement in a romantic relationship, including kissing and touching the partner without sexual intercourse, and age at sexarche (i.e. first peno-vaginal intercourse).

Statistical analysis:

Univariate and backwards stepwise linear regression analysis were used to identify factors associated with cosmetic outcome. Intercorrelated variables were evaluated for the presence of confounding and/or effect modification in stratified analysis. Effect modification was twice identified: between 'level of confluence', and 'number of surgeries' , and between 'level of confluence', and 'age at first surgery'. We looked for a significant contribution of the interaction effects to the predictive ability of the model by adding the interaction effects to the main effects (backwards stepwise linear regression analysis).

Comparisons between groups were assessed with descriptive statistics; the chi square test for nominal/ordinal variables, Student's t-test for normally distributed

continuous variables, and the Wilcoxon signed–rank test for paired variables with skewed distributions. Comparisons of continuous variables with skewed distributions were evaluated with the Mann-Whitney U test (two groups) or the Kruskal Wallis test (three or more groups).

A p-value <0.05 (two sided) was considered a significant difference. As patients were free to refuse parts of the gynaecological examination and the psychological assessment, number of participants may vary across analyses.

Results

Patient Group

Forty of the 89 invited women participated (response rate: 45%). The remaining 49 either declined participation (45%), or could not be reached personally (i.e. by phone, email or post mail). Characteristics from the non-responders and participants are depicted in table 1. By inspection, it seems that the groups did not significantly differ in the medical variables. Of the 40 participants, 38 fulfilled the criteria of 21 hydroxylase deficiency (32 had salt wasting CAH (SW-CAH), 6 had simple virilizing CAH (SV-CAH)), and two had 11 Beta-hydroxlyase deficiency. The median age at participation was 29 years (range 15 to 46).

Characteristics	Participant	Non- Responders	P- values
Mean age (range)	29 (15-46)	30 (18-54)	0.75[1]
Type			0.88[2]
SW	32	37	
SV	6	9	
LO	2	3	
LOC			0.51[2]
High	8	10	
Low	14	15	
No	5	12	
Unknown	13	12	
Mean number of surgeries (range)	1.8 (0-4)	1.5 (0-5)	0.15[3]

Table 1 Age and medical characteristics of non-responders and participants. We did not find statistical differences between the two groups. Abbreviations: SW = salt wasting, SV= simple virilizing, LO= late onset, LOC= level of confluence. 1 Students t test, 2 Chi square test, 3 Mann Whitney U test.

	Age (years)	BMI	Height (SDS)	Partner	CAH Type	Therapy Glucocorticoids	Mineralcorticoids	Menarche
1	36	23.4	-1.64	No	SW	Hc 15-0-20mg	Fc 62.5-62.5 ug	13
2	37	28.3	-1.02	No	SV	Dexa 0.25 mg		14
3	28	23.9	-0.86	Yes	LO	Hc 10-5-5mg		13
4	37	24.8	-0.40	Yes	SW	Hc 10-0-25mg	Fc 62.5 ug	15
5	32	36.3	-0.71	No	SW	Hc 10-5-5mg	Fc 62.5-94 ug	17
6	23	25.7	2.07	Yes	SW	Hc 15-0-20mg	Fc 125-125 ug	15
7	29	26.3	-1.33	Yes	SW	Hc 7.5-5-2.5mg	Fc 62.5-62.5 ug	16
8	22	27.8	-2.72	No	SW	Hc 10-10mg	Fc 62.5-62.5 ug	13
9	38	32	-2.10	Yes	SW	Dexa 0.125-0.125mg	Fc 125-125 ug	15
10	36	23.3	-1.17	No	SW	Dexa 0.25-0.25mg	Fc 62.5 ug	11
11	18	25.4	-3.03	No	SW	Dexa 0.25-0.25mg	Fc 62.5 ug	12
12	21	27	-1.64	No	SW	Hc 15-10mg	Fc 30-30 ug	12
13	28	22.6	1.61	No	SW	Hc 5-5-15mg	Fc 25-25 ug	16
14	22			No	SW	Hc 10-10mg	Fc 50-50 ug	
15	19	24.6	0.06	Yes	LO	Hc 10-10mg		15
16	24			Yes	SW	Hc 10-5-10mg	Fc 62.5-62.5 ug	
17	30			Yes	SW	Hc 15-5-5mg	Fc 62.5-62.5 ug	
18	40	29.1	-0.40	Yes	SW	Dexa 0.50mg	Fc 62.5 ug	18
19	40	25.3	-1.02	Yes	SW	Hc 10-10mg	Fc 125 ug	12
20	26			Yes	SW	Hc 10-15mg	Fc 62.5-62.5 ug	
21	23			Yes	SW	Dexa 0.25-0.25mg	Fc 45-45 ug	
22	19	28.2	-1.17	No	SW	Dexa 5mg	Fc 62.5 ug	16
23	34			Yes	SV	Hc 20-0-10mg		
24	44	29.3	-1.33	Yes	SV	Dexa 0.5mg		13
25	24	35.5	-2.88	Yes	SW	Dexa 0.5-0.5mg	Fc 100-100 ug	11
26	16	21.8	-0.94	No	SW	Hc 8-4-8mg	Fc 62.5-62.5 ug	14
27	19			Yes	SV	Hc 10-10mg		
28	38	19.6	-2.10	No	SW	Hc 10-10mg	Fc 100 ug	14
29	46	26.1	-3.34	Yes	SW	Cortisone 5-5-5mg	Fc 62.5 ug	12
30	22	22.6	-2.72	Yes	SW	Hc 20mg, Dexa 1,5mg	Fc 100-50 ug	15
31	46	29.7	-1.17	Yes	SW	Hc 25mg	Fc 62.5 ug	13
32	44	22.8	-0.86	Yes	SW	Hc 15-15mg	Fc 62.5-62.5 ug	15
33	35	26.7	-2.72	No	SW	Hc 7.5-5-12.5mg	Fc 125-125 ug	12
34	18	20	-2.41	No	SV	Hc 12mg, dexa 0.2mg		12
35	19	22.6	-0.17	No	SV	Hc 10-10-10mg		16
36	46	37.3	-3.88	No	SW	Hc 25mg	Fc 62.5-62.5 ug	14
37	25	26	-1.95	No	SW	Hc 15-10mg, Dexa 0.10mg	Fc 40 ug	17
38	32	23.6	-1.10	Yes	SW	Hc 10-10mg	Fc 62.5- 62.5 ug	15
39	45	28.8	-2.10	Yes	SW	Hc 15-15mg	Fc 75-75 ug	17
40	20	19.7	-4.34	No	SW	Hc 6-3-3mg	Fc 62.5-62.5 ug	15

Table 2 Main features of the participants. All visited the endocrinologist on a regular basis. Only one had hirsuitism (patient number 30, Ferriman Gallway Score for hirsuitism was 25). In general the women were small, with 12 women below -2 SDS in height. Abbreviations: SW= salt wasting, SV= simple virilizing, LO= late onset, Hc= Hydrocortisone, Dexa= Dexamethasone, Fc= Fludrocortisone.

Most patients with SV-CAH, and those with SW-CAH had been diagnosed within the first year of life; only three SW–CAH, and two SV-CAH patients were diagnosed after the age of one (mean age 3.3 and 4.0 respectively). Those with late onset CAH (n=2) were diagnosed at a mean age of 12 years, and were included because they attended

the clinic with virilized genitalia, they both had clitoral hypertrophy. Main features of the 40 women are presented in table 2.

Height was below -2SDS in 12 of the women, calculated on Dutch reference data for age 21 years. [20] Family history was positive for CAH in 13 patients, and parental consanguinity was present in 3 patients, all of whom had SW-CAH.

Surgical procedures

Thirty six of the 40 patients had undergone feminizing surgery of the external genitalia. Figure 1 gives an overview of the surgical procedures performed.

In 13 of those 36 patients, surgery consisted of a single stage clitoro- and vaginoplasty. The median age at surgery was three years (range 0-17 yrs). Seven patients (7/13, 54%) needed re-surgery later in life. In 20 patients (median age 2 yrs, range 0-19) first surgery comprised only clitoroplasty. Additional vaginoplasty was performed in 16 patients (16/20, 80%) at the median age of 13 (range 4-22 yrs). Several patients (see figure 1) had more than one additional surgical procedure.

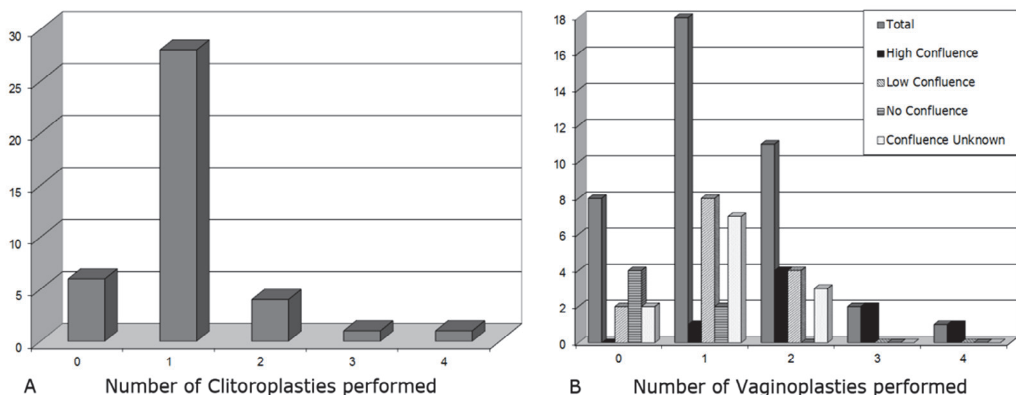

Figure 1 Numbers of Clitoroplasties and Vaginoplasties performed in the total patient group (n=40). A: Number of Clitoroplasties performed. B: Number of Vaginoplasties performed and total number of vaginoplasties with break down by severity of virilization.

In three patients (median age 11, range 2-16 yrs) first surgery comprised only vaginoplasty. One of these women requested additional clitoroplasty at the age of 17 even though clitoromegaly was only mild.

A redo-operation was performed in twenty-five of the 36 patients (69%). Eleven of these patients had their first surgery before the age of 13 months, and 14 patients had their first surgery between 13 months of age and 6 years.

In almost all cases (n=32) clitoroplasty comprised reduction of the clitoris with preservation of the neurovascular bundle and glans. Two patients had a clitorectomy;

for one patient that was standard procedure at that time (42 years ago), the other patient underwent clitorectomy because of persisting painful erections. The total number of surgical procedures in the patients assigned to the high confluence group (n=8) was significantly higher than that in the patients assigned to the low confluence group (n= 14) or that in patients with no confluence (n= 5) (p=0.004). As expected, the total number of clitoral or vaginal surgeries was higher in the SW group than the SV group, but, given the small sample size, the difference did not reach statistical significance (p= 0.381 and p=0.092 respectively).

Gynaecological examination

The results of the anatomical assessment during gynaecological examination are presented in table 3. Assessment data were available for 17 (Hegar examination) and 29 (labia majora) women. Characteristics between patients who refused gynaecological examination compared to who did not were not different for CAH type (i.e. SW or SV), or number of surgeries (1.8 (1-4) vs 1.8 (0-4)), but the groups did differ in age of attendance. The patients who participated were significantly older with a mean age of 32.6 years (18-46) compared to a mean age of 23.1 years (16-34) for those who did not participate (p=0.03). The clitoris was scored absent in two cases (due to clitorectomy), small in five patients, normal in 15, and enlarged in six patients. Visible labial scarring was present in 15 patients; only in 1 woman the scars were very pronounced. One patient had a vagina shorter than 6 cm after a pull-through procedure. In four patients, digital examination was possible with one finger only; they were virgins i.e. had never had sexual intercourse. The Hegar width of the vagina was in the normal range in almost all patients; in only one woman it was smaller than 20 mm. Thirteen women refused speculum examination. Speculum examination was physically impossible in five patients. One patient was diagnosed with vaginism, while another had recently undergone a vaginoplasty.

Only two patients showed abnormalities (increased vaginal secretions and atrophy respectively). Seven patients had vaginal strictures. Although variations in genital appearance were established during gynaecological examination, appearance was in the normal range in the majority of women. Again, differences between the SW-CAH and SV-CAH groups did not reach statistical significance due to small sample size (see table 3).

Outcome	SW (n=32)	SV (n=8)[1]	Total (n= 40)	*P* value*
	Result			
Anatomical assessment				
Clitoris				0.918
Absent	2	0	2	
Small	4	1	5	
Normal	12	3	15	
Large	5	1	6	
Labia Majora				# 0.126
Normal	13	5	18	
Abnormal	11	0	11	
Scrotal effect	9		9	
Labia Minora				0.393
Normal	9	3	12	
Abnormal	14	2	16	
Absent	5		5	
Small	5		5	
Labial scarring				0.502
absent	10	3	15	
present	13	2	15	
Satisfactory	6	0	6	
Neutral	6	2	8	
Not satisfactory	1	0	1	
Vagina				
Length[2]				0.531
Short	1	0	1	
Normal	15	6	21	
Digital examination				0.191
1 finger	4	0	4	
2 fingers	13	6	19	
Introitus (Hegar)				0.588
<15 mm	0	0	0	
15-20 mm	1	0	1	
20-25 mm	3	0	3	
25-30mm	6	1	7	
> 30mm	4	2	6	
Speculum examination[3]				0.714
physically impossible	4	1	5	
normal	15	5	20	
abnormal	2	0	2	
Stricture[4]				
Minimal	2	2	4	0.147
Moderate	3	0	3	
Meatus externus urethrae				0.694
Normal	6	2	8	
Superficial	3	1	4	
Deep	14	2	16	

Table 3 Anatomical Assessment.
*Overall p - value between SW and SV (Chi- Square Test or # Fisher's exact test).[1] includes patients with CYP11B1 deficiency. [2] Short = 0-6cm, normal > 6cm. [3] Physically impossible for the following reasons: virgin, recent operation, vaginism. Abnormal: too much fluor, atrophy.[4] 2-3 cm = minimal, 1-2 cm = moderate

Cosmetic ratings

Cosmetic results of surgical and medical treatment was evaluated in 28 patients. Both the patient and the gynaecologist who did the examination of the patient scored cosmetic outcome for various parts of the external genitalia on a 10 point scale. The median total score of all parts was 7 for patients and 7 for gynaecologists, which reflects overall satisfaction with cosmetic outcome (p= 0.467 for comparison of patients and gynaecologists (N=27 Wilcoxon signed-rank test). 25% (7/28) of the patients vs 21% (6/28) of the gynaecologists scored the cosmetic outcome as insufficient (i.e a mean score <6).

Age at first surgery and level of confluence were significantly associated with the mean cosmetic outcome score (univariate analysis, p=0.021 and p<0.01 respectively (n=27, Kruskall- Wallis test and one way ANOVA). Stratified analysis revealed that number of operations and age at first surgery were effect modifiers of the association between level of confluence and cosmetic outcome.

Multiple regression analysis (Table 4) showed that after adjustment for the effect modifiers, only level of confluence had a significant effect on cosmetic outcome. The adjusted impact of the level of confluence on cosmetic outcome is 1.055 – 0.987*number of surgeries. The adjusted R^2 of this model was 0.335.

Multiple regression model for cosmetic appearance			
	B	Standard Error	*P* value
Constant	4,631	1.278	0.002
Level of Confluence (1)	1.055	0.489	0.045
Number of Surgeries (2)	0.181	0.305	0.560
Age at First Surgery	0.078	0.056	0.180
(1) x (2)	-0.987	0.539	0.084

Table 4 Multiple regression model for cosmetic appearance with mean gynecological score as dependent variable. Adjusted R^2: 0.335. Only level of vaginal confluence had a significant effect on mean gynecological score. The adjusted impact of level of confluence depends on the number of surgical procedures performed.

Functional assessment

Assessment of sexual function consisted of an interview with a gynaecologist – as part of the gynaecological check-up - and an interview and questionnaire assessment with a psychologist. The questionnaire results are shown in Table 5.

The gynaecological interview was completed by 32 women. Twenty of the 32 (62.5%) women had experienced vaginal penetration (median 28.5 years, 19-46 years), and twelve (37.5%) had not (median 33.5 years, 16-46 years). Two of the latter considered

their vagina too narrow; two were embarrassed by the look of their body; two considered themselves too young (ages 16 and 19); three had sexual experiences in female homosexual relationships only, whereas three patients did not give a reason. Eighty three procent of women reported they were able to achieve orgasm. Eight women reported dyspareunia (8/33, 24%), all of whom had experienced vaginal penetration. Neither width nor length of the vagina correlated with dyspareunia (median Hegar width: 25.5 with dyspareunia vs. 26.0 without dyspareunia, p= 0.86; median length 10.0 cm vs 10.0cm, p=0.152). Physical examination revealed vaginal strictures in two of the eight women who reported dyspareunia. Whereas five other patients with vaginal strictures did not report dyspareunia, two of them were virgins. Seven out of 33 women reported urinary incontinence (3 stress incontinence, 3 urge incontinence, and 1 both). None of them had sought medical attention for the incontinence. They lost small amounts of urine and sometimes used panty liners for this. Two women reported incontinence that started after genital surgery.

All but three women filled in the FSFI questionnaire and all but five the FSDS-R questionnaires. However many questions remained unanswered. 27% of the women (10/37) did not have sex regularly. Sexually experienced women with CAH had passed sexual milestones later than the Dutch reference sample (romantic engagement 17.7 years vs. 15.6 years, p=0.04; sexarche 18.9 years vs. 18.3 years, p=0.36). At the time of follow-up, half of the participant group (22/40) reported to be in a stable relationship.

	Control		CAH		p-value
Desire	4.0 (0.8)	n= 108	3.5 (1.2)	n=37	0.017*
Arousal	5.3 (0.8)	n=108	4.4 (1.6)	n= 31	0.004**
Lubrication	5.7 (1.0)	n=108	5.0 (1.1)	n= 26	0.004**
Orgasm	5.1 (1.1)	n=108	4.6 (1.6)	n= 26	0.131
Satisfaction	5.4 (0.8)	n=108	5.2 (1.0)	n=22	0.442
Pain	5.7 (0.8)	n=108	4.5 (1.7)	n=13	0.025*
Total FSFI	31.2 (3.9)	n=108	29.0 (4.2)	n=11	0.107
Total FSDS	5.1 (6.4)	n=108	8.8 (8.8)	n=35	0.02*

Table 5 Mean scores (SD) on the different domains of the FSFI (range 0-6), total FSFI (range 0-36), FSDS-R (range 0-52) comparing CAH women and a non-affected reference group [18] (independent samples t-tests). $^{**}p<0.01$, $^{*}p<0.05$. SD = standard deviation; FSFI = Female Sexual Function Index; FSDS-R = Female Sexual Distress Scale – Revised; CAH= Congenital Adrenal Hyperplasia

A Total FSFI score can be calculated for women who had sexual intercourse in the last 4 weeks; in our patient group only 11 women had been sexually active during this period. The mean score of 29 (range 19.9-33.7) on the total FSFI did not differ significantly from the score of the Dutch reference group of 108 non-affected women

with a partner. All but one had had feminizing surgery and had no confluence or low confluence of the vagina into the UGS at birth. However, when considering the separate FSFI domains, women with CAH had significantly lower functioning on the subscales of desire, arousal, lubrication, but also pain compared to the reference group. No differences were found for satisfaction, or orgasm. On the FSDS-R, women with CAH indicated to experience more sexual distress in comparison to the reference group (Table 5). 29% (10/35) had a score above the clinical cut-off of 11. When combining the (valid) FSFI and FSDS-R data, only one woman suffered from a sexual dysfunction, as defined in DSM-IV-TR. Kruskal-Wallis tests showed no significant differences between women with a high or low confluence level, or no confluence, possibly because of the small sample sizes. In addition no significant difference existed in perception of sexual function between SW and SV women as tested with Mann-Whitney U-tests.

Discussion

We report on genital anatomy and ratings of cosmetic, and functional outcome in women with CAH with, and without feminizing surgery. We integrated gynaecological, and psychosexual outcome with the aim to add new insight in the long term outcome of genital surgery in these women.

Level of virilisation, assessed as level of confluence, proved to be the most important factor in cosmetic outcome; cosmetic appearance was judged as less favourable in case of a high vaginal confluence. In addition patients with a high vaginal confluence underwent significantly more surgical procedures than the remainder of the women. Our regression model clearly showed that the adjusted impact of level of confluence on cosmetic outcome is modified by the number of surgical procedures. As shown in table 4, the impact of the level of confluence on cosmetic outcome is larger or smaller, depending on the number of surgeries performed. More than half of the patients who had single – stage surgery initially, underwent additional procedures. Our results are in line with Nordenström *et al.* [21], who reported that surgery is more extensive in severely virilised patients, whereas other groups have reported either disappointing, or reasonably good outcomes after re-surgery in puberty [6, 15-16]. A practical advice is that parents should be informed on the fair chance of re-operation in adolescence.

In this study, patients and gynaecologists both rated genital appearance as sufficient. In contrast, Wisniewski et al. [10] and Nordenström et al. [21] reported that patients were more negative than doctors. This might be due to a different composition of the group, or different approaches towards surgery. Female genital

self- image, assessed using a 4 point scale (FGSIS), was shown to be related to sexual function [22]. Although further research is needed, especially in patients born with genital anomalies like CAH, this scale might be useful in a clinical setting to create talking points that enhance patient – doctor communication and to better understand requests for additional surgery [22, 23].

Our findings probably underestimate the delay in experience of romantic and sexual encounters since there was a considerable number of virgins, and a considerable number of non-responders.

Comparisons using the FSFI scores were limited to women who were sexually active in the last four weeks, having a partner and having penile-vaginal intercourse as described by Brotto *et al*. [18] Only one (1/11) of the women suffered from sexual dysfunction according to DSM-IV-TR criteria [17]. We suspect that the real number is considerably higher. Women with CAH achieved sexual milestones later than Dutch reference women and a substantial part never had been engaged in a sexual relationship. Women with CAH experienced a significantly less satisfactory sexual functioning (desire, arousal, lubrication, pain), and experienced more sexual distress compared to a Dutch reference group of healthy females. These data are in line with findings from Wisniewski *et al.* [10] and Gastaud *et al.* [7]. In our study, 24% of the CAH women reported dyspareunia (deep and superficial). In a large Dutch study 5.4% of 2024 healthy females reported dyspareunia, while 29.6% sometimes experienced pain during intercourse [24]. In females who have undergone vaginal surgery, dyspareunia might be related to vaginal stenosis [25], but in our study only two of the eight patients who reported dyspareunia had vaginal strictures. 83% reported they were able to achieve orgasm, although with more difficulty compared to healthy refecrence women. Other authors have [6, 21] reported that the clitoral sensitivity was affected in nearly all the women who had had surgery. These findings indicate that clitoral surgery may affect genital sensitivity. However, additional factors are likely to play a role as well, for example, degree of virilization, and psychological factors. They are likely to be interrelated and little is known about their effects on outcome. For example, women with CAH reported high satisfaction levels. One possible explanation, suggested by Minto and colleagues, is that living with CAH has contributed to low expectations of sexual functioning, that is, the women might have felt that sexual difficulties were to be expected and that they should not be dissatisfied [26]. Additionally, the paternalistic attitude of physicians, and past practice of secrecy relating to the DSD, especially experienced by the older women in this study, might have resulted in inadequate information and lack of opportunity for discussion. Alternatively, infrequent sexual contact, associated with sexual dissatisfaction might indicate a

withdrawal from sexual intercourse and a relief from facing problems of sexual difficulties [27].

This study has drawbacks that need to be addressed. First, the study has a cross–sectional design, and part of the data have been retrieved retrospectively from medical files. Therefore the study was limited by information available in these files. Second, 55% of the CAH women who had been under medical treatment in childhood, adolescence and adulthood in our hospitals did not participate in the follow-up study. Most refused to participate, and in some cases we were unable to contact them. We did not find significant differences, demographical and medical, in characteristics between responders and non-responders. However, we cannot exclude the possibility that the non-responders may have worse functioning than the participants. Women may not want to participate because participation meant a confrontation with a painful and distressing aspect of their disease, i.e. sexual functioning. Women who participated in the gynaecological examination were significantly older compared to those who did not. We cannot explain this difference, but assume that women, as they become older, for medical purposes have undergone several gynaecological examinations before and got familiar with this type of examination. Familiarity probably makes them feel more comfortable to participate in a gynaecological examination with a research goal. Third, to measure sexual functioning we selected FSFI and FSDS-R. These scales are considered the first choice screening tool because they have excellent psychometric qualities, are easy to administer, and are available in many languages [15, 19]. Its utility has been demonstrated in diverse conditions, for example, vaginoplasty in females with Mayer-Rokitansky-Küster- Hauser Syndrome [28]. However, the FSFI will artificially inflate scores towards the sexual dysfunction pole in females who are not sexually active or who did not experience vaginal penetration in the 4 weeks prior to the test [18]. The results in our study revealed that only 55% of the women had a partner. The Dutch reference group only included women who had a sexual partner. In order to improve applicability of the scales in patient groups, the scales need adaptation. Additional data in different patient and reference populations including females suffering from different types of somatic, emotional, and sexual problems should be collected.

Clinical implications

We showed that cosmetic appearance, and functional outcomes are associated with the degree of virilization at birth, and that the impact of level of confluence on cosmetic outcome depends on the number of surgical procedures performed. These data might be used in the discussion whether or not to treat pregnant women at risk

of carrying a child with CAH with dexamethasone. Incidence of reported dyspareunia is high in women with and without vaginal strictures, stenosis, or other visible anomalies. Parents and patients need to be informed extensively about the multiple aspects that contribute to outcome after feminizing surgery. They need to be informed that there will be a fair change that re-operations will be necessary in adolescence. Despite their sexual problems, only a few women reported sexual problems to a gynaecologist or psychologist, or had sought help from a sexologist. We would like to make a plea for assessing sexual well-being at follow up visits and to discuss sexuality, and work towards acceptance of the genital anatomy that may always remain different from the perceived norm. Ultimately, referral to a sexologist may be needed.

Conclusions

The level of confluence appeared to be the major determinant for cosmetic outcome and the impact depends on the number of surgical procedures performed. The outcome of corrective genital surgery is positive with respect to genital appearance but less favorable with respect to sexual functioning. Vaginoplasties to improve sexual function should only be performed after consulting a multidisciplinary DSD team and after ample vaginal examination and counseling. We would advocate that surgeons inform and discuss these aspects with parents and patients so they can make a balanced decision.

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CHAPTER 3

Long term outcomes in males with Disorders of Sex Development

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Abstract

Purpose:

Indications that prenatal action of testosterone in the brain is an important determinant of gender development and improved reconstructive techniques caused a shift in male gender assignments in patients with 46, XY disorders of sex development (DSD). We report long term outcome data on psychosexual development and sexual function of these individuals in a cross-sectional study.

Materials and Methods:

Physical status of 14 men with a mean age of 25 years with DSD was assessed using a structured interview and physical examination. Psychosexual outcome was evaluated by means of questionnaires and compared with a control group of 46 healthy age-matched men.

Results:

A total of 13 men underwent 1 to 6 (mean 2) genital surgeries. Mean age at first surgery was 2.7 years. The mean penile length was 6.6 cm. All men reported erections and were able to experience orgasms. Ejaculatory dysfunction was reported by 7 men. Mean penile length was 7.9 cm in patients who were able to achieve penetrative intercourse and 4.9 cm in those who were not. Meatus was glandular in 5, coronary in 7 and at the distal shaft in 1. Compared to controls, men with disorders of sex development were less satisfied with the appearance of the penis and scrotum but not with total body image. These patients reported decreased sexual desire and activities.

Conclusion:

Outcome in this group of men with disorders of sex development was poor regarding penile length, ejaculation, satisfaction with external genitalia and frequency of sexual activity. Other aspects, such as overall body image and psychosexual functioning, showed no difference from controls.

Introduction

Disorders of Sex Development (DSD) are defined as congenital conditions involving atypical development of chromosomal, gonadal or anatomical sex [1]. The underlying chromosomal constitution of infants with markedly ambiguous genitalia may be 46,XY, 46,XX or a mosaic pattern. Of patients with disorders of sex development and 46,XY hypovirilization syndromes a specific diagnosis can be made in only 20% [2].

Improved reconstructive techniques and observations of gender dysphoria and a wish for a gender role change in patients with 46,XY hypovirilization raised as girls resulted in more male sex assignments in the last decades, particularly in patients with less severe hypovirilization [1, 3-7].

The aim of masculizing surgery in patients with disorders of sex development is to improve cosmesis and function of the external genitalia, to enable sexual intercourse and to avoid stigmatization. Therefore, it is important to assess the functional and sexual outcome of these patients.

There have been a limited number of outcomes studies in males with disorders of sex development, including those with an undefined 46,XY DSD [5, 8-12]. However, studies with combined data on urological and in-depth psychological examination in relation to surgical history are scarce. We investigated the long term physical, functional and psychosexual outcomes in males with disorders of sex development in a cross-sectional study.

Methods

A total of 37 males older than 14 years with DSD identified at 2 university hospitals between 2007 and 2009 were invited to participate. Of these individuals 14 (37%) participated, including 9 from Erasmus MC Rotterdam and 5 from Radboud University Nijmegen Medical Center. Inclusion was based on diagnosis of DSD and phenotype (i.e. proximal hypospadias and unilateral/bilateral cryptorchidism). The study was approved by the medical ethics committees of both centers. All participants were informed about the study and signed a written consent form. Participant responses to the Male Sexual Health Questionnaire were compared with those of a control group of 46 male students with a median age 21.5 years (range 18 to 36) who volunteered to participate in the study.

Data on genital appearance at birth and genital surgeries were retrospectively collected from the medical files. Subsequently participants underwent a urological examination, hormonal analysis and psychological assessment between 2007 and 2009. LH, FSH and serum testosterone levels were determined as described previously [13].

Surgeries were divided in hypospadias repair and additional procedures. Patients were grouped based on diagnosis. Cases without a molecular diagnosis were classified as undefined 46,XY DSD. Standardized urological examination consisted of visual inspection (general impression, testes, localization and shape of meatus, penile curvature, distortion, penoscrotal transposition) and measurements (testis volume, penile circumference, stretched penile length, self-measured degree of curvature). The examiners had not been involved in the medical care of these patients.

Psychosexual functioning and satisfaction with genital image were assessed by questionnaires and a semistructured interview administered by psychologists not involved in the care of the patients. The MSHQ is a validated, self-administered instrument for assessing problems in the primary domains of erection, ejaculation and sexual satisfaction in men [14]. Aspects of sexual functioning and problems, as well as satisfaction with (surgical) treatment and impact of treatment on psychosexual functioning were addressed in the interview. Satisfaction with body appearance and appearance of the external genitalia was assessed using a 5-point Likert scale [15].

Comparisons between groups were done using chi-square test for categorical variables and Student t-test for continuous variables. MSHQ scores were compared using a Mann-Whitney U test (not normally distributed). A p value of less than 0.05 was considered significant.

Results

Participants

Of the 38 men invited to participate 14 agreed (response rate: 37%). Mean age was 25 years (range 14 to 32). There were no significant differences between the participants and non- responders regarding age (p=0.81), diagnosis (p=0.5) or number of hypospadias repairs (p=0.97).

Characteristics of the participants are presented in the Appendix. Median age at participation was 25 years (range 14 to 38). One patient with 45,X/46,XY DSD who presented with bilateral cryptorchidism without hypospadias at age 8 months, was excluded from the functional and psychosexual analyses. The others had been diagnosed at birth with hypospadias and cryptorchidism. Family history was positive in 3 patients and consanguinity in 2. Based on Dutch reference data for age 21 years, height was below -2SDS in 5 men and borderline (-1.98 SDS) in one [16].

Of the six men with undefined 46,XY DSD, 1 had a positive family history of proximal hypospadias. This patient was diagnosed with morbid obesity (body mass

index 44 kg/m^2), hypergonadotropic hypogonadism and small testes. Semen analysis revealed azoospermia. Medical history consisted of unilateral testicular torsion, utricular cyst and epididymitis. Hormonal analysis including hCG, ACTH and GnRH tests was normal. Genetic analysis of the androgen receptor was negative.

One man with undefined 46,XY DSD was suspected of a 5α-reductase deficiency. Sequencing of the *SRD5A2* gene did not reveal any abnormalities, but enzyme function was impaired in in-vitro studies in fibroblasts. In the remaining 4 patients with undefined 46,XY DSD, testosterone synthesis disorders were excluded and sequencing of the androgen receptor gene did not reveal any abnormalities.

Surgeries and Urological examinations

A total of 13 men underwent hypospadias correction involving a mean of two surgeries (range 1 to 6). Mean age at first surgery was 2.7 years (range 3 months to 6 years). The undefined 46,XY DSD group differed from other diagnostic groups with respect to a larger variety in age at first surgery. No other differences between groups were found. The groups were too small to assign statistical significance. One man with undefined 46,XY DSD had undergone 6 surgical procedures for hypospadias repair. One patient in the mixed GD group did not have hypospadias. Repeat surgery was needed in another patient with mixed GD at age 17. The other surgeries consisted of hypospadias repair in 2 planned sessions.

The numbers of additional procedures are listed in table 1. Both patients with PAIS had had a gynaecomastia correction, of whom 1 needed correction of a recurrence after 1.5 years. Gonadectomy was performed in 4 patients. In the ovotesticular DSD group, all patients had a histologically confirmed ovotestis. One of these patients had undergone gonadectomy on the right and orchidopexy on the left side plus biopsy. In 2 patients a streak gonad was found, both of whom had a mosaic chromosomal constitution. Histological review of gonadal tissue did not reveal signs of malignancy.

The results of the urological exam are listed in the supplementary table (http://jurology.com/). Mean penile length was with 6.6 cm (range 4.2 to 10.5), which was below -2.5 SDS. Four men had a curvature of more than 10 to 60 degrees during erection. The meatus was glandular in 5 patients, coronary in 7 and on the distal shaft in 1.

	Total	PAIS	Mixed GD	Ovotesticular DSD	Undefined 46XY DSD
Mean yrs age at first hypospadias repair (range)	2.7 (0.3-6.0)	3.3 (1.5-5.0)	3 (2.5-3.5)	3.2 (2.0-5.0)	2.3 (0.3-6.0)
Mean No. hypospadias repair (range)	1.9 (0-6)	1 (1-1)	1.3 (0-2)	2 (1-3)	2.5 (1-6)
No. additional procedures:	28	10	4	7	7
Hernia inguinales	7	2	1	2	2
Orchidopexy	7	2	1	2	2
Penoscrotal transposition	2	2	0	0	0
Gynaecomastia	3	3	0	0	0
Gonadectomy	4	0	2	2	0
Fistula repair	3	1	0	0	2
Utriculus excision	2	0	0	1	1

Table 1 Surgical procedures.

Functional assessment

All men reported erections and were able to experience an orgasm. Abnormal ejaculation was present in 7 men, of whom 3 were on androgen replacement. Two patients experienced dry ejaculations most of the time, 1 had dry ejaculations half of the time. Two men complained about the small amount of ejaculate (a couple of droplets), and 2 experienced weak ejaculation. The 8 men who reported achieving penetrative intercourse had a mean penile length of 7.9 cm which was significantly greater than in the 4 not achieving penetrative intercourse (4.9 cm, p=0.028).

Serum LH, FSH and testosterone measurements demonstrated hypergonadotropic hypogonadism in the 3 patients with mixed GD (LH: 18.7, 22.3 and 27.3 IU/L; FSH: 46.6, 66.0 and 54.4 IU/L; Testosterone: 7.0, 4.2 and 2.7 nmol/L respectively). All of these patients were on testosterone replacement therapy (Sustanon® or testosterone undecanoate) after gonadectomy and thus had signs of under treatment. The remaining patient (with ovotesticular DSD) who was on testosterone replacement after gonadectomy had slightly elevated FSH values (11.1 IU/L), but normal LH (1.15 IU/L) and testosterone (19.5 nmol/l) values. The two patients with PAIS had high levels of LH (19.8 and 12.4 IU/L) and testosterone (63.7 and 28.7 nmol/L). The 6 men with undefined DSD had normal male values (2 patients) or elevated FSH values and normal LH and testosterone levels (4 patients). There was no relation between libido and testosterone values (p=0.892).

Men with a sufficient urine volume (greater than 100 cc) during flow measurement (8 patients) showed a mean maximum flow rate of 20.6 ml per second. One had a plateau curve and the others had a normal curve.

Men with DSD were significantly less satisfied with the appearance (p<0.001), color (p<0.001), thickness (p=0.03), and size (flaccid (p <0.001) and erected (p<0.001)) of their penis, as well as the scrotum (p<0.001) and of the testes size (p<0.001), compared to the control group. Participants were dissatisfied regarding penile size (small), scrotum (asymmetry) and testes (too small). Satisfaction with the amount or appearance of pubic hair did not differ between participants and controls. There was also no difference in satisfaction with secondary sex characteristics (voice, body hair, facial hair, breasts, hips and Adams' apple (p=0.489)) and non-sex characteristic body parts (p=0.267) or total body image between participants and controls (p =0.098). Six of the 10 men who answered this question perceived that their sexual development was negatively influenced by their genital appearance. Only 3 men felt sexually hampered, especially regarding penile size.

Psychological assessment

Detailed psychosexual functioning was assessed in 11 participants. One adolescent (14 years) had no sexual experience; and 1 man (19 years), refused to fill in the MSHQ. Mean age at first coitus was 18 years (range 15 to 23). Frequency of sexual activity significantly differed between patients and controls. Of the controls 76% had been involved in sexual activities more than 6 times in the month preceding the study, compared to 18% of the patients (p<0.001, Table 2). One man (18 years) never had sex due to the absence of sexual desire and arousal, difficulties with erection and orgasm and lack of partner. Another man tried to avoid sex as much as possible, but gave no further explanation. These statements suggest that both men might be asexual. Others reported difficulties with erection and orgasm (1 patient), low arousal (1) or having no partner (1).

	Control men	Pts with DSD	p Value
Sexual activity/mo			
No. 0 (%)	0 (0)	3 (27)	
No. 1-6 (%)	11 (24)	6 (55)	
No. >6 -almost daily (%)	35 (76	2 (18)	
mean (SD) frequency	4.0 (1.0)	2.6 (1.4)	0.002**
mean (SD) distress	1.6 (0.9)	1.8 (1.3)	0.900
Partner			
No. with partner (%)	21 (46)	5 (45)	0.805
Mean (SD) Satisfaction°	4.4 (1.0)	4.4 (0.8)	0.610
Mean (SD) Desire			
Frequency	3.9 (0.6)	3.3 (0.9)	0.016*
Level	3.8 (0.7)	3.4 (0.9)	0.121
Distress	2.0 (0.8)	1.9 (1.0)	0.534
Mean (SD) Erection			
Frequency	4.7 (0.6)	4.2 (1.2)	0.155
Frequency of maintaining erection	4.6 (0.6)	4.2 (1.3)	0.465
Firmness of erection	4.7 (0.6)	3.8 (1.2)	0.006*
Distress	1.4 (1.1)	1.3 (0.6)	0.988
Mean (SD) Ejaculation			
Frequency	4.8 (0.5)	3.7 (1.8)	0.052
Frequency dry orgasm	1.2 (0.6)	1.5 (1.2)	0.342
Frequency delayed ejaculation	1.5 (0.7)	1.2 (0.9)	0.222
Force	4.7 (0.6)	3.8 (2.1)	0.395
Volume	4.8 (0.5)	4.2 (2.2)	0.583
Pain	1.2 (0.5)	1.4 (1.6)	0.394
Distress	1.2 (0.6)	1.3 (0.6)	0.786

Table 2 Male Sexual Health Questionnaire results.

* P < 0.05 (2-tailed Mann-Whitney U test)

° based on 5 items (satisfaction with the overall sexual relationship, quality of sex life, frequency of sexual activity with partner, communication about sex with partner and affection during sex).

The distress related to the frequency of sexual activity did not significantly differ between patients and controls. The same held true for the proportion of men involved in a steady relationship, and satisfaction with sexual relationship. Ten out of 11 men had a heterosexual orientation, one a homosexual. None reported gender dysphoria. Men with a DSD reported on average more difficulties than the men without a DSD, but this did not reach significance, except regarding the frequency of desire to have sex, and problems with firmness of the erection (Table 2).

Discussion

This study evaluates the long-term outcome in male patients with DSD. We found that mean penile length of post pubertal DSD patients was below -2SDS and that penile length was correlated to the ability to achieve penetrative intercourse. Moreover, abnormal ejaculations are common. The incidence of male gender assignment is increasing due to, the awareness of prenatal testosterone effect on gender development, improved surgical techniques and change in attitude towards toleration of genital anomalies [1, 5, 6, 12]. Generally, males with DSD need extensive genital surgery. The severity of hypospadias is correlated with adult penile length [17]. In our study all but 1 patient had a history of hypospadias repair. The patient without hypospadias repair presented with undescended testis and slight scrotal asymmetry, which triggered chromosomal analysis that revealed 45,X/46,XY mosaic DSD.

Mean penile length was below -2.5 SDS. Mean adult penile length is 13.3 cm (SD 1.6)[18]. Reilly *et al.* reported the absence of a relation between sexual functioning and penile length and ability of men with a microphallus to achieve satisfactory sexual intercourse [19]. In our study, the men who were able to achieve penetrative intercourse had a significantly greater mean penile length than men who were unable to do so. Phalloplasty might be an option for men who fail to have penetrative intercourse and are dissatisfied with this. However, there is little experience with this procedure and long term results are lacking [20].

Men with DSD were less statisfied than controls with penile appearance, color, size and girth. This finding is in line with other studies demonstrating that small penile size is a major cause of dissatisfaction [9, 21]. Sircili *et al.* found that penile length is not significantly related with satisfaction with surgical results [11]. Patients in our study were significantly more dissatisfied with their genital image in comparison to controls, and those with a shorter penile length were most dissatisfied. Interestingly, satisfaction with secondary sex characteristics and more neutral body parts did not significantly differ. This suggests that genital image and acceptance needs special attention during psychosexual counselling of boys with DSD.

Our study confirms that male DSD patients in general experience erections and orgasms. However, half of our studypopulation reported abnormal ejaculation as described previously [22, 23]. Schönbucher *et al.* reported an increased incidence of sexual dysfunction and an overall low sexual quality of life in male 46,XY DSD patients [24]. In our study equivalent numbers of men with DSD and controls were involved in a steady relationship and satisfaction with the partner and sexual relationship did not differ between these groups. Men with DSD tended to be less active sexually, and reported more erection problems, but no significant differences were found in terms of distress or dissatisfaction. The frequency of desire was significantly reduced in men with DSD, but not the intensity. Finally, sexual orientation was heterosexual in all but 1 patient, this is in line with previous studies [9, 24].

Our study has some limitations that need to be addressed. First, the study had a cross–sectional design, and part of the data was retrospectively retrieved from medical files. In addition, 61% of the men with DSD declined participation for unknown reasons. Therefore our data may not be representative for the total group. Furthermore, sexual functioning was measured with the MSHQ, which is a questionnaire that asks about sexual problems in the past four weeks only, and was designed to assess sexual problems in males. Regrettably there are no validated questionnaires focussing on sexual impairment due to a DSD. Lastly, due to the small sample size, the power of statistical analysis was limited and associations might therefore not reach significant difference.

In conclusion, the outcome in this group of men with DSD was poor regarding penile length, ejaculation, satisfaction with external genitalia and frequency of sexual activity. Other aspects such as overall body image and psychosexual functioning showed no difference with controls.

APPENDIX

	Karyotype	Diagnosis	mutation found	family history
1	46 XY	PAIS	AR +	Positive
2	46 XY	PAIS	AR +	Positive
3	45X/46XY	mixed GD (streak)	no	Negative
4	45X/46XY	mixed GD (streak)	no	Negative
5	45X/46XY	mixed GD (streak)	no	Negative
6	46 XX	ovotesticular DSD	SRY sequences	Negative
7	46 XX	ovotesticular DSD	no	Negative
8	46 XX/ 46 XY	ovotesticular DSD	no	Negative
9	46 XY	Undefined 46 XY DSD	no	Negative
10	46 XY	Undefined 46 XY DSD	no	Positive
11	46 XY	Undefined 46 XY DSD	no	Negative
12	46 XY	Undefined 46 XY DSD	no	Negative
13	46 XY	Undefined 46 XY DSD	no	Negative
14	46 XY	Undefined 46 XY DSD	no	Negative

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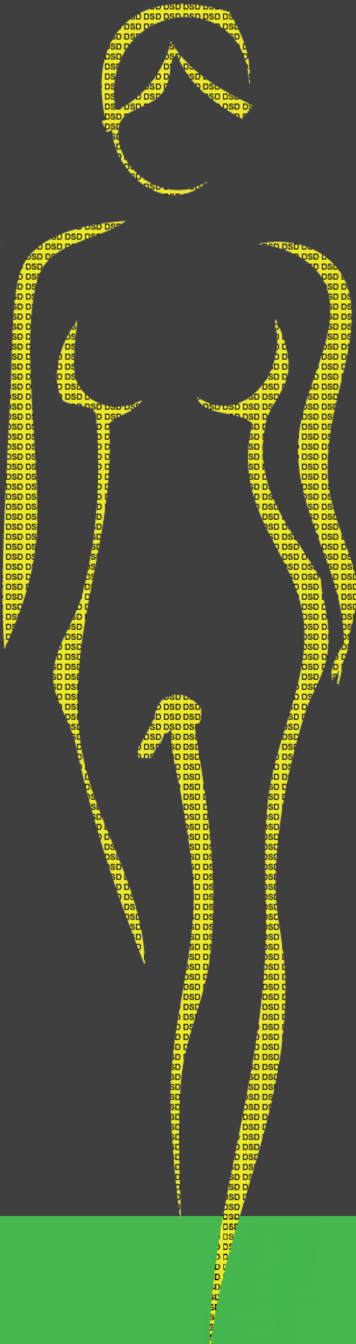
Supplementary table

	Height (SDS)	BMI	hair growth	position testis	volume testis (ml) left/right	circumference penis (cm)	stretched penile length (cm)	curvature penis	distortion penis	meatus	shape meatus	penoscrotal transposition
1	-1.55	23	feminine	inguinal	10/15	-	4.2	non	non	coronary	irregular	no
2	0.05	23	absent	scrotal	15/20	6	5	non	non	coronary	irregular	no
3	-3.39	27	male	scrotal	8/20	-	5.4	45° ventral	no	coronary	irregular	yes
4	-3.67	20	feminine	scrotal	0/8	8.5	7	30° lateral	30° left	coronary	irregular	no
5	-1.98	27	male	scrotal protheses	10	-	10.5	non	non	top glans	vertical slit-shaped	no
6	-2.97	25	male	absent	0/<1	-	7	non	non	distal shaft	vertical slit-shaped	no
7	-2.12	18	male	scrotal protheses	10	9.2	9.7	10° dorsal	non	top glans	vertical slit-shaped	no
8	-	-	feminine	scrotal	12/8	-	-	-	-	-	-	-
9	-2.40	20	male	scrotal	10/0	-	8	10° ventral	no	low-glandular	irregular	no
10	-0.28	44	male	scrotal	8	9	5	non	10° left	midglandular	irregular	yes
11	-0.99	28	male	scrotal	10	8	6	non	non	midglandular	vertical slit-shaped	no
12	-1.20	27	male	high scrotal	8/25	9.5	5	20° dorsal	non	coronary	horizontal	no
13	0.01	24	male	scrotal	10	12.5	6	60° ventral	no	low glandular	irregular	no
14	0.15	21	feminine	scrotal	30/35	10	7.5	non	40° right	top glans	vertical slit-shaped	no

Supplementary table 1 Physical examination.

CHAPTER 4

Management of Disorders of Sex Development in the Netherlands; Changes over time



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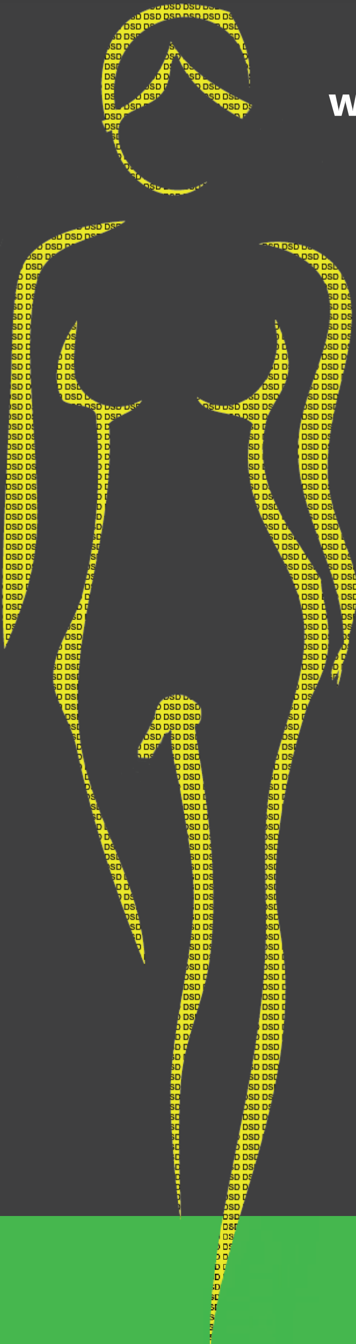
CHAPTER 5



A 46,XY female DSD patient with bilateral gonadoblastoma, a novel SRY missense mutation combined with a WT1 KTS splice-site mutation.

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Abstract

Patients with Disorders of Sex Development (DSD), especially those with gonadal dysgenesis and hypovirilization are at risk of developing malignant type II germ cell tumors/cancer (GCC) (seminoma/dysgerminoma and non-seminoma), with either carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesion. In 10 -15% of 46,XY gonadal dysgenesis cases (i.e. Swyer syndrome), SRY mutations, residing in the HMG (High Mobility Group) domain, are found to affect nuclear transport or binding to and bending of DNA. Frasier syndrome (FS) is characterized by gonadal dysgenesis with a high risk for development of GB as well as chronic renal failure in early adulthood, and is known to arise from a splice site mutation in intron 9 of the Wilms' tumor 1 gene (*WT1*). Mutations in *SRY* as well as *WT1* can lead to diminished expression and function of SRY, resulting in sub-optimal *SOX9* expression, Sertoli cell formation and subsequent lack of proper testicular development. Embryonic germ cells residing in this unfavorable micro-environment have an increased risk for malignant transformation. Here a unique case of a phenotypically normal female (age 22 years) is reported, presenting with primary amenorrhea, later diagnosed as hypergonadotropic hypogonadism on the basis of 46,XY gonadal dygenesis with a novel missense mutation in *SRY*. Functional *in vitro* studies showed no convincing protein malfunctioning. Laparoscopic examination revealed streak ovaries and a normal, but small, uterus. Pathological examination demonstrated bilateral GB and dysgerminoma, confirmed by immunohistochemistry. Occurrence of a delayed progressive kidney failure (focal segmental glomerular sclerosis) triggered analysis of *WT1*, revealing a pathogenic splice–site mutation in intron 9. Analysis of the *SRY* gene in an additional five FS cases did not reveal any mutations. The case presented shows the importance of multi-gene based diagnosis of DSD patients, allowing early diagnosis and treatment, thus preventing putative development of an invasive cancer.

Introduction

Disorders of Sex development (DSD) are congenital conditions of incomplete or disordered gonadal development leading to discordance between genetic sex, gonadal sex, and phenotypic sex [1]. DSD occurs with an estimated incidence of 1:5000 [1]. Individuals with an underlying DSD, especially those with specific Y chromosomal material in their karyotype, have an increased risk for developing a type II germ cell tumor/cancer (GCC) [2]. GCCs arise from primordial germ cells (PGC) or gonocytes and can be subdivided into seminomas/ dysgerminomas and non-seminomas with carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesions [3, 4]. GCC risk varies, but is estimated to be over 30% in patients with complete gonadal dysgenesis and is often bilateral [2].

Frasier syndrome (FS), currently classified as 46,XY DSD, complete gonadal dysgenesis, is characterized by gonadal dysgenesis, a high risk for development of a GCC and chronic renal failure in early adulthood. Usually patients with complete gonadal dysgenesis are not diagnosed at birth because of their normal female appearance of external genitalia. However, these patients will not develop secondary sex characteristics at pubertal age, and will generally attend the clinic because of primary amenorrhea, with hormonal analysis showing hypergonadotropic hypogonadism because of lack of gonadal function.

Wilm's Tumor 1 (WT1) is an important regulator of early gonadal and kidney development [5]. It is expressed earlier in time than SRY in the urogenital ridge, from which the gonads and kidneys are derived. All known WT1 isoforms share four C-terminal zinc fingers which are necessary for DNA/RNA binding. The two major WT1 isoforms are produced by alternative splicing, resulting in an insertion (+KTS) or exclusion (-KTS) of lysine, threonine and serine between zinc fingers three and four. The –KTS isoform mainly plays a role in transcription and *AMH* transcriptional activation in Sertoli cells [6]. The +KTS isoform is involved in RNA processing, and in the mouse plays a role in *Sry* regulation *in vivo* [7].

Essential in the process of sex determination is the presence of the sex determining region on the Y chromosome (SRY) gene [8-10]. *SRY* mutations residing in the HMG (High Mobility Group) domains are found in 10 -15% of the 46,XY gonadal dysgenesis cases and affect binding to and bending of DNA or nuclear transport [11-14]. As a consequence these mutations can lead to an early error in the process of sex determination preventing proper formation of a testis. Specific intron 9 splice site mutations in *WT1* resulting in a decreased WT1+KTS isoform are typically found in FS patients, leading to a diminished expression of SRY and subsequently SOX9, thereby

disturbing testicular development [15]. Furthermore, knockout mice for the +KTS isoform showed sex reversal in males [16]. Thus both *SRY* and *WT1* mutations can cause (complete) sex reversal.

A highly informative marker for the presence of type II GCCs (i.e. GB, CIS and their invasive counterparts dysgerminoma and seminoma as well as embryonal carcinoma) is the transcription factor OCT3/4, also known as POU5F1 [17]. OCT3/4 is involved in the regulation of pluripotency, is expressed in PGCs and gonocytes during normal gonadal development, is required for PGC survival, and is lost after maturation to pre-spermatogonia in males and oogonia in females [17-20]. In DSD patients OCT3/4 positivity of the germ cells might be due to maturation delay and not due to malignant transformation. To distinguish between these, Stem Cell Factor (SCF, also known as KITLG) has been shown to be informative [21]. GB arises in the context of granulosa cells, staining positive for FOXL2 and negative for SOX9 (a Sertoli cell marker), this in contrast to the precursor lesion arising in a testicular environment, being CIS, in which the supportive (Sertoli) cells are negative for FOXL2 and stain positive for SOX9 [22].

Here, we present a unique case with bilateral GB and dysgerminoma in an adult woman presenting with primary amenorrhea at the age of 22 years, who was initially diagnosed with 46,XY gonadal dysgenesis. Mutation analysis identified a novel missense mutation (c.383A>G, p.Lys128Arg) in the HMG domain of the *SRY*, which did not have a significant effect on transcriptional activation and nuclear import *in vitro*. Laparoscopy revealed streak ovaries with GB and dysgerminoma on both sides. During follow-up the patient developed progressive renal failure based on focal glomerulosclerosis. Subsequent analysis of the *WT1* gene revealed a splice site exon 9 mutation (IVS9 +5 G>A) resulting in the final diagnosis FS. Sequence analysis of DNA from five additional FS patients with a proven *WT1* mutation for *SRY* mutations did not reveal any variants, indicating that the presence of mutations in both genes in FS patients is rare. To our knowledge this is the first case describing a patient with a mutation in both *WT1* and *SRY*, and underlines the importance of proper diagnosis, especially in patients with an increased risk for GCC, allowing early diagnosis and treatment, thus preventing the development of invasive cancer.

Materials and methods

Tissue samples

Anonymized tissue samples were collected from our diagnostic archives and diagnosed according to WHO standards [23] by an experienced pathologist in gonadal

pathology, including GCC (JWO). Use of tissue samples for scientific reasons was approved by the Medical Ethical Committee ErasmusMC (MEC 02.981 and CCR2041). Samples were used according to the "Code for Proper Secondary Use of Human Tissue in The Netherlands" as developed by the Dutch Federation of Medical Scientific Societies (FMWV (Version 2002, updated 2011).

Immunohistochemical staining

Immunohistochemical staining was performed on formalin fixed paraffin embedded samples of 3 µm thickness. The antibodies directed against OCT3/4, c-KIT (CD117), Stem Cell Factor (SCF), Testis Specific Protein on the Y chromosome (TSPY), SOX9 and FOXL2 have been described before [21, 22]. Briefly, after deparaffinization and 5 min incubation in 3% H_2O_2 for inactivating endogenous peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 0.9 bar in an appropriate buffer. After blocking endogenous biotin using the Avidin/Biotin Blocking Kit (SP-2001; Vector Laboratories, Burlingame, CA, USA), sections were incubated either overnight at 4°C (SCF, TSPY) or for 2h at room temperature (OCT3/4, SOX9, FOXL2) and detected using the appropriate biotinylated secondary antibodies and visualized using the avidin–biotin detection and substrate kits (Vector Laboratories).

SRY sequencing

Direct sequencing of the *SRY* gene on peripheral blood DNA from the patient was performed at the department of clinical genetics (reference sequence: NM_003140.1). For the additional samples DNA was isolated from either peripheral blood lymphocytes (4 patients) or from formalin fixed paraffin embedded material (from two independent blocks, 1 patient) according to standard procedures. SRY was PCR amplified, analyzed on a 1% agarose gel, purified using the Agencourt AMPure XP kit (Beckman Coulter genomics, Danvers, MA, USA) and Sanger sequencing was done according to standard procedures.

SRY transactivation assay

DNA encoding wild type SRY, mutant SRY and SF1 were cloned into the pcDNA3 mammalian expression plasmid (Clontech, Mountain View, CA, USA), and sequence verified. To test for SRY activation of TESCO, *in vitro* luciferase assays were performed on a human embryonic kidney carcinoma cell line (HEK293T, ATCC, CRL-11268). Cells were cultured in DMEM, High Glucose, GlutaMAX media (Invitrogen, Life Technologies, Paisley, UK) containing 10% Fetal Bovine Serum, 1% sodium pyruvate and 1% penicillin-streptomycin. Cultures were grown at 37°C with 5% CO2. Cells were seeded

in serum-free media 24 hours prior to transfection in 96-well tissue culture plates at a density of 30,000 cells per well.

Cells in each well were co-transfected with the reporter constructs TESCO-E1b-*Luc* (10 ng) or the empty vector E1b-*Luc* (8 ng), together with 40 ng of each of the expression constructs pcDNA3-SF1 and either pcDNA3-hSRY (wild-type) or pcDNA3-SRY-K128R (mutant). The reporter constructs contained the minimal E1b promoter driving a luciferase gene. pRL-TK-Renilla (Promega, Madison, WI, USA; 1 ng) was added to each well as an internal control. pcDNA3 and pUC DNA were added to make up a total of 100 ng DNA per well, and transfection was performed with 0.38 μl of FuGENE6 Transfection Reagent (Roche, Basel, Switzerland) following manufacturer's instructions. Cells were lysed 48h after transfection and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Six independent assays were performed, each in triplicate. Firefly luciferase activity (Luc) was normalized against that of Renilla luciferase (Ren). Luc/Ren readings for TESCO-E1b-*Luc* were further normalized against that of E1b-*Luc* to obtain the fold change of TESCO activity over that of the empty vector. Fold change of the mutant SRY-K128R construct was then normalized against that of wild-type SRY. Data are therefore represented in the form of mean percentage of wild-type SRY fold change. Statistical analysis was performed by conducting an unpaired t-test.

SRY nuclear import assay

pcDNA3-FLAG-SRY plasmid has been described previously [13]. pcDNA3-FLAG-K128R was created using site-directed mutagenesis. All constructs were verified by sequencing.

HEK293T cells seeded in 6-well plates were transfected with 2 μg/well of either pcDNA3-FLAG-SRY wild-type or pcDNA3-FLAG-K128R mutant using Fugene 6 (Roche). After transfection, immunohistochemistry was carried out using mouse monoclonal antibody against FLAG tag (1:400). The secondary antibody used was Alexa 488-conjugated donkey antimouse IgG (1:500, Molecular Probes, Life technologies). DNA was stained with 0.1 μg/ml of 4',6-diamidino-2-phenylindole (Molecular Probes, Life technologies). Image analysis was performed by using NIH ImageJ (public domain software). Briefly, measurements were taken of the density of fluorescence from the cytoplasm and the nucleus with the background fluorescence subtracted from the equation: $Fn/c = (n - bkgdn)/(cp - bkgdcp)$, where n = nucleus and bkgdn = background in the nucleus, cp = cytoplasm and bkgdcp = background in the cytoplasm.

WT1 mutation analysis
Mutation analysis was performed at the department of clinical genetics of the Amsterdam Medical Center. Briefly: Exon 9 of the WT1 gene (NM_024426, but with the translation initiation codon starting at c.395), including flanking intronic sequences, was amplified by PCR followed by direct sequencing using Bigdye v1.1 chemistry and an ABI3100 sequencer (Life Technologies, Carlsbad, CA, USA). Sequences were analyzed using Codoncode Aligner (CodonCode Corporation, Dedham, MA, USA).

Results

Patient clinical history
A phenotypically normal female presented at the outpatient clinic with primary amenorrhea at the age of 22. She reported to have had some vaginal bleeding at the age of 13 and 14 years which she thought was the start of menarche. This together with the fact that she grew up in different families was the reason of her late clinical presentation. Patient history mentioned migraine and severe asthma for which she was treated with corticosteroids. Physical examination showed normal female external genitalia, with Tanner stage III breast development and stage II pubic hair development. She had a scoliosis and 2.5 cm difference in length of her legs. Hormonal analyses at the age of 22 and 23 years revealed low oestradiol: 11 and <10 pmol/L (normal 100-1000 pmol/L), testosterone: 1 nmol/L (normal 0.5-3 nmol/L), high FSH: 215 and 219 IU/L (normal 1-8 IU/L), and high LH: 78 and 75 IU/L (normal 2-8 IU/L) levels, indicating hypergonadotropic hypogonadism. Furthermore an increase in serum creatinine levels 111-217 umol/L (normal 90 umol/L) was found over the course of ten months suggestive of impaired kidney function, although not diagnosed at the time of presentation. Chromosome analysis on peripheral blood lymphocytes showed the presence of a 46,XY karyotype, and mutational analysis of the *SRY* gene revealed an, at that time, unclassified variant K128R (c.383 A>G, p.Lys128Arg). Based on these results the patient was diagnosed with 46,XY gonadal dysgenesis. Laparoscopic examination showed streak ovaries and a normal, but small, uterus. Because of the known tumor risk in these patients, both ovaries were removed during this intervention (for histology, see below).

Two months after gonadectomy the patient visited the emergency room with complaints of agonizing headache, which were caused by severe hypertension; her blood pressure was 200/127 mmHg, with a good response to treatment with Amlodipine. In addition, blood analyses showed severe renal failure and additional examinations showed that the progressive renal failure was due to primary focal

glomerulosclerosis. The rapid progression of kidney failure together with the diagnosis of 46XY gonadal dysgenesis and bilateral GB and dysgerminoma (for histology, see below) triggered investigation for a *WT1* mutation. The patient is currently on haemodialysis and awaits kidney transplantation, which has to be postponed for five years (until 2014) due to the treatment of the GCC.

Histological and Immunohistochemical analysis

Histological examination of both gonads showed that GB and dysgerminoma was present in a dysgenetic histological context. The lesions on both sides were restricted to the gonad. A representative image of the hematoxylin and eosin (H&E) staining is shown in Figure 1A. In agreement with this diagnosis, the germ cells showed positive staining (shown only for the left GB lesion) for OCT3/4 (Figure 1B), TSPY (Figure 1C) and SCF (Figure 1D). In addition the supportive cells stained positive for FOXL2 (granulosa cell marker, Figure 1E) and were negative for SOX9 (Sertoli cell marker, Figure 1F). The GB removed from the other side showed a similar staining pattern for all markers investigated (data not shown). Both gonads showed multiple micro-calcifications (microlithiasis), represented in the images of Figure 1.

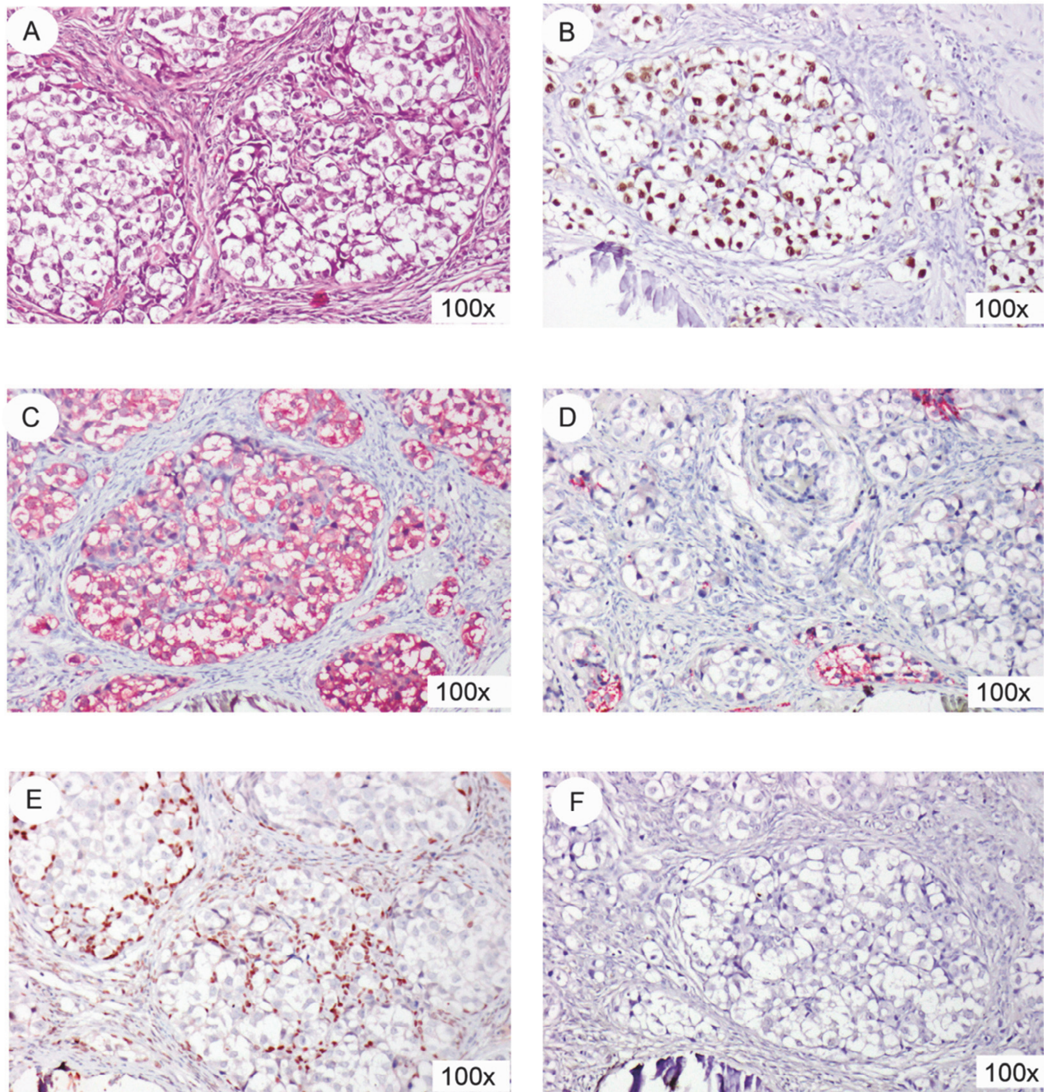

Figure 1 Immunohistochemical staining of the left GB lesion. (A) representative hematoxylin and eosin (HE) staining. The germ cells present in the GB stain positive for OCT3/4 (B, brown staining), TSPY (C, red staining), and SCF (D, red staining). Supportive cells in the GB stain positive for FOXL2 (E, brown staining), while SOX9 (F) is negative. All slides are counterstained with hematoxylin. Magnification 100x for all.

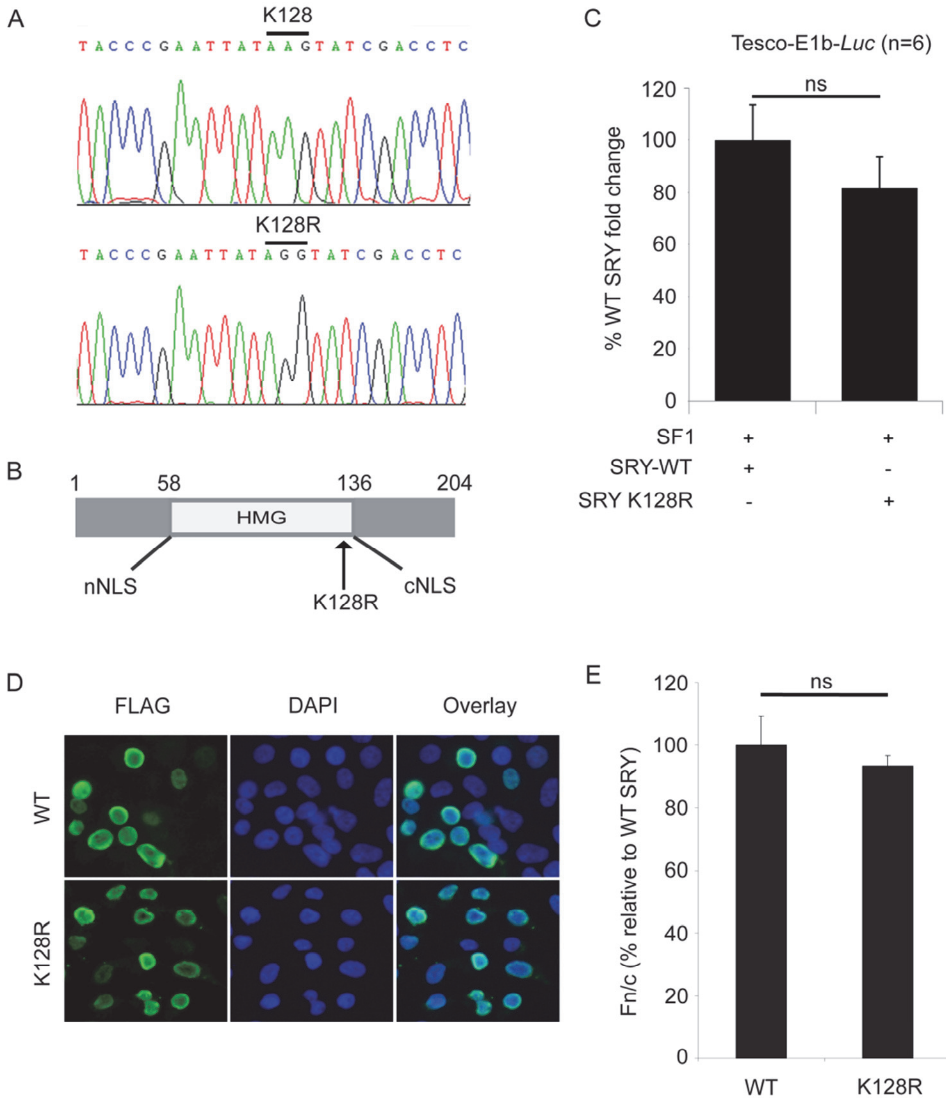

Figure 2 Mutational analysis of SRY and WT1. (A) Wild type (upper panel, control) and mutated sequence (lower panel, patient) of SRY. (B) Schematic representation of the SRY protein. The K128R mutation resides in the HMG domain, just before the cNLS. (C) In vitro luciferase assays of SRY-WT (wild-type) and SRY-K128R (mutant) in HEK293T cell line. Cells were co-transfected with TESCO-E1b-Luc, SF1 and WT or mutant SRY to assess for activation of TESCO. The mean percentages of fold change of luciferase activity of TESCO-E1b-luc over the empty vector, relative to WT SRY levels from six independent assays (each performed in triplicate) are shown. Error bars represent standard error of the mean (SEM). (D) pcDNA3-FLAG-SRY wild-type (WT, 2 µg) or pcDNA3-FLAG-SRY mutant (K128R, 2 µg) were transiently transfected into HEK293T cells using Fugene 6. Exogenous SRY (WT or K128R) expression was detected using a FLAG antibody and a green fluorescent Alexa-488 dye coupled secondary antibody. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Both wild type and mutant SRY show strong nuclear staining.

(E) SRY fluorescence was quantified as previously described [24]. Nuclear accumulation of SRY (WT or K128R) expressed as fluorescence in the nucleus over that in the cytoplasm (Fn/c) were background fluorescence has been subtracted. Measurements represent the average of 3 independent transfections. Results are relative to WT transfected cells (Fn/c given value of 100%). The number of cells analyzed is n=111 (WT) and n=121 (K128R). Error bars represent the standard error of mean values. Two-tail t-Test of unpaired sample means was performed between WT transfected cells and mutant transfected cells and showed no significant differences. P=0.49. (F) Mutated sequence (upper panel, patient) and wild type sequence (lower panel, control) of *WT1*, showing the heterozygous +4C>T change.

Mutation analysis and functional analysis of SRY

Direct sequencing of the *SRY* gene showed the presence of a single nucleotide change at position 383 (A to G, see Figure 2A), resulting in a missense substitution (Lysine (K) to Arginine (R) amino acid change) at position 128 in the SRY protein (hemizygous pattern). A K128R missense mutation in SRY has not been reported to date. The K128R sequence variant is located within the HMG domain of SRY next to the C-terminal nuclear import signal (cNLS) (Figure 2B).

SRY activates *SOX9* expression together with SF1 via a testis-specific enhancer called TESCO, which is located approximately 13 kb upstream of *SOX9* [25]. The ability of the SRY K128R mutant form of SRY to activate *SOX9* via TESCO was analyzed. Results show that the K128R mutation of *SRY* did not significantly affect TESCO activity *in vitro* compared to wild-type SRY (Figure 2C), although a reduction of about 20% was observed.

As the K128R substitution is located next to the cNLS, the effect on nuclear import was also investigated using expression plasmids encoding wild-type and mutants full-length SRY transfected in HEK293T cells. The subcellular localization of SRY was determined 48 h after transfection using indirect immunofluorescence and quantified using image analysis (Figure 2D and E). Wild type SRY efficiently accumulated in the nucleus. The mutant K128R also showed a slight reduced but non-significant difference in nuclear accumulation compared to the wild type protein, indicating that the K128R mutation does not affect the nuclear import function of SRY.

Mutation analysis WT1 and additional FS samples analyzed

As the patient had 46,XY gonadal dysgenesis together with renal failure (focal segmental glomerulosclerosis), and GB with dysgerminoma, without Wilm's tumor, all pointing to FS, the *WT1* gene was analyzed. Direct sequencing of the *WT1* gene showed a single nucleotide change at the start of intron 9 at the position +4 (IVS9 + 4C > T) in a heterozygous state (Figure 2F), characteristic for FS.

To determine if *SRY* mutations together with *WT1* mutations were present in other DSD cases with the same clinical characteristics a review of the literature was done (supplementary Table S1 and S2), showing that this has not been investigated to date. Therefore an additional five DNA samples from FS patients with a proven *WT1* mutation were analyzed for *SRY*, showing no aberrations in *SRY* in addition to the *WT1* mutation.

Discussion

Sex determination and specifically testis differentiation in males is critically dependent on transcriptional regulation of a selective number of genes including *WT1, SRY*, and *SOX9* [26, 27]. Expression of the Y-chromosome located *SRY*, above a threshold and in a critical time window, is crucial in triggering testis formation. SRY will upregulate SOX9 which will orchestrate the formation of the pre-Sertoli cells and further regulates testis development. *WT1* is expressed in the gonadal ridges before the onset of SRY, and plays an important role in testicular as well as kidney formation. It has been suggested that the WT1+KTS isoform functions in terminal Sertoli cell differentiation and homeostasis through the maintenance of a critical level of *SRY* and *SOX9* expression [15].

SRY mutations play a role in 46,XY sex reversal (46,XY DSD) and in about 15% of 46,XY gonadal dysgenesis cases mutations are found [28]. The majority of mutations reside in the HMG domain, which is involved in the binding and bending of DNA. Besides these, mutations located in one of the NLSs have been reported, resulting in a reduced nuclear import of SRY. The K128R mutation described here does not lead to a statistically significant reduction in transactivational activity as ascertained by an *in-vitro* assay, although a minor reduction (about 20%) was observed. In addition, although located adjacent to the cNLS of SRY, the mutation does not result in a significant reduction in nuclear import of the protein. This suggests that the phenotype of the patient is not due to a nuclear import defect as has been observed in other cases [13, 14, 29]. Although the lysine on position 128 is conserved between man and mouse, mutation of lysine on position 128 to arginine does not affect regulation of SRY subcellular distribution by (de-)acetylation via p300 [30]. Taken together, the results show that the mutation has little effect on the *in vitro* transactivation and nuclear import assays available. Therefore it is unlikely that the SRY K128R mutation has a significant effect on the (gonadal) phenotype of the patient has, although a more dramatic effect of the mutation in an *in vivo* situation cannot be ruled out.

Reviewing the literature shows that almost all gonadal dysgenesis cases with a proven *SRY* mutation (86 cases in total, Table S1) show a female phenotype (n=81, 94%). Only a few cases show ambiguous genitalia (n=4), and one patient has a male phenotype with ambiguous genitalia (respectively 5% and 1%). In a total of 61 cases gonadal histology was analyzed: 18 showed a GB (30%), one a dysgerminoma (1%) and two GB along with dysgerminoma (3%). This strongly shows the known increased GCC risk in these patients (34% in this cohort). Only a limited number of papers describe the functionality of *SRY* mutations (20 in total, 23%), and the effects range from completely abolished DNA binding to no differences in DNA binding when compared to wild type SRY. Based on these data, no genotype-phenotype correlation can be gathered (Table S1). In some cases the mutations described are also present (in mosaic form) in male family members, with one showing hypospadias and cryptorchidism, one diagnosed with a testicular seminoma, and one without GCC and a normal male phenotype [31-33] (refs 14, 15 and 54 in Table S1). Whether this is also the case in the patient described here, or the mutation arose *de novo,* cannot be investigated because family members are not available for analysis (see above).

The patient described here was initially diagnosed as a 46,XY DSD complete gonadal dysgenesis and a (until now unclassified) mutation in *SRY* was found (i.e. Swyer syndrome), associated with GB and dysgerminoma. However, upon follow-up the diagnosis of progressive renal failure based on focal segmental glomerulosclerosis, prompted analysis of the *WT1* gene. Initially the mild renal impairment found at presentation was not considered to be indicative to screen *WT1* for mutations.

Mutations in *WT1* play a role in 46,XY DSD (i.e. FS, Denys-Drash syndrome, and WAGR-syndrome), and those found in FS consist of *WT1* intron 9 splice-site mutations. These patients have complete 46,XY sex reversal, late onset kidney failure (between 10-20 years), focal segmental glomerulosclerosis, streak gonads, and a high risk for GB, but not Wilm's tumors [34]. Sequence analysis of the *WT1* gene in the patient described here revealed a classic FS mutation in the intron 9 splice-site (IVS9 +4 C>T). This ultimately results in the decrease of the +KTS isoform and it is known that the subsequent reversion in +KTS/-KTS ratio causes defects in the development of glomerular podocytes and male sex-determination, ultimately leading to nephritic syndrome and male-to-female sex reversal, respectively [34, 35]. Careful review of the literature revealed that this is the first patient described having both a *WT1* as well as a *SRY* mutation; however in almost all cases described a mutation screen of both *SRY* and *WT1* was not performed. Analysis of five additional FS patient samples with a proven *WT1* mutation by conventional Sanger sequencing of the *SRY* gene did not reveal any mutations. The majority of FS patients described in literature are

phenotypically females (n=48, 96%) and only two phenotypically males are presented (4%, Table S2). It also underlines the high incidence of GB and/or dysgerminoma in this patient group; 18 out of 39 patients with described gonadal histology showed GB (46%), in one patient carcinoma *in-situ* (CIS) is described, the precursor lesion of GCC in the testis, and in one patient GB next to CIS is described. Five patients had an invasive dysgerminoma next to the GB, one patient is described as having GB and a metastatic tumor, and one patient is mentioned as having dysgerminoma. In the other patients with described gonadal histology, the majority show streak gonads (n=17, 44%), in one it is described as a dysgenetic gonad and in one no gonadal tissue could be found. For the other patients no gonadal histology was analyzed (n=11).

It has been described that *SRY* and *SOX9* expression can be diminished in FS [15] and one could speculate that in the case presented here the effects from reduced *SRY* expression by a mutated *WT1* were exacerbated by the presence of the SRY K128R mutation, although a reduced SRY function could not be shown conclusively *in vitro*. This situation may have contributed to the maldevelopment of the gonads, thereby creating the micro-environment in which embryonic germ cells can survive, and are prone to become malignant. However, screening an additional five FS patients with a proven *WT1* mutation did not reveal any sequence variants in *SRY*. Although this is a limited series of these unique cases, it indicates that presence of *SRY* mutations in FS is rare.

To our knowledge this is the first patient described with a mutation in *SRY* together with a classical FS *WT1* mutation, and thus seems to be a rare condition. Nonetheless, in this patient an optimal diagnosis could have been made, if a screening for *WT1* mutation was performed at an earlier time point. The patient is currently on haemodialysis and awaits kidney transplantation, which has to be postponed for five years (until 2014) due to the GCC in her history. This case clearly demonstrates the significant role of proper diagnosis of the variants of DSD, especially in those with an increased risk for GCC, allowing early diagnosis and treatment, thus preventing the development of invasive cancer. The presence and type of *WT1* mutation has major consequences for the patient. We therefore suggest that *WT1* mutation screening should be performed in all patients with 46,XY gonadal dysgenesis, especially in case of an unclassified SRY variant, and not vice versa. In addition, careful evaluation of kidney function at early stage is recommended in these patients.

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Supplementary Information

Histology description		Phenotype	Mutation	Functionality	Reference
GB	dysgenetic gonads, right microscopic GB	Female (complete GD)	p.E89K	almost completely abolished SRY DNA binding activity	[1]
GB	proliferation atypical germ cells admixed with GB	Female (complete GD)	Q57R	NA	[2]
GB	GB left	Female (complete GD)	C>G pos 184 (arg>gly)	NA	[3]
GB	GB (both patients)	Female (complete GD)	C>T pos 220 (glu>stop)	NA	[3]
GB	GB bilateral	Female (complete GD)	T>C pos 233 (met>thr)	NA	[3]
GB	Li Streak, right GB	Female (complete GD)	A>T pos259, codon 87 Asn>Tyr, (nucl pos 3,258 in HMG box)	NA	[4]
GB	Streaks, right GB	Female but little clitoromegaly	A>G pos 271, codon91 Ser>Gly, (publ: pos 681), S91G	Reduced ability of mutant protein to bind specifically to DNA	[5]
GB	GB	Female (complete GD)	A>T pos 274, codon 92 Lys>Stop	NA	[6]
GB	Streak with bilateral gonadoblastoma in oldest sister (16)	Female (complete GD), 2 sisters (newborn and 16)	C>T pos 289, codon 97 Gln>Stop	Incomplete DNA binding domain	[7]
GB	GB	Female (complete GD)	Y127F, A>T pos 380	Abolished binding capacity	[8]
GB	Bilateral GB	Female (complete GD)	Subst C at +352, A118P	Greatly reduced binding activity	[9]
GB	Bilateral GB	Female (complete GD)	G>T pos 209, W70L	50% reduction nuclear import	[10]
GB	Streaks, right GB	Female (complete GD)	A113T, G>A pos 337, ala>thr, codon113, (publ: pos 747)	NA	[11]
GB	1. bilateral GB +metas 2. encaps GB + streak	Females (2x), (complete GD)	F67V, (pos 199,T>G)	NA	[12]
DG	1. dysgerminoma right. 2.ovarial stroma differentiation of streak gonad, no follicular cells. 3.no histology	Family. Female (all complete GD)	P125L, C>T pos 374, codon 125 Pro>Leu, (publ: pos 681)	Reduced ability of mutant protein to bind specifically to DNA	[5]
GB DG	1. Right GB, Left Dysgerminoma. 2. streak no malignancy	Female (complete GD), 2 sisters	T>A pos 488, codon 163 Leu>stop	NA	[13]
SE GB DG	1. Seminoma. 2. A. left GB, ri DG, B. bilateral GB	Family. 1. father, mosaic state c.72del: hypospadia, cryptorchidism. 2. 2 sisters, Complete GD	c.71delA codon24. stop pos 60	NA	[14]
GB SE	1. bilateral streaks + GB. 2. testicular seminoma	Family. 1. turner, 45x 79%, 46XY 21%. 2. father, mutation in mosaic form	Del C>frame shift L94fsX180	NA	[15]
GB	1. Gonadal dysgenesis. 2. bilateral GB.	Family. 1. and 2. Female (complete GD). Father, mutation in mosaic form	c.347T>C p.Leu>Ser	Strong reduction in DNA affinity.	[54]
YST	Yolk sac tumor (pT4) with chorionic giant cells	Female (complete GD)	G>A pos 284 codon 95 Gly>Glu	NA	[16]
OT	Ovotestis	Female. Ambiguous genitalia. (Ovotesticular DSD)	T>C pos 237 codon 79 Ala>Ala. (silent)	NA	[17]
OT	Ovotestis	Female. Ambiguous genitalia. (Ovotesticular DSD)	T>A pos 302, codon 101, Leu>His	NA	[17]
OT	Ovotestis (unilateral)	Female. (true hermaphroditism)	T>C codon 60 pos 179, Val>Ala	NA	[18]
S	Bilateral streaks	Female. (Turner, 45X) (2x)	S18N, G>A pos 53	NA	[19]
S	Streak gonads	Female (complete GD)	G>A pos 209 codon 70 (Trp>Stop)	NA	[20]
S	Streak	Female (complete GD)	C>A pos 306 (mr in table pos 226), Ser>Arginine pos 76	NA	[21]
S	Streak	Female (complete GD)	C>G pos 270, codon 90 Ile>Met, (publ : nucl pos 680)	N.a. (but in other study by Harley et al 1992 Science, this mutation à reduced DNA binding	[22]
S	Streak	Female (complete GD)	A>T pos 317, codon106 Lys>Ale (publ: nucl pos 727)	NA	[22]
S	Streak	Female (complete GD), 46XY9h+	Deletion A pos 324, codon 108, frameshift. (publ: nucl pos 734)	NA	[22]
S	Streak, no malignancy	Female (complete GD)	G>A pos 320 codon 107 Trp>stop	NA	[23]
S	Streak, no malignancy	Female (complete GD)	AGAG deletion pos 363-366, codon 121-122, frameshift	1/3 protein affected, non functionality is supposed	[24]
S	Gonadal streak removed	Female (complete GD)	Tyr129stop	NA	[25]
S	Ultrasound: streak	Female (complete GD)	A>T +275, K92M	Reduced binding activity	[9]

Table S1 *SRY* mutation literature overview (table continued on next page). NA.: not available, GB: gonadoblastoma,

DG: dysgerminoma, SE: seminoma, YST: yolk sac tumor, OT: ovotestis, S: streak, DS: dysgenetic testis, T: testis, ov st: ovarian stroma, GD: gonadal dysgenesis, POF: Premature Ovarian Failure

	Histology description	Phenotype	Mutation	Functionality	Reference
S	Streak, No GB	Turner, 45x (80%), 46 XY (20%)	Insertion T, frame shift. N82X	NA	[26]
S	Streak, No GB	Turner, 45x (86%), 46 XY (14%)	Insertion A frameshift. L159fsX167 downstream HMG	NA	[26]
S	Streak, No GB	Turner, 45x (89%), 46 XY (11%), also ambiguous genitalia	G>C codon 74, Q74H	NA	[26]
S	Streak, No GB	Female (complete GD)	C>T pos 132, Arg>Gly	NA	[27]
S	Bilateral Streak, no GB	Female (complete GD)	Deletion A in codon 82 at position +244> frame shift	NA	[28]
S	streak	Family, 1. complete GD, 2. healthy father	Pos 8 C>T third codon, S3L	Protein remodelling: could disrupt N terminal a helix	[29]
S DS	Streak (R), dysgenetic testis (L), wollfian and mularian ducts	Male, (ambiguous genitalia, partial GD)	G>A pos 53, S18N	NA	[30]
S T	1 and 2. streak with overialinke stroma and absence GC left, right: prepubertal testis. 3. both gonads ovarian like stroma and absence of GC	Family, Different phenotypes: (compete (1,3) à partial (2) GD)	R30I	affects mainly SRY PKA phosphorylation, therefore reducing DNA-binding activity	[31]
S	Right side consisted entirely of fibroadipose tissue. The left gonad small amount of ovarian sromalike tissue. No follicles were seen; cluster of tubular structures was present. One of the tubules was ciliated, reminiscent of epididymis.	Female, (POF)	Gln2Stop	No protein due to stop codon	[32]
S	Streak gonads with ovarian stroma, no germ cells	Female (complete GD)	G>A pos 192, codon 64 (Met64Leu)	NA	[33]
	Cystic gonad with ovarian stroma and no germ cells	Female (complete GD)	G>C pos 178, codon 60 (Val60Leu)	NA	[33]
ov st	Fragments of mesothelial tissue with a highly fibrotic stroma and cystic epithelial inclusion	Female (complete GD)	GG>AT pos 224-225, codon75 (Arg>Asn)	NA	[34]
ov st	ovarian like stroma, absence germ cells, hilus cell hyperplasia	Female (complete GD)	N65H	No DNA binding activity in vitro	[31]
ov st	Ovarian stroma, no tubules	Female (complete GD)	T>C pos 203 codon 68 (Ile>Thr)	NA	[35]
ov st	Ovarian stroma, no tubules	Female (complete GD)	T>A pos 381 codon 127, (Tyr>stop)	NA	[35]
NA	Germ Cells neg	Female (complete GD)	C>T pos 277, codon 93 (Gln>stop)	NA	[36]
NA	No GB	Female (complete GD)	C>T pos 397 (Arg>Trp)	NA	[3]
NA	No GB	Female (complete GD)	S143C	NA	[2]
NA	NA	Female (complete GD)	I90M	NA	[37]
NA	NA	Female (complete GD)	p.G95R	electrostatic repulsion caused by the proximity of positive charges that could destabilize the tip of helix 2	[1]
NA	NA	Female (complete GD)	T>A pos 12 (Tyr>stop)	NA	[38]
NA	NA	Female, (45x, 47XYY)	T deletion pos 12 (Tyr>stop)	NA	[39]
NA	NA	Female (complete GD)	pos 191 T>G, M64R	NA	[40]
NA	NA	Female (complete GD)	codon 43 pos 127 lys>stop, K43X	NA	[40]
NA	NA	Female (complete GD)	pos 199 T>G, F67V	NA	[40]
NA	NA	Female (complete GD)	G>A pos 209 codon 70 (Trp>stop)	NA	[41][42]
NA	NA	Female (complete GD)	G>C pos 283 codon 95 (Gly>Arg)	NA	[41]
NA	NA	Female (complete GD)	C>T pos 256, codon 86 Arg>Stop	NA	[43]
NA	NA	Female (complete GD)	T>C, pos 326, codon 109 Phe>Ser	No differences in DNA binding compared	[44]
NA	NA	Female (complete GD)	A>G pos 380, codon 127 Tyr>Cys	Abolished SRY protein binding ability (binding specificity in paper..)	[45]
NA	NA	Sex reversal, Partial GD	T>A pos 385, Y129N	Lower degree of cooperativity in binding	[46]
NA	NA	Female (complete GD)	C254T>C, M85T	NA	[47]
NA	NA	Female (complete GD)	c.391C>T, R130X	NA	[47]
NA	NA	Female (complete GD)	A>G codon Glu38Gly	NA	[48]
NA	NA	Female (complete GD)	T>A, nucl pos 387	NA	[48]
NA	NA	Female (complete GD)	T>A codon 129 pos 387, Tyr>stop	NA	[49]
NA	NA	Female (complete GD)	c.294G>A, Trp98stop	NA	[50]
NA	NA	Family. 3 females (complete GD)	c.334G>A, Glu112Leu	NA	[50]
NA	NA	Female (complete GD)	Three base pair deletion promotor SRY in one of the Sp1 binding sites	Abolished Sp1 binding in vitro	[51]
NA	NA	Female (complete GD)	insA 103 stop, F67L	NA	[52]
NA	NA	Female (complete GD)		NA	[53]

Table S2 *SRY* mutation literature overview (continued (previous page))

References SRY mutation overview

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	Histology description	Phenotype	Mutation	Functionality	Reference
GB	Bilateral streak with GB. Right TE (smooth muscle, cartilage, nerve tissue and glial elements)	Female (pure GD)	NA	NA	[1]
GB	Bilateral streak with right side GB	Female (pure GD)	NA	NA	[1]
GB	Streak gonad (Right), GB Left side (removed 19 yrs). Unspecific glomerular changes	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+4 C>T	Shift in +/-KTS ratio to 0.39	[2]
GB	Streak gonads GB. FSGS	Female, normal external genitalia	IVS9+4 C>T	Shift in +/- KTS ratio	[3]
GB	Right GB, Left Streak	Female, hypotrophic uterus	IVS9+4 C>T	NA	[4]
GB	Streak gonads, Microscopic GB	Female	IVS9+5 A40>G	NA	[5]
GB	GB (bilateral)	Female, normal external genitalia	IVS9+5 G>A	NA	[6]
GB	Streak gonads, Bilateral GB	Female	Mut not given, described as a mutation in intron 9	NA	[7]
GB	Left: GB, Right dysgenetic gonad	Female	IVS9+4 C>T	NA	[8]
GB	GB	Female	Not further specified, intron9 mut	NA	[9]
GB	Streak gonads, bilateral GB. FSGS	Female	IVS9+4 C>T	NA	[10]
GB CIS	Bilateral GB and right side CIS. Testis and sex cords. FSGS	Male (perineal hypospadias, urogenital sinus, bifid scrotum and bilateral cryptorchid testes)	IVS9+4 C>T	Less +KTS (shift in +/-KTS ratio from 2.2 to 0.25)	[11]
GB DG	Complete GD, streak gonads, GB DG FSGS	Female (complete GD), Hypoplastic uterus, normal fallopian tubes	IVS9+4 C>T	NA	[12][13]
GB DG	Left: GB and DG. Right: ovarian stroma (streak).	Female, normal external genitalia (slightly hypertrophied clitoris), normal; vagina, prepubertla uterus	Not assessed	NA	[14]
GB DG	GB and DG	Female	IVS9+5 G>A	NA	[15]
GB DG	Left: GB, Right: DG	Female, hypoplastic uterus	Mut not given, described as a mutation in intron 9	NA	[16]
GB DG	Bilateral GB and left DG(11 yr). Developed Pilocytic Astrocytoma (17yr)	Female, normal external genitalia, normal appearing vagina and uterus	IVS9+5 G>A	NA	[17]
GB	Complete GD, streak gonads, GB and metastatic tumor. FSGS	Female (Complete GD), Vestigial uterus, primitive ovarian tissue (left side), no Wolffian structures	IVS9+5 G>A	Shift in +/-KTS ratio to ~0.5	[12][18]
CIS	Testicular atrophy, CIS, arrest at spermatocyte stage, LC nodules	Male (glandular hypospadias, herniotomy)	IVS9+5 G>A	Less +KTS, less *SRY* and *SOX9* expression	[19]
DG	Right DG (13yr, 20x25cm), Left gonad removed, gonadal dysgenesis Retroperitoneal DG 15yr	Female	IVS9+4 C>T	NA	[20]

Table S2 WT1 mutation literature overview (table continued on next page).

GB: gonadoblastoma, CIS: carcinoma in situ, DG: dysgerminoma, d.g.: dysgenetic gonad, S: streak, NGT: no gonadal tissue, NA.: not available, GD: gonadal dysgenesis, FSGS: focal segmental glomerulosclerosis, LC: leydig cell

	Histology description	Phenotype	Mutation	Functionality	Reference
d.g.	Left and right: dysgenetic gonad	Female	IVS9+4 C>T	NA	[8]
S	Streak gonads, Focal Glomerular Sclerosis	Female, normal external genitalia	IVS9+2 T>C	Shift in +/- KTS ratio	[3]
S	Complete GD, streak gonads	Female (complete GD)	IVS9+4 C>T	NA	[12]
S	Complete GD, streak gonads	Female (complete GD)	IVS9+4 C>T	NA	[12]
S	Complete GD, streak gonads. FSGS and segmental hyalinosis	Female (Complete GD)	IVS9+4 C>T	NA	[12]
S	Streak gonads (removed 17yrs). Retriperitoneal myofibroblastic tumor (23 yrs). FSGS	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+4 C>T	NA	[2]
S	Streak gonads. FSGS	Female, normal external genitalia	IVS9+4 C>T *	Shift in +/- KTS ratio	[3]
S	Streak gonads. FSGS	Female, normal external genitalia	IVS9+4 C>T *	Shift in +/- KTS ratio	[3]
S	Streak gonads (tubules and isolated germ cells? Mentioned in text). Wilms' Tumor 3 yr, no sclerosis of glomeruli	Female, normal external genitalia, hypoplastic uterus	IVS9+4 C>T	NA	[13]
S	Complete GD, streak gonads. FSGS	Female (Complete GD)	IVS9+5 G>A	NA	[12]
S	Complete GD, streak gonads	Female (Complete GD)	IVS9+5 G>A	Shift in +/-KTS ratio to ~0.5	[12][21]
S	Complete GD, streak gonads. FSGS	Female (Complete GD)	IVS9+5 G>A	NA	[12][22]
S	Complete GD, streak gonads. Glomelar Sclerosis	Female (Complete GD), Infantile uterus	IVS9+5 G>A	NA	[12][23]
S	Streak gonads (removed 24 yrs). FSGS	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+6 T>A	Shift in +/-KTS ratio to 0.48	[2]
S	Streak gonads	Female, normal external genitalia, vagina, uterus	IVS9+4 C>T	NA	[24]
S	Streak gonads, no malignancy. FSGS	Female	IVS9 +5 G>A	NA	[10]
S	Streak gonads	Female	IVS9+5 G>A	NA	[25]
S	Streak gonads, no signs of malignancy	Female, anfantile uterus	IVS9+4 C>T	NA	[26]
NGT	No gonadal tissue. Bilateral testosterone hilar cell adenoma.	Female, normal external genitalia, small uterus	IVS9+4 C>T	NA	[27]
NA	Focal Glomerular Sclerosis	Female, normal external genitalia	IVS9+5 G>T	Shift in +/- KTS ratio	[3]
NA	Nephrotic syndrome at 9 months. Diffuse mesangial sclerosis	Female, normal external genitalia, Small uterus	IVS9+5 G>A	Shift in +/-KTS ratio to 0.4	[28]
NA	FSGS	Female	IVS9+5 G>A	NA	[29]
NA	Orchidectomy 11 months, testicular tissue?	Female, normal external genitalia, bicornate uterus, normal vagina	IVS9+5 G>A	NA	[30]
NA	NA	4 patients	?	?	[31]
NA	NA	Female	Not further specified, intron9 mut	NA	[9]
NA	NA	Female	IVS9+5G>A	NA	[29]
NA	FSGS	Female (Uterus)	IVS9+5G>A	Shift in +/-KTS ratio to 0.67 (1.35 and 1.42 in parents)	[32]

Table S2 WT1 mutation literature overview (continued (previous page).

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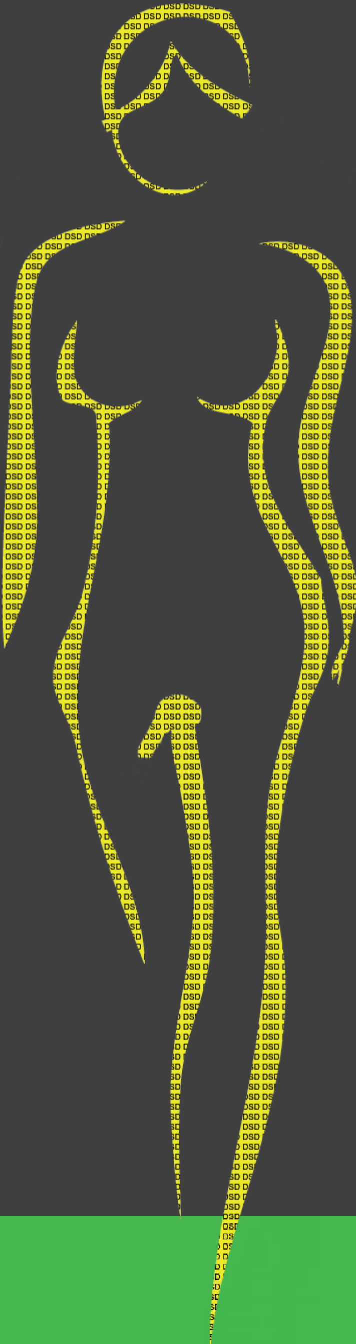
CHAPTER 6

MAP3K1 is a testis cancer susceptibility gene



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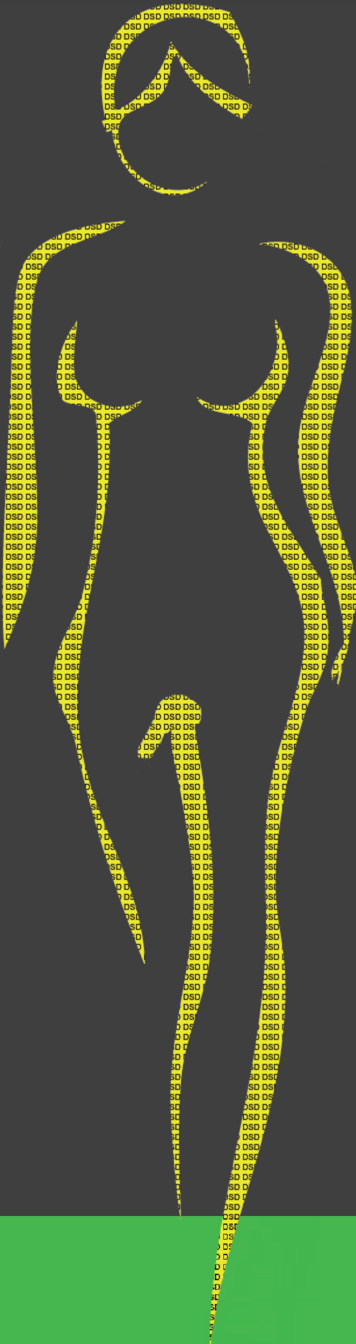
CHAPTER 7

A novel AMH missense mutation in a patient with persistent Müllerian duct syndrome



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Abstract

Persistent Müllerian Duct Syndrome (PMDS) is characterized by the presence of a uterus, fallopian tubes, and the upper part of the vagina in phenotypic normal male patients.
Here, we report a patient diagnosed with PMDS with a novel homozygous missense mutation in anti-Müllerian hormone (AMH) (single nucleotide insertion (C) at position 208 (c.208dup, p.Leu70fs) leading to a frameshift and the introduction of a premature stop codon.
Biopsy of both gonads revealed that germ cells were present in an irregular distribution. However, the absence of OCT3/4, PLAP and c-KIT expression indicated physiological maturation.

Introduction

Sexually dimorphic development of the reproductive system is the result of three sequential processes: initial or chromosomal sex determination, differentiation of the bipotential gonad into either testis or ovary, and finally sex-specific development of the reproductive tracts and external genitalia under influence of hormones produced by the gonads [1, 2]. Testosterone produced by testicular Leydig cells will induce the differentiation of the male reproductive tract, i.e. the Wolffian ducts into epididymis, vas deferens, and seminal vesicles. Anti-Müllerian hormone, produced by the testicular Sertoli cells, is responsible for the regression of the Müllerian ducts. In the absence of AMH the Müllerian ducts will develop into normal female internal organs [3].

Persistent Müllerian Duct Syndrome (PMDS) is characterized by the presence of a uterus, fallopian tubes and the upper part of the vagina in phenotypic normal male patients, and is usually discovered at surgery for cryptorchidism or inguinal hernias. AMH, a member of the transforming growth factor β (TGFβ) family, signals through a heterodimeric receptor complex consisting of a specific type II receptor (AMHRII) and shared type I receptors (ALK2, 3, and 6) [4, 5].

Mutations within the *AMH* and *AMHR2* genes are in approximately 85% of the cases responsible for this error in sex differentiation [6]. Mutations in the three type I receptor genes have not been detected [6], and thus for the remaining 15% of the cases the causative genes remain to be identified. Patients with mutations in *AMH* or *AMHR2* represent with a similar phenotype. However, when assessed before puberty, levels of circulating AMH are usually extremely low or undetectable in patients with *AMH* mutations, whereas normal levels are observed in patients carrying *AMHR2* mutations [6].

We report here a novel missense mutation within *AMH* due to a single basepair insertion leading to a premature stop codon. The mutation was homozygously present in the patient due to inheritance from both parents.

Materials and Methods

Case report

A phenotypically normal male presented at birth with bilateral nonpalpable testis. There was no parental consanguinity. Ultrasonography showed that the testis were high scrotal and inguinal respectively, therefore the policy was to wait and see if descent would occur spontaneously. At the age of two, ultrasound showed that both

testes (0.5 cc in volume) were abdominally located. Karyotyping of peripheral blood lymphocytes showed a normal 46,XY karyotype.

Laparoscopic exploration at the age of two revealed a uterus and tubae next to the testes and epididymides at the level of the internal abdominal ring. A biopsy was taken from both gonads and after subsequent dissection of the Fallopian tubes bilateral orchidopexy was performed. The Müllerian structures were left *in situ*.

Histology and immunohistochemistry

Immunohistochemical staining was performed on formalin fixed paraffin-embedded slides of 3 μm thickness. The antibodies directed against VASA, TSPY, OCT 3/4, c-KIT and PLAP have been described before [7, 8]. Briefly, after deparaffinization and 5 min incubation in 3% H2O2 to inactivate endogenous peroxidase activity, sections were subjected to heat-induced antigen retrieval under pressure of up to 0.9 bar in the appropriate buffer. After blocking endogenous biotin using the Avidin/Biotin Blocking Kit (SP-2001 Vector Laboratories, Burlingame, CA, USA), sections were incubated either overnight at 4°C (VASA, TSPY, PLAP, c-KIT) or for 2 h at room temperature (OCT 3/4). Next, sections were incubated with the appropriate biotinylated secondary antibodies, followed by avidin-biotin complex. Peroxidase activity was developed with 3,3-diamino-benzidine (DAB) and Alkaline Phosphatase activity was developed with New Fuchsine (Vector Laboratories).

Hormonal follow up

Serum determinations of Inhibin B, AMH, testosterone, LH and FSH were performed in the endocrine laboratory of Erasmus MC (Rotterdam) as described previously [9]. Serum FSH and LH were determined with the Immulite assay (Diagnostic Products Corporation, Los Angeles, USA). Serum testosterone was determined using a Coat-a-Count radioimmunoassay purchased from Siemens (Los Angeles, CA). Inhibin B was measured by ELISA purchased from Serotec Ltd, Oxford, UK. An in-house AMH ELISA, with a detection limit of 6.3 pg/ml, (commercially available through Beckman-Coulter, Marseille, France) was used to measure AMH [10].

A

a

b

c

a

b

c

c.208dup

B

```
               68 69  70 71 72 73 74 75 76 77 78 79 80
                S  P   L  R  V  V  G  A  L  S  A  Y  E
Wild type:     TCCCCC-CTGCGGGTGGTGGGGGCTCTAAGCGCCTATGAG
               |||||| |||||||||||||||||||||||||||||||||
Mutant:        TCCCCCCCTGCGGGTGGTGGGGGCTCTAAGCGCCTATGA
                S  P  P  A  G  G  G  G  S  K  R  L  *
               68 69 70 71 72 73 74 75 76 77 78 79
```

Figure 1 Mutational analysis of DNA from the patient and both parents. A) Pedigree of the patient and sequence chromatograms of part of AMH corresponding to position c.208 and flanking sequences of the parents (a and b) and the patient (c).

Molecular analysis

DNA from the patient and parents was isolated from peripheral blood leucocytes using Magnetic Separation Module 1 from Chemagen (Baesweiler, Germany), after informed consent from both patient and his parents was received. All five exons of the AMH gene, including flanking sequences, were amplified by PCR using specific primers (Table 1). Direct sequencing was performed on an ABI prism 3100 automatic capillary sequencer (Applied Systems, Nieuwerkerk aan den IJssel, The Netherlands). In the laboratory of Clinical Genetics the mutation was confirmed and parental DNA was analyzed for the presence of the pathogenic variant using direct sequence analysis using an independent primerpair (Table 1) (ABI 3730XL automated sequencer). The AMH sequence with accession number NG_012190.1 was used as a reference sequence. For nucleotide numbering we followed the recommendation of the Human Genome Variant Society (HGVS) nomenclature, where nucleotide 1 is the A of the ATG-translation initiation codon (http://www.hgvs.org/mutnomen/recs-DNA.html) [11].

Results

At the age of two the patient showed a normal basal testosterone (0.10 nmol/L) and Inhibin B (91 ng/L) production. However, AMH levels were extremely low (0.2 μg/L). Measurements at the age of 10 years showed normal basal prepubertal levels of LH (0.2 U/L), FSH (1.7 U/L), and testosterone (0.10 nmol/L). Inhibin B levels were also in the normal prepubertal range (149 ng/L), whereas AMH levels remained extremely low (0.2 μg/L).

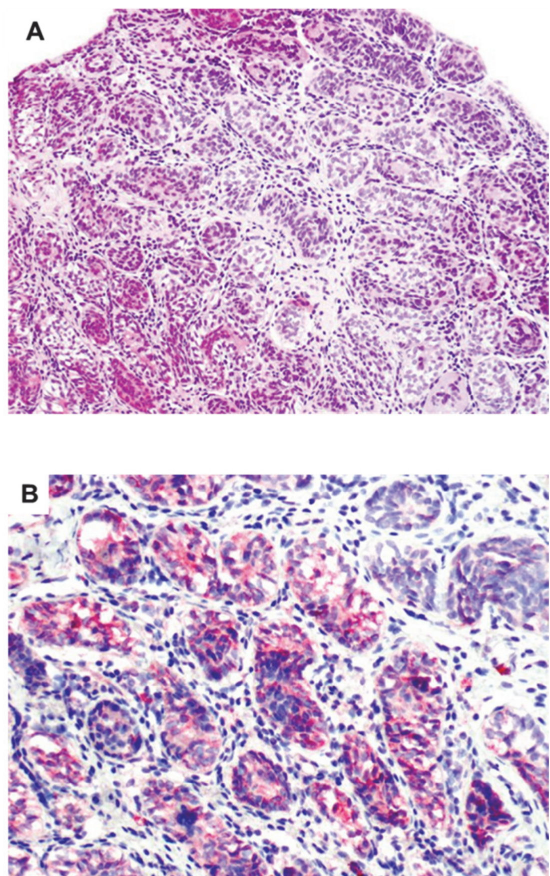

Figure 2. Morphological analysis of testicular tissue of the patient at the age of two. A) Representative hematoxylin and eosin (HE) staining showing normal testicular tissue. B) Germ cells (VASA positive) are present and show an irregular distribution pattern. Magnification HE 100x and VASA 200x.

Since AMH levels were extremely low, a mutation in the *AMH* gene was suspected. Direct sequencing revealed the presence of a novel homozygous single basepair insertion at position 208 in exon 1 (c.208dup, p.Leu70fs) (Figure 1). The insertion of an additional C leads to a frameshift and the introduction of a premature stop codon. Both parents were heterozygous for this mutation (Figure 1).

Histological examination of testicular biopsies showed the presence of normal testicular tissue in both gonads (Figure 2A). Germ cells were present but in an irregular pattern, confirmed by immunohistochemistry for VASA (Figure 2). There were tubules that contained germ cells but also tubules that were empty and therefore showed no VASA positive staining. Comparison with tissues of normal descended testis of the same age group as the patient showed no difference in number and distribution of spermatogonia. Healthy boys show remarkable variation in the number of spermatogonia until puberty. Immunohistochemistry for the markers OCT 3/4, PLAP, and c-KIT was negative, thus there were no indications for the presence of an invasive germ cell tumor or an *in situ* lesion in the patient.

Discussion

Here, we describe a patient with PMDS with a novel pathogenic mutation in the *AMH* gene. An insertion of an additional C at basepair position 208 results in a frameshift, and consequently in the introduction of a premature stopcodon. *AMH* maps to chromosome 19 band p13.3, and consists of five exons, encoding for a glycoprotein of 535 amino acids [12]. A number of pathogenic mutations spanning the *AMH* gene have been described. Most of the mutations, including the novel mutation we identified, are located in exon 1 or exon 5 [6], although hotspots for mutations have not been observed. Interestingly, at the position where we detected the novel mutation, a previous study has reported a basepair change T>C, leading to a L70P amino acid change [13], which suggests that also in the *AMH* gene certain positions may be more prone to mutations. Furthermore, most of the reported mutations were homozygously present due to parental consanguinity. In our patient, the mutation was present in a homozygous pattern due to inheritance from both non-consanguineous parents.

Surgical procedures in men with PMDS are still under debate. Odi *et al* claim that surgery should take place in two separate procedures: 1) testis reposition into the scrotum with hernia repair and testis biopsy; 2) orchidectomy upon indication for atrophic testis or when orchidopexy cannot be performed [14].

Germ cell tumors, mostly seminomas, have been reported in patients with PMDS, although the incidence is similar to that in non-affected males with cryptorchidism [15]. Manassero *et al* described a patient with PMDS and transverse testicular ectopia with the presence of a mixed germ cell tumor (teratoma and embryonal carcinoma) in a testis in the scrotal position after orchidopexy at the age of 5 years [16], suggesting that orchidopexy early in life does not decrease the risk of malignancy in this patient group. Therefore it has been suggested that orchidectomy should be performed [15].

Our patient had normal testicular tissue in both biopsies. Germ cells were present but showed an irregular distribution pattern. However, comparison with normal descended testis tissue of the same age group as the patient showed no difference in number and distribution of spermatogonia, due to the remarkable variation in number of spermatogonia in this age group. Bilateral orchidectomy is a definite procedure leading to life-long androgen substitution, induction of puberty and loss of fertility. We suggest that with the markers for germ cell tumors known to date [17], a more individual tailored approach is possible. Therefore evaluation of both testis and of potential fertility problems will be part of clinical follow up [15, 18].

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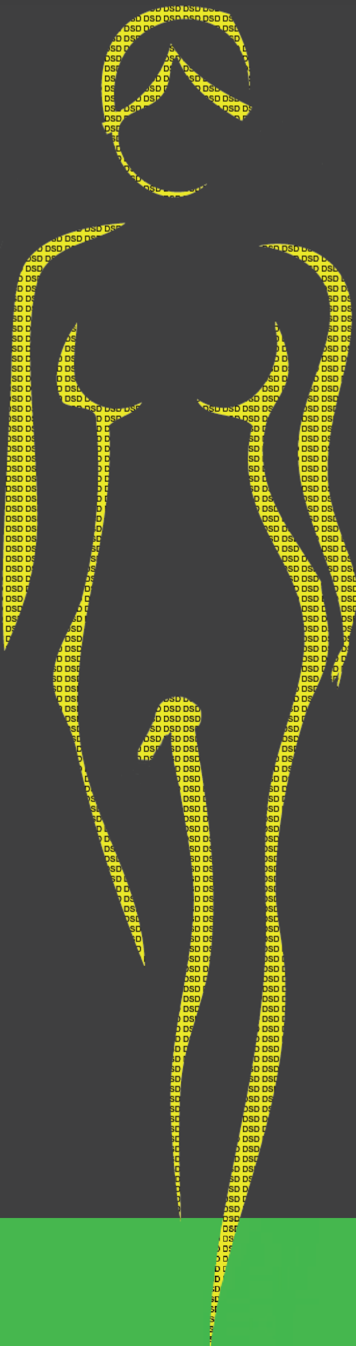
CHAPTER 8

Role of epigenetics in etiology of Germ Cell Cancer



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Abstract

Embryonic development is strictly controlled by functionality of genes, both protein encoding- and noncoding-, in which the existing networks can act both on transcription and translation regulation. During the process of differentiation, defined epigenetic modifications occur, associated with irreversible loss of pluripotent potential. In adult organisms, under normal physiological conditions, only the germ cells can regain pluri/totipotent potential after fertilization.

Germ cell cancers (GCC) are unique because of a number of characteristics. In spite of their clinical presentation, i.e., predominantly after puberty, they arise from primordial germ cells/gonocytes that have failed appropriate maturation to either pre-spermatogonia (male) or oogonia (female). In addition, GCC mimic embryonal development to a certain extent, including capacity for totipotency. This knowledge has allowed the identification and application of informative diagnostic markers, including OCT3/4 (POU5F1), NANOG, SOX2 and SOX17. An additional marker is the overall demethylated status of the genome. Genetic mutations in GCC are rare, which is exceptional for solid cancers. Our hypothesis is that a disturbed epigenetic regulation (through combined interaction of genetic or environmental parameters; referred to as genvironment) affect physiological embryonic germ cell development, resulting in delayed or blocked maturation, and potentially progression to an invasive GCC. In this respect, studies of patients with various forms of Disorders of Sex Development (DSD) have increased our knowledge significantly. In fact, genvironmental influences can lead to retention of existence of embryonic germ cells, the first step in the pathogenesis of GCC, resulting into the precursor lesions gonadoblastoma or carcinoma in situ. Identification of epigenetic alterations could lead to better understanding these processes and development of specific markers for early detection, eventually leading to development of targeted treatment. This review describes an interactive model related to the role of epigenetics in GCC pathogenesis, focusing on DNA methylation, histone modifications, epigenetic memory and inheritance, as well as environmental factors.

Key Words:
Epigenetics, Germ Cell Cancer, methylation, histone modifications, Genvironment

Introduction

Type II (testicular) germ cell tumors, referred to as Germ Cell Cancers (GCC), are the most common malignancy in Caucasian adolescents and young adults and their incidence is still rising [1, 2]. GCC arise from primordial germ cells (PGC) or gonocytes and are subdivided into seminomas/dysgerminomas and non-seminomas with carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesions [3] . Non-seminomas can be further categorized into embryonal carcinoma, which can differentiate into somatic lineages and extra – embryonic tissues (teratoma vs yolk sac tumor and choriocarcinoma respectively) [3]. PGC have the intrinsic capacity for pluri/totipotency, reflected in GCC, in which even the germ line can be formed in non–seminomatous tumors [4].

Regulation of pluripotency

Embryonic development is controlled by highly orchestrated patterns of gene expression (both temporal and tissue specific). It is essential to understand these networks in order to gain insight into disturbed development, including cancer. A number of genes are known to play critical roles in establishing and maintaining pluripotency, including OCT3/4, NANOG, SOX2 [5-8]. As such they are candidates for involvement in GCC development. Indeed, OCT3/4 has been shown to be an important diagnostic marker for the different histological elements of GCCs, including the precursor lesions [9]. Expression of SOX2 is seen in non-seminomas, especially and consistently in embryonal carcinomas, and more heterogeneously in the other components, whereas expression of a related gene, SOX17, is present in normal germ cells, CIS/GB and seminomas [10, 11].

Genetics and Epigenetics in GCC

Although much knowledge has been gained over the last decades, the exact role of the various risk factors involved in development of GCC is still unclear. Several genetic loci have been linked to increased risk of GCC [12], and the familial predisposition (i.e., increased risk of brothers and sons) [13] supports a significant genetic component in the pathogenesis of this type of cancer. So far, somatic mutations are rare in GCC [14], with a few notable exceptions such as *KIT* and *KRAS2* [15-17]. It is therefore likely that epigenetic factors are involved in GCC development as well. This is not unexpected as a clear role for epigenetic regulatory processes exist in both the mechanisms of initiating and protecting pluripotency of embryonic stem cells as well as in maintaining the identity of differentiated cell types [18]. Deregulation of this process may alter

chromosomal stability, specifying properties of stem cells, self-renewal and the potential to differentiate, leading to initiation and/or progression of cancer. Disrupted epigenetic regulation might therefore be one of the underlying factors in the origin and biology of GCC. As such, epigenetic alterations could be candidates for specific diagnostic and prognostic markers. This review gives an overview of the rapidly growing field of understanding the impact of epigenetics in normal and disrupted development, specifically in the pathogenesis of GCC.

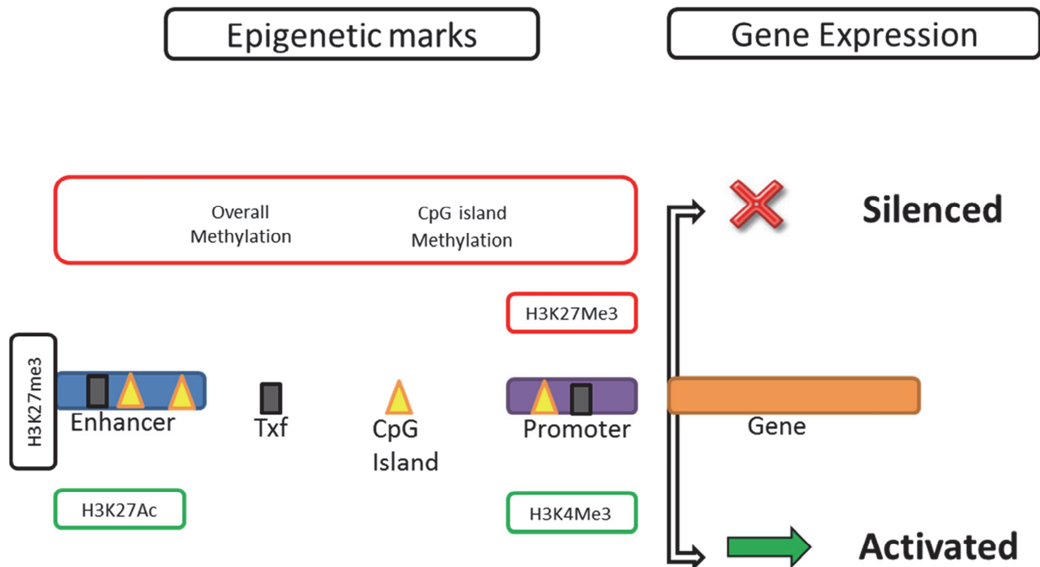

Figure 1 Schematic representation of the role of epigenetic changes in regulating gene expression, controlled by DNA methylation and histone modification. This will determine whether a gene is susceptible for transcription or not, related to the presence of appropriate transcription factors. In general, DNA methylation (indicated in the red box) is associated with gene silencing. It can occur at enhancer and promoter sites, as well as at CpG islands/shores. In this scheme the enhancer site is located in front of the gene, but can be located several Mb away (up- or downstream) from the gene. Histone modifications occur at enhancer or promoter sites and can be either activating or silencing (indicated in red and green box, respectively). In general, H3K4Me3 is active, whereas H3K27Me3 in the promoter region is inactive or silenced. The presence of both represents a bivalent state. A high H3K27Ac mark at an enhancer site is indicative for an active enhancer state. The presence of H3K27me3 together with the absence of H3K27ac will poise the enhancer for activation or repression at a later stage in development.

Gonadal Development

PGC are the progenitor cells of gametogenesis in later life, first recognized at day E6.5 in mice and 5-6 weeks gestational age (GA) in humans [19]. These PGC are characterized by their alkaline phosphatase reactivity, which is used as a specific marker [20]. Immediately prior to this, pre-PGC begin to express *BLIMP1* mRNA and protein, which maintains the pluripotent state [21]. During migration PGC proliferate extensively. Once they reach the genital ridge (around E9-10 in mice and 6-8 weeks GA in human, then called gonocytes), they are under the influence of the sex determination process of the bipotential gonad, under control of SOX9 and FOXL2, amongst others [22, 23], into either testis or ovary. This determines germ cell fate towards either the male or female direction. Male germ cells continue to proliferate until they differentiate to pre-spermatogonia, that then enter mitotic arrest.

Epigenetics and (germ cell) development

Epigenetics is commonly defined as inheritable changes affecting gene regulation that are not due to alterations in primary DNA sequence. The epigenome is highly dynamic, and can change depending on cell type and developmental stage within a single organism. The epigenetic processes work together to establish and maintain both global as local chromatin states e.g. open or condensed which determines gene expression (see Figure 1 for an overview [24]). Epigenetic modifications are relatively stable in somatic cells. In germ cells, however, the epigenome is reprogrammed on a genome-wide level. By E12.5/10 weeks GA most DNA methylation is lost [25] and *de novo* methylation is initiated in males at E14.5, leading to highly methylated mature gametes. This allows re-establishment of parental imprints in germ cells, the erasure of epimutations, and the generation of toti- or multipotent cells [26-28]. Genomic imprinting results in the silencing of one of the parental alleles in a subset of genes, and is different between different tissues and cell types [29]. In addition, epigenetic modifications play essential roles in transmitting transcriptional memory; *i.e.* the phenomenon that epigenetic marks can be inherited across more than one generation [30]. Our knowledge of the epigenome has increased enormously due to next – generation sequencing techniques for mapping DNA methylation and chromatin modifications. A number of relevant issues in this context will be presented in the next paragraph.

Epigenetic memory and inheritance

Epigenetic modifications play essential roles in transmitting transcriptional memory to daughter cells following mitosis [30]. In rare cases GCC development depends on known genetic alterations, however, abnormal cellular memory or epigenetic changes that lead to aberrant gene expression patterns are also critical for tumor initiation and progression [31].

In general, epigenetic marks are cleared and re-established with each generation *i.e.* reprogramming in PGC to ensure the totipotency of cells of the early embryo. However, the environment can stably influence epigenetic marks, which suggest that transgenerational epigenetic inheritance exists. The first evidence that epigenetic marks could indeed be inherited across more than one generation involved transgenes [32-34]. More recently, Lang-Mladek *et al.* showed in plants that, after extreme temperature or UV-B stress, a silent transgene and several transposable elements were activated, and these changes were heritable for two generations [35]. This suggests that some epigenetic marks may avoid erasure during early development, including the germ line. Some repetitive elements show incomplete erasure, which may be essential for chromosome stability and for preventing activation of transposons to reduce the risk of germline mutations. It was shown in mice, that partial deficiency of Apobec1 cytidine deaminase in the maternal germ-line led to suppression of teratomas in both partially and fully deficient males, and significantly reduced teratoma risk in a transgenerational manner among wild-type offspring. These heritable epigenetic changes persisted for multiple generations, and could be fully reversed [36]. Aberrant epigenetic reprogramming in the germ line would cause the inheritance of epimutations, that may have consequences for human diseases [27, 37]. The same is true for histone modifications. It was initially thought that all histones were cleared and replaced by protamines [38], however in sperm, part of the haploid genome remains packed into nucleosomes. These histones are enriched at gene promoters important for development, as well as imprinted genes [39]. This provides these genomic loci with the ability to convey instructive epigenetic information to the zygote. Indeed, genome-wide analysis in fertile and non-fertile men showed that there are moderate differences at these loci, which may have a cumulatively detrimental effect on fecundity [40].

Recently the focus of epigenetic inheritance research has moved to the role of small RNAs e.g. miRNAs, endo-siRNAs and piRNAs. These are present in both sperm and oocytes, and are known to be involved in gene silencing [41-45]. Interestingly, small RNAs are associated with the production of a mobile signal that can travel

between cells and over long distances in plants and nematodes [46]. Three recent papers suggest that piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans,* and that it might be involved in scanning the germline transcriptome for foreign sequences, while endogenous germ-line-expressed genes are actively protected from piRNA-induced silencing [41, 43, 44]. Although there could be a role in GCC development and inheritance via aberrant piRNA regulation, the mechanisms and biology remain poorly understood. The role of RNAs in transgenerational inheritance is extensively reviewed elsewhere [30, 47, 48].

DNA Methylation

DNA can be modified by the addition of a methyl group to cytosine residues, generating 5-methylcytosine (5mC). Three genes are known to be involved in the methylation of cytosine residues. *DNMT3A* and *DNMT3B* are *de novo* DNA methyltransferases, and together with the maintenance methyltransferase *DNMT1* are necessary for DNA methylation essential for embryonic development [49, 50]. Other functions for these enzymes have been reported recently, but will not be discussed here [51]. In germ cells, DNMT3L is expressed in testes at the stage of *de novo* methylation, and interacts with DNMT3A and B [52]. Recently, DNMT3L was described as a novel Embryonal Carcinoma (EC) marker, shown to be essential for growth of these cells [53]. DNA methylation was primarily thought to occur at CpG islands [54], but recent studies based on whole genome analyses have shown the existence of so-called CpG "shores". This refers to methylation areas located adjacent to CpG islands in regions of less dense CpG dinucleotides [55]. In addition, almost 25% of methylation in Embryonic Stem (ES) cells is found outside of CG motifs, being lost upon differentiation [56]. Hypermethylation of CpG islands (strongly associated with gene promoters), and associated gene silencing by transcriptional repression due to inhibition of transcription factor binding is the most extensively studied epigenetic mechanism in cancers [24, 57, 58] including GCC [59-61]. DNA hypomethylation is generally associated with gene activation. Therefore, DNA methylation is a critical part of the control of gene expression, and as such regulatory of differentiation. It is a key part of embryonic development, chromosome stability and genomic imprinting [57]. A complete lack of methylation can only occur in ES cells, but has not been seen in cancer cells [62, 63]. Of specific interest is the fact that imprinting-free ES cells can result in malignant transformation, by conferring cellular immortality [64], including a seminomatous-like cancer.

During epigenetic reprogramming of germ cells, the genome becomes demethylated, and reprogramming by *de novo* methylation will be initiated during

later stages of development. Here, TET1 was found to have an important role, as it catalyzes the oxidation of 5mC into 5‑hydroxymethylcytosine (5hmC) and therefore might play a role in the removal of 5mC. Indeed, repression of TET1 correlated with a reduction in 5hmC levels [65], and high level of Tet1 expression is detected in PGC [66]. The role of TET1 and 5hmC in the epigenome is extensively reviewed elsewhere [67].

Origin of GCC

All GCC originate from a common precursor, the PGC/gonocyte [68], this is in line with findings on Alkaline phosphatase [69]. It has been shown that global methylation status of GCC subtypes differ according to the time point of their developmental arrest; more differentiated cells showed a higher degree of methylation [70-72]. It is also known that CIS/GB cells show very little DNA methylation [71-73]. Seminomatous GCC subtypes showed more global hypomethylation and almost no CpG island methylation, whereas non– seminomas showed more methylated DNA, both globally and at CpG islands. A targeted analysis of 15 promoter regions confirmed that there are differences in methylation profiles between the different GCC [74]. Moreover, Ellinger *et al*. showed that detection of hypermethylated cell – free circulating DNA is feasible in most patients with GCC who undergo orchiectomy [59]. The diagnostic information received from cell – free methylated DNA by testing multiple gene sites seemed to be superior to that of conventional markers. Figure 2 shows the assessment of global 5mC methylation, DNMT3L mediated methylation and maintenance methyl transferase DNMT1 by immunohistochemistry for embryonic testis, different histological elements of GCC and their precursors. This confirms the presence of different methylation profiles in the GCC subtypes, with the more differentiated being more methylated, associated with different methyltransferases.

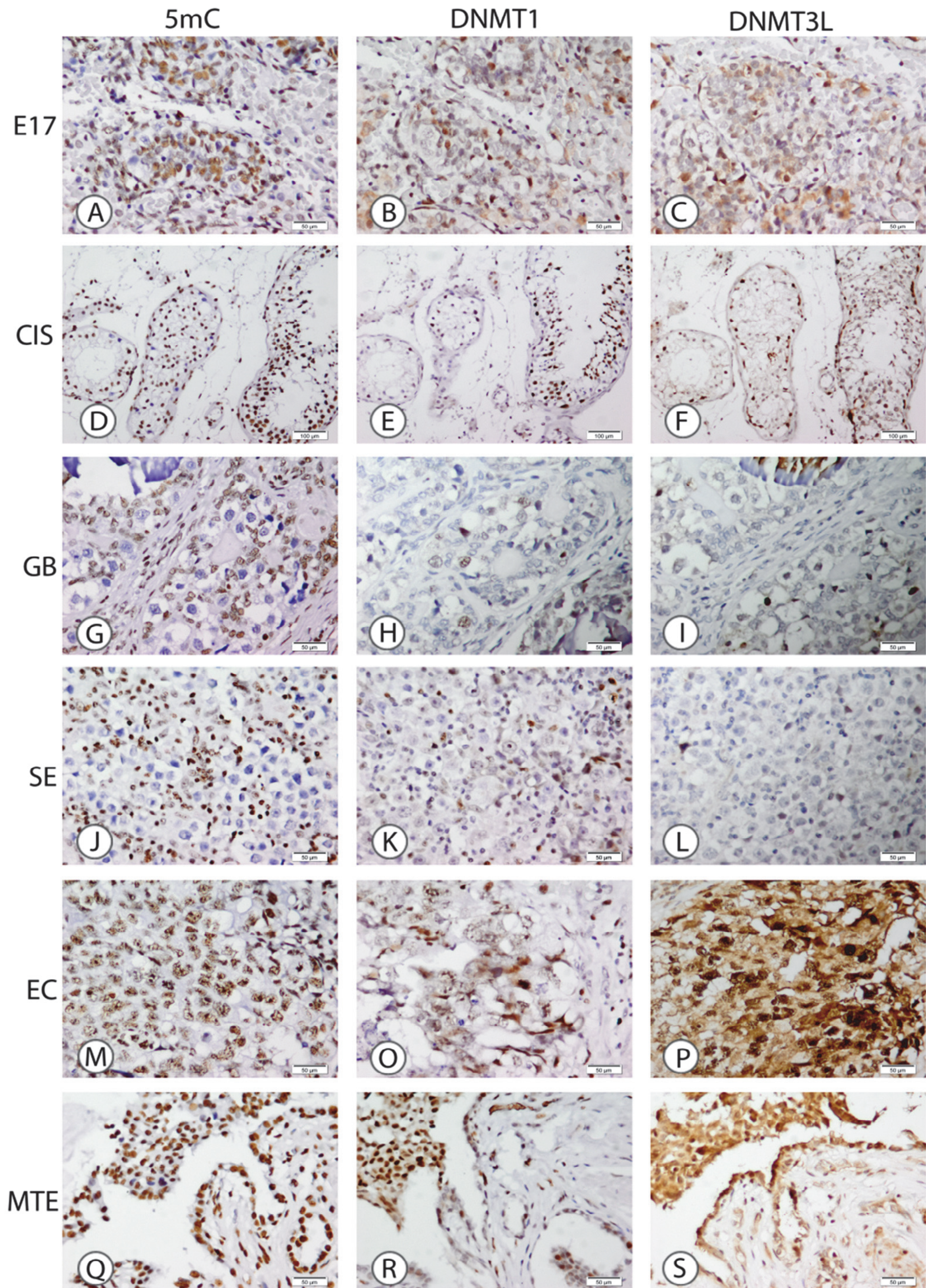

Figure 2 Immunohistochemical staining. Staining for 5mC (left column), DNMT1 (middle column) and DNMT3L (right column) on an embryonic testis, at the 17th week of development (E17) (first lane), the different GCC and their precursor lesions (2nd-6th lane).

In the embryonic testis the germ cells are negative for 5mC (A), negative to weak positive for DNMT1 (B) and DNMT3L; Carcinoma *in situ* cells are negative for 5mC (D), positive for DNMT1 (E) and positive for DNMT3L (F); Gonadoblastoma stains negative for 5mC (G), DNMT3L (I), and negative to weak positive for DNMT1(H); Seminoma/Dysgerminoma (SE/DG) stain negative for 5mC (J), DNMT1 (K) and DNMT3L (L); Embryonal Carcinoma (EC) shows a heterogeneous pattern for both 5mC (M), DNMT1 (O) and positive for DNMT3L (P); Mature Teratoma (MTE) stains positive for all markers (Q;R;S). All slides are counterstained with hematoxylin. Magnification 200x for all but 100x for CIS.

X inactivation and XIST expression

Acquired numerical chromosomal changes in X chromosomes are commonly observed in GCC [75]. Inactivation of one of the X chromosomes in female mammalian cells is necessary to balance the increased dosage of X-linked genes compared with male cells [76]. This process is initiated by the RNA gene *XIST*, expressed exclusively by the inactive form of the X chromosome, and results in hypermethylation of specific sites of genes to be silenced. In males, *XIST* is only detectable in germ cells of the normal male testis [77]. In GCC XIST is expressed only in tumors derived from the germ cell lineage with supernumerical X chromosomes: seminomas, nonseminomas, and spermatocytic seminomas. Although low *XIST* expression is present in testicular parenchyma with spermatogenesis, it is expressed at a higher level in parenchyma with CIS [78].

Kawakami *et al*. showed that *AR*, *FMR1* and *GPC3* (all X-linked genes) remained unmethylated in both seminomas and non-seminomas with *XIST* expression [79], normally methylated in inactive X chromosomes. Identification of unmethylated XIST DNA fragments in male plasma might serve as a diagnostic marker for GCC, although these findings need to be confirmed in independent studies.

Repetitive elements

A significant fraction of the genome (approximately 42%) is composed of repetitive elements [80]. Two major classes can be identified; long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements (SINEs). LINE1 and SINEs of the Alu family are the most prominent elements, and both are highly methylated in normal tissues [80, 81]. Ushida *et al.* showed that both LINE1 and Alu repeats are unmethylated in seminomas, whereas only LINE1 is unmethylated in non-seminomas and EC [82]. Thus the degree of demethylation of the repetitive elements is more pronounced in seminomas compared to non-seminomas, and GCC were more demethylated compared to cancers originating from somatic tissues [82].

Cisplatin sensitivity

Another factor that distinguishes seminomas and non-seminomas compared to other solid cancers is their overall responsiveness to cisplatin, a chemotherapeutic agent which binds to DNA, inducing crosslinks, which ultimately triggers apoptosis [83]. Seminomas are usually sensitive to chemotherapy with cisplatin, whereas the response of non-seminomas differ according to their histology. Teratomas, which are the most differentiated GCC, show the highest degree of methylation and are cisplatin resistant [84, 85]. In addition, cisplatin-resistant GCC showed different overall methylation profiles than non – resistant forms [72, 73, 86]. Hypermethylation might therefore be a diagnostic marker as well as a predictive marker of treatment response. Exposure of TCam-2, a highly cisplatin resistant seminoma cell line, to the demethylation agent 5-aza-cytidine resulted in an increased sensitivity to cisplatin. Subsequent analysis of CpG island methylation showed that there were different methylation profiles in the treated and non-treated cells. For example, the promoter region of the *CFLAR* (c-FLIP) gene was hypermethylated in the treated cell line [72]. CFLAR has an important role in regulation of apoptosis via the caspase pathway and therefore could be a therapeutic target [87], something that needs further evaluation. In addition, it has been shown that CFLAR can identify GCC patients with cisplatin resistance, based on genome-wide expression analysis [88]. Induction of c-FLIP has been reported in EC cell lines, as expected this results in resistance to cisplatin [89].

Histone modifications

Histones and associated chromatin proteins control the accessibility of genes and genomic elements. DNA is folded into nucleosomes; histone octameres consisting of two copies of each of the four histone proteins H2A, H2B, H3 and H4, wrapped with approximately 147 bp of DNA. A wide range of histone modifications, including methylation, acetylation and phosphorylation of specific amino acid residues, have been identified [90]. These are often associated with genomic regions with regulatory potential, and it has been proposed that different combinations of histone modifications can be linked to specific types of functional elements [91].

Technological advances have allowed the mapping of diverse histone modifications in a large number of cell types [92-94]. In this context a number of revelant DNA segments must be recognized, which will be discussed in more detail in the next paragraphs.

Promoters

Specific promoter-associated chromatin signatures involved in regulating proliferation have been identified. In general, H3 lysine 4 trimethylation (H3K4Me3) is associated with active regions, whereas H3 lysine 27 trimethylation (H3K27Me3) is associated with inactive or silenced loci. The presence of both represents a bivalent state, and has been identified in cells with pluripotent potential such as ES cells [95, 96]. Hawkins *et al.* showed that modifications at promoters remain largely invariant during differentiation, except at a small number of promoters where a dynamic switch between acetylation (H3K27Ac) and methylation at H3K27 marks the transition between activation and silencing of gene expression. This suggests a hierarchy in cell fate commitment over most differentially expressed genes [18].

Enhancers

Enhancers are defined as sequences, often outside the gene body, that regulate when and where a gene is expressed. Several histone marks have been associated with enhancers, including H3K4me1/2 and H3K27ac/me3. The current model in human ES cells is that high H3K27Ac is indicative for an active enhancer state. Loss of H3K27Ac and presence of H3K27Me3 will poise the enhancer for activation or silencing during differentiation [97].

Germ Cell Development and GCC

PGC actively suppress somatic differentiation programs by epigenetic modifications, a mechanism which might also account for CIS and seminoma [98]. In mice, PGC increase levels of H3K27me3 by E8.5-E9.5, with levels reduced at E10.5-11 [99]. Utx, an H3K27 demethylase, regulates efficient induction of pluripotency. In the absence of Utx, PGC showed aberrant, cell-autonomous germ cell development during their embryonic maturation *in vivo,* as well as aberrant epigenetic reprogramming [100]. CIS cells showed low levels of the repressive histone modifications H3K9me2 and H3K27me3, but high levels of activating marks H3K9Ac, H3K4me and H2A.Z [101]. This permissive chromatin structure is in accordance with the high levels of RNA polymerase II activity and proliferation that were observed in CIS cells. Epigenetic patterns similar to that of CIS cells were observed in human gonocytes present within sex cords in foetal testes and corresponds to migrating primordial germ cell in mice [101]. CIS cells therefore have a permissive and foetal-like chromatin structure, which is associated with high transcriptional and proliferative activity.

Ohinata *et al.* showed that Blimp1, a known transcriptional repressor, has a critical role in the development of the mouse germ cell lineage, as disruption of Blimp1

causes a block early in the process of PGC formation. Blimp1-deficient mutant embryos form a tight cluster of about 20 PGC-like cells, which fail to show the characteristic migration, proliferation and consistent repression of homeobox genes that normally accompany specification of PGC [21]. BLIMP1 and PRMT5 were expressed, and dimethylation of histones H2A and H4 was detected in human male gonocytes at weeks 12–19 of gestation, indicating a role of this mechanism in human fetal germ cell development [102]. In addition, this study also showed that BLIMP1/PRMT5 and histone H2A and H4 arginine 3 dimethylation are present in CIS and most seminomas, and less in EC and other nonseminomas. Recently, Schuster-Böckler and Lehner showed that chromatin organization has a major influence on regional mutation rates [103]. Mutation rates were positively correlated with heterochromatin related marks, of which histone modification H3K9Me3 was most important, and accounted for more than 40% of the somatic mutation variants [103]. The open chromatin in regions involved in embryonic development showed a negative association, which is in line with the fact that mutations are rarely found in GCC and the idea that the germ line is protected for mutations (immortal strand). In fact it points towards a regulatory role for histone modifications and chromatin structure on germline mutation rates, as the chromatin organization in the germline is substantially different to that in somatic cells [27].

Histone modification in GCC cell lines

Relatively little is known about histone modifications involved in GCC. We therefore initiated a study to explore the epigenetic differences between GCC subtypes, using the cell lines TCam-2 and NCCIT as representatives of seminomas and non-seminomas respectively (van der Zwan, in preparation). As depicted in Figure 3, our initial analysis matched the classification of the cell-lines. SOX17 was strongly enriched for H3K4me1 and H3K27ac in TCam-2 compared to NCCIT cells, whereas the opposite pattern was observed for SOX2. Additional analysis of the epigenetic differences between the two cell lines, along with the extension of these studies to cancer tissues, will contribute to our understanding of the role of histone modifications in GCC.

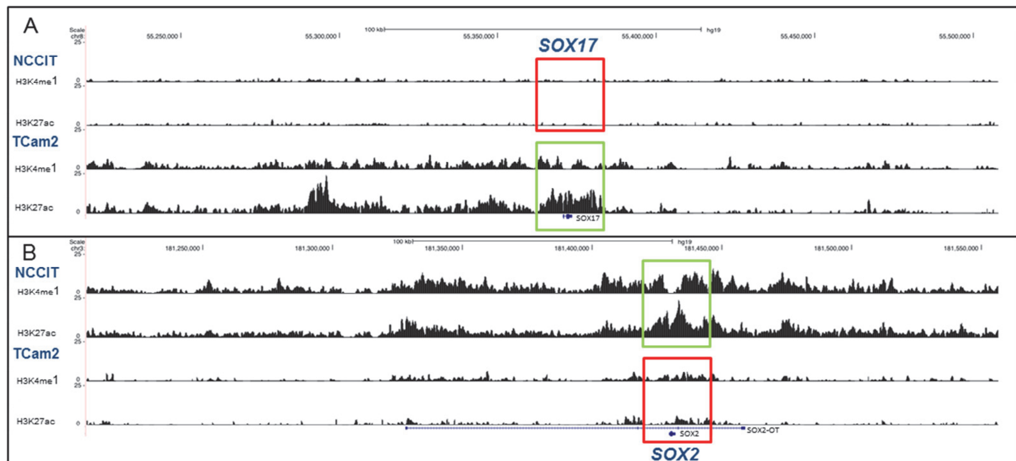

Figure 3 Display of H3K4me1 and H3K27ac tracks for both NCCIT and TCam-2. The red box indicates repressive state and the green box an active state. Figure derived from the UCSC Genome Browser. A. Genomic region around the *SOX17* gene, showing strong enrichment for both H3K4me1 and H3K27ac in TCam-2 (representative of seminoma) vs NCCIT (representative of Non-seminoma) cells. Enrichment outside this region is likely indicating regulatory elements such as enhancers. SOX17 is used as a diagnostic marker for differentiating seminoma from non-seminoma. B. Genomic region around the *SOX2* gene, showing strong enrichment for both H3K4me1 and H3K27ac in TCam-2 vs NCCIT cells. SOX2 is used as a diagnostic marker for differentiating non-seminoma from seminoma.

Environmental factors

Environmental factors can influence epigenetic processes, and may therefore be related to cancer development [35, 104]. There are indications that endogenous factors such as the hormonal balance between estrogens and androgens might play a role [26]. It is known that diethylstilbestrol (DES), used widely to reduce the risk of abortions, led to a higher risk of hypospadias, cryptorchidism and poor semen quality in male offspring [105]. The use of DES was abandoned after it was associated with cervical cancer [106]. It was shown that DES exposure in the third generation still leads to an increased risk of hypospadias[107]. The risk of GCC development in this patient group is controversial, and a slightly increased risk has been reported [108].The transgenerational effect of DES is however suggestive for heritable epigenetic alterations. In addition, exogenous estrogen may interrupt the maturation of primitive germ cells by reducing the secretion of Anti- Müllerian hormone from the Sertoli cells [109]. Hormonal factors can also induce methylation of promoter regions of certain genes [110].

Major geographic differences in incidence are consistent with environmental influences. Increased use of endocrine disruptors has been suggested to be one of the environmental factors responsible for the increasing incidence of testicular GCC in

testicular dysgenesis syndrome (TDS) [111]. A meta–analysis confirmed the link between estrogen exposure and GCC [112]. Developmental disturbances of the micro-environment could result in inadequate maturation of the germ cells. This may result in a foetal epigenetic profile which, upon hormone stimulation during puberty, leads to an aberrant induction of transcription and proliferation, ultimately leading to GCC later in life [101].

In this context, patients with Disorders of Sex Development (DSD) form an intriguing model to study the impact of intrinsic and environmental factors on normal and abnormal gonadal development. Indeed the diagnosis of 46, XY and chromosomal DSD is a risk factor for the development of GCC, with higher risk associated with an earlier block in differentiation. Other risk factors include anatomical position of the gonad, the presence of Y-chromosomal material (GBY region), genetic and epigenetic anomalies [113, 114].

It has been shown that seminomas are more often found in abdominal testes compared to scrotal testes [115]. This might also explain the occurrence of dysgerminomas in the ovary and dysgenetic gonads, which are both located in the abdomen. Indeed, seminoma and dysgerminomas are similar in morphology and gene expression profile, and therefore might have the same epigenetic profiles [116-118]. Bens *et al.* showed that *HOXA5* represents a candidate gene of androgen-mediated promoter methylation, by studying patients with Complete or Partial Androgen Insensitivity Syndrome (CAIS and PAIS respectively) [119]. *HOXA5* was significantly hypermethylated in CAIS patients compared to normal male controls, whereas PAIS patients could be both hyper- or hypo methylated. This suggests that *HOXA5* promoter methylation is at least partly controlled by androgen receptor activity and could possibly explain PAIS heterogeneity. In addition, TSPY was found to be a repressor for androgen signaling due to entrapping of the cytosolic androgen receptor, even in the presence of androgens. Androgen treatment stimulated cell proliferation and TSPY expression was found to be reduced in more malignant GCC [120]. Together, these results underline the theory that the androgen-estrogen balance is important in the etiology of GCC, in both AIS patients as well as ‘healthy’ men.

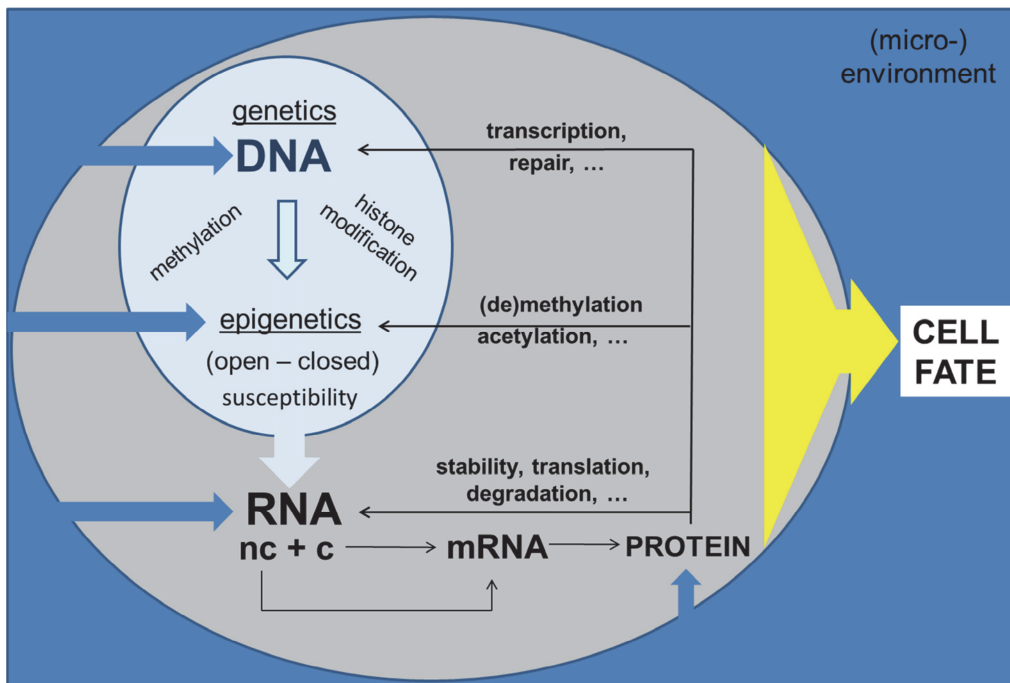

Figure 4 Final cell fate depends on interaction of a large number of parameters, including genetics, epigenetics, transcription and translation, in interaction with the (micro)environment. This is referred to as Genvironment. At the genetic level, DNA functionality can be influenced by various mechanisms, for instance transcription or repair. Epigenetics (*i.e.* DNA methylation and histone modifications) will determine chromatin structure, and as such susceptibility of the genome for transcription. The process of gene transcription and translation is dependent on these parameters, the presence of appropriate transcription factors, as well as stability, translation and degradation of enzymes, RNA and protein. In fact, (micro)environmental factors can impact on all these levels.

Perspectives and concluding remarks

Next generation sequencing has made it possible to study epigenetic processes in a genome-wide matter. Mapping and integration of these data with information from genetic and protein experiments will lead to a broader knowledge of cancer initiation and progression. Figure 4 shows a pathogenetic model for GCC based on the different aspects discussed in this review. Genetic, epigenetic and environmental factors ('genviroment') play essential roles in normal gonadal development. After fertilization the PGC completely erase their biparental genomic imprint. Gonocytes undergo different epigenetic modifications (i.e. increased methylation and more condensed chromatin structure) during their differentiation along the male (testis) or female (ovary) pathway. The process of testis formation is referred as 'testicularization' of the

gonad. The micro-environment of the PGC is essential for physiological maturation, and disruption of this process may lead to delayed maturation and possibly malignant transformation. In this respect, studies of DSD patients have increased our knowledge. Genetic factors, epigenetic aberrant reprogramming end/or environmental factors, referred to genvironmental parameters, can block the PGC in a fetal-like state, allowing proliferation. This block in maturation of the PGCs or gonocytes can therefore initiate a pathogenetic pathway, leading to the precursor lesions GB or CIS that can eventually progress to invasive GCC. Additional research may allow epigenetic profiles to identify risk groups, predict clinical outcomes and allow the development of targeted therapies in patients with high risk for development of GCC.

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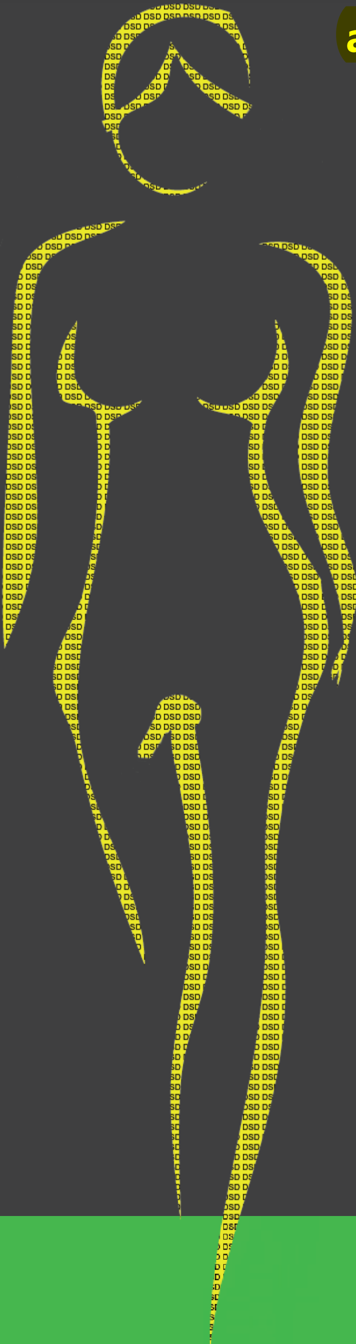
CHAPTER 9

Seminoma and embryonal carcinoma footprints identified by analysis of integrated genome-wide epigenetic and expression profiles of germ cell cancer cell lines.



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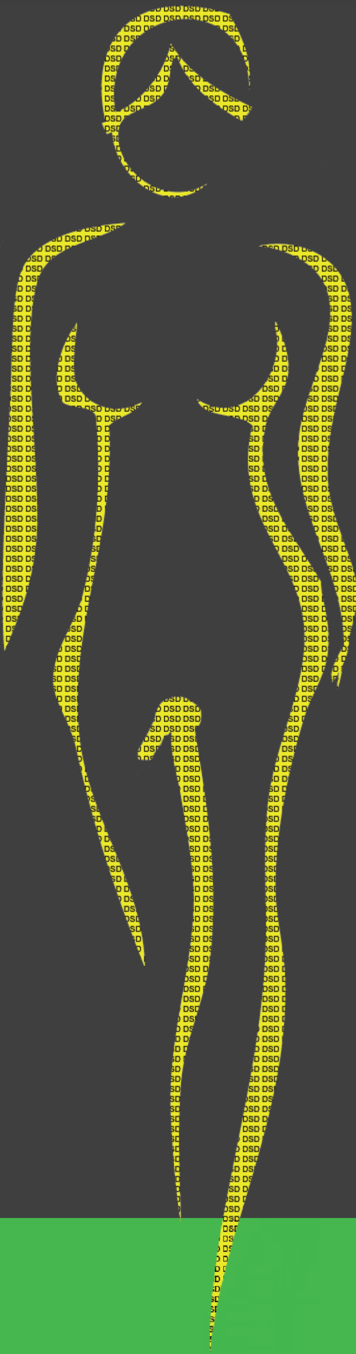
CHAPTER 10

General Discussion



Introduction

Disorders of Sex Development (DSD) are defined as congenital conditions of incomplete or disordered gonadal and/or anatomical sex and subdivided in three major groups based on karyotype; 46,XX DSD, 46,XY DSD and chromosomal DSD [1]. This classification, based on the 2006 Chicago consensus meeting, stimulated research in this field with focus on the multidisciplinary aspects of DSD and the need for skilled multidisciplinary teams for diagnosis and treatment [2-6]. Research has focussed on sex assignment, psychosexual and surgical outcomes and development of markers to estimate germ cell cancer (GCC) risk in order to individualize the need for prophylactic gonadectomy [7-28]. However, outcome data was mostly retrospectively collected and findings are inconclusive. Therefore lack of evidence based treatment protocols regarding surgery and psychosexual support exists. The risk of malignant transformation of germ cells is seemingly highly heterogeneous and dependent on a number of parameters of which the presence of defined Y-chromosomal material is essential. The process of normal sex development is strictly controlled by functionality of genes, both in time and place, in which the existing networks can act both on transcription and translation. To develop markers to estimate GCC risk and to create guidelines for management/treatment strategies of DSD patients it is important to understand the molecular etiology underlying normal gonadal development. As this is in fact the overall topic of this thesis, the results will be discussed in light of the current rapid scientific developments and with a broad view towards the future.

Management of patients with DSD

Outcome studies are important to evaluate the management of patients with DSD and to develop evidence based guidelines for future management. In recent years, a number of outcome studies have been performed. Mostly data was retrospectively collected and show contradictory results. The necessity and timing of feminizing surgery are still being debated. In **Chapter 2 and 3** we describe long term outcomes in women with CAH and DSD males respectively.

Long term outcomes in women with CAH

In **Chapter 2** we report on genital anatomy and ratings of cosmetic and functional outcome in women with CAH with and without feminizing surgery. We showed that cosmetic appearance, and functional outcomes are associated with the degree of virilization at birth, and that the impact of level of confluence on cosmetic outcome

depends on the number of surgical procedures performed. These results are in line with Nordenström *et al.* [23], who reported that surgery is more extensive in severely virilised patients, whereas other groups have reported either disappointing, or reasonably good outcomes after re-surgery in puberty [27, 29]. In total, 24% of the CAH women reported dyspareunia (deep and superficial). In a large Dutch study 5.4% of 2024 healthy females reported dyspareunia, while 29.6% sometimes experienced pain during intercourse [30]. In females who have undergone vaginal surgery, dyspareunia might be related to vaginal stenosis [31], but in our study only two of the eight patients who reported dyspareunia had vaginal strictures. Of the patients, 83% reported they were able to achieve orgasm, although with more difficulty compared to healthy reference women. In addition, other authors have reported that the clitoral sensitivity was affected in nearly all the women who had had surgery [23, 29]. These findings indicate that clitoral surgery may affect genital sensitivity. However, additional factors are likely to play a role as well, for example, degree of virilization, and psychological factors. They are likely to be interrelated and little is known about their effects on outcome. Parents and patients need to be informed about the multiple aspects that contribute to outcome after feminizing surgery and it should be emphasized that there is a fair chance that re-operations will be necessary in adolescence. Women with CAH achieved sexual milestones later than Dutch reference women and a substantial part never had been engaged in a sexual relationship. Women with CAH experienced a significantly less satisfactory sexual functioning (desire, arousal, lubrication, pain), and experienced more sexual distress compared to a Dutch reference group of healthy females. These data are in line with findings from Wisniewski *et al.* [32] and Gastaud *et al.* [33]. Despite their sexual problems, only a few women reported sexual problems to a gynecologist or psychologist, or had sought help from a sexologist.

Long term outcomes in DSD men

The incidence of male gender assignment is increasing due to the awareness of prenatal testosterone effect on gender development, improved surgical techniques and a change in attitude towards toleration of genital deviations [1, 34-36]. The study presented in **Chapter 4**, confirmed a shift occurring around 1990 towards more frequent male sex assignment in the Dutch DSD population – specifically in patients with 46,XY DSD and chromosomal DSD. Generally, male patients with DSD need extensive genital surgery. Outcome in Dutch men with DSD, described in **Chapter 3**, was poor regarding penile length, ejaculation, satisfaction with external genitalia and

frequency of sexual activity. Other aspects like overall body image and psychosexual functioning showed no difference with controls, suggesting that genital image and acceptation needs special attention during psychosexual counseling of boys with DSD. Mean penile length was below -2.5 SDS. Mean adult penile length is 13.3 cm (SD 1.6)[37]. The severity of hypospadias is correlated with adult penile length [38]. Reilly *et al.* reported absence of a relation between sexual functioning and penile length and ability of men with a microphallus to achieve satisfactory sexual intercourse [39]. In our study, the men who were able to achieve penetrative intercourse had a significant larger mean penile (7.9 cm) length than men who were unable to do so (4.9 cm). Phalloplasty might be an option for men who fail to have penetrative intercourse and are dissatisfied with their incapability. However, there is little experience with this procedure and long term results are lacking [40, 41].

Changes in management in the Netherlands

Important to keep in mind is that outcome is found to be related to cultural factors as we showed in a collaborative study with a DSD center in Indonesia [42]. In addition Ismail *et al*. showed that social components had a substantial contributing impact on gender outcome in Egyptian patients, even more than religion [43]. Care of DSD patients has changed over the last decades due to insights in molecular biological mechanisms underlying these disorders, screening for CAH, advanced surgical techniques and GCC risk awareness. The study presented in **Chapter 4** underlines the progress in management of DSD patients, a younger age at diagnosis, a shift towards male gender assignment in patients with ambiguous genitalia. Several reasons may account for this shift: the incorporation of the increasing knowledge in the field of DSD, the start of the neonatal screening, and start of Advocacy groups in the care of DSD patients. Gonadectomy was performed at a younger age, based on a better knowledge about GCC (discussed below). The incidence of GCC was 11/67 in 46,XY patients at risk, being 16,4%. It is known that patients with Y chromosomal material, known as the GBY region, in their karyotype are at increased risk of a malignancy. Patients with CAIS are an exception, most likely because the majority of germ cells with embryonic characteristics will not survive. In addition, upon puberty, androgen stimulation leads to proliferation/progression of the blocked embryonic germ cells to precursor lesions and eventually invasive cancer. Since androgens do not have an effect in CAIS patients, most likely the germ cells left will not progress to cancer in these patients. Our results support this theory; none of the patients with CAIS in our

group had a malignancy. Moreover, our results are consistent with a previously published risk prediction [7] with respect to disorders of androgen synthesis and PAIS.

Overall we can conclude from these studies that level of virilization is the most important determinant of outcome in our population, in both CAH women as DSD men. However, impact depends on the number of surgeries performed in CAH women. For the DSD men no sufficient numbers were available and therefore the power for statistical analyses was limited. In addition part of the data, especially surgical, was retrospectively collected and hard to subdivide in different procedures performed. This is a common problem and in the future, evaluation of long term outcomes for patients with DSD need systematic recording in a prospective fashion, preferably in a multicenter/national/ worldwide setting, over a period from birth till late adulthood. In addition, it is important to concentrate DSD care at centers of expertise; currently being established in Rotterdam. Important responsibility of this expertise center is to take care of up to date and understandable patient information but also to teach clinicians/nurses and provide them tools to understand the principles of normal sex development as well as DSD. A big step was made with the development of a DSD animation, developed for this last group of professionals, which will be discussed at the end of this chapter. In addition, clinical implications and future perspectives will be discussed in more detail and integrated with gonadal development and germ cell cancer risk at the end of this chapter.

Abnormal gonadal development and germ cell cancer risk

What explains the increased risk of GCC in patients with DSD? The other way around, GCC mimics embryogenesis to a certain extend. What can we learn about normal gonadal development when studying these cancers? Our findings will be discussed in relation to the spectrum of gonadal differentiation (i.e. micro-environment) present in DSD patients. We hypothesize that disturbed (epi)genetic regulation (through combined interaction of genetic or environmental parameters; referred to as genvironment) affect physiological embryonic germ cell development (either direct or indirect via the supportive cells), resulting in delayed or blocked maturation, and potentially progression to an invasive GCC. The results will be discussed in light of this hypothesis and finally we will propose a model for early detection of GCC and follow-up in a clinical setting.

Germ Cell Cancer

GCC arise from primordial germ cells (PGC) or gonocytes and are subdivided, into seminomas and non-seminomas. Non-seminomas can be further categorized into embryonal carcinoma, which can differentiate into somatic lineages and extra – embryonic tissues (for additional information, see Introduction Chapter) [44]. The precursor lesion is carcinoma *in situ* (CIS) in the testis and Gonadoblastoma (GB) in the dysgenetic gonad/ovary and both are derived from an embryonic germ cell blocked in its maturation [45, 46]. The germ cell lineage is important to pass genetic information from one generation to the next, and is derived from the pluripotent epiblast cells [47]. After fertilization the germ cells must generate totipotency to enable the formation of all the different cell lineages to form the fetus. This suggests that there is a balanced system between underlying totipotency and differentiation programs. When germ cells enter the bipotential gonad, they start interacting with the somatic cells and commit to male/female development after which either mitotic arrest or meiosis occurs. This process involves downregulation of pluripotency factors, expression of differentiation markers and epigenetic reprogramming which takes places during a critical sensitization window.

Diagnostic markers for GCC

These processes allowed the identification of informative markers for embryonic germ cells, which indicate related germ cell cancer risk when present after a certain developmental window. PGC are characterized by their alkaline phosphatase expression, which could be used as a diagnostic marker on fresh (frozen) tissue [48]. However, more informative for GCC detection is OCT3/4, a pluripotency factor, which is positive in CIS/GB, seminomas and embryonal carcinomas [49]. To differentiate between seminomas and embryonal carcinomas, SOX17 and SOX2 proved to be informative. SOX17 is positive in CIS/GB and seminomas, as well as normal spermatogonia, whereas SOX2 is positive in embryonal carcinoma [50]. Together, this set of markers will allow diagnosis of these GCCs. The SCF-c-KIT pathway is important for proliferation and survival of germ cells and for the migration of PGC to the bipotential gonad [51]. Genome Wide Association Studies (GWAS) found a strong association between GCC and Single Nucleotide Polymorphisms (SNPs) within KITLG (SCF) [52-54]. Activating mutations in *c-KIT* are involved in the development of seminomas and found to be present in 93% of the bilateral GCC cases compared to 2% in the unilateral GCCs [55]. c-KIT activates a number of downstream targets, including the PI_3K pathway involved in pluripotency. *PTEN*, a tumor suppressor gene,

inhibits the activity of PI_3K and loss of PTEN was found to be involved in tumor progression [56]. In addition, *c-KIT* mutations were found to be present in ovarian GCC from non – DSD patients, whereas TSPY was expressed in ovarian GCC with underlying DSD syndromes (thus presence of the GBY region, see below) [57]. Important to notice is that in patients with cryptorchidism or DSD, delayed maturation of germ cells can occur. These germ cells express OCT3/4 after the age of one; therefore this could lead to overdiagnosis of CIS. To distinguish germ cells delayed versus blocked in their maturation, SCF was found to be informative. SCF is expressed in CIS/GB cells but negative in cells showing maturation delay [58]. Since DSD can cause abnormalities in the level of testicularization at different stages, this could lead to either development to the male (testis) as well as female (ovary) pathway. To distinguish GB of the undifferentiated gonad and carcinoma in situ of the testis, use can be made from the markers FOXL2 (positive in granulosa cells) and SOX9 (positive in Sertoli cells) [59].

Genetic aberrations as underlying cause for DSD and GCC

In the 46,XY constitution, the sex – determining region on the Y-chromosome (*SRY*) initiates sex determination towards the male pathway [60]. SRY is expressed in the supportive cells starting around week 6 GA. Several transcription factors, like SF1, GATA4 and WT1 (+KTS) have been implicated in initiation, sustention and amplification of SRY expression [61-63]. In addition, signaling molecules involved in receptor tyrosine kinase activity (i.e. MAP3K1 and Map3k4) in the supportive cells are also important for the initial stages of sex determination [64-66]. Transient expression of SRY, specifically in Sertoli cell precursors, activates the downstream target SOX9 [67]. In turn, SOX9 orchestrates the genetic cascade of testis development. Aberrations in these genes are known to be involved in DSD [68], here we focus on the following genes:

SRY and WT1

SRY mutations residing in the HMG (High Mobility Group) domains are found in 10 - 15% of the 46,XY gonadal dysgenesis cases and affect binding to and bending of DNA or nuclear transport [69-72]. As a consequence these mutations can lead to an early error in the process of sex determination preventing proper formation of a testis, explaining a complete female phenotype in a 46,XY individual. **Chapter 5** describes a woman with bilateral GB and dysgerminoma presenting with primary amenorrhea at the age of 22 years, who was initially diagnosed with 46,XY complete gonadal dysgenesis. Mutation analysis identified a novel missense mutation (K128R) in the

HMG domain of the *SRY*, which did not have a significant effect on transcriptional activation and nuclear import *in vitro* as described in other cases [71-73]. During follow-up the patient developed progressive renal failure based on focal glomerulosclerosis, which triggered the analysis of the *WT1* gene. Mutations in *WT1* play a role in 46,XY DSD i.e. Frasier Syndrome, Denys-Drash syndrome, and WAGR-syndrome. Frasier syndrome is characterized by complete 46,XY sex reversal, late onset kidney failure (between 10-20 years), focal segmental glomerulosclerosis, streak gonads, and a high risk for GB, but not Wilm's tumors [74]. Sequence analysis of the *WT1* gene in the patient described in **Chapter 5** revealed a classic Frasier syndrome mutation in the intron 9 splice-site (IVS9 +4 C>T). This ultimately results in the decrease of the +KTS isoform and it is known that the subsequent reversion in +KTS/-KTS ratio causes defects in the development of glomerular podocytes and male sex-determination, ultimately leading to nephritic syndrome and male-to-female sex reversal, respectively [74, 75]. It has been described that *SRY* and *SOX9* expression can be diminished in Frasier syndrome [76] and it could be that the effects of reduced *SRY* expression by a mutated *WT1* were exacerbated by the presence of the SRY K128R mutation, although a reduced SRY function could not be shown conclusively by the *in vitro* assay applied. This situation may have contributed to the maldevelopment of the gonads, thereby creating the micro-environment in which embryonic germ cells can survive. However, screening an additional five Frasier syndrome patients with a proven *WT1* mutation did not reveal any sequence variants in *SRY*. This case underlines the importance of a proper and timely diagnosis, and stretches the importance of *WT1* mutation screening in all patients with 46,XY gonadal dysgenesis, especially in case of an unclassified SRY variant. It is expected that with implementation of next generation sequencing techniques in a diagnostic set – up multiple genetic aberrations contributing to the phenotype could be determined and studied in more detail.

<u>*MAP3K1*</u>

As described above, abnormalities in the level of testicularization, thereby creating a micro-environment in which a PGC/gonocyte blocked in his maturation may survive can be the underlying cause of GCC. This is supported by the fact that patients with Testicular Dysgenesis Syndrome (TDS) and patients with specific forms of DSD have an increased risk to develop GCC [7, 77, 78]. Mutations in the protein kinase gene *MAP3K1* have been reported to be a significant cause of 46,XY DSD, being identified in 18% of cases [64]. This results together with *SRY*, *AR*, *SF1* and *SRD5A2* mutations to about 40% of explained cases. In one of the two families studied there were three

affected cases that also developed a GCC. There was a wide degree of severity of clinical features seen in 46,XY individuals with the genetic mutation, ranging from micropenis combined with cryptorchidism to complete gonadal dysgenesis. In **chapter 6** we provide evidence that MAP3K1 variants are predisposing factors for GCC development in patients without (recognized) signs of TDS or DSD, suggesting that these variants only mildly affect the degree of testicularization. Exome analysis of blood-derived DNA from a bilateral GCC patient with unilateral cryptorchidism identified a missense variant in exon 9 of the *MAP3K1* gene. In addition, the index patient had a monozygotic twin brother, who had been diagnosed with a GCC two years earlier. DNA analysis of that tumor confirmed the presence of the same variant in the index patient and the brother. Subsequent analysis of 95 GCC samples, all from patients without DSD characteristics, identified two additional, missense MAP3K1 variants. In this study we demonstrate that the testicular somatic tissue surrounding the tumor showed SOX9 staining and was negative for FOXL2, although the patients carried germline variants in *MAP3K1*. In addition, there were no clinical signs of undervirilization, further supporting the conclusion that there was no DSD in these patients. All variants were predicted to be deleterious, and have not been reported in genetic variation databases. *Ex vivo* analysis of mouse embryonic testis showed that pharmacological inhibition of the MAPK pathway lead to a significantly increased percentage of germ cells failing to enter mitotic arrest.

The *MAP3K1* variants we identified in non-DSD GCC cases showed a lower ratio (4/97) than the DSD cases (4/18). Three of the four mutations were present in the amino third of the MAP3K1 protein. This region contains the domain that is thought to bind to RHOA, a protein that positively regulates MAP3K1 kinase activity [79]. Indeed, functional analysis of MAP3K1 variants in DSD patients showed increased RHOA binding to MAP3K1, as well as altered kinase activity of MAP3K1, resulting in increased phosphorylation of the target proteins p38 and ERK1/2 [64]. We have also examined the effect of disrupting the MAPK pathway in the developing mouse testis during the developmental period that germ cells are considered to be vulnerable to tumor initiation. In this study it was demonstrated that inhibition of MEK1/2 signaling disrupts germ cell mitotic arrest, a key characteristic of mouse germ cells that form teratomas in mouse germ cell tumor models which proves its relevance in the normal development of germ cells [80-82]. Defects in the MAPK pathway are associated with a large number of cancers [83-87], and targeting the MAPK pathway is a significant therapeutic strategy [88, 89]. The most comprehensive sequence analysis of kinase genes in GCC to date was targeted at 518 protein kinases [90]. This study was

specifically focused on detecting somatic mutations present only in the tumor, so will not have identified any germ line *MAP3K1* variants. The MAPK family has previously been implicated in GCC susceptibility by GWAS [53, 54], as two of the genes identified (*KITLG* and *SPRY4*) are directly involved in the MAPK signaling pathway. Genetic variants in *MAP3K1* and upstream factors, such as *MAP2K4,* are therefore compelling candidates for GCC susceptibility and progression, and may be potential targets for therapeutic intervention. Our data suggests that the effect on germ cells is indirect, through a diminished support of the Sertoli cells. This is in line with other aberrations found, i.e. SRY also exerts its effect via the supportive cells. In addition, it might explain the low number of mutations present in GCC. Finally, our data are also supportive for a role of epigenetic processes underlying its etiology.

AMH

Functional Sertoli cells produce AMH which causes regression of the Müllerian ducts. In the absence of AMH the Müllerian ducts will develop into normal female internal organs [91]. Persistent Müllerian Duct Syndrome (PMDS) is characterized by the presence of a uterus, fallopian tubes and the upper part of the vagina in phenotypic normal male patients, and is usually discovered at surgery for cryptorchidism or inguinal hernias. AMH, a member of the transforming growth factor β (TGFβ) family, signals through a heterodimeric receptor complex consisting of a specific type II receptor (AMHR2) and shared type I receptors (ALK2, 3, and 6) [92, 93]. Mutations within the *AMH* and *AMHR2* genes are in approximately 85% of the cases responsible for this error in sex differentiation [94]. Mutations in the three type I receptor genes have not been detected [94], and thus for the remaining 15% of the cases the causative genes remain to be identified. Patients with mutations in *AMH* or *AMHR2* present with a similar phenotype. However, when assessed before puberty, levels of circulating AMH are usually extremely low or undetectable in patients with *AMH* mutations, whereas normal levels are observed in patients carrying *AMHR2* mutations [94]. In **Chapter 7** we describe a patient with PMDS caused by a novel missense mutation within *AMH*, due to a single basepair insertion (c.208dup, p.Leu70fs) leading to a premature stop codon. Biopsy of both gonads showed irregular distribution of germ cells but no signs of abnormal maturation. GCC, mostly seminomas, have been reported in patients with PMDS, although the incidence is similar to that in non-affected males with cryptorchidism [95]. Manassero *et al.* described a patient with PMDS and transverse testicular ectopia with the presence of a mixed GCC (teratoma and embryonal carcinoma) in a testis in the scrotal position after orchidopexy at the

age of 5 years [96], suggesting that orchidopexy early in life does not decrease the risk of malignancy in this patient group. Therefore it has been suggested that orchidectomy should be performed in these patients [95]. Bilateral orchidectomy is a definite procedure leading to induction of puberty, life-long androgen substitution and loss of fertility. We suggest that with the markers for GCC known to date [7], a more individual tailored approach is possible. Therefore evaluation of both testis taking potential fertility problems in consideration should be part of clinical follow up [95, 97].

Differentiation to either the female or male path of gonadal development not only involves downregulation of pluripotency genes but also upregulation of differentiation markers, which for testis development are human specific TSPY and VASA amongst others [98, 99]. Interestingly, only DSD patients with Y-chromosomal material in their karyotype, more specifically presence of the GBY region, are known to have an increased GCC risk [7, 100]. TSPY is the main candidate within the GBY region involved in the initiation of GCC and is expressed in spermatogonia, CIS and GB and can be used as a general immunohistochemical marker for the presence of germ cells [101]. It is suggested that TSPY plays a role in entrapment of the cytosolic androgen receptor, thereby repressing AR signaling and creating an androgen – insensitive micro-environment [102]. This needs to be verified independently. Together with studies in PAIS/CAIS patients which showed that *HOXA5* promoter methylation is at least partly controlled by androgen receptor activity underline the theory that androgen-estrogen balance is important in the etiology of GCC [103]. These environmentally related epigenetic effects, their role in GCC etiology and the possibility to include epigenetic profiles in future management protocols were the topic of **Chapter 8 and 9** will be discussed below in more detail.

Epigenetics and Germ Cell Cancer

The differentiation of each cell type is achieved without changes in DNA sequence, but through the coordinated expression of specific genes regulated by epigenetic mechanisms as methylation and histone modification. The epigenetic processes act together to establish and maintain both global as local chromatin states e.g. open or condensed which determines gene expression [104]. Epigenetic modifications are relatively stable in somatic cells, although influenced by age. In germ cells, however, the epigenome is reprogrammed on a genome-wide level. By E12.5/10 weeks GA in mice/humans most DNA methylation marks are lost [105] and *de novo* methylation is

initiated in males at E14.5, leading to highly methylated mature gametes. This allows re-establishment of parental imprints in germ cells, the erasure of epimutations, and the generation of toti- or multipotent cells [106-108]. Genomic imprinting results in the silencing of one of the parental alleles in a subset of genes, and differs between different tissues and cell types [109]. In addition, epigenetic modifications play essential roles in transmitting transcriptional memory; *i.e.* the phenomenon that epigenetic marks can be inherited across more than one generation [110]. This is referred to as transgenerational effect (TGE). These issues are described in detail in **Chapter 8**. It has been shown that global methylation status of GCC subtypes differ according to the time point of their developmental arrest; more differentiated cells showed a higher degree of methylation [111-113]. This was confirmed and is described in **Chapter 8.** It is also known that CIS/GB cells show a relatively low level of DNA methylation [112-114]. Seminomas show overall hypomethylation and almost no CpG island methylation, whereas non–seminomas showed more methylated DNA, both globally and at CpG islands. PGC actively suppress somatic differentiation programs by epigenetic modifications, a mechanism which might also account for CIS/GB and seminoma [115]. In mice, PGC increase levels of H3K27me3 by E8.5-E9.5, with levels reduced at E10.5-11 [116]. Utx, an H3K27 demethylase, regulates efficient induction of pluripotency. In the absence of Utx, PGC showed aberrant, cell-autonomous germ cell development during their embryonic maturation *in vivo,* as well as aberrant epigenetic reprogramming [117]. CIS/GB cells showed low levels of the repressive histone modifications H3K9me2 and H3K27me3, but high levels of activating marks H3K9Ac, H3K4me3 and H2A.Z [118]. This permissive chromatin structure is in accordance with the high levels of RNA polymerase II activity and proliferation that were observed in CIS cells. Epigenetic patterns similar to that of CIS cells were observed in human gonocytes present within sex cords in fetal testes and correspond to migrating PGC in mice [118]. CIS cells therefore have a permissive and fetal-like chromatin structure, which is associated with high transcriptional and proliferative activity. Recently, Schuster-Böckler and Lehner showed that chromatin organization has a major influence on regional mutation rates [119]. Mutation rates were positively correlated with heterochromatin related marks, of which histone modification H3K9Me3 was most important, and accounted for more than 40% of the somatic mutation variants [119]. The open chromatin in regions involved in embryonic development showed a negative association, which is in line with the fact that mutations are rarely found in GCC [90] and the idea that the germ line is protected for mutations (immortal strand). In fact it points towards a regulatory role for histone modifications and chromatin structure on germline mutation rates, as the chromatin

organization in the germline is substantially different to that in somatic cells [107]. Relatively little is known about histone modifications involved in GCC and in **Chapter 8** we show that initial analysis on cell lines of NCCIT (representative for nonseminoma, i.e., embryonal carcinoma) and TCam-2 (representative for seminoma) matched the classification of the cell lines. SOX17 was strongly enriched for H3K4me1 and H3K27ac in TCam-2 compared to NCCIT cells, whereas the opposite pattern was observed for SOX2. Additional analysis and integration with methylation and expression data was performed and is described in **Chapter 9**.

Link between different epigenetic mechanisms

Several histone modifications serve to coordinate $5^{m}C$ and are associated with chromatin condensation. Acetylation of lysine residues is thought to neutralize the positive charge of histones and contributes to a relaxation of the interaction between DNA and histones [120]. Chromatin condensation plays a crucial role in silencing genes and stabilizing chromosomal structure. There are numerous tissue-dependent differentially methylated regions in the mammalian genome. DNA methylation profile is specific to cell and tissue type and can therefore be used as an identification tag for cells; a change in DNA methylation profile will cause alteration in cell properties [120, 121]. DNA methylation status cooperates closely with histone modifications to regulate gene expression. In **Chapter 9** we provide insight into the interaction between gene expression, DNA methylation and histone modifications in GCC. Two well characterized cell lines were used: one representative for seminoma (TCam-2) and the other for EC (NCCIT). Two types of epigenetic modifications were investigated: absence of CpG DNA methylation, and presence of activating histone modifications (H3K4Me3 and H3K27ac) in relation to genome wide expression analysis. An extensive analysis of the differences between the NCCIT and TCam-2 epigenetic landscape and it's relation to expression was performed. Histone modification patterns were assessed using chromatin immunoprecipitation combined with high throughput sequencing (ChIP-seq). Alterations in H3K4me3 and H3K27Ac were investigated, which are markers for promoter activation (transcription start site (TSS), H3K4me3 and H3K27Ac) and enhancer activation (primarily H3K27Ac) [122, 123]. H3K4Me3 and H3K27Ac do not show preferential enrichment near transcription start sites, which allowed us to combine the histone modification results in subsequent analysis. H3K4Me3 and H3K27Ac enrichment patterns, methylation and expression patterns in TCam-2 and NCCIT are in accordance with known SE / EC markers specificity. In addition to the analysis of (differential) histone modification patterns, motif enrichment of the

modified regions was investigated and compared between the cell lines. Germ cell markers AP2α and AP2γ are the only top ranked motifs in TCam-2, while embryonic stem cell specific motifs SOX2/OCT4/SOX2/TCF/NANOG are enriched in both cell lines.

Our results support known germ cell markers, not only regarding expression but also at the epigenetic landscape. The primordial germ cell origin of GCC is supported by the overall erased imprinting in these cells. DMRs were significantly enriched for imprinted genes, and 59% (51/86) of all imprinted genes showed loss of methylation around their TSS in one or both cell lines. In an unbiased way, this study also compared the cell lines regarding their epigenetic and transcriptional profile and integrated this data. As expected, pluripotency regulation was important in both cell lines, although more dominant in NCCIT. Androgen related regulation was overrepresented in both cell lines as well, but more strongly in TCam-2. Also these cells showed stronger enrichment of functions related to mature germ cells. In conclusion, the results support a pluripotent, early germ cell origin of the SE / EC like cells and position NCCIT before TCam-2 in the timing of their separation from normal germ cell maturation, strongly influenced by genvironmental factors.

Environment

Environmental influences early in embryonic development, thereby affecting normal testis development have been suggested to be responsible for TDS, which combines features like cryptorchidism, hypospadias, subfertility and GCC [124]. This seems to be an effect on the supportive cells rather than a direct effect on the PGC [125]. In addition, there are indications that endogenous factors such as the hormonal balance between estrogens and androgens might play a role in altering DNA methylation and histone modification patterns, resulting in epigenetic phenotypes [106, 126-129]. A meta–analysis confirmed the link between estrogen exposure and GCC [130]. Developmental disturbances of the micro-environment might result in inadequate maturation of the germ cells and subsequently this may result in a foetal epigenetic profile which, upon hormone stimulation during puberty, may lead to an aberrant induction of transcription and proliferation, ultimately leading to GCC later in life [118]. The estrogen receptor is differentially expressed in the normal testis and the β subunit is present in spermatogonia, spermatocytes and spermatids and supportive cells [131]. Therefore, testicular estrogen action seems to be important. Indeed, it has been shown that estrogens are important for survival and development of the male germ line. This observation also provides a mechanism by which endocrine disrupters could exert an effect at the gonadal level. Ferlin *et al.* studied the association of GCC with

polymorphisms in the estrogen receptor but could not confirm a relation between the two [132]. It is known that diethylstilbestrol (DES), used widely to reduce the risk of abortions, led to a higher risk of hypospadias, cryptorchidism and poor semen quality in male offspring [133]. The use of DES was abandoned after it was associated with cervical cancer [134]. It was shown that DES exposure in the third generation still leads to an increased risk of hypospadias [135]. The risk of GCC development in this patient group is controversial, and a slightly increased risk has been reported [136]. The TGE of DES is however suggestive for heritable epigenetic alterations. In addition, exogenous estrogens may interrupt the maturation of primitive germ cells by reducing the secretion of AMH from Sertoli cells [137]. Several studies have indicated that children of mothers with high parity have a decreased risk of GCC when compared to children of mothers with only one child. It has been suggested that maternal endogenous estrogen levels are higher in first pregnancies compared to subsequent ones [138] although a higher risk of GCC was not confirmed in a meta-analysis performed by Cook *et al.* [139]. Fraga *et al.* provided evidence of environmental modification of the epigenome, by measuring global CpG methylation as well as acetylation on H3 and H4 histones in monozygotic twins of various ages and living in different environments. Twins are epigenetically indistinguishable during the early years of life but older twins exhibit remarkable differences in their overall content and genomic distribution of DNA methylation and histone acetylation. In addition, these data clearly showed a more important difference of the epigenome between older twins and between twins who have lived separately more than 50% of their lifetime [140]. In **Chapter 6** we described the role of *MAP3K1* germ-line mutations in GCC in an identical twin, both of whom had the same variant and developed GCC at the age of 26 and 28 respectively.

Androgens are also important in GCC etiology. During puberty, upon hormonal stimulation, replication and spread of blocked PGC will occur, which will lead to CIS/GB eventually resulting in GCC. The AR receptor is expressed by CIS cells and patients with partial androgen insensitivity syndrome are known to have an increased GCC risk [7, 141]. A disrupted AR function could lead to a relative androgen insensitive environment with high levels of circulating testosterone, where embryonic germ cells are able to survive. In addition, low postnatal testosterone/gonadotropin ratios were found to be predisposing factors for GCC [142]. This is in line with the fact that low fertility or even infertility is reported, associated with the same ratio's, in patients who are later in time diagnosed with GCC [142]. Baldness en severe acne used as surrogates for increased androgen levels were shown to be negatively correlated to GCC [143] The other way around, GCC patients were significantly less likely to report

hair loss en history of acne compared to controls [144]. In addition, we propose similar roles in the etiology of GCC in patients with DSD who form an intriguing model to study the impact of intrinsic and environmental factors on normal and abnormal gonadal development. Indeed the diagnosis of 46,XY and chromosomal DSD is a risk factor for the development of GCC, with a higher risk associated with an earlier block in differentiation. Other risk factors include anatomical position of the gonad, the presence of Y-chromosomal material (GBY region), genetic and epigenetic anomalies as described above and in **Chapter 8** [7, 145].

Genvironment

As discussed in this thesis, genetic, epigenetic and environmental factors, referred to as genvironment play essential roles in normal and abnormal gonadal development. After fertilization the PGC completely erase their biparental genomic imprint. Gonocytes undergo different epigenetic modifications (i.e. increased methylation and more condensed chromatin structure) during their differentiation along the male (testis) or female (ovary) pathway. The process of testis formation is referred as 'testicularization' of the gonad. The micro-environment of the PGC is essential for physiological maturation, and disruption of this process may lead to delayed maturation and possibly malignant transformation. In this respect, studies of DSD patients have increased our knowledge significantly. Genvironmental parameters, during the process of testicularization, thus during a specific window of sensitization, may block the PGC in a fetal-like state, allowing proliferation. During this window, the above mentioned genetic (high risk SNPs, mutations), epigenetic (methylation, histone modification) and environmental factors (estrogen-androgen balance) may influence this process. This will determine clinical characteristics like TDS and DSD. A block in maturation of the PGC or gonocytes can initiate a pathogenetic pathway, leading to the precursor lesions GB or CIS that can eventually progress to invasive GCC. In addition, GCC mimic embryonal development to a certain extent, including capacity for totipotency. This knowledge has allowed the identification and application of informative diagnostic markers, including OCT3/4 (POU5F1), NANOG, SOX2 and SOX17. The genomic constitution, i.e. the presence of susceptibility alleles (high risk SNPs) might determine the sensitivity for the other factors. This conclusion is supported by the fact that in the general Asian population the risk to develop GCC is lower than in the Caucasian population, possibly associated with the lower frequency of high risk alleles, but in the Asian DSD population it is as common as in Caucasian

DSD patients. Again underlining that DSD is a dominant risk factor for the development of GCC [146].

Taken together, it could be argued that a spectrum of abnormal testis development underlies GCC. This ranges from a low risk, resulting from minor changes that lead to GCC in males who have apparently normal testes, to an increased GCC risk in TDS patients with affected testis development that is not clinically assignable as a DSD, and to the highest GCC risk in patients with clinically identifiable DSD. Thus a link is made between the level of testicularization and clinical observations and environmental factors, with an important role of the testicular micro-environment in supporting germ cell development.

Clinical implications

When a child is born with DSD, care by a multidisciplinary team based on an optimal (evidenced based) diagnostic approach is essential. The studies performed in this thesis underline the multiple aspects to be involved; thereby we focused on long term outcomes with respect to surgical, hormonal and psychosexual management and on underlying normal/abnormal gonadal development and GCC risk.

We learned that parents and patients with CAH need to be informed extensively about the multiple aspects that contribute to outcome after feminizing surgery. They need to be informed that there will be a fair chance that re-operations will be necessary in adolescence. For DSD men, the mean penile length was below -2.5 SD and ejaculatory dysfunction was common. The ability to penetrate during intercourse was correlated with penile length. Despite their sexual problems, only a few, both men and women, reported sexual problems to a gynecologist/urologist or psychologist, or had sought help from a sexologist. We would like to make a plea for assessing sexual well-being at follow up visits and to discuss sexuality, and work towards acceptance of the genital anatomy that may always remain different from the perceived norm. Ultimately, referral to a sexologist may be needed. Some of the participants did not have full disclosure of their condition when growing up and had to find out later.

These studies again confirmed the fact that this has major impact on outcome, mainly due to self-esteem problems. Counseling of parents and patients, providing information and explanation of the underlying condition and involving parents and patients in an individualized treatment plan (regarding surgery, psychology/sexology, fertility and search for a molecular biological diagnosis) will help to accept and to find a way to deal with a condition as DSD. Written information and links to internet based

information should be provided. In addition, this underlines the fact that there should be a good transition from pediatric to adult care. A structural and secure database system is essential for good outcome studies; we used a structured data entry (SDE) system developed within our group [147]. One of the goals was to design a database for DSD patients in which retrospective and prospective data could be collected and the possibility to merge with other databases like the international DSD (I-DSD) database [148]. Since DSD is a rare condition, future research in a large international cohort is important and will require collaboration and data exchange in a virtual research environment (VRE). As for all databases, there is a privacy issue and data should be entered anonymously with informed consent. In addition, data entry and maintenance of the database is pivotal. When these issues are taken care of this VRE could be the center of collaboration between all the different clinical specialist involved in DSD, more basal researchers and bioinformaticians in the participating centers worldwide. In fact, the first papers resulting from this collaboration are published recently [149].

More information about (epi) genetic causes will improve our understanding of normal and abnormal gonadal development which in turn will improve our diagnostic and prognostic ability regarding DSD. Epigenetic research in this field is still in the discovery phase and aims at mapping the functional genome. This could lead to identify epigenetic risk profiles for GCC. In addition, next generation sequencing (NGS) will add significantly to find underlying genetic aberrations. Although still costly, due to the large amount of data that needs to be analyzed and interpreted, implementation in the clinic is ongoing. An absolute requirement is informed consent. Pre-test counseling could cover issues as unexpected findings. In addition, involvement of a multidisciplinary team is essential to discuss the meaning of new findings. Ongoing is also how to charge the costs, for children this is covered by their health insurance. However to be able to interpret the data, preferably also the parents need to be screened, do they have to pay for that themselves? NGS allows a rapid screen of known DSD genes and an interesting pool of data to study etiology in patients in which no known mutations are found. In the research setting there is a code of good conduct (Federa, www.federa.org), allowing rest-material to be used but gives a couple of rules to fulfill manly focusing on informed consent, protection of privacy and well-considered policy on incidental findings. This stretches the importance of a multidisciplinary team in a research setting. Since often no mutations are found in coding regions, we expect a shift towards the functional regions and combining epigenetic and functional data. Because specific genetic effects can have different effects in individual DSD patients, no definite answer relating to GCC risk and

treatment options regarding gonadectomy can be given. The risk of malignant transformation depends on a number of parameters related to the micro-environment/level of testicularization of the gonad and survival and proliferation of embryonic germ cells. These parameters include the presence of Y-chromosomal material (GBY region) and genvironmental factors (e.g. SNPs and hormonal exposure). Germ cells residing in a disturbed environment can be delayed or blocked in maturation; in this respect diagnostic markers including OCT3/4, SCF and TSPY are informative. In addition, to be able to distinguish between SE and NS, SOX17 and SOX2 (respectively) are informative. FOXL2 and SOX9 were shown to discriminate between granulosa and Sertoli cells, depending on the level of testiculization a GB or CIS will form as precursor lesion. Combining all factors will allow individualized risk assessment and the appropriate treatment could be offered. This ranges from no action at all, surveillance, irradiation or prophylactic gonadectomy.

There are still questions unanswered, specifically regarding timing of prophylactic gonadectomy. Is it save to leave the gonads *in situ* in patients with complete androgen insensitivity syndrome? This would allow spontaneous puberty and possibly even fertility. What to do with patients with testosterone synthesis disorders, now often raised as males? No hard data on GCC exist, do we advise prophylactic gonadectomy?

Overall, it can be concluded that interaction of the different specialists, both clinical and in research, is essential to provide good clinical care for DSD patients. In addition, research can be focused on clinical issues encountered by the team and allows integration of new techniques in a diagnostic setting. Figure 1 shows the different team members involved, together a diagnostic and treatment plan can be made, gender assignment can be advised, psychological support can be offered and at a later stage psychosexual and transition to adult care could be discussed. Each of these steps need input from the different expertise fields since factors like surgery, hormonal treatment and psychosocial/sexual aspects are interrelated.

The future will teach us how the implementation of NGS influenced our understanding of underlying genetic defects in DSD patients and our ability to deal with the data generated, both in a clinical as well as research setting. In addition, education about the different forms of DSD and their relation with GCC is essential for the medical profession (specialist in training, nurses, and researchers amongst others). An important step was made with the development of a DSD animation. A web based model was designed within our group to generate a structured educational environment to understand the principles of normal sex development as well as DSD.

It fills a niche in current available visually based information represented in a structured, clear and understandable format. The design is in principle based on two separate areas. The first is "Normal Sex Development", representing the different steps in the process of normal sex development, in chronological order. It is the baseline on which information on the main variants of DSD are represented, under the second area, referred to as "Disorders of Sex Development (DSD)". In addition, an informative section is presented regarding "Parameters related to Germ Cell Cancer Development" (www.espe-elearning.org).

For further research in this field, close world-wide collaborations are needed to create sufficient numbers for the different subcategories of DSD to be able to identify the risk of GCC and provide markers to individualize treatment. Thereby patient support groups must be included. In addition the numbers are needed to get reliable outcome data regarding surgery, hormonal treatment and gender assignment on which we could refine our treatment protocols. Additional research may allow linkage of genetic and epigenetic profiles, possibly even with environmental cues. These profiles could then be used to identify risk groups, predict clinical outcomes and allow the development of targeted therapies in patients with high risk for development of GCC.

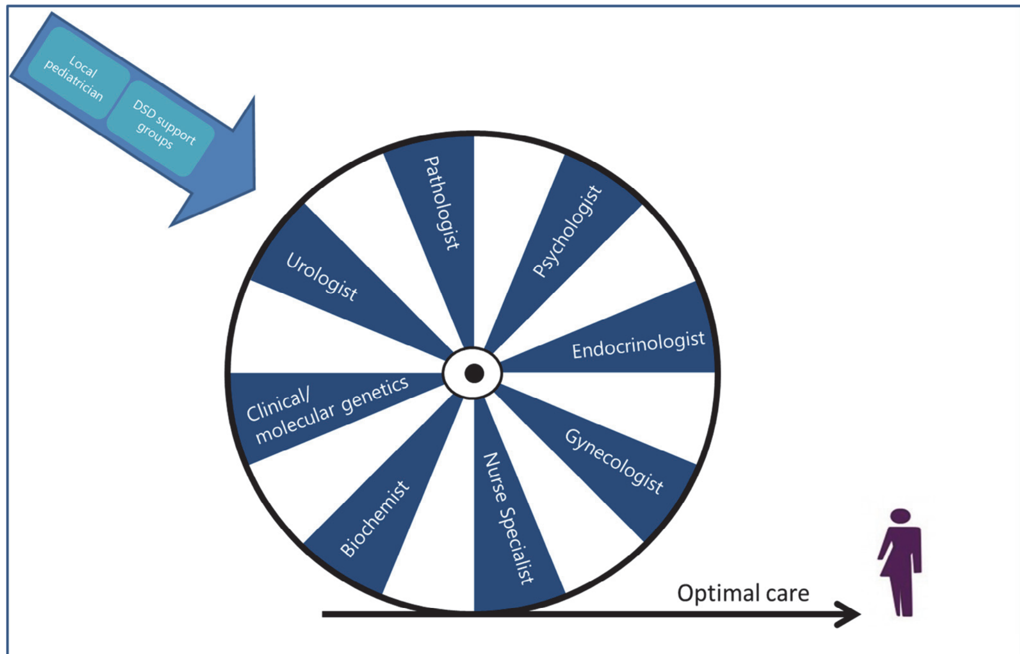


Figure 1 For optimal care regarding diagnosis, treatment and follow-up a multidisciplinary team is essential, here represented as spokes of a wheel. When one of them falls out, it will still go forwards but not as stable as before.

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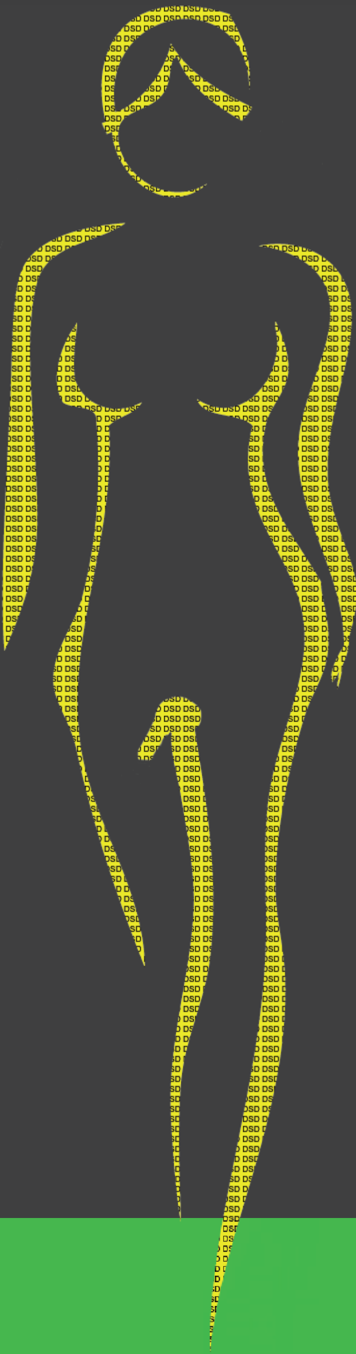
CHAPTER 11

Summary/Samenvatting



Summary English

Chapter 1

This chapter provides an introduction in normal and abnormal gonadal development with their underlying genetic and epigenetic pathways. First the processes of sex determination, sex differentiation (both male and female) and germ cell development are discussed. Next, abnormal gonadal development, including Testicular Dysgenesis Syndrome (TDS) and the various Disorders of Sex development (DSD) are explained. Germ cell cancer is introduced and related to TDS and DSD. In addition, the importance of clinical outcome studies in patients with DSD is stretched and explained in the context of recent findings. Finally the aims and outline of this thesis are presented.

Chapter 2

In this chapter we report on long term outcome data in women with CAH. Forty women were studied of which 36 had undergone vaginal surgery, 25 of them had surgery more than one time. Anatomical assessment showed reasonable results and cosmetic evaluations did not differ between women and gynecologists. We show that level of confluence is the major determinant for cosmetic outcome; the impact dependent on the number of surgeries performed. Only 20 women had experience of intercourse, eight of those reported dyspareunia. The women evaluated their sexual functioning and functional outcome less favorable than the reference group. Based on these results we advise that vaginoplasties to improve sexual function should only be performed after consulting a multidisciplinary DSD team and after ample vaginal examination and counseling. Surgeons need to inform parents and patients and discuss these aspects so that they can make a balanced decision.

Chapter 3

Long term outcomes in males with DSD are discussed. Fourteen men were studied; the control group comprised 46 healthy age-matched men. Thirteen men underwent 1 to 6 (mean 2) genital surgeries with a mean age at first surgery of 2.7 years. Mean penile length was 6.6 cm; all men reported erections and were able to experience orgasms. We show that penile size is related to the ability to have penetrative intercourse e.g. mean penile length was 7.9 cm in patients who were able to achieve penetrative intercourse compared to 4.9 cm in those who were not. Compared to controls, men with DSD were less satisfied with the appearance of the penis and scrotum but not with total body image. These patients reported decreased sexual desire and activities. Based on this study we can conclude that outcome in this group of men was poor regarding penile length, ejaculation, satisfaction with external genitalia and frequency

of sexual activity. Other aspects such as overall body image and psychosexual functioning showed no significant difference from controls.

Chapter 4

Our knowledge about DSD has greatly increased in the past years. Improved reconstructive techniques and the awareness of testosterone effect on gender identity resulted in more male sex assignments in the past decades. In this chapter we analyzed retrospective data of 485 DSD patients from 4 large centers in the Netherlands; 229 patients with 46,XY DSD, 201 with 46,XX DSD and 55 with chromosomal abnormalities. Age at presentation differed significantly between the various DSD groups as well as over time. Nineteen patients changed gender, 17 from male to female; 2 from female to male. In addition we found that there was indeed a shift towards more male sex assignments over the years. We describe data about mutation screening and number of surgeries performed in the different DSD groups and showed that age at gonadectomy became lower over the decades. The incidence of GCC was 11/67 in 46,XY patients at risk. In conclusion, our results underline that the management of DSD has improved.

Chapter 5

DSD can, in a number of cases, be explained by an underlying mutation. Here we describe a phenotypically normal female, presenting with primary amenorrhea at the age of 22 years, later diagnosed as hypergonadotropic hypogonadism based on 46,XY gonadal dysgenesis with a novel missense mutation in *SRY*. The K128R mutation found in the HMG domain did not show convincing protein malfunction *in vitro*. Pathological examination of the gonads demonstrated bilateral gonadoblastoma and dysgerminoma. Late onset progressive kidney failure triggered analysis of the *WT1* gene and identified an intron 9 splice-site mutation, known to be the cause of Frasier Syndrome (FS), in addition to the *SRY* mutation. Additional SRY mutation screening in five FS cases was negative. This case demonstrate that a proper diagnosis (multi-gene based) of DSD patients is important, allowing early diagnosis and treatment of (precursor lesions of) GCC.

Chapter 6

Interaction with the micro-environment is essential for normal maturation of a PGC. Deregulation of the supportive cells, thereby not properly nourishing the germ cell could be the underlying cause of GCC development. Here we describe *MAP3K1* as a

GCC susceptibility gene. Using next generation sequencing we identified a novel, germline missense variant in *MAP3K1* in a mongozygotic twin pair, both of whom had developed SE, but lack clinical features of DSD. Changes in this gene have previously been associated with 46,XY DSD, which is a known risk factor for GCC. Subsequent analysis in 95 GCC samples, all from patients without DSD characteristics, identified two additional *MAP3K1* variants. Analysis of matched testis showed reduced Sertoli cell numbers in parenchymal tissue next to the SE. *Ex vivo* analysis of mouse embryonic testis showed that inhibition of the MAPK pathway led to decreased Sertoli cell proliferation and an increased percentage of germ cells failing to enter mitotic arrest. We propose that failed MAPK signaling leads to insufficient testis cord formation and function. This leads to an unfavorable environment for the PGC which compromises its differentiation and could eventually lead to GCC development.

Chapter 7

Functional Sertoli cells produce AMH which causes regression of the Müllerian ducts. This chapter describes a patient with persistent Müllerian duct syndrome, characterized by the presence of a uterus, fallopian tubes, and the upper part of the vagina in phenotypic normal male patient. A novel homozygous missense mutation in the *AMH* gene was identified (single nucleotide insertion (C) at position 208) leading to a frame shift and the introduction of a premature stop codon. Biopsy of both gonads revealed that germ cells were present in an irregular distribution. However, the absence of OCT3/4, PLAP and c-KIT expression indicated physiological maturation.

Chapter 8

Mutations are rarely found in GCC, which is exceptional for solid cancers. Therefore, epigenetic deregulation, possibly caused by environmental factors, could be one of the underlying mechanisms. This chapter describes the role of epigenetics in the etiology of GCC with focus on DNA methylation, histone modifications, epigenetic memory and inheritance as well as environmental factors. Our hypothesis is that a disturbed epigenetic regulation affect embryonic germ cell development, resulting in delayed or blocked maturation and potentially progression to GCC. Identification of epigenetic alterations could lead to better understanding these processes and development of specific markers for early detection, eventually leading to development of targeted treatment.

Chapter 9

It has been proposed that a disrupted epigenetic constitution, either primarily or caused by risk factors, might be involved as a mechanism in the pathogenesis of GCC. In this chapter, we provide insight into the interaction between gene expression, DNA methylation and histone modifications in GCC. Two well characterized cell lines were used: one representative for seminoma (TCam-2) and the other for EC (NCCIT). Our results support known germ cell markers, not only regarding expression but also at the epigenetic landscape. The primordial germ cell origin of GCC is supported by the overall erased imprinting in these cells. In an unbiased way, this study also compared the cell lines regarding their epigenetic and transcriptional profile and integrated this data. As expected, pluripotency regulation was important in both cell lines, although more dominant in NCCIT. Androgen related regulation was overrepresented in both cell lines as well, but more strongly in TCam-2. Also these cells showed stronger enrichment of functions related to mature germ cells. Our results support a pluripotent, early germ cell origin of the SE / EC like cells and position NCCIT before TCam-2 in the timing of their separation from normal germ cell maturation, strongly influenced by genvironmental factors.

Chapter 10

In the general discussion we discuss the main findings of the studies described in this thesis which will be related to the spectrum of gonadal differentiation present in DSD patients. First lessons learned from clinical outcome studies will be discussed and related to recent literature. The second topic is abnormal gonadal development and germ cell cancer risk. We hypothesized that disturbed (epi) genetic regulation affect proper germ cell maturation; retention of embryonic germ cells is the first step in the pathogenesis of GCC. Results will be discussed in light of this hypothesis and we propose a model for early detection of GCC and follow-up in a clinical setting. Finally, we present a DSD animation, designed to generate a structured educational environment to understand the principles of normal sex development as well as DSD and GCC development.

Samenvatting Nederlands

Hoofdstuk 1
Dit hoofdstuk geeft een introductie in normale en abnormale gonadale ontwikkeling, met onderliggende genetische en epigenetische regulatie mechanismen. Als eerste worden de processen van sex determinatie, sex differentiatie (zowel mannelijk als vrouwelijk) en kiemcelontwikkeling besproken. Vervolgens wordt een toelichting gegeven over abnormale gonadale ontwikkeling, waaronder Testicular Dysgenesis Syndrome (TDS) en de verschillende aandoeningen die onder de noemer van geslachtsdifferentiatie stoornissen (Disorders of Sex Development (DSD)) vallen. Kiemcel kanker wordt geïntroduceerd en gerelateerd aan TDS en DSD. Bovendien wordt het belang van het klinisch onderzoek bij patiënten met DSD besproken en toegelicht in de context van recente bevindingen. Als laatste worden de doelen en opzet van dit proefschrift gepresenteerd.

Hoofdstuk 2
In dit hoofdstuk beschrijven we de lange termijn uitkomsten bij vrouwen met Adreno-Genitaal Sydroom (AGS). Veertig vrouwen werden onderzocht, waarvan 36 vaginale chirurgie ondergingen, 25 van hen meer dan een keer. Anatomische beoordeling bleek redelijke resultaten te geven en evaluaties van genitale cosmetiek verschilde niet tussen vrouwen en gynaecologen. We zien dat het niveau waarop de urethra en vagina samenkomen (level of confluence) de belangrijkste determinant is voor het cosmetische resultaat, het effect is afhankelijk van het aantal operaties dat uitgevoerd is. Slechts 20 vrouwen hadden ervaring met geslachtsgemeenschap, acht van hen ervaarde dyspareunie. De vrouwen evalueerden hun seksueel functioneren en functionele resultaat als minder gunstig ten opzichte van de referentiegroep. Op basis van deze resultaten adviseren wij dat vaginoplasties ter verbetering van seksuele functie alleen na overleg met een multidisciplinair team DSD en na voldoende begeleiding en gedegen vaginaal onderzoek moet worden uitgevoerd. Chirurgen moeten deze aspecten met de ouders en patiënten bespreken, zodat zij een evenwichtige beslissing kunnen maken.

Hoofdstuk 3
De lange termijn uitkomsten bij veertien mannen met DSD worden besproken. De controlegroep bestond uit 46 gezonde mannen met dezelfde leeftijd. Dertien mannen ondergingen 1 tot 6 (gemiddeld 2) genitale operaties met een gemiddelde leeftijd bij de eerste operatie van 2,7 jaar. De gemiddelde penislengte was 6,6 cm; alle mannen rapporteerden de mogelijkheid om erecties en een orgasme te krijgen. De studie laat zien dat penis lengte gerelateerd is aan de mogelijkheid tot het hebben van

(penetratieve) gemeenschap; de gemiddelde lengte was 7.9 cm bij patiënten die in staat zijn om geslachtsgemeenschap te hebben in vergelijking tot een lengte van 4,9 cm bij degenen die dat niet konden. Vergeleken met controles, waren mannen met DSD minder tevreden met het uiterlijk van de penis en het scrotum, dit in tegenstelling tot het algehele lichaamsbeeld. De mannen met DSD meldden een verminderd seksueel verlangen en activiteit. Op basis van dit onderzoek kan er geconcludeerd worden dat de uitkomst in deze groep mannen slecht was wat betreft penislengte, ejaculatie, tevredenheid met de uitwendige geslachtsorganen en de frequentie van seksuele activiteit. Andere aspecten zoals algemeen lichaamsbeeld en psychoseksueel functioneren toonde geen significant verschil met controles.

Hoofdstuk 4

Onze kennis over DSD is sterk toegenomen de afgelopen jaren. Verbeterde reconstructieve technieken en het feit dat testosteron een effect heeft op genderidentiteit, heeft geresulteerd in meer mannelijk geslacht toewijzingen in de afgelopen decennia. In dit hoofdstuk analyseerden we retrospectief de gegevens van 485 DSD patiënten uit 4 grote centra in Nederland; 229 patiënten met een 46,XY DSD, 201 met 46,XX DSD en 55 met chromosomale afwijkingen. Leeftijd bij presentatie was significant verschillend tussen de DSD diagnostische groepen als ook over tijd. Negentien patienten zijn van geslacht veranderd, 17 van man naar vrouw; 2 van vrouw naar man. Daarnaast vonden we dat er inderdaad een verschuiving was naar meer mannelijke geslacht toewijzingen door de jaren heen. We beschrijven gegevens over mutatie screening en het aantal operaties uitgevoerd in de verschillende DSD groepen en toonden aan dat leeftijd bij gonadectomie lager werd in de afgelopen decennia. De incidentie van GCC was 11/67 in de 46, XY risicopatiënten. Concluderend onderstrepen onze resultaten dat management van patienten met DSD is verbeterd over de afgelopen decennia.

Hoofdstuk 5

DSD kan, in een aantal gevallen, worden verklaard door een onderliggende mutatie. Hier beschrijven we een fenotypisch normale vrouw, die zich presenterde met primaire amenorroe op de leeftijd van 22 jaar, later gediagnostiseerd als hypergonadotroop hypogonadisme op basis van 46,XY gonadale dysgenesie met een nieuwe missense mutatie in SRY. De K128R mutatie gevonden in het HMG-domein heeft geen overtuigende functionele invloed op het eiwit *in vitro*. Pathologisch onderzoek van de gonaden toonde bilateraal gonadoblastoom en dysgerminoom. Late-onset progressief nierfalen triggerde analyse van het WT1-gen en identificeerde een intron 9 splice-site

mutatie, waarvan we weten dat dit de oorzaak is van Frasier Syndrome (FS), naast de SRY mutatie. Additionele SRY mutatie screening in vijf FS gevallen was negatief. Deze patiënt laat zien dat een juiste diagnose (multi-gen gebaseerd) van DSD patiënten belangrijk is en bijdraagd aan vroege diagnose en behandeling van (voorloper stadia van) kiemceltumoren.

Hoofdstuk 6

Interactie met de micro-omgeving is essentieel voor normale ontwikkeling van een primordiale kiemcel. Deregulatie van de ondersteunende Sertoli cellen, waardoor ze de kiemcel niet juist kunnen ondersteunen, kan de onderliggende oorzaak van GCC ontwikkeling zijn. Hier beschrijven we *MAP3K1* als risico gen voor het ontstaan van GCC. Met behulp van next generation sequencing identificeerden we een nieuwe, kiembaan missense variant in *MAP3K1* in een mongozygotische tweeling, die beiden SE hadden ontwikkeld, maar de klinische kenmerken van DSD missen. Mutaties in dit gen zijn eerder geassocieerd met 46,XY DSD, een bekende risicofactor voor GCC. Analyse in 95 GCC samples, allemaal van patiënten zonder DSD kenmerken, leverde twee extra MAP3K1 varianten op. Analyse van gematched testis materiaal toonde verminderde Sertoli cel aantallen in parenchymweefsel naast het SE. Uit *ex vivo* analyse van muis embryonale testis bleek dat onderdrukking van de MAPK pathway leidde tot een verminderde Sertoli cel proliferatie en een verhoogd percentage van kiemcellen dat niet in mitose ging. Onze hypothese is dat veranderde MAPK pathway leidt tot onvoldoende testis ontwikkeling en verminderde functie. Dit leidt tot een ongunstige omgeving voor de primordiale kiemcellen, waardoor de differentiatie gecompromiteerd wordt en de kiemcel zich kan ontwikkelen tot GCC.

Hoofdstuk 7

Functionele Sertoli cellen produceren anti-müllerian hormone (AMH) dat regressie van de buizen van Müller veroorzaakt. Dit hoofdstuk beschrijft een patiënt met Persistant Müllerian Duct Syndrome (PMDS), gekenmerkt door de aanwezigheid van een baarmoeder, eileiders, en het bovenste deel van de vagina in fenotypisch normale mannelijke patiënt. Een nieuwe homozygote missense mutatie in het *AMH* gen werd geïdentificeerd (single nucleotide insertie (C) op positie 208) dat leidde tot een frameshift en de aanwezigheid van een voortijdig stopcodon. Biopsie van beide gonaden liet zien dat kiemcellen aanwezig zijn in een onregelmatige verdeling. De afwezigheid van OCT3/4, PLAP en c-KIT expressie geven aan dat het hier om fysiologische maturatie gaat.

Hoofdstuk 8

Mutaties worden zelden gevonden in GCC, wat uitzonderlijk is voor solide tumoren. Daarom kan epigenetische deregulatie, mogelijk veroorzaakt door omgevingsfactoren, een van de onderliggende mechanismen zijn. Dit hoofdstuk beschrijft de rol van epigenetica in de etiologie van GCC met focus op DNA-methylatie, histonmodificaties, epigenetisch geheugen en overerving, als ook omgevingsfactoren. Onze hypothese is dat een verstoorde epigenetische regulatie embryonale kiemcellen beïnvloed in zijn ontwikkeling, resulterend in vertraagde of geblokkeerde uitrijping en potentieel progressie naar GCC. Identificatie van epigenetische veranderingen kunnen leiden tot het begrijpen van deze processen en ontwikkeling van specifieke markers om vroegtijdige opsporing te verbeteren, wat uiteindelijk zou kunnen leiden tot de ontwikkeling van gerichte behandeling.

Hoofdstuk 9

Een verstoorde epigenetische constitutie, primair of veroorzaakt door risicofactoren, kan een onderliggend mechanisme zijn in de pathogenese van GCC. In dit hoofdstuk geven we inzicht in de wisselwerking tussen genexpressie, DNA methylatie en histon modificaties in GCC. Twee goed gekarakteriseerde cellijnen werden gebruikt: TCAM-2 voor seminoom en NCCIT voor EC. Onze resultaten zijn in overeenstemming met de bekende kiemcel markers, niet alleen met betrekking tot expressie, maar betreffende het epigenetische landschap. De primordiale kiemcel origine van GCC wordt ondersteund door de in het algemeen gewiste imprinting in deze cellen. Deze studie is ook, unbiased, vergeleken met betrekking tot hun epigenetische en transcriptie profiel en integreerde deze gegevens. Zoals verwacht, regulatie van pluripotency was belangrijk in beide cellijnen, hoewel meer dominant in NCCIT. Androgeen gerelateerde pathways waren oververtegenwoordigd in beide cellijnen, maar sterker in TCAM-2. Ook vertoonden deze cellen een sterkere enrichment van functies met betrekking tot volwassen kiemcellen. Onze resultaten ondersteunen een pluripotente, vroege kiemcel orsprong van de SE / EC cellen en timen de positie van NCCIT voor dat van TCam-2 betreffende de timing van hun scheiding van normale kiemcel uitrijping, sterk beïnvloed door 'genvironmental factors'.

Hoofdstuk 10

In de algemene discussie bespreken we de belangrijkste bevindingen van de in dit proefschrift, gerelateerd aan het spectrum van gonadale differentiatie die bij DSD patienten aanwezig is. Eerst worden de resultaten van de klinische uitkomst studies besproken en gerelateerd aan recente literatuur. Het tweede onderwerp is abnormale gonadale ontwikkeling en het risico op kiemcel kanker. Onze hypothese is dat verstoorde (epi) genetische regulatie uitrijping van kiemcellen beïnvloedt; behoud van embryonale kiemcellen is de eerste stap in de pathogenese van GCC. De resultaten zullen in het licht van deze hypothese worden besproken en bespreken we een model voor vroegtijdige opsporing van GCC en voor follow-up in een klinische setting. Tot slot, presenteren we een DSD animatie, ontwikkeld om een gestructureerd educatieve omgeving te creëren om de principes van normale gonadale ontwikkeling, DSD en GCC te begrijpen.

CHAPTER 12

Dankwoord
List of publications
PhD portfolio

Dankwoord

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List of Publications

Callens, N*., **van der Zwan, Y. G*.**, Drop, S. L., Cools, M., Beerendonk, C. M., Wolffenbuttel, K. P., Dessens, A. B. on behalf of the Dutch Study group on DSD **Do surgical interventions influence psychosexual and cosmetic outcomes in women with Disorders of Sex Development?** ISRN endocrinology. 2012;2012:276742. Epub 2012/03/31.

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Hersmus, R.*, **van der Zwan, Y. G.*** , Stoop, H., Bernard, P., Sreenivasan, R., Oosterhuis, J. W., Bruggenwirth, H. T., de Boer, S., White, S., Wolffenbuttel, K. P., Alders, M., McElreavy, K., Drop, S. L., Harley, V. R., Looijenga, L. H. **A 46,XY female patient presenting with bilateral gonadoblastoma showing a novel SRY missense mutation affecting transactivation together with a WT1 KTS splice-site mutation.** PLoS One. 2012;7(7):e40858. Epub 2012/07/21.

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Cheng, Q., Fan, H., Ngo, D., Beaulieu, E., Leung, P., Lo, C. Y., Burgess, R., **van der Zwan, Y. G.**, White, S. J., Khachigian, L. M., Hickey, M. J., Morand, E. F. **GILZ Overexpression Inhibits Endothelial Cell Adhesive Function through Regulation of NF-kB and MAPK Activity.** J Immunol. 2013 May 31

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Van der Zwan Y.G.*, de Boer S.*, Rossello F.J., Stringer J., Gillis A.J.M., Stoop H., Notini A.J., Belluoccio D, Lee C.Y., Watkins D.N., Western P.S., Looijenga L.H.J.^, White S.J.^. **MAP3K1 is a testis cancer susceptibility gene.** Submitted.

Rijlaarsdam M.A.*, **van der Zwan Y.G.***, Dorssers L.C.J., Looijenga L.H.J. **DMR1^{+}: identifying Differentially Methylated between unique samples using array based methylation profiles.** Submitted.

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PhD Portfolio

Summary of PhD training

- Department of Pediatrics, subdivision of Endocrinology, Erasmus MC Rotterdam, the Netherlands
- Department of Pathology, Laboratory of Experimental Patho-Oncology, Erasmus MC Rotterdam, the Netherlands
- Centre for Genetic Diseases, Monash Institute of Medical Research, Monash University, Clayton, Australia

Research School:	Molecular Medicine Postgraduate School (MolMed)
PhD period:	January 2010 – December 2013
Promotors:	Prof. Dr. L.H.J. Looijenga
	Prof. Dr. S.L.S. Drop

General courses	**Year**
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2010
CPO – minicursus - Methodologie van patiëntgebonden onderzoek en voorbereiding van Subsidieaanvragen	2010
Thomson Reuters Endnote	2010
Basic introduction course on SPSS, MolMed	2010
Course on Statistics & Survival Analysis, MolMed	2011
Biomedical English writing and communication, MolMed	2012

Specific courses	
Workshop 'moleculaire diagnostiek voor dokters', MolMed	2010
Introduction course genetics, MolMed	2010
Biomedical research techniques, MolMed	2010
SNP's and human diseases, Molmed	2010
Adobe Photoshop and Illustrator CS5, MolMed	2011
Adobe InDesign CS5, MolMed	2011
Translational Endocrinology, MolMed	2011
Research management, MolMed	2012
NVvO (Nederlandse vereniging voor Oncologie) Basiscursus Oncology	2013

Seminars and workshops	
Weekly research meeting, department of Pediatric Endocrinology, Erasmus MC- Sophia	2010-2013

Weekly research meeting, department of Pathology, Laboratory of Experimental Patho-Oncology, Erasmus MC	2010-2013
Weekly research meeting, Josephine Nesfkens Institute, Erasmus MC	2012-2013
Weekly research meeting, Monash Institute of Medical Research, Monash University	2011-2012
Ghent –Rotterdam DSD workgroup meetings	2010-2013
Annual Pediatric research day, Erasmus MC-Sophia	2010, 2013
Annual MolMed Day, MolMed, Erasmus MC	2011
Jonge Onderzoekersdag NVK (Nederlandse Vereniging voor Kindergeneeskunde)	2012-2013
Federa Scientific Day – Next-generation DNA sequencing: impact on clinical care and society	2013

International conferences

4th International symposium Disorders of Sex Development, Glasgow, UK (oral presentation en poster)	2013
9th Joint meeting of Paediatric Endocrinology (ESPE), Milaan, Italy (oral)	2013
World Congress on Reproductive Biology & Society for Reproductive Biology, Cairns, Australia (poster)	2011
3rd International Symposium on Disorders of Sex Development (DSD): From Gene to Gender, University of Lübeck, Germany (2 posters)	2011
22nd Congress of the European Society for Paediatric Urology (ESPU), Copenhagen, Denmark (2 posters)	2011
7th Copenhagen Workshop on CIS, testis and Germ Cell Cancer, Copenhagen, Denmark (poster)	2010
36th meeting of the International Academy of Sex Research (IASR), Prague, Czech Republic (2 posters)	2010

Teaching activities

Co-supervisor Master Thesis (Medicine) of Patricia Koot	2010
Supervising Master Thesis (Molecular medicine) of Cristina Tommasi	2012-2013

LIST OF ABBREVIATIONS

AIS	Androgen Insensitivity Syndrome
AMH	Anti-Müllerian Hormone
CAH	Congenital Adrenal Hyperplasia
CAIS	Complete Androgen Insensitivity Syndrome
CIS	Carcinoma in situ
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DSD	Disorders of Sex Development
EC	Embryonal Carcinoma
ES	Embryonic Stem (cells)
FS	Frasier Syndrome
FSDR	Female Sexual Distress Scale
FSFI	Female Sexual Function Index
GA	Gestational Age
GB	Gonadoblastoma
GCC	Germ Cell Cancer
GD	Gonadal Dysgenesis
GO	Gene Ontology
GWAS	Genome Wide Association Studies
HE	Hematoxylin-Eosin
HMG	High mobility Group
HPG-axis	hypothalamic-pituitary-gonadal
MSHQ	Male Sexual Health Questionnaire
NS	Non-seminoma
PAIS	Partial Androgen Insensitivity Syndrome
PGC	Primordial germ cell
PMDS	Persistent Müllerian Duct Syndrome
SE	Seminoma
SNP	Single Nucleotide Polymorphism
SV-CAH	Simple virilising CAH
SW-CAH	Salt wasting CAH
TDS	Testicular Dysgenesis Syndrome
TGE	Transgenerational effect
TSS	Transcription Start Side
UGS	Urogenital Sinus

CURRICULUM VITAE

Yvonne van der Zwan was born on October 7th, 1984 in Hellendoorn, The Netherlands. After passing her secondary school exam at Reggesteyn in Nijverdal in 2003, she started medical training at the Erasmus University in Rotterdam. Here, she graduated in 2010.

During her medical studies she developed a special interest for patients with Disorders of Sex Development (DSD) and initiated a research project which ultimately resulted in an appointment as a Ph.D. candidate at the department of Pediatric Endocrinology of the Erasmus MC – Sophia Children's Hospital (supervisor Prof. dr. S.L.S. Drop). Initially, her research focused on outcome in patients with Disorders of Sex Development.

After obtaining a research fellowship from the European Society of Pediatric Endocrinology (ESPE), she had the possibility to extend her project with the investigation of the molecular and genetic processes underlying normal and abnormal gonadal development at the department of Pathology (Laboratory of Experimental Patho-Oncology, LEPO, supervisor Prof. dr. L.H.J. Looijenga). For seven months her fundamental training took place at the Monash Institute of Medical Research in Melbourne, Australia (supervisor Dr. S.J White). Here, she performed various experiments of which the results are included in this thesis.

In January 2014 she will start as a pediatric resident at the Sophia Children's Hospital (Prof.dr. M de Hoog). Yvonne lives together with Jeroen Baan in Rotterdam, the Netherlands.

UNFOLD FOR LIST OF ABBREVIATIONS