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Patogeneze vzniku germinálních nádorů: Využití současných poznatků ve včasné diagnostice u pacientů s poruchou pohlavního vývoje.

Pathogenesis of germ cell tumor development: Application of current knowledge in early diagnostics in patients with disorders of sex development.

Disertační práce

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CONTENT

ABBREVIATIONS

AP2-gamma	Transcription factor AP2-gamma
AR	Androgen receptor
CAIS	Complete androgen insensitivity syndrome
c-KIT	A type III tyrosine kinase receptor
DSD	Disorders of sex development
EMS	External masculinization score
ESC	Embryonic stem cells
ESPE/LWPES	European Society of Pediatric Endocrinology/Lawson Wilkins Pediatric Endocrinology Society
FOXL2	Forkhead transcription factor 2
GB	Gonadoblastoma
GBY	Gonadoblastoma locus on Y chromosome
GCT	Germ cell tumors
GD	Gonadal dysgenesis
HE	Hematoxylin and eosin
IGCNU	Intratubular germ cell neoplasia unclassified
KITLG	c-KIT ligand (also SCF)

NANOG   Homeobox transcription factor NANOG

OCT3/4   Octamer binding transcription factor 3/4 (also POU5F1)

PAIS   Partial androgen insensitivity syndrome

PGS   Primordial germ cells

PLAP   Placental alkaline phosphatase

POU5F1   POU domain class 5 transcription factor 1 (also OCT3/4)

RSPO1   R-spondin family member 1

SCF   Stem cell factor (also KITLG)

SOX9   SRY-related HMG-box gene 9

SRY   Sex-determining region on Y (also TDF)

TDF   Testis determining factor (also SRY)

TDS   Testicular dysgenesis syndrome

TSPY   Testis-specific protein Y-encoded

UGT   Undifferentiated gonadal tissue

WNT4   Wingless-type MMTV integration site family member 4

1. INTRODUCTION

Patients with disorders of sex development (DSD) with a specific part of Y chromosome in their karyotype are at a higher risk for development of gonadal Type II germ cell tumors (GCT), i.e., seminoma/nonseminoma in the testis and their counterparts dysgerminoma/nondysgerminoma in the dysgenetic gonad. According to current knowledge, the risk ranges between 0.8 and 60% in different DSD subgroups (Cools *et al.* 2006; Page 1987). Therefore, almost all the patients undergo prophylactic gonadectomy, usually soon after the diagnosis is made. Gonadectomy is an invasive and psychologically stressing procedure, moreover, it may in some cases prevent a spontaneous course of puberty, and instead result in a life-long need for hormonal substitution. Thus, a crucial question rises, is gonadectomy necessary in all the cases?

In this context, the aim of my thesis is to contribute to the improvement of a decision-making process that would enable us to better determine patients with a need for prophylactic gonadectomy, and on the contrary, to protect others from this procedure and preserve their endogenous hormonal production and in some cases even fertility. Luckily, the recent research on pathogenesis and diagnostics of GCT has provided us with tools to identify not only non-invasive tumor precursors, but also premalignant lesions and germ cells at risk for neoplastic transformation (Looijenga *et al.* 2010; van de Geijn *et al.* 2009).

I focused on a better determination of patients at risk within two ethiological DSD subsets. The first study is targeted on patients with complete form of androgen insensitivity syndrome (CAIS), the second study deals with patients with 45,X/46,XY gonadal dysgenesis (GD). Immunohistochemical detection of markers of early germ cell changes (especially OCT3/4 and KITLG) was applied for the purpose of the thesis.

2. THEORETICAL BACKGROUND

2.1. Germ cell tumors

GCT represent a group of neoplasms that originate from germ cells and occur throughout the whole life, from neonatal period to the old age. They are classified into five groups according to their origin and biological characteristics (Table 1). In the rest of the thesis, I will talk about the so called Type II GCT which the most typically reside in gonads and account for the most prevalent solid tumors in Caucasian men between 20 and 40 years of age. Lifetime risk for GCT development reaches 1% in Danish and Swiss men, in whom the prevalence is the highest. However, the occurrence of Type II GCT is even more frequent in patients with specific types of DSD (Cools *et al*. 2006a; Oosterhuis and Looijenga 2005).

Table 1: Classification of germ cell tumors (adapted from Oosterhuis and Looijenga 2005).

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype
I	testis/ovary/ sacral region/ retroperitoneum/ mediastinum/ neck/middle brain/ other rare sites	(Immature) teratoma yolk-sac tumor	Neonates and children	Early primordial germ cell/ gonocyte	Biparental, partially erased	Diploid (teratoma) Aneuploid (yolk-sac tumor): gain of 1q, 12(p 13) and 20q; loss of 1p,4 and 6q
II	Testis	Seminoma/ non-seminoma	> 15 years	Primordial germ cell/ gonocyte	Erased	Aneuploid (+/- triploid): gain of X, 7, 8, 12p, 21; loss of Y, 1p, 11, 13, 18
	Ovary	Dysgerminoma/ non-dysgerminoma	> 4 years			Aneuploid
	Dysgenetic gonad	Dysgerminoma/ non-dysgerminoma	Congenital			Diploid/tetraploid
	Mediastinum	Seminoma/ non-seminoma	Adolescents			Diploid/tri-tetraploid
	Middle brain	Germinoma/ non-germinoma	Children			Diploid/tri-tetraploid
III	Testis	Spermatocytic seminoma	> 50 years	Spermatogoni-um/ spermatocyte	Partially complete paternal	Aneuploid: gain of 9
IV	Ovary	Dermoid cyst	Children/ adults	Oogonia/ oocyte	Partially complete maternal	(Near) diploid, diploid/ tetraploid (gain of X,7,12,15)
V	Placenta/uterus	Hydatiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)

Based on the morphology, Type II GCT are divided to seminomas and non-seminomas (i.e., yolk-sac tumor, embryonal carcinoma, choriocarcinoma, and teratoma) in the testis. Their counterparts in ovary and dysgenetic gonad are called dysgerminomas and non-dysgerminomas (Cools *et al.* 2006a; Oosterhuis and Looijenga 2005). All testicular Type II GCT share a common non-invasive precursor, intratubular germ cell neoplasia unclassified (IGCNU, also known as carcinoma *in situ* of the testis) (Oosterhuis and Looijenga 2005; Skakkebaek 1972). The non-invasive precursor in dysgenetic gonad is termed gonadoblastoma (GB) (Cools *et al.* 2006b).

While IGCNU is made up of neoplastic cells located within the context of seminiferous tubules, GB is composed of a mixture of different cell types including malignant germ cells and supporting (granulosa) cells arranged in well circumscribed nests (Figure 1) (Kersemaekers *et al.* 2005). Therefore, it was initially hypothesized that IGCNU and GB were two basically distinct entities. Later, immunohistochemical profiling of both lesions indicated a tight similarity of their malignant cells. The difference in overall morphology may be only a result of a different gonadal microenvironment. Moreover, the theory of a common origin of IGCNU and GB is also supported by the resemblance of the subsequent invasive tumors in morphology, mRNA expression, and miRNA profiling (Hersmus *et al.* 2008; Gillis *et al.* 2007; Kersemaekers *et al.* 2005; Looijenga *et al.* 2010).

The noticeable similarity in morphology and protein expression suggests that the neoplastic cells of IGCNU, GB, and invasive tumors are derived from fetal germ cells (primordial germ cells/gonocytes) arrested in an early stage of development (Dieckmann and Skakkebaek 1999; Rajpert-De Meyts 2006; Sonne *et al.* 2009).
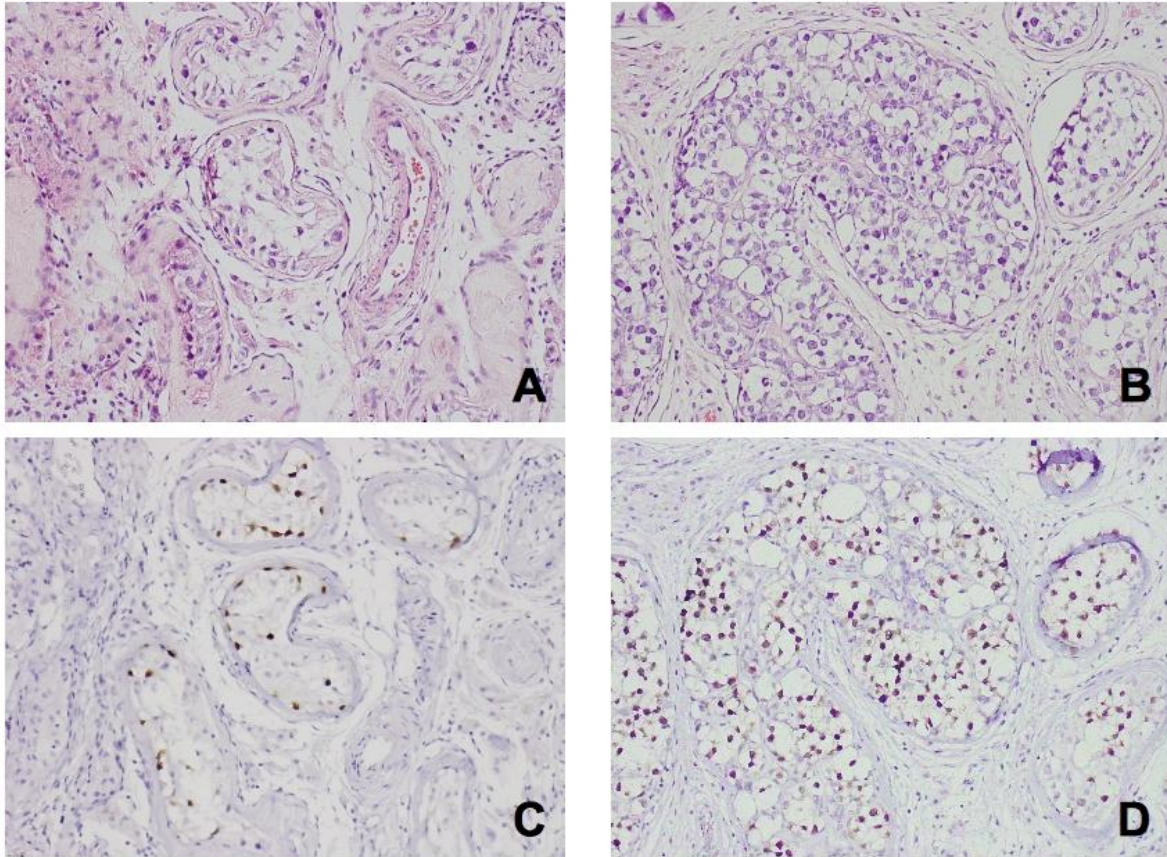
Figure 1: Intratubular germ cell neoplasia unclassified (IGCNU) and gonadoblastoma (GB). A - IGCNU stained with HE; B - GB stained with HE; C - IGCNU cells stained for presence of OCT3/4 (brown); D - GB stained for presence of OCT3/4.

2.2. Physiological germ cell development

To better explain the developmental process of GCT, it is necessary to understand specific basic issues of the development of gonadal tissue and germ cells themselves. Our knowledge in this field is mainly based on research in mice, but is (with some exceptions) also applicable to humans.

Primordial germ cells (PGC) arise from embryonic stem cells (ESC) and can be detected in humans for the first time at week 5-6 of gestation in the yolk sac as they stain positive

for PLAP (Placental Alkaline Phosphatase) and OCT3/4 (Octamer-binding Transcription Factor, also termed POU5F1), among others (Hersmus *et al.* 2008a; Looijenga *et al.* 2010). OCT3/4 is a transcription factor which is physiologically expressed only in ESC and PGC to provide them with ability of pluripotency, survival and proliferation (Cheng *et al.* 2006; Matin *et al.* 2004; Niwa *et al.* 2000). NANOG (Homeobox Transcription Factor NANOG), another pluripotency regulatory transcription factor, shows similar function and temporospatial expression as OCT3/4 (Hart *et al.* 2005; Hoei-Hansen *et al.* 2005). Moreover, OCT3/4 as well as NANOG prevents PGC from apoptosis (Kehler *et al.* 2004; Yamaguchi *et al.* 2009).

PGC migrate from the yolk sac along the hindgut towards the genital ridges. Among others, KITLG (c-KIT ligand, also know as SCF) and its tyrosine kinase receptor c-KIT are responsible for the proper migration, proliferation and survival. c-KIT is expressed in germ cells whereas KITLG serves as a chemo-attractant (Hersmus *et al.* 2008a; Molineaux and Wylie 2004). As soon as the PGC arrive to the genital ridges the structures are called indifferent gonads while PGC are termed gonocytes despite the unchanged morphology and expression profile (Looijenga *et al.* 2007).

The indifferent gonad has a potential to develop either as a testis or as an ovary. Its fate depends on the genetic constitution, i.e., the combination of sex chromosomes (XY in males and XX in females) (Wilhelm *et al.* 2007). In the presence of the Y chromosome, SRY (Sex-determining region on Y, also known as TDF) expression occurs in supportive (pre-Sertoli) cells during human embryogenesis at week 7 of gestation and initiates the expression of a cascade of down-stream genes (among others *SOX9*) which orchestrate testicular differentiation (Fleming and Vilain 2004; Wilhelm *et al.* 2007). During this process pre-Sertoli cells and gonocytes form cord-like structures (sex cords), and eventually seminiferous tubules (Wilhelm *et al.* 2007). Initially, germ cells, still with characteristics of

gonocytes, are located in the centre of the tubules while Sertoli cells are situated at the periphery. Germ cells then start migrating gradually towards the periphery. Once they reach the basal lamina, they mature to pre-spermatogonia, their morphology changes and expression of gonocyte markers (OCT3/4, NANOG, AP-2gamma, PLAP, c-KIT, *etc.*) ceases (Hoei- Hansen *et al.* 2004; Hoei-Hansen *et al.* 2005; Honecker *et al.* 2004; Pauls *et al.* 2006). Testicular tissue of normal neonates hardly contains any OCT3/4 positive cells and none of these cells can be detected in infants older than 6 months (Cools *et al.* 2005). On the contrary, TSPY (Testis-specific protein Y-encoded) expression is almost exclusively restricted to (pre-)spermatogonia and appears firstly during the migration of the gonocytes towards the perifery of the tubules (Figure 2) (Honecker *et al.* 2004).

In females, absence of *SRY* allows the differentiation towards an ovary (Fleming and Vilain 2004; Wilhelm *et al.* 2007). Germ cells (oogonia) arrange in cyst-like structures with supportive (pre-granulosa) cells, then enter the first step of meiosis, and form primitive follicles. At that time they lose OCT3/4 expression, and from then on they are called oocytes (Figure 2) (Stoop *et al.* 2005; Rajpert-De Meyts *et al.* 2004; Wilhelm *et al.* 2007). The process is no more believed to be a simple default pathway, since several indispensable genes (e.g. *FOXL2, WNT4, RSPO1*) have been identified (Mandel *et al.* 2008; Parma *et al.* 2006; Uhlenhaut *et al.* 2009). Recently, the importance of FOXL2 in maintenance, instead of only induction of ovarian phenotype of the gonad in mice, was demonstrated as the loss of FOXL2 expression leads to up-regulation of SOX9 and subsequently to a change of gonadal morphology, i.e., testis formation. FOXL2 and SOX9 are expressed in supporting cells (i.e., in granulosa and Sertoli cells, respectively) in a mutually exclusive manner (Hersmus *et al.* 2008b; Uhlenhaut *et al.* 2009).
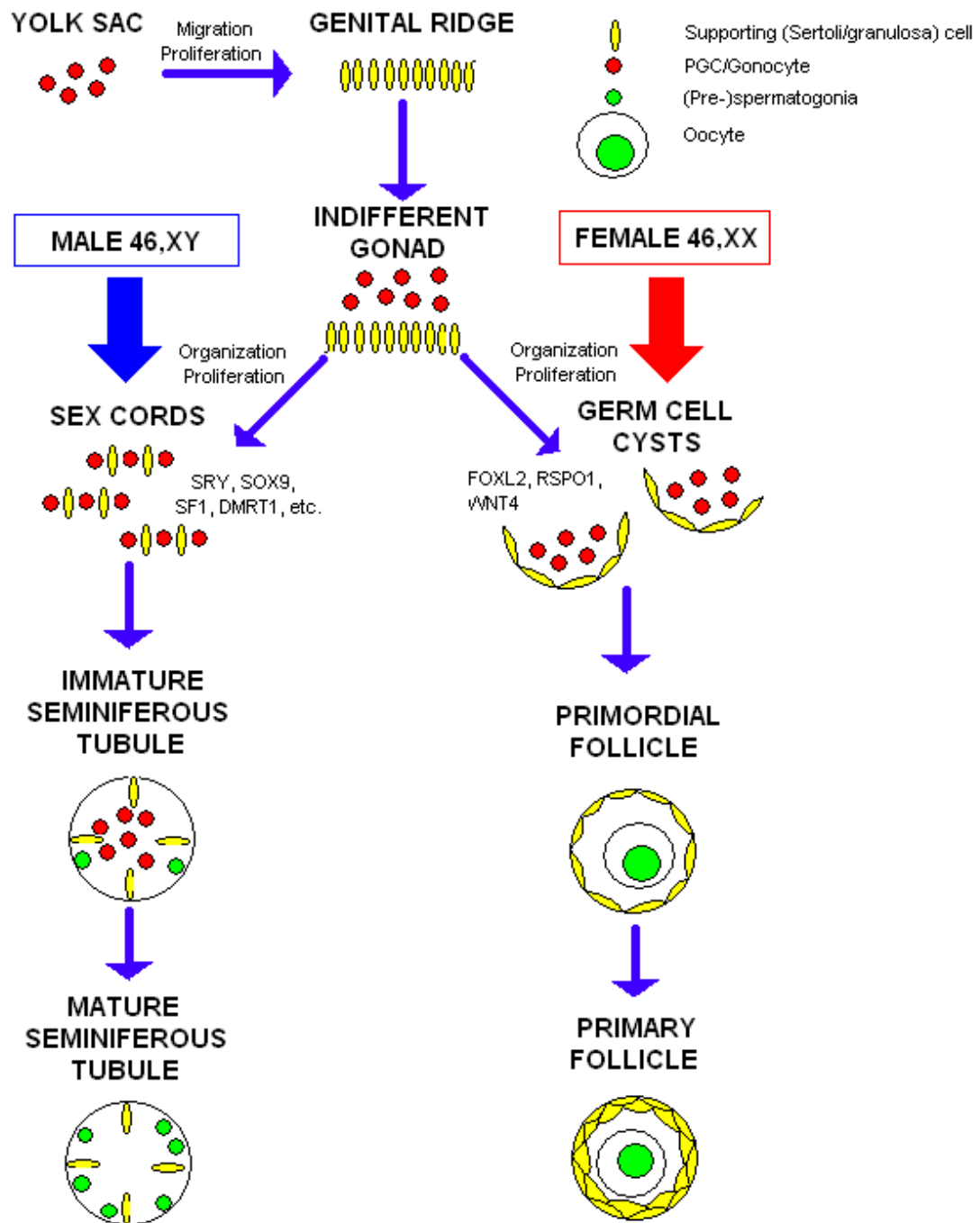
Figure 2: Normal male and female gonadal differentiation. Primordial germ cells migrate from the yolk sac to the gonadal ridge which is then called indifferent gonad. Subsequent development can follow either a male (sex cords, seminiferous tubules in the testicle) or a female (germ cell cysts, follicles in the ovary) differentiation pathway according to the chromosomal constitution and genes expressed.

2.3. Pathogenesis of germ cell tumors

Skakkebaek and co-workers postulated a clinical condition termed testicular dysgenesis syndrome (TDS). It is an umbrella entity for phenomena as testicular cancer, poor semen quality, maldescended testicles and hypospadias, which frequently coincide in a single patient. The rapid increase of incidence of reproductive disorders indicates that environmental factors are the likely cause in most of the cases, although it is supposed that some genetic aberrations or polymorphisms might be involved (Skakkebaek *et al.* 2001; Wohlfahrt-Veje *et al.* 2009). The forms of DSD with increased risk for GCT development could be considered as an extremely escalated cases of TDS. Indeed, genetic background in DSD patients seems to play a major role.

In gonads of predisposed individuals (including DSD patients) immature germ cells, which resemble PGC/gonocytes, persist after birth as the insufficiently differentiated supporting cells (Sertoli/granulosa cells) are not able to provide a satisfactory milieu to induce maturation to either pre-spermatogonia or oocytes. Immature germ cells can be identified by the positivity for factors typically expressed in early fetal germ cells (OCT3/4, PLAP, *etc.*) (Cools *et al.* 2005). The prolonged expression of OCT3/4 is believed to be one of the crucial factors in GCT development as it allows germ cells to survive and proliferate (Cools *et al.* 2009). Thanks to its similar function and temporospatial expression pattern, NANOG supposedly has the similar impact on tumorigenesis as OCT3/4 (Hart *et al.* 2005; Hoei-Hansen *et al.* 2005).

Another piece of the GCT pathogenetic puzzle seems to be an abundant expression of TSPY in germ cells. A special role of TSPY in human GCT development in general, but in DSD patients specifically, is depicted by the fact that it is the most likely candidate for the GBY region (Gonadoblastoma locus on Y chromosome). Existence of such a gene was

previously postulated by Page who based his hypothesis on genetic studies in DSD patients with GB or invasive tumor (Page 1987). Only patients with this part of the Y chromosome in their karyotype have a higher risk to develop a Type II GCT. The physiologic function of TSPY is not fully understood, but it is thought to be involved in control of germ cell mitotic proliferation in normal testis (Lau *et al.* 2009). *In vitro* TSPY potentiates cell proliferation by promoting cell cycle progression via cyclin B (Oram *et al.* 2006). An aberrant expression of TSPY has been related to increased proliferation of germ cells and to oncogenic activity (Lau 1999; Lau *et al.* 2000; Lau *et al.* 2003; Li *et al.* 2007a; Li *et al.* 2007b; Oram *et al.* 2006; Tascou *et al.* 2003). TSPY overexpression in germ cells may contribute to their survival and proliferation in an unfavourable environment which would otherwise result in a depletion of germ cell population.

Finally, KITLG/c-KIT system may play an important role in GCT development. KITLG is a ligand of the proto-oncogene c-KIT which acts as a tyrosine kinase (Strohmeyer *et al.* 1995). This system is indispensable not only for fetal germ cell migration as mentioned above, but also for their proliferation and apoptosis as well as for regulation of adult spermatogonia proliferation and maintenance (Feng *et al.* 1999; Hoei-Hansen 2008; Maduit *et al.* 1999; Stoop *et al.* 2008; Strohmeyer *et al.* 1995). In the adult testis KITLG is synthesized by Sertoli cells in two splice variants: the membrane-bound form acts in establishing and maintenance of germ cells; the soluble form induces the testosterone production in Leydig cells (Maduit *et al.* 1999; Stoop *et al.* 2008). c-KIT mutations have been identified in numerous GCT (Biermann *et al.* 2007; Hoei-Hansen 2008; Looijenga *et al.* 2003a; Tian *et al.* 1999). Additionally, an association between specific polymorphisms within KITLG and the risk for Type II GCT has been reported (Kanetsky *et al.* 2009; Rapley *et al.* 2009), but still the detailed pathogenetic role of KITLG in tumorigenesis remains to be elucidated.
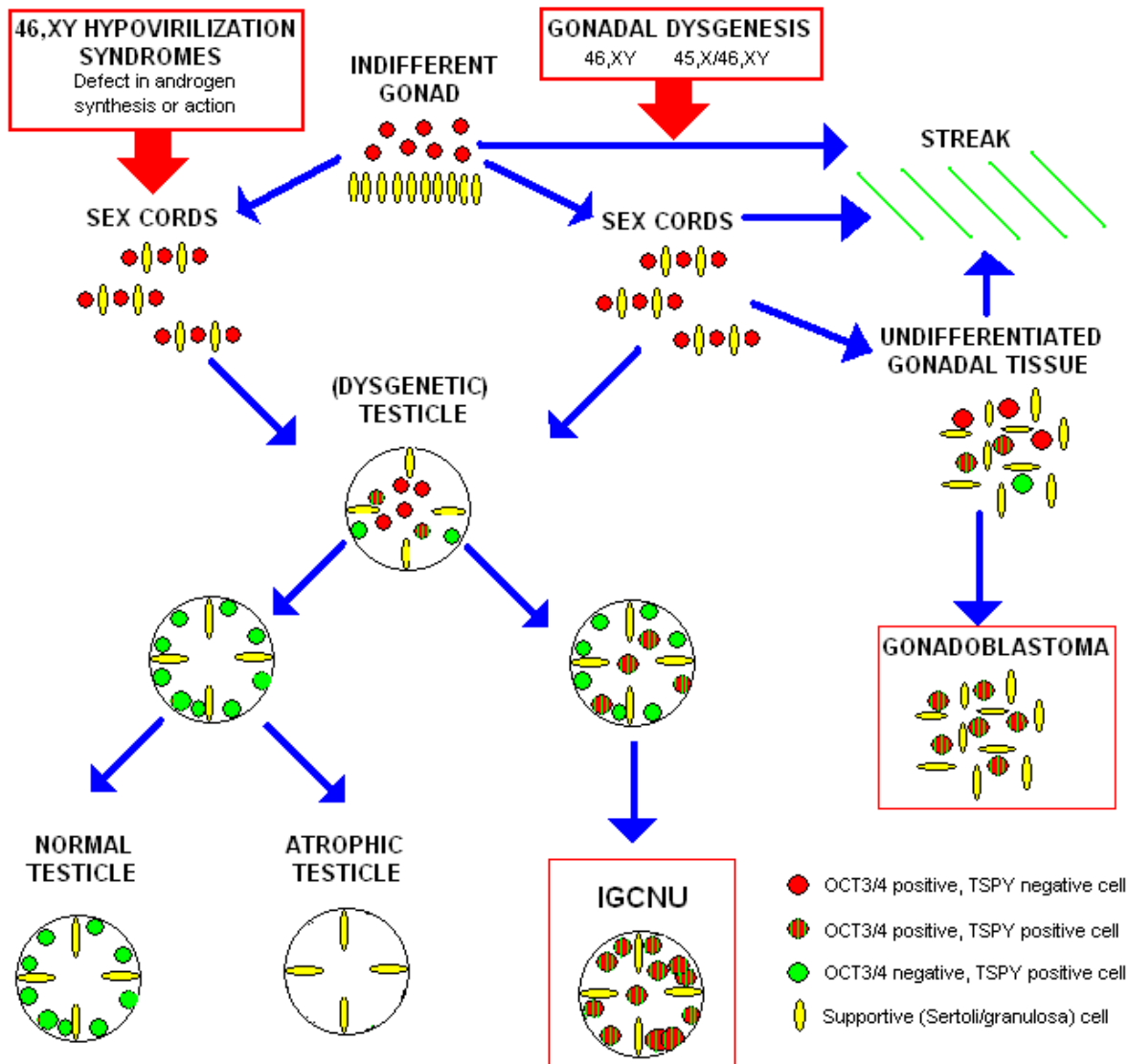
Figure 3: Intratubular germ cell neoplasia unclassified (IGCNU) and gonadoblastoma (GB) development. In hypovirilization syndromes the gonad develops as a testicle, whereas in case of gonadal dysgenesis final morphology can vary from streak, over undifferentiated gonadal tissue (UGT) to a dysgenetic testicle according to the step of differentiation in which the development was disrupted. IGCNU arises from testicular tissue in which OCT3/4 positive germ cells persist and escape normal development while expressing TSPY at the same time. Similar persisting cells in UGT give rise to gonadoblastoma. Supporting cells in IGCNU and GB have characteristics of Sertoli and granulosa cells, respectively.

The gonad in which the IGCNU/GB or GCT arise may be of various phenotypes. IGCNU and (non)seminomas develop in context of testicular tissue both in normal males and certain types of DSD (hypovirilization syndromes and GD). GB is encountered in patients with GD and seems to develop mainly in context of undifferentiated gonadal tissue (UGT), which remarkably resembles the developmental stage of sex cords. Also streak tissue can harbor GB (Cools *et al.,* 2006b). Interestingly, supporting cells in GB display significant FOXL2 (a marker for granulosa cells) and no or very low SOX9 (Sertoli cell marker) expression, indicating that GB most likely arises in the gonads which failed to follow the male differentiation pathway (Hersmus *et al.* 2008b). The highest risk for GCT development is attributed to UGT and to the testis displaying dysgenetic changes (Figure 3)(Cools *et al.* 2009).

2.4. Currently known markers for germ cell tumors and precursor lesions used in the thesis

In the last decades several markers for detection of malignant germ cells in IGCNU/GB and GCT have been established (e.g. PLAP, c-KIT, OCT3/4, AP-2gamma, NANOG). None of them is detectable in normal gonads few months after birth (de Jong *et al.* 2005; Hoei-Hansen *et al.* 2004; Hoei-Hansen *et al.* 2005; Hoei-Hansen 2008; Honecker e*t al.* 2004; Jones *et al.* 2004; Looijenga *et al.* 2003b). OCT3/4 appears to be the most reliable for detection of IGCNU/GB cells thanks to its consistent strong nuclear staining with weak or no background (Figure 4A) (Cools *et al.* 2005; Kersemaekers *et al.* 2005). It serves as a highly specific and sensitive marker for both IGCNU/GB and pluripotent types of GCT (i.e. seminoma/dysgerminoma and embryonal carcinoma) (Cools *et al.* 2009; Looijenga *et al.* 2003b).

When assessing gonadal tissue in DSD patients, a more cautious approach should be

applied due to possible maturation delay of the germ cells which frequently occurs in DSD gonads and may lead to overdiagnosis and overtreatment (Cools *et al.* 2005). Immature germ cells express similar markers as early fetal germ cells and as IGCNU/GB cells which are believed to develop from them (Cools *et al.* 2005; Dieckmann and Skakkebaek 1999; Rajpert-De Meyts 2006). Cools *et al.* proposed additional criteria which should help to distinguish between maturation delay and early neoplasia in patients with hypovirilization syndromes (Cools *et al.* 2005). The criteria are based on knowledge of fetal germ cell development. OCT3/4 positive cells which are located in the centre of the seminiferous tubules and are scattered throughout the whole gonad are believed to be delayed in their maturation (Figure 4B) (Cools *et al.* 2005). On the other hand, larger number of cells attached to the basal lamina is considered to be abnormal and is suspicious from future IGCNU and GCT development.

As mentioned above, TSPY is the most probable candidate for GBY and is abundantly expressed in germ cells in DSD gonads and in IGCNU/GB cells (Cools *et al.* 2005; Kersemaekers *et al.* 2005; Lau *et al.* 2009; Schnieders *et al.* 1996). Its expression is normally almost exclusively restricted to (pre-)spermatogonia (Honecker *et al.* 2004; Pauls *et al.* 2006). Therefore, it is only sporadically co-expressed with OCT3/4 within a single cell under the physiological conditions (Cools *et al.* 2006). Double-staining for OCT3/4 and TSPY is informative for the detection of cells, which escape normal development (Kersemaekers *et al.* 2005). In case of IGCNU, the OCT3/4-positive cells are attached to the basal lamina and may strongly co-express TSPY (Figure 4C) (Cools *et al.* 2006a; Oosterhuis *et al.* 2011). Double-positive cells in the luminal site are likely to be only delayed in their maturation (Figure 4D).
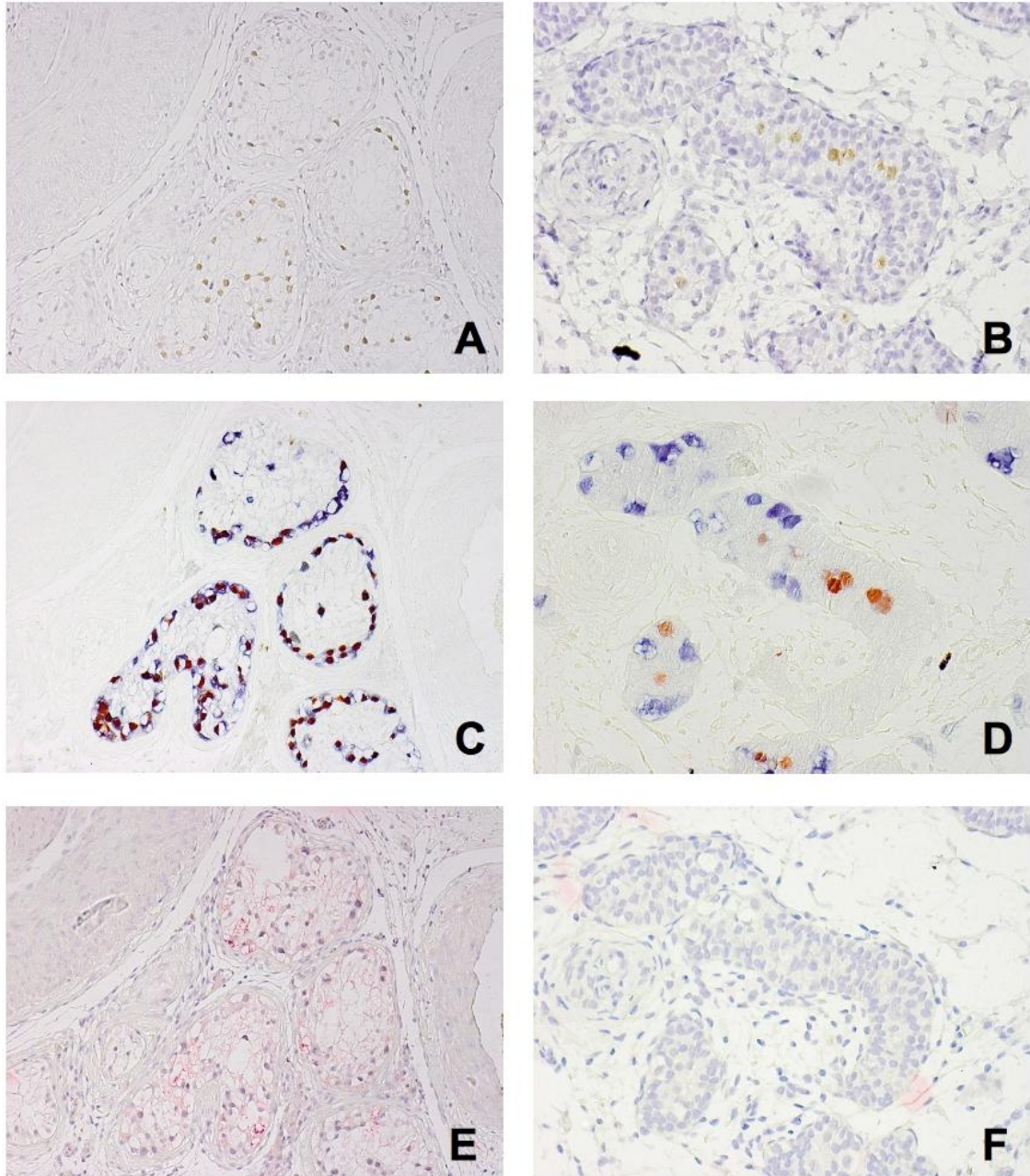
Figure 4: Intratubular germ cell neoplasia unclassified (IGCNU) and delayed maturation of the germ cells - various immunohistochemical stainings. A - IGCNU in adult testis stained for OCT3/4 expression (brown), IGCNU cells are attached to the basal lamina, 100x; B - delayed maturation of germ cells in a testis of 10-month-old patient with complete form of androgen insensitivity syndrome stained for OCT3/4 expression, positive cells are located in the center of the tubules, 200x; C - same area as in A stained for expression of both OCT3/4 (orange) and TSPY (blue), IGCNU cells are mostly double-positive, 100x; D - same area as in B stained for expression of both OCT3/4 and TSPY, OCT3/4-positive germ cells are either TSPY-negative or only weakly TSPY-positive, 200x; E - same area as in A and C positive for KITLG (red), 100x; F - same area as in B and D negative for KITLG, 200x.

KITLG seems to be a useful additional tool in distinguishing between immature germ cells and early malignant cells (Stoop *et al.* 2008, van de Geijn *et al.* 2009). KITLG is undetectable in both normal fetal and postnatal testes using immunohistochemical methods. However, KITLG positivity in IGCNU/GB is convincingly consistent (Figure 4E) (Sandlow *et al.* 1996; Stoop *et al.* 2008). KITLG staining in invasive GCT is heterogeneous. Interestingly, DSD gonads which harbor germ cells displaying OCT3/4 positivity but not fulfilling the criteria for IGCNU cells (i.e., cells with maturation delay), are KITLG-negative (Figure 4F) (Stoop *et al.* 2008). This gives KITLG a unique position among other IGCNU/GB markers.

2.5. Disorders of sex development

2.5.1. Definition and classification

DSD are defined as congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical. The overall occurrence of genital anomalies is estimated to be one in 4,500 births. DSD represent a group of disorders with a highly heterogeneous genetic background. The very last and worldwide accepted DSD classification is a result of consensus made by members of ESPE/LWPES (European Society of Pediatric Endocrinology/Lawson Wilkins Pediatric Endocrinology Society) in Chicago in 2005. It divides the disorders into three subgroups according to the karyotype: i) disorders with aberrant sex chromosomes; ii) 46,XY DSD; iii) 46,XX DSD. Details are stated in Table 2 (Hughes *et al.* 2006).

Table 2: Currently used classification of DSD (adapted from Hughes *et al.* 2006).

DSD WITH DISORDERS OF SEX CHROMOSOMES	46,XY DSD	46,XX DSD
A. 47,XXY (Klinefelter syndrome and variants)	**A. Disorders of testicular development**	**A. Disorders of ovarian development**
	1. Complete or partial gonadal dysgenesis (e.g. *SRY,SOX9, SF1, WT1, DHH, etc.*)	1. **Gonadal dysgenesis**
	2. **Ovotesticular DSD**	2. **Ovotesticular DSD**
	3. **Testicular regression**	3. **Testicular DSD** (e.g. *SRY+, dup SOX9, RSPO1, WNT4, etc.*)
B. 45,X (Turner syndrome and variants)	**B. Disorders of androgen synthesis and action**	**B. Excess of androgens**
	1. Disorders of synthesis - mutation of LH receptor - Smith-Lemli-Opitz syndrome - mutation of StAR (Steroid acute regulatory protein) - mutation of enzyme cleaving side- chain of cholesterol (*CYP11A1*) - mutation of 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*) - mutation of 17α-hydroxylase/ 17,20-lyase (*CYP17*) - mutation of p450 oxidoreductase (*POR*) - mutation 17β-hydroxysteroid dehydrogenase (*HSD17B3*) - mutation 5α-reductase 2 (*SRD5A2*) - *ect.*	**1. Fetal origin** - mutation of 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*) - mutation of 21-hydroxylase (*CYP21A2*) - mutation of p450 oxidoreductase (*POR*) - mutation of 11β-hydroxylase (*CYP11B1*) - mutation of glucocorticoid receptor - *etc.*
	2. Disorders of action - androgen insensitivity syndrome (*AR*) - drugs and environmental disruptors influencing the sex development	**2. Fetoplacental origin** - mutation of aromatase (*CYP 19*) - mutation of oxidoreductase (*POR*)
		3. Maternal origin - tumors with androgen production - drugs with androgen action
C. 45,X/46,XY (Mixed gonadal dysgenesis)	**C. Others**	**C. Others**
	1. Disorder as a part of a syndrome (e.g. cloacal anomalies, Robinow sy, Aarskog sy, Hand-Foot-Genitalia sy, *etc.*)	**1. Disorder as a part of a syndrome** (e.g. cloacal anomalies)
	2. Syndrome of persisting müllerian ducts	**2. Agenesis of müllerian duct structures**
	3. **Vanishing testis syndrome**	**3. Uterus anomalies** (e.g. MODY5)
	4. **Isolated hypospadia**	4. **Vaginal atresia**
	5. **Congenital hypogonadotropic hypogonadism**	5. **Labial adhesions**
	6. **Cryptorchidism**	
	7. **Environmental influences**	
D. 46,XX/46,XY (Chimerism)		

2.5.2. Current view on prevalence of germ cell tumors in disorders of sex development

As mentioned above, only patients carrying GBY region in their karyotype are endangered by GCT development (Page 1987; Lau *et al.* 2009). Multiple studies endeavour to establish the risk for GCT in particular DSD subgroups. However, one has to reckon with bias due to i) inconsistent DSD classifications ii) inaccurate DSD diagnostics iii) heterogeneous criteria for IGCNU/GB diagnosis iv) possibly preferential reporting of tumor cases v) lower incidence of malignancies in later series due to early prophylactic gonadectomy.

In the last few years, the most extensive meta-analysis was performed by Cools and colleagues (Cools *et al.* 2006a). They divided DSD patients into several risk groups for development of Type II GCT. Patients with hypovirilization syndromes have a normal male karyotype and their gonads develop into normal testicles. These disorders are caused by a defect in either synthesis or action of androgens. The most numerous are patients with androgen insensitivity syndrome.

The overall prevalence of IGCNU and invasive GCT in this group is estimated to be 5.5%. There is, however, an important difference between patients with complete (CAIS) and partial (PAIS) androgen insensitivity syndrome in which malignancies occur in 0.8% and 15%, respectively (Cools *et al.* 2006a). Other hypovirilization syndromes are very rare. Malignancies in 17% of patients with 17β-hydroxysteroid dehydrogenase were mentioned in one small series (Cools *et al.* 2005), while only one case of seminoma in a patient with 5α-reductase deficiency and no tumors in patients with Leydig cell hypoplasia have been reported so far (Cools *et al.* 2005; Sasaki *et al.* 2003).

Patients with gonadal dysgenesis (with either a 46,XY or 45,X/46,XY karyotype) seem to

be the most endangered subgroup, although the prevalence in different series is rather incoherent being reported in 15-100% of cases (Cools *et al.* 2006a; Slowikowska-Hilczer *et al.* 2001). After the rational interpretation of the available data, Cools *et al.* rated the total occurrence at 12%, and possibly more than 30% if gonadectomy would not have been performed. Particularly in patients with mosaic karyotype the prevalence ranges between 15 and 40%, while in those with 46,XY karyotype it reaches approximately 30% (Cools *et al.* 2006a). In a series of patients with a defect of the *WT1* gene, malignancies were reported in 60% of patients with Frasier syndrome (Joki-Erkkilä *et al.* 2002) and in 40% of patients with Denys-Drash syndrome (Pelletier *et al.* 1991).

In patients with ovotesticular DSD, previously referred to as true hermaphroditism, the occurrence of neoplasia is estimated at 2.6% (Table 3) (Cools *et al.* 2006).

Table 3: Prevalence of Type II GCT in various forms of DSD (adapted from Cools *et al.* 2006a).

Risk	Type of DSD	Prevalence
High	GD in general	12%*
	46,XY GD	30%
	Frasier syndrome	60%
	Denys-Drash syndrome	40%
	45,X/46,XY GD	15-40%
Intermediate	PAIS	15%
	17β-hydroxysteroid dehydrogenase defficiency	17%
Low	CAIS	0.8%
	Ovotesticular DSD	2.6%
Unknown	5α-reductase	?
	Leydig cell hypoplasia	?

GD – gonadal dysgenesis; CAIS – complete androgen insensitivity syndrome; PAIS – partial androgen insensitivity syndrome; * might reach more than 30%, if gonadectomy would not be performed.

2.5.3. DSD subtypes selected for the studies

2.5.3.1. Complete androgen insensitivity syndrome

Androgen insensitivity syndrome is caused by an inactivating mutation in androgen receptor (AR) gene. It is an X-linked recessive disorder and thus affects only 46,XY individuals (Galani *et al.* 2008; Hughes *et al.* 2012). Overall incidence is estimated to be 1:40,800-99,000 (Boehmer *et al.* 2001). Clinical presentation may vary between normal infertile male and female with blindly ended vagina and missing internal genitalia. Gonads have a character of testis in all cases. Phenotypical variability led to a subdivision of the syndrome into mild/minimal, partial and complete form (Quigley *et al.* 1995). Patients with CAIS are raised as girls and are usually recommended for gonadectomy. As it has been stressed before, this procedure prevents spontaneous pubertal development, if provided in childhood, and leads to a need for hormonal substitution.

Typical changes in gonadal histology of patients with CAIS have been described. They develop with rising age and may be influenced by two major factors: abnormal gonadal location and decreased AR activity (Giwercman *et al.* 1989; Hannema *et al.* 2006). However, precise contribution of the factors is not yet fully elucidated.

Among others, germ cell anomalies are of crucial importance. The occurrence of GCT has been reported to be up to 22% in adult patients in different studies (Deans *et al.* 2012). Invasive GCT are very rare in childhood and adolescence (Hurt *et al.* 1989); however, non-invasive precursor lesions characterized as IGCNU have been repeatedly described in this age group (Cools *et al.* 2005; Hannema *et al.* 2006). Interestingly, the occurrence of IGCNU in pediatric patients, which was present at a maximum of 6% of cases, does not reach the frequency of GCT in adulthood, reported in most literature (Cools *et al.* 2005;

Hannema *et al.* 2006; Cassio *et al.* 1990; Müller and Skakkebaek 1984; Bangsbøll *et al.* 1992).

The prevalence of IGCNU appears to be remarkably higher (15%) in pediatric patients with PAIS than in patients with CAIS (Cools *et al.* 2006a). Hannema and colleagues described that the expected residual activity of AR has a positive effect on the development of Wolffian structures and the enlargement of seminiferous tubules during puberty in patients with CAIS (Hannema *et al.* 2004; Hannema *et al.* 2006). Whether the residual AR activity also has an impact on the survival of normal and/or atypical germ cells has not yet been reported.

2.5.3.2. 45,X/46,XY gonadal dysgenesis

Sex chromosome mosaicism (45,X/46,XY and variants) occurs with an estimated incidence of 1,5/10 000 (Chang *et al.* 1990) and may be due to loss of the Y chromosome through anaphase lag or to interchromosomal rearrangements with final loss of a structurally abnormal Y chromosome. The clinical spectrum is highly heterogeneous, with no obvious correlation between the phenotypic appearance and the respective cell line counts on routine peripheral blood karyotyping (Chang *et al.* 1990; Grumbach *et al.* 2003; Telvi *et al.* 1999) or even on the basis of gonadal cell line counts (Cools *et al.* 2007). Up to 95% of individuals may live undiagnosed as normal males (Chang *et al.* 1990). However, ambiguous genitalia in a newborn, but also mild undervirilization (e.g. hypospadias) in boys or even typical Turner syndrome in girls may be associated with 45,X/46,XY mosaicism (Telvi *et al.* 1999).

Because of the presence of GBY in their karyotype, the patients are endangered by

aberrant expression of TSPY and consequently by increased tumor risk. The recent change in attitude towards clinical management of DSD patients, with increased emphasis on a conservative approach and the delay of irreversible surgery until adulthood (Hughes *et al.* 2006; Hughes 2010; Wiesemann *et al.* 2010; Creighton *et al.* 2004) has brought along uncertainty concerning the optimal approach with regard to gonads at risk for malignant transformation, e.g. in individuals with 45,X/46,XY mosaicism and male gender. Evidently, gonadectomy is not the treatment of choice in these patients, but on the basis of a review of the relevant literature, tumor risk in 45,X/46,XY individuals has been reported to be around 15% (Cools *et al.* 2006a). However, clinical experience suggests a much lower incidence in 45,X/46,XY Turner syndrome girls. On the other hand, recent research has identified UGT, for which 45,X/46,XY is a known risk factor, as the precursor lesion for GB (Cools *et al.* 2006b; Looijenga *et al.* 2010).

3. HYPOTHESES AND AIMS

3.1. Study 1: Complete androgen insensitivity syndrome

Impaired gonadal position and androgen action very likely cause histopathological changes of the gonads in CAIS, however, precise contribution of the two factors has never been elucidated. With respect to the fact that risk of development of gonadal GCT in PAIS is strikingly higher than in CAIS, patients with CAIS and residual AR activity may have higher tumor risk than CAIS patients with no AR activity.

Hypothesis:

1. Level of AR activity and gonadal position influence the histopathological changes in gonads of patients with CAIS.

2. CAIS patients with expected residual AR activity are at higher risk of GCT development than patiens with no residual activity of AR.

Aims:

1. To assess gonadal histology including germ cell pathology in patients with CAIS.

2. To compare it with expected level of AR activity and gonadal position.

3.2. Study 2: 45,X/46,XY gonadal dysgenesis

Clinical experience in patients with 45,X/46,XY mosaicism indicates that there exist differences in risk for development of gonadal GCT between individuals with different phenotypes. However, evidence based work is missing.

Hypothesis:

Tumor risk differs between patients with different clinical phenotype in individuals with 45,X/46,XY karyotype.

Aims:

1. To assess the level of masculinization of 45,X/46,XY individuals.

2. To assess the gonadal type and presence of (pre-)malignant germ cells in gonads of the subjects.

3. To compare clinical phenotype and risk for GCT development.

4. MATERIAL AND METHODS

4.1. Patients and histological material

4.1.1. Study 1: Complete androgen insensitivity syndrome

We investigated 37 testes from 19 patients with the complete form of androgen insensitivity syndrome, i.e., patients with an unambiguously female phenotype who were diagnosed based on having AR gene mutation identified by direct sequencing in a diagnostic set up. Patients ranged in age from 3 months to 18.5 years (mean = 9.2 years). The samples were included only if the activity of AR could be inferred from the sequencing results or if a reference about the functional studies existed. Based on the type of mutation (frame shift mutations, mutations leading to the introduction of a stop codon after internal initiation-of-translation sites) and a search of the literature (point mutations with zero activity in ligand-binding or transactivation studies), no residual activity of AR was expected in 11 patients (Brüggenwirth *et al.* 1996; Brüggenwirth *et al.* 1998; Mowszowitz *et al.* 1993; Weidemann *et al.* 1996). In 5 patients, a point mutation in the ligand-binding domain resulted in a mutated AR with some residual activity in transactivation studies (Hannema *et al.* 2004; Knoke *et al.* 1999; Beitel *et al.* 1994; Marcelli *et al.* 1994). In the remaining 3 patients, the activity of AR was uncertain: mutation of an intronic region led to abnormal splicing of AR in one sibling pair (Brüggenwirth *et al.* 1997); AR truncated at the amino terminal side was detected as a result of an early introduced stop-codon in the third case (Zoppi *et al.* 1993). One or both gonads were originally positioned in the inguinal region in 6 patients and were relocated into the abdominal cavity at hernioplasty during infancy or early childhood. Only a single gonad was situated in the labial region and was grouped with inguinal gonads for statistical analysis (Table 4). The gonads were removed

as a prophylactic measure in all cases.

This study was approved by the local Ethics Committee of the University Hospital Motol, Prague (EC 237/2009), and the 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 (Federa, 2011).

Table 4: Patients information for age, mutation (Reference sequence: hg19, NM 000044) (Gotlieb *et al.* 2012), expected AR residual activity and gonadal location.

	Age (yrs)	Mutation nucleotide (HGVS)//amino acid level	Expected residual AR activity, (method used)	Reference	Gonad	Gonadal location
S1	0.25	c.1769-11T>A//p.? *Sister of S5*	??? TA (0% activity with 10 nM R1881)	Brüggenwirth *et al.* 1997	right left	inguinal inguinal
S2	0.80	c.2566C>T//p.Arg856Cys	No LBA (0% ligand binding activity)	Weidemann *et al.* 1996	right left	inguinal inguinal
S3	1.20	c.1721C>A//p.Ala574Asp	No TA (0% activity with 10 nM R1881)	Brüggenwirth *et al.* 1998	right left	inguinal inguinal
S4	1.25	c.1000insT//p.Gly334Valfs*7 *Sister of S9*	No		right left	inguinal abdominal
S5	2.50	c.1769-11T>A//p.? *Sister of S1*	??? TA (0% activity with 10 nM R1881)	Brüggenwirth *et al.* 1997	right left	inguinal inguinal
S6	2.75	c.2343G>A//p.Met781Ile	Yes TA (100% activity with 3 nM R1881)	Knoke *et al.* 1999	right left	inguinal inguinal
S7	3.00	c.178C>T //p.Gln60*	??? TA (30% with 2 nM DHT)	Zoppi *et al.* 1993	right left	NA inguinal
S8	3.20	c.2522G>A//p.Arg841His	Yes TA (60% activity with 2.2 nM mibolerone)	Beitel *et al.* 1994	right left	labial inguinal
S9	6.00	c.1000insT// p.Gly334Valfs*7 *Sister of S4*	No		right left	abdominal abdominal
S10	9.50	c.2197G>A//Asp733Asn *Sister of S17*	Yes TA (120% activity with 10 nM mibolerone)	Hannema *et al.* 2004	right left	i/a (1 y) i/a (1 y)
S11	13.66	c.832-833dupGC//p.Val279Leufs*18 *Sister of S18*	No		right left	i/a (6 y) i/a (6 y)
S12	15.20	c.1847G>A//p.Arg616His	No TA (0% activity with 10 nM mibolerone)	Mowszowitz *et al.* 1993	right left	abdominal abdominal
S13	15.50	c.2567G>A//p. Arg856His	Yes TA (80% activity with 2 nM mibolerone)	Marcelli *et al.* 1994	right left	inguinal inguinal
S14	15.66	c.2546dupA//p.Asn849Lysfs*32 *Sister of S15*	No		right left	abdominal i/a (0 y)
S15	15.66	c.2546dupA//p.Asn849Lysfs*32 *Sister of S14*	No		right left	i/a (0 y) abdominal
S16	16.20	c.1774C>T//p. Gln592*	No		right left	abdominal abdominal
S17	16.50	c.2197G>A//Asp733Asn *Sister of S10*	Yes TA (120% activity with 10 nM mibolerone)	Hannema et al. 2004	right left	i/a (1 y) i/a (1 y)
S18	16.66	c.832-833dupGC//p.Val279Leufs*18 *Sister of S11*	No		right left	i/a (3 y) i/a (3 y)
S19	18.50	c.159-171del13//p.Leu54Serfs*117	No		right left	abdominal abdominal

TA- transactivation; LBA - ligand binding activity; ??? - uncertain; i/a (x y) - original location inguinal, relocated to abdominal cavity at x years; NA - not available.

4.1.2. Study 2: 45,X/46,XY gonadal dysgenesis

Eighty seven gonadal tissue samples from 48 patients with 45,X/46,XY (or variants) GD, obtained by biopsy or gonadetomy, were available. Clinical data were recorded by treating physicians and reviewed. An overview of the patients and samples is provided in Table 5. The group of individuals with female phenotype is overrepresented which probably relates to the finding that most 45,X/46,XY cases live undiagnosed as normal males (Chang *et al.* 1990). Surgery is delayed in this group due to later diagnosis (mostly because of short stature) than in groups with mild undervirilization and ambiguous phenotype. Eighty four gonadal samples were considered for further analysis; one sample was excluded because it was a gonadectomy specimen of a previously biopsied gonad and revealed no new findings. In one patient who received a left biopsy and a right gonadectomy clinical information was insufficient.

Patients were classified into three groups, based on the EMS (External masculinization score), which represents a clinical scoring system (based on the position of the gonads, length of the phallus, presence of scrotal fusion and position of the urethral meatus) to quantitatively assess the degree of undervirilization in DSD patients (Ahmed *et al.* 2000). The EMS was calculated from data of the first clinical presentation: Group 1: mild undervirilization, EMS 7-12, Group 2: ambiguous phenotype, EMS <7, and Group 3: female phenotype, representing in fact girls with Turner syndrome (without clitoromegaly).

The study was approved by the medical ethical committees of the University Hospital Ghent (MEC 2008/098), the Erasmus Medical Center Rotterdam (MEC 02.981) and the Ethics Committee of the University Hospital Motol, Prague (EC 237/2009). The 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 2011).

Table 5: Overview of the Study 2 - population and available samples.

	Mild undervirilization (EMS ≥7)	Ambiguous phenotype (EMS < 7)	Female phenotype (Turner syndrome)
Patients	10	14	23
Gonadal samples	15	24	46
	Biopsy 9 Gonadectomy 6	Biopsy 6 Gonadectomy 18	Gonadectomy 46
Sex of rearing	Male 10 Female 0	Male 10 Female 4	Female 23
Mean age at surgery (years)	4.0	2.2	12.2

EMS and sex of rearing unknown in 1 patient. EMS - external masculinization score.

4.2. Methods

4.2.1. Immunohistochemistry

All gonadal samples were fixed in 10% formalin for 24 hours and embedded in paraffin. After antigen retrieval (120°C, 0.9 Bar), 3 to 4-µm-thick sections were evaluated for the presence of OCT3/4 (sc-5279, Santa Cruz Biotechnology, CA, USA, dilution 1:350-1000, incubated 120' at room temperature), TSPY (the antibody was kindly provided by prof. Dr. C. Lau, Department of Medicine, VA Medical center, University of California, San Francisco, CA, USA; dilution 1:3000-4000, incubated overnight at 4°C), and KITLG (sc-1302, Santa Cruz Biotechnology, CA, USA, dilution 1:250-500, incubated overnight at 4°C). Detection and visualization was conducted using biotinylated secondary antibodies and avidin-biotin-complex conjugated with horseradish peroxidase for OCT3/4 (Vectastain ABC kit Elite pk-6100 Standard) or alkaline phosphatase for TSPY and KITLG (Vectastain ABC kit pk-5000 AP). Diaminobenzidine/H_2O_2 (for OCT3/4) and New Fuchsine/Naphtol

ASMX phosphate (for TSPY and KITLG; N500 Sigma, Steinheim, Germany) were used as substrates. Adult testicular tissue with IGCNU was used as a positive control for all staining.

In case of Study 1, tissue samples from 36 gonads (22 gonads from patients lacking AR activity; 9 gonads from patients with expected residual AR activity; 5 gonads from patients with uncertain AR activity) were available for immunohistochemical staining.

All included samples were available for the staining in Study 2.

4.2.2. Double-staining

Samples with OCT3/4-positive cells were investigated for co-expression of OCT3/4 and TSPY. Sections were pretreated with H_2O_2, pressure cooked and incubated with primary antibodies against TSPY (overnight, at 4°C) and OCT3/4 (sc-8629, dilution 1:350, incubated 120' at room temperature). TSPY was detected using the avidin-biotin-complex conjugated with alkaline phosphatase complex and Fast Blue/Naphtol ASMX phosphate as a substrate. OCT3/4 was detected with avidin-biotin-complex conjugated with horseradish peroxidase complex and 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany)/H_2O_2 as a substrate. In between the two stainings, free biotin was blocked (Vector Laboratories, Burlingame, CA USA). The adult testicular tissue containing IGCNU was used as positive control. This technique was performed only in Study 1.

4.2.3. Gonadal histology assessment

Study 1: At least one section from each gonad was stained with hematoxylin and eosin (HE) and assessed for overall organization of the gonadal tissue, markers of pubertal

maturation, and abnormal histological phenomena. The fraction of seminiferous tubules containing germ cells was calculated in at least 200 cross-sections of tubules in each gonad. The proportion of OCT3/4-positive cells allocated to the basal lamina was assessed on twenty cross-sections of tubules, if available.

Delayed maturation of germ cells was defined as a presence of OCT3/4-positive germ cells with round nuclei located centrally in the seminiferous tubules of individuals over 6 months of age. Only occasionally are weakly OCT3/4-positive cells at the basement membrane of the tubules accepted as indicators of delayed maturation. Such cells are considered to be in the process of turning off OCT3/4 expression. KITLG is not expressed in gonads with delayed maturation germ cells (Cools *et al.* 2005; Stoop *et al.* 2008). For the diagnosis of IGCNU, the presence of at least one cross-section of a seminiferous tubule containing a homogeneous population of atypical germ cells with angulated nuclei was required. These cells must show either homogeneous double expression of OCT3/4 (nuclear) and TSPY (cytoplasmic and membranous) or homogenous expression of OCT3/4 (nuclear) in the absence of TSPY. The involved tubule must show expression of KITLG, which usually presents as irregular spots associated with the cytoplasm of Sertoli cells. A diagnosis of pre-IGCNU was made when the findings fall short of the criteria for IGCNU and are beyond those that are acceptable for the diagnosis of delayed maturation. KITLG may be expressed in testes containing pre-IGCNU (Oosterhuis *et al.* 2011; Stoop *et al.* 2008).

Study 2: Based on the general morphology, as assessed on HE staining, samples were categorized as (dysgenetic) testis, UGT, ovary, streak, or a combination of these. The sample was considered to be at risk for GCT development if either an *in situ* neoplastic lesion (GB or IGCNU) or one or more indices for pre-malignancy (UGT, OCT3/4-positive cells on the BL of testis tubules, positive KITLG staining) were present.

4.3. Statistics

4.3.1. Study 1: Complete androgen insensitivity syndrome

Influence of age, gonadal location at the time of gonadectomy, and expected level of AR activity on particular histological features was analyzed. The results were assessed using Mann-Whitney and Chi-square tests, as well as logistic, multivariate and linear regressions. Correlation between original gonadal location and AR activity was evaluated by a Chi-square test. Correlations between AR activity and age and between gonadal location and age were analyzed by a Mann-Whitney test. A value of $p<0.05$ was considered significant. All analyses were performed using SYSTAT 10.0.

4.3.2. Study 2: 45,X/46,XY gonadal dysgenesis

Results were analyzed with the SPSS software (version 15.0), comparison of categorical variables was performed using a Fisher exact test.

5. RESULTS

5.1. Study 1: Complete androgen insensitivity syndrome

The original anatomical gonadal location was significantly dependent on expected AR activity ($p < 0.05$). Whereas half of the gonads were originally situated in the abdominal cavity in patients with expected no residual activity, one gonad was labial and the rest were inguinal in patients with expected residual activity of AR. Labial or inguinal location of the gonads possibly contributed to relatively earlier clinical diagnosis and therefore earlier gonadectomy ($p < 0.01$) in our study; however, age of gonadectomy did not differ significantly ($p > 0.05$) between patients with and without residual activity of AR.

Intertubular stroma appeared edematous (Figure 5A) mostly in younger patients whereas in the older individuals it was rather fibrotic ($p < 0.001$) (Figure 5B). Combination of both edematous and fibrotic stromal changes within a single gonad was also observed, mainly in prepubertal patients. Areas of ovarian-like stroma (Figure 5C) were present in the gonads of one sibling pair (S4 and S9). Remarkably dilated lymphatic vessels (Figure 5D) were observed in 67% of gonads throughout the entire cohort. Hamartomatous nodules (Figure 5E), i.e., well circumscribed nodules composed of Sertoli cell-only tubules and Leydig cells in between the tubules, were observed in all pubertal and postpubertal patients (13-year-old and older), and therefore, their development was significantly dependent on age ($p < 0.001$). Tubular atrophy presented as fibrosis of the tubules (Figure 5F) was observed in 47% of patients older than 6 years of age and was significantly dependent on higher age ($p < 0.001$). Diffuse Leydig cell hyperplasia (Figure 5G), which was present in all pubertal and postpubertal patients, was also dependent on older age ($p < 0.001$). Scattered Sertoli cells with eosinophilic granules in the cytoplasm (Figure 5H), so-called Hürtle cell-changes (a result of altered mitochondria), were identified in 14% of
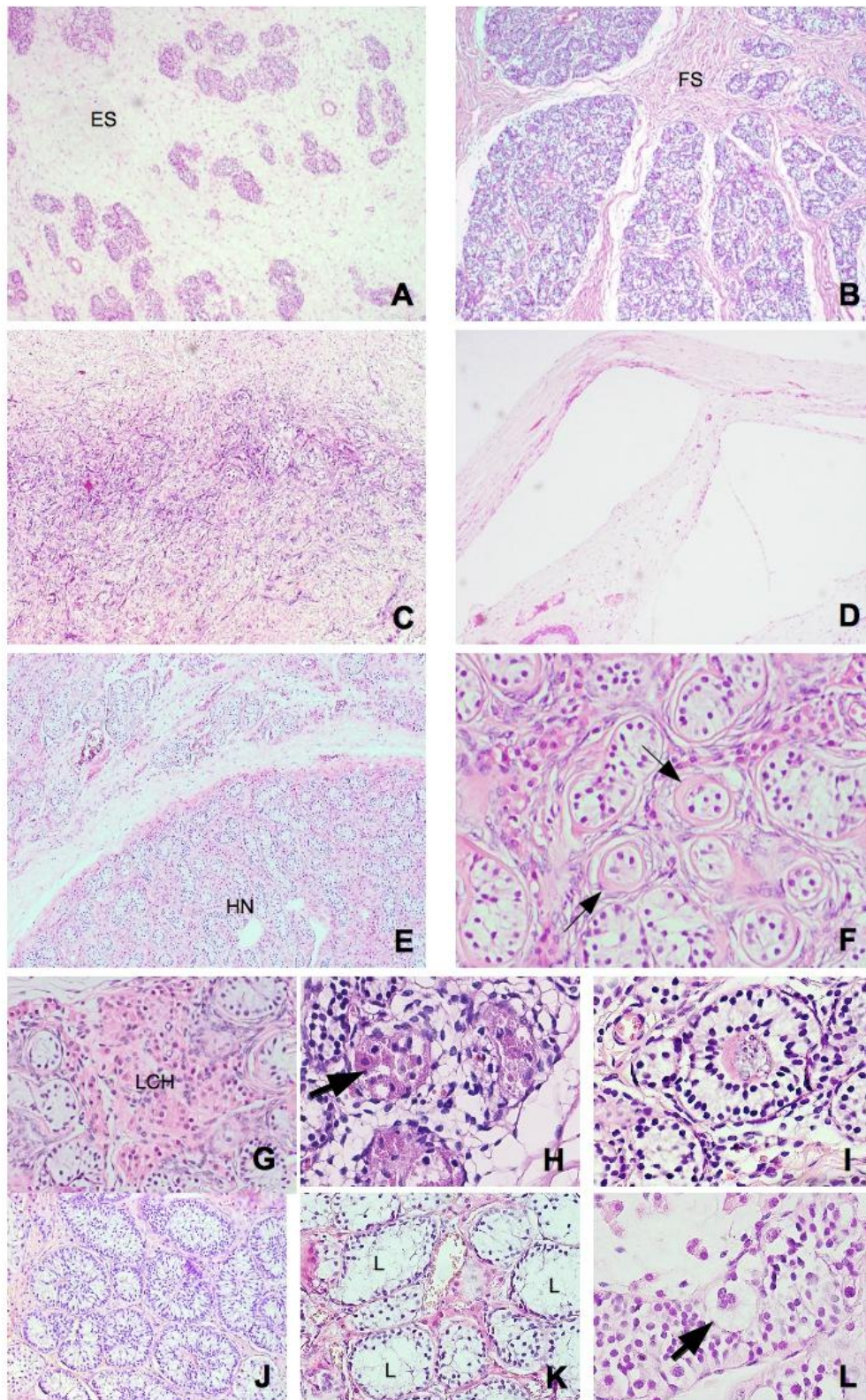
Figure 5: Different histopathological changes of the testis in CAIS, HE staining. A - edematous stroma (ES), 50x; B - fibrotic stroma (FS), 50x; C - ovarian-like stroma, 50x; D - dilation of lymphatic vessels, 50x; E - hamartomatous nodule (HN), 50x; F - fibrotic atrophy of the seminiferous tubule (arrow), 200x; G - Leydig cell hyperplasia (LCH), 200x; H - eosinophilic granular changes of Sertoli cell cytoplasm (arrow), 400x; I - Sertoli cell nodule with central hyalin deposit, 400x; J - Sertoli cell nodules without hyaline deposit, 100x; K - sporadic lumen (L), 200x; L - multinucleate germ cell (arrow), 400x.

gonads, all in patients older than 9 years. Sertoli cell nodules, either with (Figure 5I) or without (Figure 5J) central hyaline deposits with partial calcification, were observed in almost 37% of patients, the youngest being 3 years old.

In regard to signs of pubertal development, tubular lumen formation was evaluated and was not consistently present in any of the cases, although sporadic lumina (Figure 5K) were observed in the majority of the patients older than 9 years. Spermatogenesis was not encountered in any of the patients.

At least one tubule with germ cells was found in 84 % of gonads. The number of tubules containing germ cells declined with age in the whole series (p<0.001). A significant difference in germ cell survival (p<0.05) between patients with and without expected residual AR activity in (post)pubertal age was observed. While the gonads of the patients without expected residual activity lacked germ cells altogether or contained only solitary tubules with germ cells, a considerable number of tubules with germ cells was identified in patients with expected residual AR activity (Figure 6). Multinucleated germ cells (Figure 5L) were found in almost two-thirds of gonads in which some germ cells remained. Gonadal location (abdominal *versus* inguinal) was not an independent influential factor in any of the above-described features when corrected for age and expected AR activity.
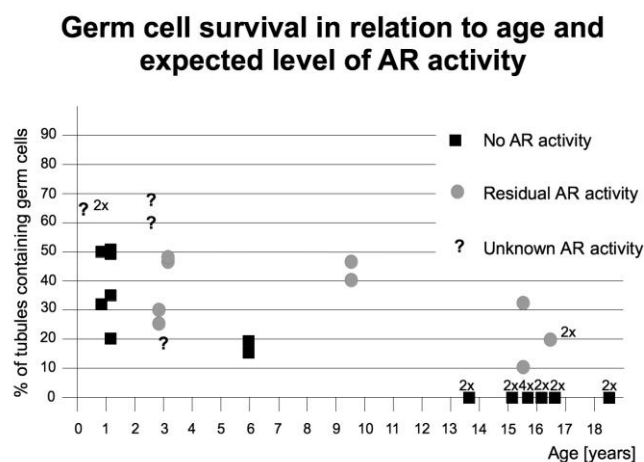

Figure 6: Germ cell survival in relation to age and expected level of AR activity.

Results of immunohistochemical analysis are represented in Table 6. Cells showing OCT3/4-positive staining were detected in 13/36 (35%) gonads from 9/19 (47%) patients. A certain continuum in their distribution within the tubules was observed with increasing age. More than 85% of the positive cells were situated in the center of the tubules in 4 young patients (age 3 months to 3 years), bilaterally in 2 of them (Figure 7A). Positively stained cells were few in all cases, had round nuclei, and were scattered throughout the whole gonad. They were TSPY-negative or only slightly positive (Figure 7B). All the gonads were negative for KITLG (Figure 7C). Thus, the histochemical and morphological pattern resembled that of fetal gonads (Honecker *et al.* 2004; Stoop *et al.* 2008). The findings were classified as delayed maturation in 3 patients who were older than 6 months.

The earliest atypical features were identified in both of the gonads of a 6-year-old patient (S9). Up to 33% of the OCT3/4-positive cells were attached to the basal lamina. Distribution throughout the testis was patchy with regions of several adjacent positive tubules. The cells were both TSPY-negative and positive regardless the location within the tubules. Additionally, the cells were morphologically atypical, made obvious by the shape of the nuclei. Some nuclei were round, and others were irregular. Some of the areas with OCT3/4-positive cells were KITLG positive. However, there were also KITLG-negative tubules containing OCT3/4-positive cells.

The changes were even more pronounced in the 4 oldest patients. OCT3/4-positive cells occupied one well circumscribed lobule in a 9-year-old patient (S10). Two-thirds of the cells were in contact with basal lamina (Figure 7D). The cells were very heterogeneous in morphology and staining pattern, again TSPY-positive or TSPY-negative in all locations (Figure 7E). KITLG expression was very strong in this case (Figure 7F).

Several areas with OCT3/4-positive cells were encountered in two 15-year-old twins (S14, S15), in both gonads in one of them. Interestingly, these were virtually the only regions

with surviving germ cells (OCT3/4-positive and negative) within the whole gonads. More than 60% of OCT3/4-expressing cells were located at the periphery of the tubules, both positive and negative for TSPY. KITLG expression was identified in most, but not all, regions.

Since the germ cells were very heterogeneous in morphology and expression within particular tubules and because normal spermatogonial cells (i.e., OCT3/4-negative and TSPY-positive) were often encountered in the same tubules as the atypical germ cells, a diagnosis of pre-IGCNU was made in patients S9, S10, S14 and S15.

Table 6: Results of immunohistochemical staining in OCT3/4-positive gonads.

	Age (yrs)	gonad	% of OCT3/4 (+) GC attached to BL	KITLG	TSPY and OCT3/4 double-staining	Pattern of double-staining	Overall evaluation
S1	0.25	right	0	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	normal development
		left	3	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	normal development
S2	0.8	right	8	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
S4	1.25	left	6	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
S8	3.2	right	13	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
		left	11	(-)	Most GC only TSPY (+); OCT3/4 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
S9	6.0	right	33	(+)	Most GC only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU
		left	19	(+)	Most GC only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU
S10	9.5	left	66	(+)	Most GC only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU
S13	15.5	right	93	(+)	Many GC only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	homogeneous in several tubules	IGCNU
S14	15.66	right	61	(+)	GC only in OCT3/4 (+) areas, some only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU
S15	15.66	right	66	(+)	GC only in OCT3/4 (+) areas, some only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU
		left	69	(+)	Many GC only TSPY (+); OCT3/4 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-IGCNU

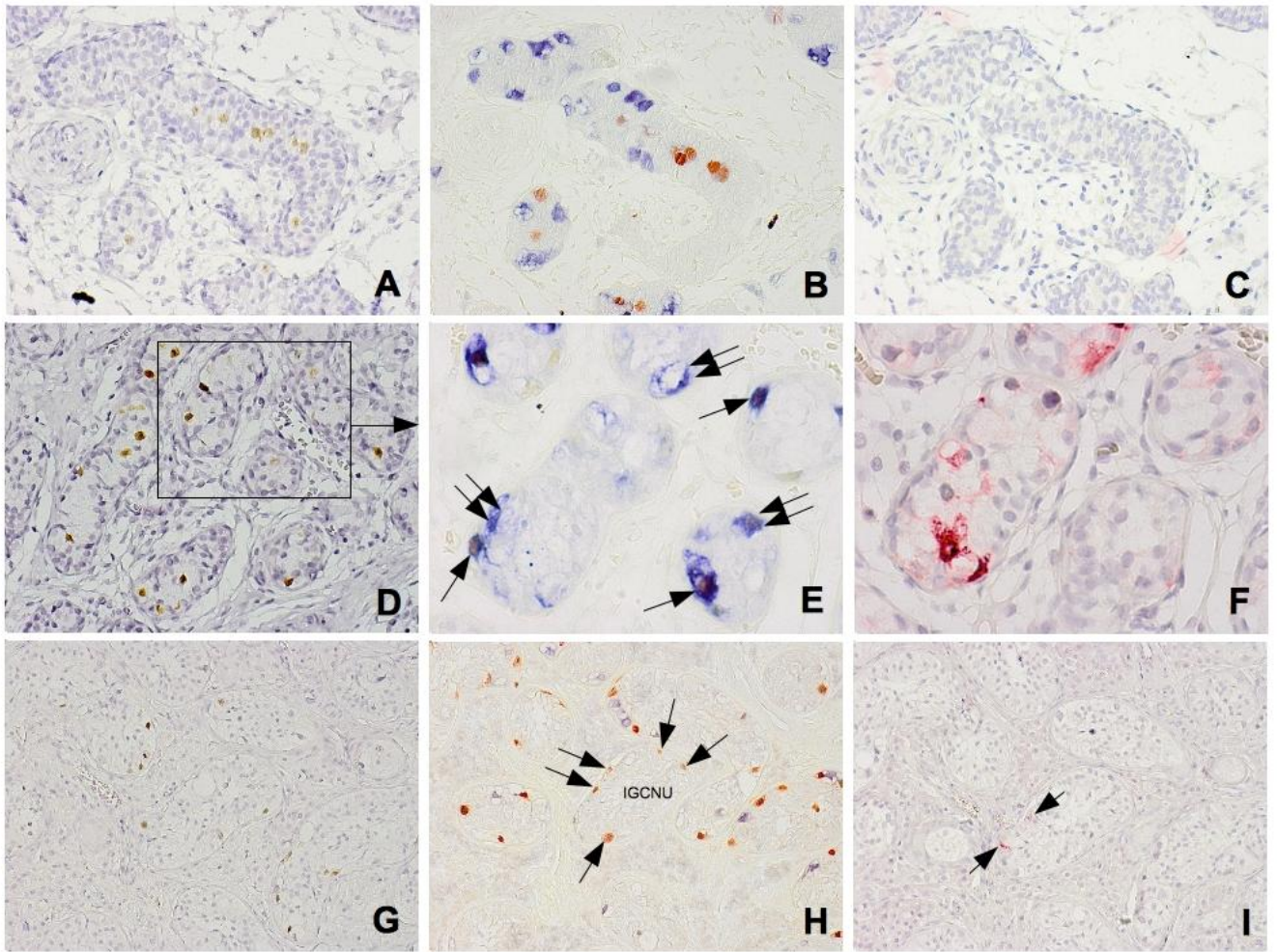GC - germ cells; BL - basal lamina; (+) - positive; (-) - negative.

Figure 7: Immunohistochemical staining of gonads positive for OCT3/4. A - delayed maturation - OCT3/4-positive germ cells (brown) all in the center of the tubules in a gonad of 10-month-old individual (S2); B - same area as in A double-stained for OCT3/4 (orange) and TSPY (blue), which are not co-expressed within the same cells; C - same area as in A and B negative for KITLG; D - pre-IGCNU - most of the OCT3/4-positive germ cells are attached to the basal lamina in a gonad of 9-year-old individual (S10); E - detail of the same area as in D double-stained for OCT3/4 and TSPY, heterogeneity of the germ cells within tubules - cells are either positive for both OCT3/4 and TSPY (arrow) or only for TSPY (double arrow); F - same area as in E strongly positive for KITLG (red); G - IGCNU - almost all OCT3/4-positive germ cells are attached to the basal lamina in a gonad from 15-year-old individual (S13); H - same area as in G double-stained for OCT3/4 and TSPY - seminiferous tubule (IGCNU) with a uniform population of germ cells which are positive for OCT3/4 only (arrow); I - same area as in G and H stained for KITLG (positivity marked with arrow).

According to the above criteria, IGCNU was present in one gonad of a 15-year-old patient

(S13). More than 90% of the OCT3/4-positive cells were attached to the basal lamina

(Figure 7G). Clonal expansion of germ cells, which were uniform in OCT3/4 and TSPY

expression, was observed in several tubules (Figure 7H). The expression of TSPY in OCT3/4-positive germ cells was heterogeneous in other regions. KITLG expression was present in some, but not all, tubules with OCT3/4-positive germ cells (Figure 7I). Many tubules contained exclusively normal spermatogonia (OCT3/4-negative and TSPY-positive) in this patient.

5.2. Study 2: 45,X/46,XY gonadal dysgenesis

In 16/87 samples (18.3%) no gonadal tissue was found; hence, for these samples, the gonadal position could not be determined. In these cases (further referred to as "vanished"), the gonad was considered to have never developed, with as a consequence, regression of the gonadal anlagen.

In the group with mild undervirilization, gonads were almost equally distributed over the scrotal (26.7%), inguinal (33.3%), and abdominal (33.3%) position; the remaining (6.7%) were vanished. In the group with ambiguous phenotype, the abdominal position was more frequent (59.1%), with 13.6% vanished gonads, whereas in the female phenotype group, if gonads were found (in 78.3%), they were invariably in the abdominal position (Figure 8A).

Figure 8B shows the distribution of the gonadal differentiation patterns. Streak and dysgenetic testis represent the majority of cases, whereas UGT and a combination of UGT and testis are less frequent. Interestingly, the finding of ovarian follicles is very rare (1/87 gonads, 1.1%), even in young 45,X/46,XY patients. No single patient presented with ovotesticular DSD. Within a streak, the stromal background displayed sometimes primitive tubule-like structures consisting of Sertoli cells, but without germ cells.

In patients with mild undervirilization, most gonads had differentiated as testes (Figure 8C). In patients with an ambiguous phenotype, testicular differentiation was found in 54% of samples, whereas the other patterns had an almost equal distribution. Vanished and streak gonads represented almost all cases in phenotypically female patients. Testicular and ovarian differentiation were each found in only one sample.

Scrotal gonads were all recognized as testes. Gonads in the inguinal position were mostly testes (72%), although also UGT (18%) and streak (9%) were encountered. Abdominal
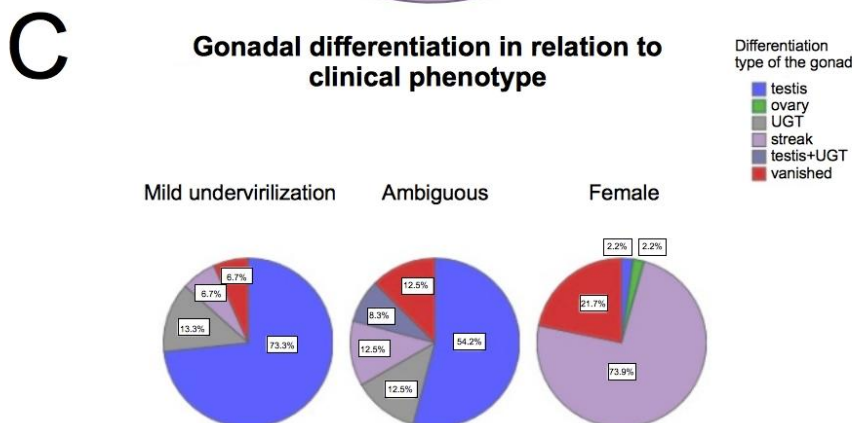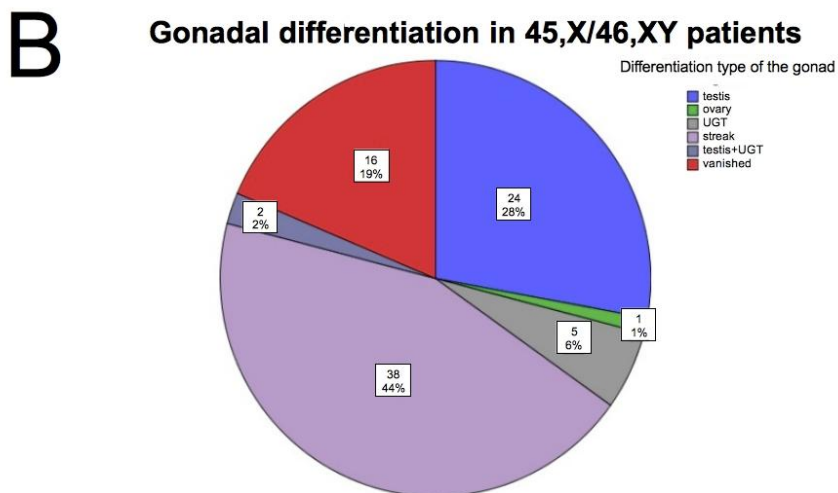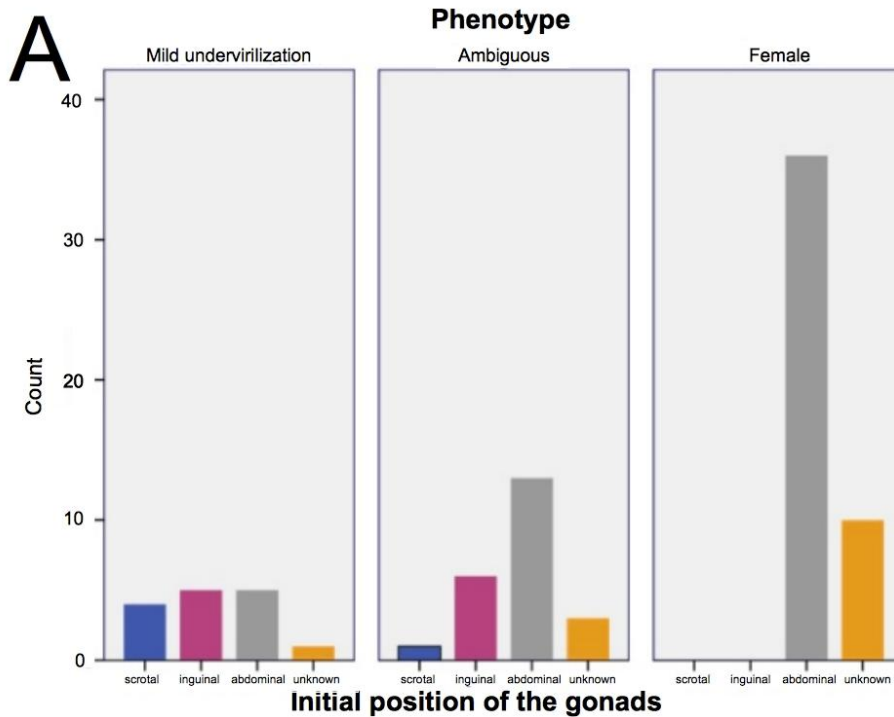
Figure 8: A - Location of the gonads in the different phenotypic groups. Samples with an unknown position contained no gonadal tissue on microscopic evaluation. B - Distribution of the encountered gonadal differentiation patterns. C - Distribution of gonadal differentiation patterns in the different phenotypic groups.

gonads mostly presented as streak tissue (68.5%), but interestingly, also testis (20.5%) or a combination of testis+UGT (3.7%) is possible. An abdominal gonad with UGT differentiation was found in 5.6%, the only gonad with ovarian differentiation was in the abdominal position.

Figure 9 shows representative examples of the gonadal differentiation patterns that were encountered in the 45,X/46,XY individuals included in the study.

In our series, an *in situ* neoplasia was found in four different patients, and no invasive tumors were encountered (Table 7). One patient with an EMS of 7.5/12 received prophylactic gonadectomy of a right abdominal gonad, which on microscopic examination contained UGT with GB. On the left side, a scrotal testis was present. One patient with an EMS of only 1/12 received prophylactic surgery at the age of 1 year and was found to have UGT with GB in the left abdominal gonad; the right abdominal testis displayed no neoplastic features. One patient (EMS 1.5/12) had a GB in a severely dysgenetic testis in inguinal position, the right abdominal gonad was a streak, surgery was at 1 year. The last patient was diagnosed with Turner syndrome after work-up for delayed puberty. None of the treating physicians noticed any clitoral enlargement. However, when prophylactic gonadectomy was performed at the age of 16, a testis containing IGCNU was found in the right abdominal gonad, while the left specimen contained no gonadal tissue.

Table 7: Summary of encountered tumors.

	MU	AP	FP	Total
No tumor	14	21	45	80
Tumor	1 (6.7%)	2 (8.7%)	1 (2.2%)	4 (4.8%)

All tumors in our series were *in situ* germ cell neoplastic lesions, discovered after prophylactic gonadectomy. There were no invasive tumors. MU - Mild undervirilization; AP - Ambiguous phenotype; FP - Female phenotype.
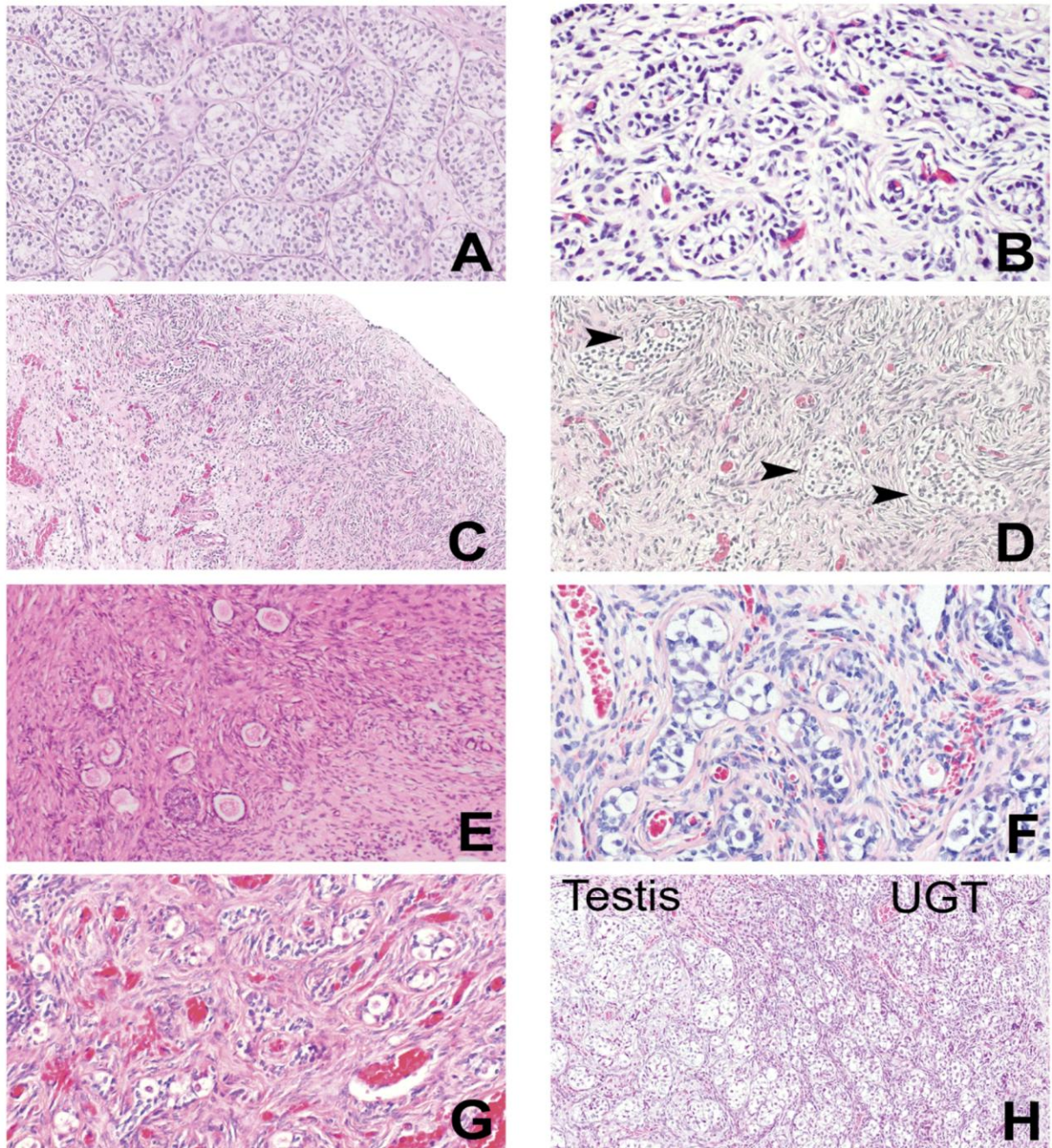
Figure 9: Representative examples of the gonadal differentiation patterns that were encountered in our series of patients with 45,X/46,XY mosacism, HE staining. A: Normal testis, 200x. B: Dysgenetic testis tubules, showing a thin basal lamina, an irregular tubular shape, and increased stromal background, 200x. C: Streak, 100x. D: Enlargement of C, clearly showing primitive testis cord-like structures (arrows), 200x. E: Ovarian follicles, encountered in only 1 gonad in our series, 200x. F and G: UGT, 200x. H: Combined pattern with testis (left) and UGT (right), 100x.

Fifteen gonads, including the four with an *in situ* neoplastic lesion, in 12 different patients, displayed premalignant characteristics. The three patients with bilateral premalignant lesions had an ambiguous phenotype. Individuals with an ambiguous phenotype had the highest prevalence of (pre)malignant characteristics (52.2%). In cases with mild undervirilization, the prevalence was lower but still considerable (13.3%). Phenotypically female individuals had a much lower prevalence (2.2%) (p < 0.001) (Table 8 and Figure 10A). Inguinal gonads displayed (pre)malignant characteristics more frequently as compared to scrotal or abdominal gonads, however, this was not statistically significant (p = 0.09) (Figure 10B and Table 9).

Table 8: Calculated risk for the development of a GCT in gonads from patients with 45,X/46,XY mosaicism, taking into account the clinical phenotype.

	MU	AP	FP	Total	X^2
No risk	13	11	45	69	
Risk	2	12	1	15	
	13%	52%	2.2%	18%	P < 0.001

MU - Mild undervirilization; AP - Ambiguous phenotype; FP - Female phenotype.

Table 9: Calculated risk for the development of a GCT in gonads from patients with 45,X/46,XY mosaicism, taking into account the gonadal position.

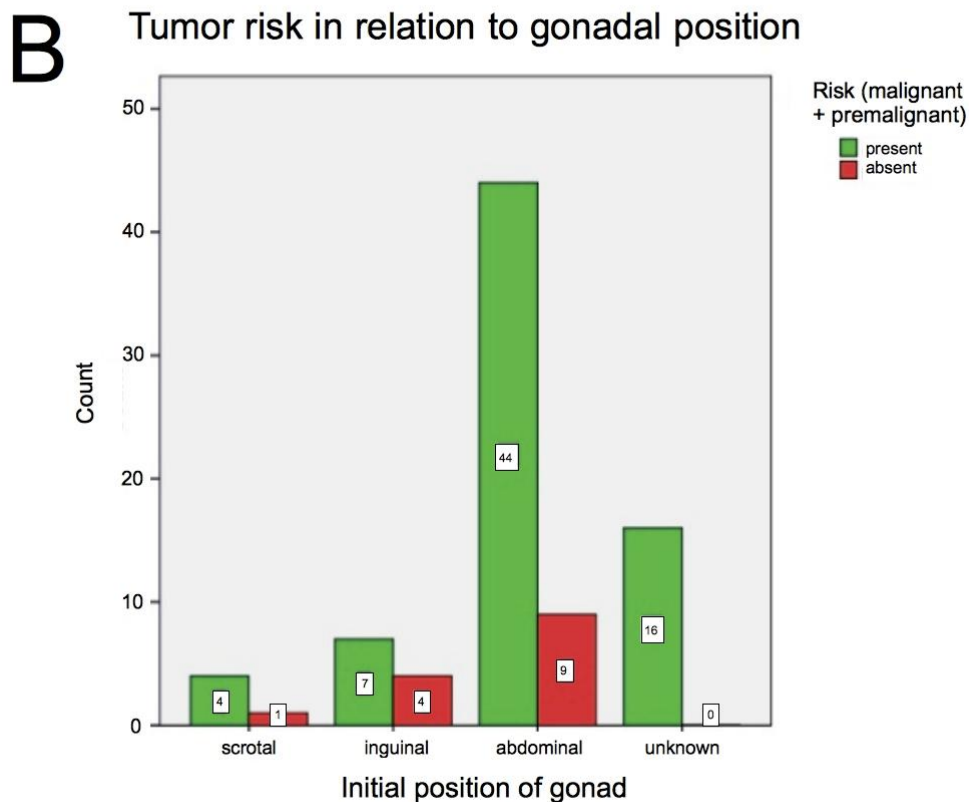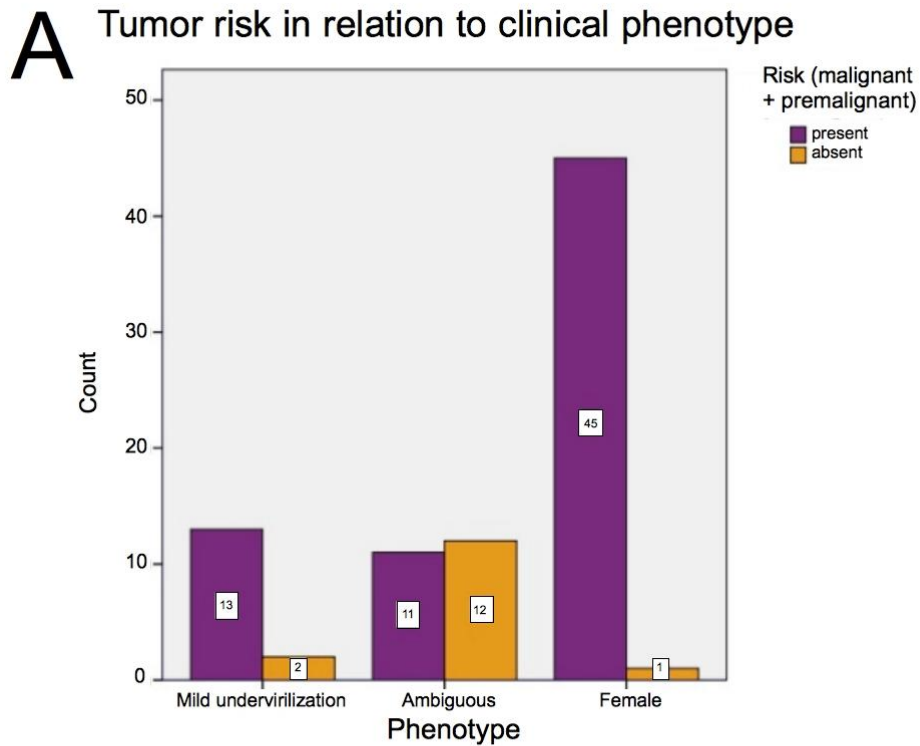|	Scrotal	Inguinal	Abdominal	Unknown	X^2
No risk	4	7	44	16	
Risk	1	4	9	0	
	20%	36%	17%	0%	P = 0.09

Figure 10: A - Presence of premalignant lesions or an *in situ* neoplasia in gonads from patients with 45,X/46,XY mosaicism categorized according to their clinical phenotype; B - Presence of premalignant lesions or an *in situ* neoplasia in gonads from patients with 45,X/46,XY mosaicism categorized according to the position of the gonad.

6. DISCUSSION

6.1. Study 1: Complete androgen insensitivity syndrome

According to current knowledge, two major factors influence postnatal testicular development: the location of the gonad and the androgen action (Giwercman *et al.* 1989; Hannema *et al.* 2006). Some histopathological changes, e.g. hamartomatous nodules or Leydig cell hyperplasia, develop during puberty as a consequence of the almost or entirely absent activity of androgens in CAIS. Other features, such as a decrease in germ cells, multinucleated germ cells, tubular atrophy, anomalous intertubular stroma, hyaline deposits, or lymphatic dilation, occur early in childhood. Therefore, their development is probably caused by abnormal (inguinal or abdominal) location of the gonads, which commonly occurs in CAIS patients (Barthold *et al.* 2000). However, differences in the effect of inguinal *versus* abdominal position of the gonads were difficult to assess due to an unequal age distribution of the study group, with inguinal gonads being more frequent in younger patients.

A positive impact of expected residual AR activity on the development of Wolffian duct structures and on the enlargement of seminiferous tubules after the onset of puberty in CAIS was described by Hannema and colleagues (Hannema *et al.* 2004; Hannema *et al.* 2006). Additionally, we observed a significant positive effect on non-malignant germ cell survival after 13 years of age. Only single tubules containing germ cells were encountered in the gonads of patients with expected no residual activity of AR, whereas up to 34% of tubules of patients from the other group contained germ cells. No clear influence of expected residual AR activity was discovered on the other above-mentioned changes.

The survival of germ cells with fetal features (i.e., OCT3/4-positive) and their progression

towards an invasive tumor in patients with CAIS are separate and very important issues. The level of risk for GCT development in CAIS remains a matter of debate. Manuel *et al.* estimated the cumulative risk to be 3.6% at 25 years of age and 33% at 50 years of age (Manuel *et al.* 1976). According to Deans *et al.*, tumors occurred in 14% of adult patients based on historical literature (Deans *et al.* 2012). The risk was estimated to be 0.8% in another meta-analysis, but this estimation was mainly based on a group of patients in which gonadectomy was carried out during childhood (Cools *et al.* 2006a). In fact, the risk is very difficult to predict because the approaches to manage CAIS patients have continually changed over time. While older reports included many adult patients and mainly reported the presence of invasive GCT (Manuel *et al.* 1976; Morris and Mahesh 1963; Rutgers and Scully 1991), more recent studies on pediatric patients refer to pre-invasive lesions, i.e., IGCNU (Cools *et al.* 2005; Hannema *et al.* 2006). IGCNU is encountered mostly in pubertal or adolescent patients, however, the risk is calculated for entire patient groups that often include very young individuals in whom IGCNU has not had time to develop. Moreover, an intermediate stage between delayed maturation and adult type of IGCNU exists (Cools *et al.* 2005). Whether this lesion is predetermined to evolve into invasive cancer in all cases is unknown. In the general population fully developed IGCNU is estimated to progress to an invasive tumor in 50% of cases during 5 years, and its prevalence is similar to that of germ cell tumors, suggesting a 100% progression to invasiveness over time (Dieckmann and Skakkebaek 1999).

A developmental continuum between germ cells with delayed maturation and IGCNU was observed in the study. OCT3/4-positive, TSPY-negative germ cells with round nuclei located in the center of the tubules, i.e., immature germ cells, were identified in at least one gonad in 4 patients whose age ranged between 3 months and 3.2 years. Cools *et al.* reported that some single OCT3/4-positive germ cells are present in testes of normal individuals 6 months of age or younger (Cools *et al.* 2005). Nevertheless, if the youngest

patient from our series is disregarded, 3 out of 7 patients (43%) between 6 months and 3.2 years of age carried germ cells with delayed maturation. OCT3/4-positive germ cells with irregular nuclei attached to the basal lamina of the tubules, which in some cases were TSPY-positive and located within KITLG-positive areas, were identified in at least one gonad in 5 out of 11 patients (45%) older than 6 years. The lesion was classified as IGCNU in one case and as pre- IGCNU in the rest of the cases. Taken together, at least one gonad in 8 out of 18 (44%) patients older than 6 months contained OCT3/4-positive germ cells, which may have given rise to an invasive tumor.

Regardless of whether we consider the proportion of the patients with (pre-)IGCNU among the older patients or the proportion of the patients older than 6 months with any kind of germ cell abnormality, the number of the gonads at risk is exceptionally high in comparison with other studies on pediatric patients. It is also high in comparison to cumulative risk for germ cell cancer in adult patients (33% at 50 years of age) estimated by Manuel (Cools *et al.* 2005; Hannema *et al.* 2006; Manuel *et al.* 1976). Such a high occurrence of atypical germ cells in this study may be incidental, but it may also indicate that not all abnormal germ cells will progress into an invasive tumor in CAIS patients. We suspect two possible mechanisms that may explain the difference between the high prevalence of germ cell anomalies in this series of pediatric/adolescent cases and the much lower prevalence of invasive tumors in adults. The first is an absolute loss of the abnormal germ cells in adulthood when the gonads would have been retained. The second is a failure of progression of the pre-invasive lesions into an invasive cancer. Both mechanisms may be caused by a lack of androgen action in CAIS, resulting in a low-androgen environment. This lack-of-androgen theory would correlate with a significantly higher risk for tumor development in PAIS patients (15% versus 0.8% in CAIS according to Cools *et al.*) (Cools *et al.* 2006a). Because we did not observe any significant differences in the persistence of OCT3/4-positive germ cells and KITLG expression in relation to the expected level of AR

activity among patients with CAIS, the residual activity of AR in CAIS is likely not powerful enough to achieve a similar effect on survival of abnormal germ cells and their progression into neoplasia as in PAIS. The result may be partly influenced by the relatively small sample size.

6.2. Study 2: 45,X/46,XY gonadal dysgenesis

Tumor risk has been estimated at 15% in 45,X/46,XY individuals (Cools *et al.* 2006a). However, in clinical practice, histological examination of prophylactically removed gonads in Turner girls with 45,X/46,XY suggests a much lower incidence, whereas no data are available for boys with 45,X/46,XY. Specifically in this group, it is of interest to preserve gonads to allow endogenous hormone production, and therefore spontaneous puberty induction and maintenance.

Categorization of gonadal differentiation patterns in 45,X/46,XY was difficult because they represent a continuum between two extremes (normal testis and normal ovary) rather than easily determinable separate entities, as is shown in Figure 9. Irrespective of the clinical phenotype, the finding of ovarian follicles was rare in 45,X/46,XY mosaicism (1/87 samples), even from tissue removed at a very young age. This is in contrast to observations in 45,X and 45,X/46,XX gonads (Borgström *et al.* 2009). Likewise, ovotestes (defined as the co-presence of testis and ovarian tissue – including follicles- in one individual) were not encountered in our population, unlike in 46,XX/46,XY chimerism. Streak tissue (in our series present in 44% of samples) by definition does not contain germ cells, but also in dysgenetic testes and UGT, germ cells were scarce. Increased apoptosis of germ cells has been attributed to a defective micro-environment and impaired meiosis of aneuploid germ cells (Kocer *et al.* 2009).

Tumor risk was significantly reflected by the clinical phenotype in this series ($p < 0.001$) allowing us to propose clinical guidelines (Table 10). The risk revealed to be very high (52%) in cases with an ambiguous phenotype. This group had the highest prevalence of UGT (20.8%), which has been recognized as the precursor lesion for GB (Cools *et al.* 2006b) (Figure 8C). Moreover, testes, if present, were severely dysgenetic in this group,

and often contained immature OCT3/4-positive cells on the basal lamina, in contrast to patients with mild undervirilization, in whom UGT was less frequently observed (13.3%) and testes had attained a more mature stage with less pronounced shape irregularity of the tubules and more frequent loss of OCT3/4 expression in germ cells that had reached the basal lamina. Moreover, testes were more often in the scrotal position in this group (Figure 8A). Cryptorchidism is known as an independent risk factor for the development of GCT (Giwercman *et al.* 1987), which has been related to maturation delay of germ cells (Rajpert-De Meyts *et al.* 1998). This risk is probably higher in inguinal than in abdominal gonads, due to early apoptosis of germ cells in the latter position (Abouzeid *et al.* 2011). In this study, inguinal gonads revealed the highest tumor risk but this was not statistically significant, maybe due to small sample size (Figure 10B). 20% of 45,X/46,XY testes with spontaneous scrotal descent revealed premalignant characteristics but this number represents in fact only 1/5 gonads, from an individual with an EMS of 5.5, so belonging to ambiguous phenotype group. While interpreting these data, it has to be kept in mind that the differentiation patterns in these gonads and the clinical phenotypes of the patients were very heterogeneous, independently influencing tumor risk, in contrast to studies in patients with simple cryptorchidism, who all are normally virilized boys with well-differentiated testes. In the phenotypically female group, tumor risk was low (2.2%). In this group, 46 gonads from 23 45,X/46,XY girls were examined, 44 of them were streak (so by definition devoid of germ cells) or had vanished (Table 5 and Figure 8C), explaining the low tumor risk. In one girl, who received gonadectomy at 10 years, ovarian follicles were found in one gonad (Figure 9E). Only 1/46 gonads (2.2%), from a 16 years old girl with Turner syndrome and a vanished gonad on the contra-lateral side, displayed testis differentiation, notably with IGCNU.

The phenotype in all the patients revealed some degree of undervirilization. Thus, due to selection bias (no gonadal specimen were available from individuals who live

undiagnosed), we are unable to predict tumor risk in normal males with a 45,X/46,XY constitution, representing in fact the largest clinical group (Chang *et al.* 1990). However, from the findings as described above, it can be hypothesized that in these individuals, the risk is low, since the clinical picture of a normal EMS score and bilaterally descended testes suggest a (close to) normal testicular differentiation and maturation process.

Table 10: Proposed guidelines for 45,X/46,XY gonadal dysgenesis.

Mild undervirilization (EMS ≥ 7)
Orchidopexy
Regular self-examination and ultrasound from puberty onwards
Biopsy pre- and post puberty to assess tumor risk by specialized immunohistochemistry
In case of pre-malignant changes (OCT3/4-positive cells on the BL / expression of KITLG / presence of UGT) or *in situ* neoplasia: gonadectomy
Ambiguous genitalia (EMS < 7)
See guidelines for Mild undervirilization
Low threshold to perform gonadectomy
Female phenotype
Elective gonadectomy (if patient is reluctant to gonadectomy: Consider to leave the gonads in place)
Cryopreservation not indicated

EMS - external masculinization score; OCT3/4 - octamer binding transcription factor 3/4; BL - basal lamina; KITLG - c-KIT ligand; UGT - undifferentiated gonadal tissue.

7. CONCLUSIONS

Many histopathological changes in testis develop with increasing age and are mostly influenced by abnormal gonadal location and impaired androgen action in CAIS patients. An independent effect of inguinal *versus* abdominal position of the gonads was difficult to assess because inguinal gonads were present primarily in the youngest individuals. Expected residual AR activity contributes to better survival of the general germ cell population in (post)pubertal age; however, it did not seem to play an important role in the survival of OCT3/4-positive germ cells and KITLG overexpression and thus appears to be unrelated to a higher cancer risk in CAIS patients. Moreover, expected residual AR activity did not prevent the development of lack-of-androgen phenomena such as hamartomatous nodules and Leydig cell hyperplasia. The high percentage of patients with germ cell abnormalities in our study suggests that most of the lesions do not progress to IGCNU and subsequent invasive germ cell tumors in CAIS. The level of risk in such cases remains to be elucidated. We observed one case with IGCNU and no invasive tumors in our study. This finding, together with other studies on pediatric patients, supports the current practice of postponing prophylactic gonadectomy to an adult age.

The data of Study 2 suggest that tumor risk in 45,X/46,XY patients is most pronounced in immature and/or poorly differentiated gonadal tissue, and that the degree of "testicularization" of the gonad (defined as the process of testicular development in its broadest sense) is reflected by the clinical phenotype. This knowledge can modify our clinical approach to the 45,X/46,XY patient, resulting in an individualized management with regard to tumor risk and gonadectomy. Future research and long-term follow-up of these patients is necessary to demonstrate the safety and benefit of this approach.

8. SOUHRN

Pacienti s poruchami pohlavního vývoje a zvýšeným rizikem rozvoje gonadálních nádorů ze zárodečných buněk většinou podstupují preventivní gonadektomii, která vždy vede k nutnosti hormonální substituce, navíc někdy pacienty zbavuje fertility. Cílem práce bylo za pomoci detekce tkáňových nádorových markerů blíže určit riziko rozvoje nádorů u kompletní formy syndromu necitlivosti k androgenům (CAIS) a 45,X/46,XY gonadální dysgeneze (GD) na základě funkčních či fenotypických charakteristik.

Pomocí barvení hematoxylinem a eosinem a imunohistochemické detekce OCT3/4, TSPY a KITLG bylo vyšetřeno 37, respektive 36 tkáňových vzorků gonád 19 pacientů s CAIS a 84 vzorků 47 pacientů s 45,X/46,XY GD. U první skupiny byly výsledky korelovány s polohou gonád a předpokládanou mírou aktivity androgenního receptoru. U druhé skupiny byla získaná data porovnána se stupněm virilizace zevního genitálu.

Vliv polohy gonád (inguinální nebo abdominální) na histologické změny nelze u pacientů s CAIS nezávisle posoudit s ohledem na nerovnoměrnou distribuci vzhledem k věku. Předpokládaná zbytková aktivita androgenního receptoru má pozitivní efekt na přežití obecné populace zárodečných buněk u (post)pubertálních pacientů, nehraje ale roli v rozvoji jejich atypických (neoplastických) změn.

U pacientů s 45,X/46,XY GD byl prokázán významný vztah mezi rizikem rozvoje gonadálních nádorů a mírou virilizace zevního genitálu. Největší riziko mají pacienti s obojetným genitálem, podstatně méně jsou ohrožení pacienti s mírnou hypovirilizací a pacientky s fenotypem Turnerova syndromu.

Klíčová slova: Kompletní forma syndromu necitlivosti k androgenům, 45,X/46,XY gonadální disgeneze, gonadální nádory ze zárodečných buněk, OCT3/4, TSPY, KITLG.

8. SUMMARY

Patients with disorders of sex development with an increased risk for development of gonadal germ cell tumors mostly undergo prophylactic gonadectomy, that always leads to a need for hormonal substitution, and moreover, in some cases prevents fertility. The aim of the thesis was to better determine the risk for tumor development in relation to the functional and phenotypical characteristics in patients with complete form of androgen insensitivity syndrome (CAIS) and patients with 45,X/46,XY gonadal dysgenesis (GD).

Hematoxiline and eosine staining and immunohistochemical detection of OCT3/4, TSPY and KITLG were used to assess 37 and 36 gonadal tissue samples of 19 CAIS patients, respectively, and 84 samples from 47 patients with 45,X/46,XY GD. The results were correlated with gonadal position and expected level of androgen receptor activity in the first group. The gained data were compared with level of virilization of external gentalia in the second group.

Due to an unequal distribution in relation to the age in patients with CAIS, it was not possible to independently assess the influence of gonadal position (inguinal *versus* abdominal) on the histological gonadal changes. Expected residual androgen receptor activity has a positive effect on survival of general germ cell population but not on the development of the atypical (neoplastic) changes of the germ cells.

A significant relation between tumor risk and level of virilization of external genitalia was demonstrated in patients with 45,X/46,XY GD. The risk is the highest in patients with ambiguous genitalia; in patients with mild undervirilization or patients with phenotype of Turner syndrome it is much lower.

Key words: Complete androgen insensitivity syndrome, 45,X/46,XY gonadal dysgenesis, gonadal germ cell tumors, OCT3/4, TSPY, KITLG.

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10. LIST OF AUTHOR'S PUBLICATIONS

10.1 Publication sources for the thesis (*in extenso*)

a) with IF

<u>Pleskacova, J.</u>, Hersmus, R., Oosterhuis, J.W., Setyawati, B.A., Faradz, S.M., Cools, M., Wolffenbuttel, K.P., Lebl, J., Drop, S.L., Looijenga, L.H.J. "Tumor risk in disorders of sex development." Sex Dev (2010) 4:259-269.
IF 2,985

Cools, M., <u>Pleskacova, J.</u>, Stoop, H., Hoebeke, P., Van Laecke, E., Drop, S.L., Lebl, J., Oosterhuis, J.W., Looijenga, L.H., Wolffenbuttel, K.P.; on behalf of the Mosaicism Collaborative Group. "Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism." J Clin Endocrinol Metab (2011) 96:E1171-1180.
IF 5,967

<u>Kaprova-Pleskacova, J.</u>, Stoop, H., Bruggenwirth, H., Cools, M., Wolffenbuttel, K.P., Drop, S.L.S., Snajderova, M., Lebl, J., Oosterhuis, J.W., Looijenga, L.H.J. "Complete androgen insensitivity syndrome: factors influencing gonadal histology with a special emphasis on germ cell pathology." Mod Pathol (2013); doi: 10.1038/modpathol.2013.193.
IF 5.253

b) without IF

<u>Pleskačová, J.</u>, Šnajderová, M., Lebl, J. "Poruchy pohlavního vývoje: Současná doporučená klasifikace a nové poznatky o poruchách vývoje gonád." Čes-slov Pediatr (2010) 65:79-85.

<u>Pleskačová, J.</u>, Šnajderová, M., Lebl, J. "Poruchy pohlavního vývoje a germinální nádory: patogeneze, prevalence, klinický přístup." DMEV (2011) 14:79-83.

10.2 Publications without relation to the thesis (*in extenso*)

a) with IF

<u>Pleskacova, J.</u>, Rucklova, K., Popelova, J., Cerny, S., Syrucek, M., Snajderova, M., Lebl, J. "Aortic dissection and rupture in a 16-year-old girl with Turner syndrome following previous progression of aortic dilation." Eur J Pediatr (2010);169:1283-1286.
IF 1,634

<u>Kaprova-Pleskacova, J.</u>, Snajderova, M., Stoop, H., Koudova, M., Kocarek, E., Novotna, D., Drop, S.L.S., Obermannova, B., Lebl, J., Oosterhuis, J.W., Looijenga, L.H.J. „45,X/46,X,psu dic (Y) gonadal dysgenesis: Influence of the two cell lines on the clinical phenotype, including gonadal histology." Accepted for publication in Sex Dev.
IF 2.27

b) without IF

Pleskačová, J., Šnajderová, M., Lebl, J. "Poruchy sexuální diferenciace s chybným vývojem gonád: dosud známé zodpovědné geny a výsledný fenotyp." Čes Gynekol (2009) 74:193-197.

Vančíková, Z., Žídová, D., Hradský, O., Pleskačová, J. "Komplikované komunitní pneumonie u dětí." Pediatr pro praxi (2009) 10:118-122.

10.3 Book chapters

Pleskacova, J., Hersmus, R., Oosterhuis, J.W., Setyawati, B.A., Faradz, S.M., Cools, M., Wolffenbuttel, K.P., Lebl, J., Drop, S.L., Looijenga, L.H.J. "Tumor risk in disorders of sex development." *In*: New concepts of disorders of sexual development. Karger (2010), ISBN 978-3-8055-9568-1

10.4 Abstracts

Pleskačová, J., Obermannová, B., Novotná, D., Šnajderová, M., Koudová, M., Kočárek, E., Zemková, D., Lebl, J. "Dysgeneze gonád s podivným chromozomem." Čes-slov Pediatr (2009) 64:324.

Pleskacova, J., Stoop, H., Snajderova, M., Cools, M., Drop, S.L.S., Lebl, J., Looijenga L.H.J. "Should we ask for routine of immunohistochemical examination of gonads in patients with disorders of sex development?" Horm Res Ped (2010) 74(suppl 3):110. *IF 1,553*

Pleskacova, J., Rucklova, K., Popelova, J., Cerny, S., Syrucek, M., Snajderova, M., Lebl, J. "Aortic dissection and rupture in a 16-year-old girl with Turner syndrome following previous progression of aortic dilation." Horm Res Paediatr (2010) 74(suppl 3):208. *IF 1,553*

Cools, M., Pleskacova, J., Wolffenbuttel, K.P., Hersmus, R., Stoop, H., Drop, S.L.S., Hoebeke, P., Looijenga, L.H.J. "Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism." Horm Res Paediatr (2010) 74(suppl 3):16. *IF 1,553*

Pleskacova, J., Stoop, H., Bruggenwirth, H., Oosterhuis, J.W., Cools, M., Wolffenbuttel, K.P., Drop, S.L.S., Snajderova, M., Lebl, J., Looijenga, L.H.J. "Gonadal histology in patients with complete androgen insensitivity syndrome (CAIS): influence of age, gonadal location and residual androgen receptor activity." Horm Res Paediatr (2012) 78(Suppl 1):123. *IF 1,553*

11.SUPPLEMENTS

1) <u>Pleskacova, J.</u>, Hersmus, R., Oosterhuis, J.W., Setyawati, B.A., Faradz, S.M., Cools, M., Wolffenbuttel, K.P., Lebl, J., Drop, S.L., Looijenga, L.H.J. "Tumor risk in disorders of sex development." Sex Dev (2010) 4(4-5):259-69. (11 stran)

2) Cools, M., <u>Pleskacova, J.</u>, Stoop, H., Hoebeke, P., Van Laecke, E., Drop, S.L., Lebl, J., Oosterhuis, J.W., Looijenga, L.H., Wolffenbuttel, K.P.; on behalf of the Mosaicism Collaborative Group. "Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism." J Clin Endocrinol Metab (2011) 96(7):E1171-80. (10 stran)

3) <u>Kaprova-Pleskacova, J.</u>, Stoop, H., Bruggenwirth, H., Cools, M., Wolffenbuttel, K.P., Drop, S.L.S., Snajderova, M., Lebl, J., Oosterhuis, J.W., Looijenga, L.H.J. "Complete androgen insensitivity syndrome: factors influencing gonadal histology with a special emphasis on germ cell pathology." Mod Pathol (2013); doi: 10.1038/modpathol.2013.193. (10 stran)