Cancer and male reproductive function- the effect on sperm DNA integrity and on birth outcomes in the offspring

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Cancer and male reproductive function

Effects on sperm DNA integrity and on birth outcomes in the offspring

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Academic Dissertation

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Abstract

Male cancer survivors (MCS) are at risk of suffering from impaired fertility. Male fertility is dependant on sperm DNA quality. The Sperm Chromatin Structure Assay (SCSA) measures the proportion of sperm with poor DNA, the DNA fragmentation index, DFI. A high DFI indicates reduced in vivo fertility and the need of ICSI (Intracytoplasmic sperm injection) to achieve pregnancy. A concern with ICSI, in which a single spermatozoon is injected into the oocyte, is the risk of transmitting defect DNA to the offspring. For MCS, due to the potential mutagenicity of radiotherapy (RT) and chemotherapy (CT), demonstrated in animal studies, this concern is even more pronounced. The aims of my thesis were to investigate the impact of cancer disease and its treatment on sperm DNA integrity and to study birth outcomes in the offspring of MCS. In papers 1 and II DFI was investigated in 96 testicular cancer patients (TC) and related to treatment and follow-up time. In paper III sperm DNA integrity was investigated in pretreatment semen from 121 cancer patients in which 58 were analyzed posttreatment as well. Cancer disease *per se* was associated with a moderate increase in DFI, especially TC and Hodgkin's lymphoma (mean DFI 17.5 and 16.5% respectively) compared to controls (mean DFI 11.5%), but for the majority of patients DFI was not at levels associated with reduced fertility. In TC patients adjuvant RT induced a transient increase in DFI, normalizing within 3-5 years, whereas intense CT induced a persisting decrease in DFI, DFI_{post} 9.1 vs. DFI_{pre} 12% (median values, $p = 0.02$). In paper III, in a group with varying diagnoses, the analysis of cryopreserved pre- vs. posttreatment semen demonstrated no change in DFI, regardless of therapy (median follow-up 3 years). Study IV was a Danish-Swedish register study investigating the impact of paternal cancer and mode of conception on birth outcomes, including 1.8 million singleton children, born 1994-2005. A moderate increase in malformation rate, 3.7 %, was seen in the 8879 children fathered by MCS, with a RR of 1.17 (95% CI 1.05, 1.31) of having an infant with a malformation, compared to non-MCS. IVF/ICSI was associated with an equivalent increased malformation rate, but did not constitute an additional risk for MCS. Treatment data was not available, but analyses of specific diagnoses, in which treatment can be anticipated, did not indicate that the increased malformation rate was treatment-induced.

Key words:
male cancer, fertility, sperm DNA integrity, SCSA, birth outcomes

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Abbreviations

ACT	Adjuvant chemotherapy
ART	Assisted reproductive technologies
BEP	Bleomycine, etoposide, cisplatin
CI	Confidence interval
CT	Chemotherapy
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
dUTP	2'-Deoxyuridine 5'-Triphosphate
EP	Etoposide, cisplatin
FCM	Flow cytometer
FISH	Fluorescence in situ hybridization
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
Gy	Gray
HCT	> 2 cycles of chemotherapy
HDS	High DNA stainability
HL	Hodgkin's lymphoma
ICD-7	International Classification of Diseases, 7th Edition
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LH	Luteinizing hormone
MCS	Male cancer survivor
NC	Natural conception
NSGCT	Non-seminomatous germ cell tumor
OR	Odds ratio
OS	Oxidative stress
ROS	Reactive oxygen species
RPLND	Retroperitoneal lymph node dissection
RR	Relative risk
RT	Radiotherapy
SCSA	Sperm chromatin structure assay
SGA	Small for gestational age
SGCT	Seminomatous germ cell tumor
SO	Surgery only
SWENOTECA	Swedish-Norwegian testicular cancer project
TDS	Testicular dysgenesis syndrome
TdT	Terminal deoxynucleotidyl transferase
TGCC	Testicular germ cell cancer
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
WHO	World health organization

List of Original Papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I: The impact of testicular cancer and its treatment on sperm DNA integrity.
Ståhl, O., Eberhard, J., Jepson, K., Spanò, M., Cwikiel, M., Cavallin-Ståhl, E. and Giwercman, A. *Cancer 2004, 100, 1137-1144.*

II: Sperm DNA integrity in testicular cancer patients.
Ståhl, O., Eberhard, J., Jepson, K., Spanò, M., Cwikiel, M., Cavallin-Ståhl, E. and Giwercman, A.
Human Reproduction 2006, 21, 3199-3205.

III: Sperm DNA Integrity in cancer patients; the effect of disease and treatment.
Ståhl, O., Eberhard, J., Cavallin-Ståhl, E., Jepson, K., Friberg, B., Tingsmark, C., Spanò, M, Giwercman, A.
International Journal of Andrology 2008, in press.

IV: Birth outcomes in the offspring of men treated for cancer- a register study comparing assisted and natural conception.
Ståhl, O., Boyd, H., Giwercman, A., Lindholm, M., Jensen, A., Krüger Kjær, S., Anderson, H., Cavallin-Ståhl , E., Rylander, L.
Manuscript

Popular scientific summary

The aims of my thesis were to investigate the effect of cancer and its treatment on the genetic material of sperm and to investigate if men with a history of cancer are at an increased risk of having children with malformations.

The number of cancer survivors is constantly growing due to the tremendous progress in the field of oncology. A major issue for young cancer survivors is the possibility of parenthood. It is well known that sperm quality can be impaired in male cancer patients, as a consequence of not only cancer treatment, but also of the disease itself. Oncological treatment, both radio- and chemotherapy, acts by inducing irreparable damage to the genetic material of the tumor cells, causing them to self-destruct. However, treatment is not tumor cell specific but will also target normal cells. Among the most sensitive cell types in the body are the germ cells and therefore there is a fear that oncological treatment can damage the genetic material of the sperm, its DNA.

Male fertility is traditionally evaluated by conventional semen analysis, including the amount, the looks and the motility of the sperm. However, it has been shown that also the DNA quality of the sperm has an impact on fertility. By means of the Sperm Chromatin Structure Assay, sperm DNA quality is rated by the DNA fragmentation Index, DFI. A high DFI, i.e. poor DNA quality, indicates that the fertilizing ability of the sperm is reduced. Among ART, assisted reproductive technologies, ICSI is specifically used in cases of male caused infertility. In ICSI, intracytoplasmic sperm injection, a single sperm, with unknown DNA quality, is injected into the egg. ICSI thereby bypasses the process of natural selection, imposing a risk of transmitting poor DNA to the offspring. This risk is further emphasized by the fact that ICSI is shown to be the superior ART in cases with high DFI. However, even though a moderately increased malformation rate is

observed among ART children in general, studies on ICSI children are fairly reassuring.

Since oncological treatment might impair fertility, even induce sterility, it is clinical practice to bank, cryopreserve semen prior to treatment. After treatment fatherhood can be achieved in several ways and luckily, most commonly by natural conception. However, male cancer survivor will suffer from infertility and benefit from ART and ICSI in a larger extent than the normal population. The concerns regarding ICSI are even stronger for cancer survivors, since treatment and the disease itself impair semen quality, possibly also sperm DNA quality.

In my thesis I have investigated the impact of cancer disease and its treatment on sperm DNA integrity. In papers I and II testicular cancer patients were investigated and I found that neither radio- nor chemotherapy induced any persisting DNA damage, but radiotherapy induced a transient increase of sperm with defect DNA. In paper III sperm DNA integrity was investigated in semen both before and after treatment in patients with varying malignant diseases. In papers I-III I conclude that cancer disease itself is associated with a moderate increase in DFI, but in most cases the sperm DNA quality is such that fecundity is not reduced. Furthermore, I conclude that oncological treatment does not result in a further withstanding impairment of sperm DNA quality.

The issue of parenthood also regards the health of the offspring. Several animal studies have shown that cancer treatment can induce DNA damage that is transmittable to the next generation. Even though studies on humans have been reassuring, failing to demonstrate any adverse effects of parental cancer on the offspring, the studies have been too small to depreciate the concern. Furthermore the specific concern of ART for male cancer survivors has not been addressed. Therefore, in paper IV, we performed a registry-based study on the offspring to male cancer survivors, investigating the impact of paternal cancer and mode of conception on birth outcomes and malformation rates. The study is the largest ever performed on the issue,

including 1.8 million Danish and Swedish children, identifying nearly 9000 children whose fathers had had cancer prior to conception.

In paper IV I conclude that male cancer survivors have a 15-20 % increased risk of having a child with a malformation, regardless of mode of conception. Treatment data was not available, but our analyses do not suggest that the increased risk is due to oncological treatment, but rather to a common tendency to develop cancer in young age and to have a child with a malformation.

In a clinical perspective the findings of the thesis are reassuring. Cancer disease *per se* was associated with a moderate impairment of sperm DNA, but intense oncological treatment did not induce any further persisting DNA damage. Although male cancer survivors were found to have an increased risk of having a malformed child, the risk was still less than 4 % and the risks associated with assisted reproduction were not higher for men with a history of cancer than for other men.

Background

Introduction

Cancer disease in childhood and young adulthood poses many therapeutic challenges. During the evolution of modern oncological treatment tremendous progress has been made, including improvement of surgery, radiotherapy and chemotherapy as well as diagnostic tools. Along with increasing treatment success, with cure rates for many of the predominating malignant diseases between 70 and 95 % (Gatta et al., 2003; Verdecchia et al., 2007), the issue of long-term effects of oncological treatment has increasingly come in focus, considering that these young, cured patients have a normal 1ifetime expectancy.

A general problem is the observation time required to gain knowledge regarding the long-term consequences. Since there is a continuous development of therapeutic tools the knowledge might be out of date already at the time of discovery. However, there are two principally different aspects to consider, first to identify potential problems for already treated patients, secondly to adapt future treatment to minimize potential negative chronic side effects.

Late effects for male cancer survivors (MCS) include several aspects, i.e. secondary malignancies, cardiovascular disease, endocrine disorders, and specifically for the young population, the issue of reproductive function.

Depending on diagnosis and treatment, fertility can be negatively affected in many ways in MCS. It might be due to;

• dysfunction in the hypothalamic-pituitary axis, as a consequence of tumor localization, surgery and/or irradiation

• ejaculatory disorders, mainly as a consequence of surgery

• germ and/or Leydig cell damage, which can be a consequence of the disease per se, chemotherapy and/or radiotherapy

In contrast to female patients, there are well-established methods to, if possible, secure the possibility of future fatherhood for male cancer patients. In the 2006 clinical guidelines of fertility preservation of the American Society of Clinical Oncology (Lee et al., 2006), it was concluded that this is an issue that should be addressed to all patients of reproductive age. In postpubertal males the standard procedure is to cryopreserve semen, most often obtained through masturbation. Alternative methods, such as testicular aspiration or extraction of sperm, should be considered if masturbation is not feasible or if no sperm are found in the ejaculate. Especially since, due to advances in assisted reproductive technologies, ART, even samples of low quantity and quality could be preserved for future use. However, data suggest that fertility issues and the possibility of semen cryopreservation is still insufficiently discussed with the patient (Schover et al., 2002b), even though this most certainly is a matter that differs widely geographically, due to availability, socio-medical and cultural differences. Regarding the situation in Sweden data are lacking. For prepubertal patients the means of preserving the possibility of fatherhood are to cryopreserve spermatogonial stem cells, which even though showing promising results in animal models, is still on an experimental level in humans, recently reviewed by Geens et al (Geens et al., 2008).

Male cancer survivors can achieve fatherhood by several means. The majority of men will maintain their fecundity and conceive naturally. Otherwise, assisted reproductive technologies, IVF (in vitro fertilization) and ICSI (Intracytoplasmic Sperm Injection) are available, with the use of either posttreatment sperm, insufficient for natural conception, or cryopreserved pretreatment semen.

Several studies indicate that the possibility of future parenthood is of great importance for MCS (Green et al., 2003a; Schover 2005; Schover et al., 2002a). The issue of parenthood has two different aspects- the possibility of fatherhood, but also the outcome, i.e. the health of the offspring.

Spermatogenesis

Testicular function, androgen production and spermatogenesis, are regulated by the gonadotropins, LH (luteinizing hormone) and FSH (follicle-stimulating hormone), produced and secreted by the anterior pituitary gland, which in turn is governed by hypothalamic gonadotropin-releasing hormone (GnRH). LH exerts its effect on the Leydig cells of the testes, stimulating testosterone production and testosterone, acting on the hypothalamus and pituitary gland, inhibits LH secretion through a negative feed-back system. Spermatogenesis is stimulated through the action of FSH on Sertoli cells, which in its turn exerts a negative feedback by the secretion of Inhibin B. Testosterone, apart from its endocrine effects, also acts locally in the testes and is required for normal spermatogenesis. Exogenous testosterone substitution inhibits testicular testosterone production as well as gonadotropin secretion, thereby inhibiting sperm production (Nieschlag 2000).

Spermatogenesis, the formation of mature spermatozoa, is a complex, multi-step procedure, involving two meiotic and several mitotic divisions, see figure 1. Spermatogonia are the diploid (2n (chromosome number), 2c (DNA content)) stem cells of spermatogenesis. Spermatogonia A represent the stem cell pool and the differentiation to spermatogonia B initiates the DNA synthesis resulting in tetraploid primary spermatocytes (2n, 4c). Thereafter follows the 1st meiotic division, which involves the cross-over of genetic material of maternal and paternal origin between the autosomal sister chromatids, resulting in secondary spermatocytes, with haploid chromosomes with diploid DNA content (1n, 2c). Shortly thereafter the 2nd meiotic division occurs, resulting in haploid round spermatids (1n, 1c) (Nieschlag 2000).

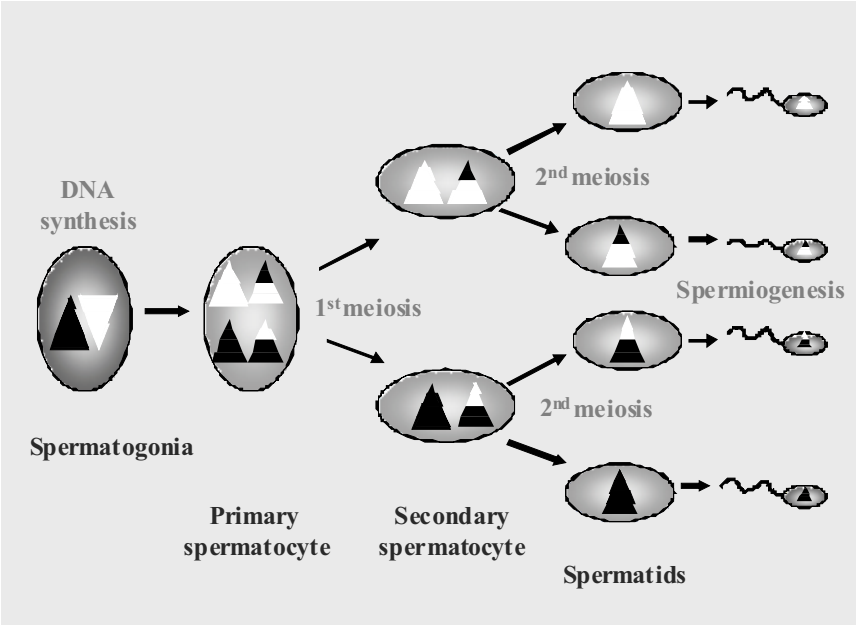

Figure 1.
Spermatogenesis

The round spermatids undergo several maturation stages, spermiogenesis, resulting in mature spermatozoa. Spermiogenesis includes the process of DNA condensation, in which DNA through several steps is reorganized. By the end of spermiogenesis the spermatids loose their ability of DNA repair (Sotomayor & Sega 2000). The end result, extremely tightly packed protamine-DNA complexes, is unique for mammalian germ cells, ensuring a safe trip for the paternal genome through the male and female reproductive tracts and contains genetic information in an accessible form for the embryo to be.

Male Fertility

Infertility, defined as a couple's inability to conceive for more than one year, is a common disorder, affecting 10-15 % of all couples (Nieschlag 2000). Approximately 50 % of all couples will be infertile due to, completely or partly, the male partner and an additional 15 % of the couples will have an unexplained infertility (WHO 2000). Male fertility is assessed by a semen

analysis. WHO has set the criteria for normal semen sample, which includes semen volume, sperm concentration, motility and morphology, shown in table 1(WHO 1999). However, due to several factors such as the heterogeneity of spermatozoa, the intraindividual variability of semen parameters and the relative subjectivity of the analysis, conventional semen analysis is an insufficient tool in fertility assessment (Aitken 2006; Bonde et al., 1998; Guzick et al., 2001; Nallella et al., 2006), leading to the development of additional means of investigating semen quality, one of which being the evaluation of sperm DNA quality.

Parameters	Reference values
Semen volume	$\geq$ 2 ml
Sperm concentration	$\geq 20 \times 10^6$/ ml
Total sperm count	$\geq 40 \times 10^6$/ ejaculate
Motility	$\geq$ 25 % rapid progressive or $\geq$ 50 % total progressive motility
Morphology	Different thresholds

Table 1. Reference values of semen parameters as defined by WHO (WHO 1999)

Sperm DNA and chromatin structure

The bulk of sperm DNA is localized in the nucleus and will forth be referred to as sperm DNA, whereas the small proportion of sperm DNA localized in the mitochondria, important for sperm movement, will not be further discussed.

In comparison to somatic cells, sperm chromatin is highly condensated, with the DNA strands tightly wrapped around protamine molecules. There are two features in the packaging of human chromatin allowing a greater variability and a higher amount of DNA strand breaks compared to other mammalian species. Firstly, specific for humans is that 15 % of the DNA is less dense, packaged by histones and secondly, whereas most other mammalian species have one type of protamine, humans have two types, the second allowing for less stable packaging due to less sufficient disulfide

cross-linking (Erenpreiss et al., 2006; Gatewood et al., 1987; Oliva 2006). Due to the less compact chromatin structure, human spermatozoa are susceptible to DNA damage, and the mature spermatozoa lack DNA repair capability (Sotomayor & Sega 2000). Several mechanisms behind the origin of DNA damage have been suggested.

During spermatogenesis there are specific steps during which DNA is thought to be more vulnerable, and crucial events for the possible formation of strand breaks have been identified. With means of TUNEL (Terminal Deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling) stage-specific presence of DNA strand breaks during spermatogenesis were detected (Marcon & Boissonneault 2004). During 1st meiosis, the process of recombination requires the formation of strand breaks, which by the completion of meiosis, are re-ligated and defect spermatocytes are aborted. Theoretically, impairment in this check-point can yield persisting strand breaks. Full maturation of spermatozoa include complete re-ligation of the breaks. The presence of strand breaks in ejaculated sperm could thus imply a defect in sperm maturation, or in DNA repair mechanisms (Sakkas et al., 1999), which could be one explanation to the age related sperm DNA damage observed (Spano et al., 1998; Wyrobek et al., 2006), with older men having impaired DNA repair capacity (Aitken 2006).

There is an excessive proliferation of spermatogonia through multiple mitotic divisions and a balance between the supportive Sertoli cell capacity and the germ cell population is maintained by testicular germ cell apoptosis (Lee et al., 1999). The function of germ cell apoptosis is to eliminate an over-population of germ cells and possibly also to eliminate abnormal spermatozoa. Germ cell apoptosis is thought to be mediated by the apoptotic cell-surface protein, Fas, orchestrated by the supportive Sertoli cells. The process initiates with the binding to the Fas ligand, which is shown to be expressed by the Sertoli cells (Lee et al., 1999). Since the Fas positive cells should be killed, the presence of Fas positive cells in the ejaculate indicates a malfunction in the apoptotic machinery, a phenomenon named

abortive apoptosis (Erenpreiss et al., 2006; Sakkas et al., 2002). It has been shown that human ejaculated spermatozoa express Fas and it is also described that semen with poorer characteristics has an increase in Fas positivity (Sakkas et al., 1999). However, Sakkas et al failed to clearly demonstrate the correlation between Fas expression and sperm DNA damage (Sakkas et al., 2002).

Yet another source of DNA damage in germ cells is that of oxidative stress (OS). OS is the process of cell damage caused by an imbalance between ROS, reactive oxygen species, and antioxidants. ROS, i.e. free radicals, are products of normal cellular metabolism, necessary for functioning spermatozoa (Agarwal et al., 2008). However, in abundance ROS cause oxidative stress, and is suggested to be a major cause of male infertility, either by damaging the sperm membrane or by inducing DNA damage. Free radicals in semen are predominantly produced by leucocytes, but also by sperm. The presence of leucocytes, specifically neutrophils, in semen correlates positively with ROS. However the seminal leukocyte concentration has not been shown to correlate with reduced semen quality (Aitken et al., 1994; Tomlinson et al., 1993). Even though ROS mostly origin from leukocytes, it has been shown that the intrinsic sperm production of ROS has the most significant impact on sperm DNA integrity (Henkel et al., 2005). There are many causes of ROS and several conditions shown to correlate to sperm DNA damage, of which the following are of specific interest in regards to cancer disease and treatment;

Testicular

A common cause of male infertility is varicocele and OS was shown to be an underlying cause of reduced fertility in these patients (Agarwal et al., 2006). Smith et al found a correlation between abnormal sperm DNA and increased ROS in both varicocele patients and men with a history of cryptorchidism, even in patients with normal conventional semen parameters (Smith et al., 2007).

Infection / systemic disease

Chronic prostatitis was shown to cause increased seminal ROS (Potts & Pasqualotto 2003). Conditions such as chronic viral infections, hepatitis B and C , as well as fever are related to an increase in systemic OS and also with impaired sperm quality, motility and/or sperm DNA damage (Sergerie et al., 2007), possibly caused by systemic OS (Tremellen 2008). Poor semen quality in Hodgkin's lymphoma patients was negatively associated with fever, elevated erythrocyte sedimentation rate and advanced stages of the disease, all factors known to correlate with increased cytokine activity and systemic OS (Rueffer et al., 2001).

Iatrogenic

In the preparation of sperm for ART procedures, the centrifugation of semen, to separate sperm from seminal plasma, has shown to increase ROS production (Aitken & Clarkson 1988). Of specific interest for cancer patients is the effect of cryopreservation on sperm DNA. An increase in ROS due to cryopreservation has been observed (Bilodeau et al., 2000). Data regarding the possible negative effect of cryopreservation on sperm DNA integrity have been somewhat conflicting (de Paula et al., 2006; Donnelly et al., 2001; Isachenko et al., 2004; Spano et al., 1999). In a recent study on 320 men, presenting for infertility investigation, the procedure of cryopreservation had an effect on sperm DNA, however dependant on pre-freeze values. The majority of semen samples increased in sperm DNA impairment, but in those samples displaying the highest pre-freeze values a decrease in DNA fragmentation was observed after thawing (Thomson et al., 2008). The effects of irradiation and chemotherapy on sperm DNA integrity will be discussed in the chapter on cancer and sperm DNA.

Sperm DNA integrity and fertility

The impact of sperm DNA integrity on fertility is well documented. Numerous studies have reported the association between reduced fertility

and impaired sperm DNA integrity (Bungum et al., 2007; Evenson et al., 1999; Sakkas et al., 2002; Spano et al., 2000). Although a moderate correlation between DNA integrity and conventional semen characteristics, especially motility and morphology, is found in many reports, sperm DNA integrity has been shown to, regardless of other semen characteristics, predict male fecundity (Boe-Hansen et al., 2006; Giwercman et al., 2003; Larson-Cook et al., 2003; Sills et al., 2004).

There are several tests assessing sperm DNA integrity, one of which being the Sperm Chromatin structure Assay, SCSA. The SCSA analysis is based on the phenomenon that the exposure of an acid-detergent causes the DNA to denaturate in sperm having altered chromatin. By adding acridine orange denaturated, single stranded DNA shifts from green to red fluorescence, whereas intact, double stranded DNA remains green. The extent of DNA denaturability is expressed as the DNA Fragmentation Index (DFI), being the ratio of red to total (red plus green) fluorescence intensity. DFI % hereby expresses the proportion of cells containing denaturated DNA, i.e. sperm with altered chromatin structure, including DNA strand breaks (Evenson et al., 2002; Spano et al., 2000). Other tests of sperm DNA integrity include the TUNEL assay, which directly measures the presence of single or double strand breaks by incorporating fluorescently labeled dUTP at DNA strand breaks, utilizing a reaction catalyzed by terminal deoxynucleotidyl transferase (Gorczyca et al., 1993; Sakkas et al., 2002). Yet another method to assess sperm DNA integrity is the Comet Assay, which, like TUNEL, specifically detects the presence of DNA strand breaks. The Comet assay is a fluorescence microscopic test, in which single sperm are cast on an agarose gel, lysed and subjected to electrophoresis. Sperm with damaged DNA will produce a “comet tail” under electrophoresis, whereas the intact DNA will remain in the nucleus. The damage is quantified by the proportion of DNA in the comet tail compared to the DNA content of the comet head, the nucleus (Morris et al., 2002).

Sperm DNA integrity, assessed by SCSA, has shown to be a powerful tool in predicting the fertilizing ability of the spermatozoa, regardless of conventional semen characteristics and DFI values above 30-40 % have shown to indicate in vivo infertility (Bungum et al., 2007; Evenson et al., 2002; Spano et al., 2000). Furthermore, the level of DFI has shown to be clinically useful also in ART.

In a study on 637 infertile couples, Bungum et al demonstrated that either IVF or ICSI was required to obtain a pregnancy if DFI exceeded 30 %, with ICSI being superior to IVF (Bungum et al., 2007). Neither with TUNEL nor with the Comet assays have such thresholds been established (Erenpreiss et al., 2006; Perreault et al., 2003).

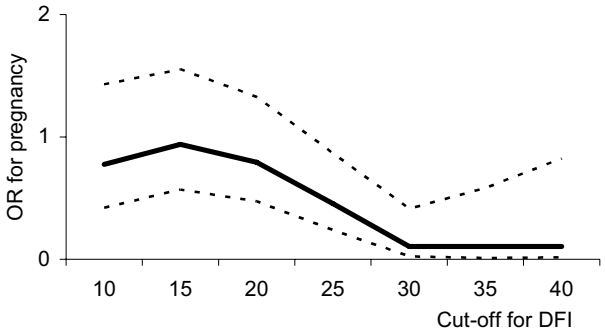

Figure 2. Odds ratios (OR) for biochemical pregnancy in relation to DFI following intrauterine insemination (IUI) adapted from Bungum et al 2007

Assisted Reproductive Technology

The definition of Assisted Reproductive Technologies (ART) is reproduction involving the handling of gametes outside the body. The oldest and least complicated method is IUI, intrauterine insemination, in which prepared semen is inseminated in the woman's uterus. In IVF both the female and male gametes are handled outside to body. After hormonal stimulation the oocyte is removed from the mature follicle and is incubated with motile sperm in a culture medium. Two to five days after successful fertilization the embryo/s are placed in the uterine cavity. Conventional IVF is performed

mainly in female factor infertility. The first IVF child was born in 1978 (Steptoe & Edwards 1978) but the problems with male factor infertility remained and lead to the development of ICSI in the early 90´s (Palermo et al., 1992). As opposed to IVF, in which the oocyte is exposed to a high number of selected spermatozoa, in ICSI a single, vital spermatozoon is injected directly into the cytoplasm of the oocyte. The indication for ICSI was primarily severe male factor infertility, but the indications have become wider and today ICSI is the dominating ART (Andersen et al., 2008). The use of IVF/ICSI has greatly increased over the last decade and ART represent between 2-4 % of all child births in the Nordic countries (Andersen et al., 2008). Even though the success with ART is undisputed, there have also been major concerns. With ICSI a single spermatozoon is chosen, thereby bypassing the process of natural selection, which has awoken concerns regarding the potential genetic risks with the procedure (Griffin et al., 2003; Tournaye 2003). This concern is further reinforced by reports on increased sperm aneuploidy in men with poor semen quality and reduced fertility (Calogero et al., 2001; Pang et al., 1999; Schmid et al., 2003) even though this finding is not consistent through all studies (Guttenbach et al., 1997).

Several studies have shown an increase in adverse birth outcomes, including malformation rates amongst ART children (Hansen et al., 2005; Hansen et al., 2002; Källén et al., 2005; Zhu et al., 2006). The increase has partly been due to the increase in multiple births with ART, multiple births *per se* being associated with adverse birth outcomes. Today, single-embryo transfer, in order to avoid multiple births, is therefore recommended in many centers (Andersen et al., 2008). Studies analyzing singleton children have found an increased malformation rate of approximately 20-30 % and the increase has mostly been attributed to the underlying causes of infertility, rather than the ART procedures *per se* (Hansen et al., 2005; Källén et al., 2005; Zhu et al., 2006). Furthermore, two meta-analyses on gestational age and birthweight in singleton ART children have demonstrated about a two-fold risk of pre-term birth and low birth weight compared to naturally conceived children

(Helmerhorst et al., 2004; Jackson et al., 2004). Apart from an increase in male urogenital malformations in boys conceived by ICSI, no difference has been observed between IVF and ICSI in terms of congenital malformations (Bonduelle et al., 2005; Källén et al., 2005). The same is true regarding chromosomal abnormalities. A slightly elevated prevalence has been observed in ART children, but ICSI was not associated with an excessive risk (Georgiou et al., 2006).

However, the youth of ICSI should be kept in mind, the oldest ICSI children being in their teens, and issues investigated so far have mostly been birth outcomes and early development, whereas morbidity in general has not yet been possible to investigate. This is further emphasized by the somewhat controversial and not consistent finding of the possible association between paternal smoking and childhood cancer (Ji et al., 1997; Pang et al., 2003; Plichart et al., 2008). Smoking is related to poor semen quality and possibly also impaired sperm DNA (Fraga et al., 1996; Sepaniak et al., 2006) and the association between paternal smoking and childhood cancer thus indicates a possible impact of paternal sperm quality on health aspects of the child.

Sperm production and cancer disease

Testicular cancer

Although rare, testicular germ cell cancer (TGCC) is the most common malignant disease in men of reproductive age, with an incidence rate of 7-8 /100 000/year in Sweden (Bray et al., 2006). TGCC patients constitute a particular challenge in terms of fertility, since fertility can be affected by both the disease and its treatment. It is well known that cryptorchidism and reduced fertility are associated with an increased risk of developing TGCC and accumulating data support that TGCC is linked with other testicular disorders. In 2001 Skakkebaek introduced the Testicular Dysgenesis Syndrome, TDS (Skakkebaek et al., 2001), in which he postulated that poor semen quality, cryptorchidism, hypospadia and TGCC all are features of one

entity, due to disruption of the gonadal development in fetal life. A prospective study on Danish men in couples with fertility problems has shown that abnormal semen, including low sperm concentration, poor motility and abnormal morphology, was associated with an increased risk of developing TGCC (Jacobsen et al., 2000). It is well established that TGCC patients suffer from reduced sperm production at diagnosis (Petersen et al., 1999b). Men with TGCC have significantly fewer children than the normal population, prior to diagnosis (Moller & Skakkebaek 1999). Epidemiological data strongly support the TDS hypothesis. The incidence of TGCC is increasing worldwide, with a distinct geographical variance, as is the frequency of cryptorchidism (Boisen et al., 2004). In the Nordic countries the incidence of TGCC increases going west, being 3 times higher in Denmark and Norway than in Finland, with Sweden in between (Richiardi et al., 2004). Correspondingly, the sperm count is highest among young men in Finland, followed by Swedes, whereas Danes have the lowest sperm count (Jorgensen et al., 2006). Furthermore, common pre- and perinatal risk factors, such as low birth weight and low parity (Akre et al., 1996; Aschim et al., 2004) have been identified for different components of TDS.

No other malignancy is as investigated in terms of fertility aspects in relation to disease and treatment as TGCC. The following observations are well established;

• TGCC per se is related to impaired sperm quality, including reduced sperm concentration (Botchan et al., 1997; Chung et al., 2004; Gandini et al., 2003; Petersen et al., 1999b).

• TGCC is associated with an increased risk of other testicular disorders, such as cryptorchidism, Sertoli-cell only syndrome, testicular atrophy, microcalcifications, cancer in situ, contralateral TGCC, all of which, in varying extent, hamper spermatogenesis (Ganem et al., 1999; Olesen et al., 2007; Virtanen et al., 2007).

• Treatment of TGCC, both radio- and chemotherapy, may affect spermatogenesis, the effect dependant of both treatment modality and dosage, but also of individual susceptibility (Eberhard et al., 2004).

Treatment of TGCC depends on histology and stage of disease. Removal of the tumor bearing testicle is always performed. Stage 1 disease can be handled in different ways; surveillance, retroperitoneal lymph node dissection, adjuvant radiotherapy and adjuvant chemotherapy are all alternatives. Disseminated disease is treated with cisplatin based chemotherapy, for current European guidelines see (Krege et al., 2008a; Krege et al., 2008b).

Other malignancies

Data on sperm production on non-TGCC malignancies are mostly retrospective analyses of cryopreserved semen. Hodgkin's lymphoma (HL) is the disease mostly investigated and several authors report severely impaired spermatogenesis, with 5-15 % prevalence rate of azoospermia (Lass et al., 1998; Ragni et al., 2003; Rueffer et al., 2001; Viviani et al., 1991). Rueffer et al found that only 30% of 158 HL patients had normal semen parameters at diagnosis. Furthermore they found a positive correlation between stage of disease and impaired spermatogenesis, leading to the hypothesis that the impact on gonadal function is due to systemic disease, with increased cytokine activity (Rueffer et al., 2001), supported by Gandini et al (Gandini et al., 2003; Sieniawski et al., 2008). However, other authors have failed to show such a correlation (Fitoussi et al., 2000; Hallak et al., 2000). Data on other hematological malignancies, Non-Hodgkin lymphomas (NHL) and leukaemias are somewhat conflicting, some studies indicating that HL has a greater impact on spermatogenesis (Lass et al., 1998), whereas others have shown corresponding semen impairment in NHL and leukemia patients (Botchan et al., 1997; Crha et al., 2008) as in Hodgkin patients. Data on solid tumors are even sparser, but there are data supporting that also these diagnoses are associated with reduced sperm production prior to treatment (Bahadur et al., 2002; Botchan et al., 1997; Crha et al., 2008; Hallak et al., 2000; Lass et al., 1998).

The effect of oncological treatment on sperm production

In the literature sperm production in cancer patients has often been evaluated by hormonal analysis, specifically serum levels of FSH. As previously mentioned, spermatogenesis is governed by Sertoli cell function, which in turn is governed by the pituitary-testicle axis and the production of FSH. FSH stimulates spermatogenesis and by a negative feed back system, spermatogenesis stimulates the production of Inhibin B in Sertoli cells, which in turn inhibits FSH release. Thus a malfunctioning spermatogenesis leads to elevated FSH and lowered Inhibin B serum levels. However, in the following text, studies in which semen analysis have been performed have been in focus, but when such data are lacking, reports on hormonal levels are referred to as well. Both radio- and chemotherapy will to a large extent induce temporary impairment of sperm production, including azoospermia, but the following text will focus on the chronic, permanent damage that can be present after oncological treatment.

It has been speculated that age, specifically prepuberty, would have a protective role in terms of gonadotoxicity. Literature does not support this theory; prepubertal boys suffer from corresponding gonadotoxic damage as adults, depending on drugs and dosages used (Howell & Shalet 2001). Furthermore, encouraged by rodent models, it has been attempted to protect the testes by inducing a pre pubertal milllieu with GnRH analogues during treatment. So far results are discouraging, but there are shortcomings in the clinical studies performed (Meistrich & Shetty 2008).

Chemotherapy

The deleterious effect of a chemotherapeutic agent on testicular tissue was first reported in a post-mortem analysis of 30 men treated with nitrogen mustard, in whom azoospermia was found in the 27/30 of men investigated (Spitz 1948), the three non-azoospermic specimens being from men who had received treatment a month or less before death. Thus, already this

report illustrated the time span between treatment and decline in spermatogenesis, a result of the kinetics of spermatogenesis. All chemotherapeutic drugs target proliferating cells. The most rapidly dividing, hence the most sensitive cell in spermatogenesis is the differentiating spermatogonia, which is initiating spermatogenesis, whereas cells in later stages in germ cell development are less sensitive to chemotherapy. Consequently, the decline in sperm counts is observed 8-9 weeks after treatment, i.e. the time required for differentiating spermatogonia to reach the stage of mature spermatozoa (Meistrich 1993a). In order to induce azoospermia, either temporarily or permanently, the damage on the germinal epithelium must be on a stem cell level. Permanent azoospermia is the result of killing of all stem cells, whereas the temporary azoospermia indicates that a stem cell population has survived. The mechanisms behind the recovery and the lag time, which can be several years, remain unknown.

The degree of damage is depending on several factors; type and dosage of chemotherapeutic drug or combination of drugs, pretreatment status of spermatogenesis, but a great inter-individual sensitivity to damage has also been observed (Eberhard et al., 2004; Relander et al., 2000). The alkylating drugs and procarbazine are the most gonadotoxic drugs (see table 2).

Most frequently investigated are the drug combinations used in treating TGCC and haematological malignancies. Lampe et al reported posttreatment sperm production in 170 men treated for TGCC (Lampe et al., 1997). With a median follow-up of 30 months 80 % of men with normal sperm count pretreatment had regained sperm production, 64 % had returned to normal, the recovery dependant on follow-up time and treatment intensity. Continued sperm recovery was observed beyond 2 years, and an 80% chance of total recovery in men treated with 4 or less courses of cisplatin-based chemotherapy was expected (Lampe et al., 1997). Several authors have reported similar dose and time dependency (Drasga et al., 1983; Palmieri et al., 1996; Petersen et al., 1994). Possibly there is a

genetic variability explaining the intra-individual sensitivity observed (Eberhard et al., 2004).

Regarding other malignancies, previously standard treatment of Hodgkin's lymphoma, MOPP (mustine, vincristine, procarbazine, and prednisolone) was shown to induce permanent azoospermia in the vast majority of cases (Anselmo et al., 1990; da Cunha et al., 1984; Viviani et al., 1991). This observation lead to the development of a less toxic but equally effective treatment with non-alkylating agents, ABVD (doxorubicin, bleomycine, vinblastin and prednisolone), now being standard treatment of good-prognosis Hodgkin' s lymphoma, with which fertility to a large extent will remain unaffected (da Cunha et al., 1984; van der Kaaij et al., 2007). Data on other malignancies, including childhood malignancies are sparser, and mirror the expected effect of drugs and dosages involved (Gerres et al., 1998; Kenney et al., 2001; Relander et al., 2000; van den Berg et al., 2004). Patients treated for Non-Hodgkin lymphomas have a lower risk of permanent azoospermia than do MOPP-treated HL patients, the effect mostly depending on the dosage of cyclophosphamide received and many men are expected to maintain their pretreatment fertility (Bokemeyer et al., 1994; Pryzant et al., 1993). Patients who have gone through high-dose chemotherapy with autologus or allogenous bone-marrow transplantation is a heterogenic group to study in relation to therapy, but will in the majority of cases suffer from permanent azoospermia. However, even 10-20 % of the patients receiving treatment including total body irradiation are reported to have a chance of regaining sperm production (Sanders et al., 1983; Sanders et al., 1996).

Agents (cumulative doses)	Expected effect on spermatogenesis
Cyclophosphamide (10 g/ m^2) Procarbazine (4 g/ m^2) Chlorambucil (1.4 g/ m^2) Melphalan (140 mg/ m^2) BCNU (1 g/ m^2) Cisplatin (500 mg/ m^2) Ifosfamide (42 g/ m^2)	Prolonged or persistent azoospermia
Carboplatin (2 g/ m^2) Cisplatin ($\leq$ 400 mg/ m^2) Doxorubicin (770 mg/ m^2) Vinblastine (50 g/ m^2) Vincristine (8 g/ m^2)	Temporary azoospermia likely
Bleomycine, dacarbazine, epirubicin, etoposide, methotrexate, 5-Fu	Only temporary effect on spermatogenesis
Oxaliplatin, irinotecan, monoclonal antibodies, tyrosine kinase inhibitors	Unknown effect on spermatogenesis

Table 2. Effects of chemotherapeutic drugs on sperm production
Adapted from Lee et al, Journal of Clinical Oncology 2006

Radiotherapy

Radiotherapy, direct or scattered, can impair spermatogenesis in two principally different ways, either by direct gonadal damage, due to testicular irradiation or by secondary gonadal failure, due to cranial irradiation, often in combination with brain surgery (Constine et al., 1993; Relander et al., 2000; Schmiegelow et al., 2001).

In clinical reports the additional effect of irradiation on spermatogenesis can be difficult to evaluate, since in many malignancies, i.e. lymphomas and childhood malignancies, treatment strategies include both radio- and chemotherapy. However, the extreme sensitivity of the germinal epithelium to irradiation was documented in a human experiment on 67 healthy men (Rowley et al., 1974). The men received testicular irradiation in single doses from 8 to 600 rad (0.08 - 6 Gy), with subsequent testicular biopsies and semen analyses performed. The effect on spermatogenesis was dose-

dependant. At all doses, even 0.08 Gy, an effect was seen on spermatogonia. Single doses above 0.8 Gy induced azoospermia, at intermediate doses of 2-3 Gy, the decline in sperm count was observed after 8-9 weeks, implying damage to spermatogonia. At higher doses azoospermia was seen earlier, thus, spermatids are less sensitive, tolerating higher doses. The recovery of spermatogenesis was also dose-dependant, with recovery after 1 Gy seen within 9-18 months, after 2-3 Gy within 30 months, and after more than 5 years, if at all, at doses of 4 Gy or more, with an increasing risk of permanent azoospermia (Rowley et al., '74). In the clinical situation testicular irradiation can be direct, for example in the treatment of testicular carcinoma in situ, most often resulting in permanent azoospermia (Classen et al., 2003), but more common due to scattered irradiation. Adjuvant abdominal irradiation was previously the standard treatment of low-stage seminomatous germ cell tumors, SGCT. Such treatment will give a measured total dose to the remaining testicle between 0.2 Gy and 0.9 Gy, depending on target volume, total dose and use of testicular lead shielding and an effect on spermatogenesis is expected. However, a return of sperm count to pretreatment values is often seen 12-30 months posttreatment (Bieri et al., 1999; Centola et al., 1994; Eberhard et al., 2004; Fossa et al., 1999; Schlappack et al., 1988; Sedlmayer et al., 1999). To date, when radiotherapy is given, a reduction of both target, excluding the ipsilateral iliac nodes, and of dosage, from 30 to 20 Gy, compared to previously, has resulted in reduced gonadotoxicity without jeopardizing the treatment outcome (Fossa et al., 1999; Jacobsen et al., 1997; Jones et al., 2005). Sixty long-term survivors of childhood leukemia receiving identical chemotherapy treatment, were analyzed according to which radiotherapy they received, craniospinal + abdominal, craniospinal or cranial only. Testicular impairment, low testicular volume and/or elevated FSH, was increasing with increasing radiotherapy target (Sklar et al., 1990). Speiser et al investigated the effect of scattered testicular radiation in ten Hodgkin patients treated with abdominal irradiation, inverted Y-inguinal field, with a

total testicular dose between 1.4 and 3 Gy in 14-26 fractions. All became azoospermic and in no patient was sperm recovery observed, indicating an upper limit after which the risk of permanent azoospermia is apparent, even though the follow-up time varied between patients (Speiser et al., 1973). However, as mentioned before, total body irradiation, with doses between 10-15 Gy, does not exclude the recovery of sperm production.

Surgery

Removing organs, indirectly or directly responsible for spermatogenesis, will, of course, result in infertility. Hypophysectomy, resulting in secondary hypogonadism and subsequent need for testosterone substitution, will render azoospermia (Relander et al., 2000). In TGCC patients, the removal of the tumor-bearing testicle may result in reduced or absent sperm production, suggesting an impaired spermatogenesis in the contra lateral testicle (Petersen et al., 1999a). Retroperitoneal lymph node dissection (RPLND) performed in TGCC patients, either as a staging procedure, or as a part of treatment, may render retrograde ejaculation. Historically, RPLND was associated with a very high risk of retrograde ejaculation, but in the 80´s nerve-sparing surgery was developed and today, depending on the extent of the procedure, i.e. uni- or bilateral dissection, the risk of developing retrograde ejaculation varies from a few per cent to almost 30 % (Donohue 2003; Krege et al., 2008a; Krege et al., 2008b).

Posttreatment fatherhood

As previously mentioned, the majority of cancer survivors will retain or regain their fertility posttreatment, and even though there are numerous studies investigating the long term consequences of cancer treatment on gonadal function, the number of studies investigating the actual end-point of male fertility - fatherhood, is limited. A Norwegian population-based study estimated the impact of cancer on the probability of having a(nother) child and reported a 24 % lower first-birth rate in men ever diagnosed with

a cancer, compared to men without cancer (Syse et al., 2007). Magelssen et al calculated the first-time parenthood probability at the age of 35 years and found no difference between male cancer patients, diagnosed at the age of 15-35 years, and the general population (Magelssen et al., 2008). A recent registry-based Finnish study on post-diagnosis parenthood among 25,874 cancer survivors found that male cancer survivors were less likely to parent at least 1 child (RR 0.46) in comparison to sibling controls, whereas the probability of having a second child was the same among cancer survivors and controls (Madanat et al., 2008). Childhood cancer survivors had the lowest probability of parenthood and furthermore, the probability differed depending on diagnosis, but was significantly lower for most of the diagnoses compared to sibling controls (Madanat et al., 2008).

Since the full extent of treatment often cannot be foreseen, and due to the great interindividual susceptibility to potentially gonadotoxic treatment, all patients should be offered to cryopreserve semen prior to oncological treatment (Lee et al., 2006). However, surprisingly little is known regarding the subsequent utilization of cryopreserved semen. The available data indicate that 5-25 % of patients use their stored semen (Blackhall et al., 2002; Kelleher et al., 2001; Magelssen et al., 2005; Neal et al., 2007). The rate of subsequent successful pregnancies varies in the different reports but is in the range of 35- 50 % (Agarwal et al., 2004; Neal et al., 2007; Schmidt et al., 2004). Furthermore, data concerning the efficiency of ART when using either pretreatment cryopreserved semen or fresh posttreatment semen in cancer patients vary, reviewed by Schmidt et al (Schmidt et al., 2007), but some studies indicate the superiority of ICSI. It has been speculated that the utilization of cryopreserved semen will increase due to improved access to this method (Agarwal et al., 2004; Rosenlund et al., 1998; Schmidt et al., 2004; Shin et al., 2005).

Cancer and sperm DNA

The issue of sperm DNA damage in male cancer survivors is of dual interest, both in terms of fertility and in terms of the risk of inducing transmittable germ cell DNA damage.

When this work was initiated there were only four studies on sperm DNA integrity in cancer, three of them assessing pretreatment sperm DNA integrity with SCSA, all demonstrating impaired DNA integrity (Evenson et al., 1984; Fossa et al., 1997; Kobayashi et al., 2001). The largest study of the three found a median DFI % of 25 in 39 testicular cancer patients, a value suggesting reduced fertility. Furthermore, these patients delivered posttreatment samples for conventional semen analyses, but pretreatment DFI could not predict sperm recovery (Fossa et al., 1997). The fourth study was on 33 childhood cancer survivors and in the 22 non-azoospermic men, sperm DNA integrity, assessed with the TUNEL, was not higher than in healthy controls (Thomson et al., 2002).

In a recent multidisciplinary workshop on human germ-cell mutagenesis it was concluded that " …we are faced with a fundamental mystery- to date no chemical or radiation has been confirmed as a human germinal mutagen. Decades of animal research have shown over 30 chemicals and ionizing radiation to be potent germ-cell mutagens…" (Wyrobek et al., 2007). Thus, numerous animal studies, mostly on rats and mice, have demonstrated the mutagenic capacity of both irradiation and several chemotherapeutic drugs (Dubrova et al., 1998; Russell et al., 1998; Witt & Bishop 1996; Wyrobek et al., 2005). In contrast to most of the chemotherapeutic drugs investigated, irradiation has shown to induce transmittable DNA damage on a stem cell level, in pre-meiotic germ cells (Dubrova et al., 1998). Otherwise, it seems that male germ cells are most sensitive to genetic damage in post-meiotic stages, in which they have less capacity of DNA repair (Meistrich 1993a; Wyrobek et al., 2007).

In humans the issue of germ-cell mutagenesis can be investigated in two, principally different, ways, either in epidemiological studies, or indirectly, by semen analyses.

In order to study acute toxicity, studies on sperm DNA are necessary. Sperm DNA damage has been assessed with different techniques. Already mentioned are the few studies performed on sperm DNA integrity at the time when my project was started. To investigate severer damage, FISH (fluorescence in situ hybridization) has been used to assess sperm chromosome abnormalities. Studies on testicular cancer and lymphoma patients, both pre-, during and posttreatment have been performed. A recent study investigating aneuploidy frequencies on the sex chromosomes, chromosomes 13 and 21 found significant increased aneuploidy rates six months after treatment, but for most part the increased rates returned to pretreatment levels. The same study observed an increase in aneuploidy rates already prior to treatment in Hodgkin patients (Tempest et al., 2008), a phenomenon that has not been observed for TGCC patients (Alvarez et al., 1999; Martin et al., 1999). Thomas et al investigated posttreatment sperm aneuploidy rates in 26 patients treated for testicular cancer or lymphoma (HL and NHL) and concluded that in most cases disomy/diploidy rates did not significantly differ from controls (Thomas et al., 2004). In the Hodgkin patient with the shortest follow-up time, assessed 6 months after treatment, an increase was observed, whereas other patients treated with the same combination of chemo-and radiotherapy, but with longer follow-up, had no such increase. A moderate increase was also observed in 4/14 testicular cancer patients, which could not be explained by follow-up time or type of treatment. The above-mentioned studies are the largest performed and other, even smaller, studies have shown the same results. Oncological treatment can induce severe sperm DNA damage, but the effect seems to be transient (Chatterjee et al., 2000; De Mas et al., 2001; Frias et al., 2003; Martin et al., 1999; Meistrich 1993b).

Thus, the few studies performed on human sperm actually seem to be in concordance with animal studies; the germ cell genome seems to be more susceptible to radio- and chemotherapy in the later stages of spermatogenesis. The combined findings of animal and human studies strongly support the general clinical advices to avoid conception during and shortly after oncological treatment and that cryopreservation of semen should be performed prior to the initiation of therapy.

In epidemiological studies exposed groups, due to occupation, medical treatment or accidents, can be identified, and by estimating, or knowing the type of exposure, different outcomes of interest in the exposed and their offspring can be assessed. Such a cohort is the Japanese atomic bomb survivors and their offspring, in whom no increase in adverse birth outcome, cancer incidence, or genetic disease has been found (Neel et al., 1990; Yoshimoto 1990). In investigations on offspring to men highly exposed to radiation at the Chernobyl accident results have been inconsistent, but there are indications that the rate of so called minisatellite mutations of paternal origin could be increased in the offspring of exposed men (Dubrova 2003). However, the clinical implications of these findings remain unclear. In 1984 a cluster of childhood leukemia was found in families living close to the Sellafield nuclear processing plant in Great Britain, and it was suggested to be caused by occupational exposure of the fathers (Gardner et al., 1990). The Sellafield observation, based on 10 cases of leukemia, initiated extensive investigations, all resulting in negative findings and in no other corresponding environment was an increased risk of childhood malignancy found (Anderson 2005).

Other highly exposed people are cancer survivors. An advantage with such cohorts is that the exposure, treatment, can be known in detail. On the other hand, oncological treatment is heterogeneous, often being a combination of multi-drug chemotherapy regimen and radiotherapy, aggravating the possibility to draw clear conclusions regarding the effect of a specific drug or radiation therapy. Furthermore, by necessity, by studying the offspring of

those treated there is a risk that the exposure investigated, the actual treatment, can be out-of date and no longer of clinical interest.

Several papers have addressed the issue of pregnancy outcome, birth defects, prevalence of genetic disease, and risk of cancer in these children. In general the findings of such studies have been reassuring both in terms of pregnancy outcome and rate of congenital malformations (see table 3) (Boice et al., 2003; Fossa et al., 2005; Green et al., 2003b; Meistrich & Byrne 2002; Nagarajan & Robison 2005; Sankila et al., 1998). The major shortcomings of these studies are in terms of statistical power, the largest study being on 1329 children, of which only 278 were fathered by men who had received " potentially mutagenic therapy" (Meistrich & Byrne 2002) and it has been estimated that to show a two-fold increase in birth defects, a minimum of 900 families is required (Wyrobek et al., 2007).

When my work was initiated several issues in terms of male cancer, sperm DNA integrity and possible consequences for the offspring remained to be elucidated. Even though studies on sperm DNA had indicated that radio- and chemotherapy did not induce any withstanding damage, the studies were few, based on a small number of patients. Likewise, studies on sperm DNA integrity prior to therapy were few, those existing indicating that cancer disease per se could be associated with impaired sperm DNA integrity.

Even though studies on the offspring to MCS had been reassuring, a specific concern had been raised with respect to ART (Morris 2002). Male cancer survivors will, to a larger extent than the general population, benefit from ART and specifically ICSI, since both cancer and oncological treatment is associated with reduced semen quality. As mentioned previously, ART is associated with adverse birth outcomes, and the possible risks of transmitting genetically damaged sperm with ICSI have been discussed. If MCS not only are subfertile, but cancer and/or its treatment also can cause defect sperm DNA, the risks of introducing negative birth outcomes in the children of male cancer survivors must be specifically addressed.

Author	*Diagnosis*	*Treatment data*	*Outcome*	*Mode of Conception*
Meistrich 2002 (Combined Byrne + Hawkins)	Childhood cancer n= 1329 (offspring)	Two categories: Potentially/ less- or non mutagenic	genetic disease: No increase	?
Fosså 2005	All diagnoses at the age of 15-45 years, 784 men, 1221 children	None	All malformations: OR 1.3 (1.0-1.7) Major malformations: No increase	?
Magelssen 2008	All diagnoses at the age of 15-45 years 487 firstborn	4 categories: Surgery, CT, RT, CT+RT	All malformations 27/487, OR 1.5 (95 CI 1.1-2.3)	39/487 IVF, no increased risk
Green 2003	Childhood cancer 2323 pregnancies, 1603 live births	complete	No adverse pregnancy outcomes, altered sex ratio	?
Byrne 1998	Childhood cancer 436 men, treated 1945-75, 916 children	Two categories: Potentially/ less- or non mutagenic	Genetic disease, malformation rates: No difference	?
Hawkins 1991	Childhood cancer, 300 men 537 children	Exposed = abdominal RT or alkylating agent vs. unexposed, same diagnosis	Malformations No difference	?

Table 3. Previous studies on birth outcomes in the offspring of male cancer survivors

Aims of the Thesis

The overall aim of my thesis was to investigate the effect of male cancer and its treatment on sperm DNA, and to investigate birth outcomes in children to men treated for cancer.

More specifically, in papers I-III, the objective was to study the impact of cancer disease per se on sperm chromatin and to discriminate the effects of different malignant diagnoses. Furthermore I intended to investigate whether oncological treatment, i.e. radio- and chemotherapy, has an impact on sperm DNA integrity. Using TGCC as a model, a specific issue was to take into account the effect of time after treatment and to investigate whether any withstanding changes in sperm DNA integrity could be observed in men treated for cancer. Finally, since the Sperm Chromatin Structure Assay is an indirect indicator of sperm DNA damage, it was of importance to confirm the results by a direct assessment of DNA strand breaks, using the TUNEL assay.

Disease or treatment-related sperm damage in male cancer survivors might impose a health risk for their offspring, especially for the children conceived by assisted reproduction. Therefore, in paper IV I aimed at investigating birth outcomes, including malformation rates, in children whose fathers have had a malignant disease and specifically in those children conceived by assisted reproductive technologies, IVF or ICSI.

Material and Methods

The details of material and methods are presented in the original papers of the thesis, and the following is a summary.

Papers I & II

The first two papers address the issue of sperm DNA integrity in TGCC patients, in relation to treatment and follow-up time. In paper I sperm DNA integrity was assessed with SCSA, whereas both the SCSA and TUNEL assays were used in paper II. These two papers will be presented together.

Study population

The papers are based on a longitudinal survey of reproductive function in males treated for TGCC, initiated in 2001, the TGCC study. All men with TGCC, below the age of 50, diagnosed less than 5 years prior to inclusion, were eligible for the study. Fixed time intervals for delivery of semen samples were defined: T_0 – after orchiectomy but prior to further therapy; T_6, T_{12}, T_{24}, T_{36} and T_{60} 6-60 months after completed treatment. Patients entered the study at any time between T_0 and T_{60} and delivered samples at the remaining intervals.

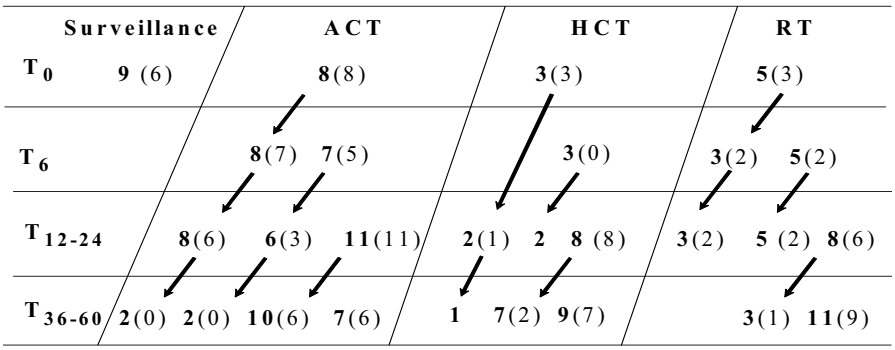

Figure 3. Flow chart of semen analyses in papers I and **II** (**bold**), depending on diagnosis and time window of delivery.

In paper I 106 semen samples delivered by 74 patients were analyzed and in the second paper 193 semen samples from 96 patients were analyzed.

Cancer treatment

The patients were treated according to the SWENOTECA protocols (Albers et al., 2005; Klepp et al., 1997), the Swedish-Norwegian Testicular Cancer collaborative cancer care program (www.ocsyd.se). Patients with non-seminomas (NSGCT) were treated with the BEP regimen (bleomycin 90 000 IU days 1,8,15, to a maximum dose of $3x10^5$ IU, etoposide 500mg/ m^2 and cisplatin 100mg /m^2 per cycle, both given days 1-5, with a 3 week interval) or a similar regimen. Patients with disseminated seminoma (SGCT) were treated with EP (BEP minus bleomycin) or BEP.

Adjuvant radiotherapy was given to SGCT stage I patients to a total absorbed dose of 25.2 Gy in 14 fractions to the clinical target volume of infradiaphragmal para-aortic and ipsilateral iliac lymph nodes. The dose to the remaining, lead-shielded testicle was measured at the start of the treatment. Based on a retrospective calculation of seven randomly selected patients in the study, the total dose to the remaining testicle was estimated not to exceed 0.5 Gy (range 0.04-0.43).

The background characteristics of the patients included in papers I and II are presented in table 4 and a flow chart of the distribution of semen samples is seen in figure 3.

The patients were allocated into groups according to treatment given;

• Surgery only (SO); patients with stage I disease, receiving no adjuvant therapy, and men from groups b-d assessed after orchiectomy, prior to further treatment

• Adjuvant chemotherapy (ACT); NSGCT stage I disease, receiving 1-2 cycles of BEP

• HCT; Disseminated disease receiving more than two cycles of CT

• RT; SGCT stage I disease, receiving adjuvant radiotherapy

• RT+CT; Disseminated disease, treated with both RT and CT

Treatment	***SO*** *n= 9 (**6**)*	***ACT*** *n=33 (**30**)*	***HCT*** *n=23 (**17**)*	***RT*** *n=29 (**20**)*	***CT+RT*** *n= 2 (**1**)*	***Total*** *n= 96 (**74**)*
NSGCT	**8** (6)	**33** (30)	**18** (12)			**59** (48)
SGCT	**1** (0)		**5** (5)	**29** (20)	**2** (1)	**37** (26)
Stage I	**9** (6)	**33** (30)	**6** (4)	**29**(20)	**2**(1)	**79** (61)
Stage II			**10** (8)			**10** (8)
Stage III			**3** (3)			**3** (3)
Stage IV			**4** (2)			**4** (2)

Table 4. TGCC patients in papers I and **II,** according to histological diagnosis, stage and treatment (**paper II in bold**)

Controls

For SCSA analysis 278 military conscripts served as controls in both papers I and II. Data on the conscripts were published elsewhere (Richthoff et al., 2002). Twenty-four of these men, corresponding to the number of patients in the SO group, not differing in SCSA from the remaining 254 men, were randomly selected as controls for TUNEL analysis.

Paper III

The aim of paper III was to investigate the effects of both cancer disease *per se* and treatment on sperm chromatin quality in patients with varying malignant diseases.

Study population

In Paper III sperm DNA integrity was assessed on cryopreserved semen from cancer patients, storing sperm prior to oncological treatment during the period 1984-2004. The men were also asked to deliver posttreatment samples for SCSA analysis. Patients who had banked semen prior to oncological treatment and with a minimum of 12 straws stored were eligible for the study. Two hundred and twenty-one men were eligible, including 58 men from the TGCC study (see Paper I and II) and a total of 121 men participated in the study. Posttreatment samples were analyzed in 71 men, of

whom eleven were azoospermic and two samples had too low sperm concentration to allow sperm DNA analysis, making a total of 58 posttreatment samples, including 30 men from the TGCC study, already published in paper II. The distribution of diagnoses is presented in table 5.

Diagnosis	Eligible	Pre-treatment sample	Post-treatment sample
Germ Cell Cancer-TGCC study	58	42	30
Hodgkin's lymphoma	40	18	5
Germ Cell Cancer	78	42	14
CNS tumor	7	3	2
NHL/ / leukemia	23	9	5
Sarcoma	8	2	1
Other	7	5	1
Total	***221***	***121***	**58**

Table 5. Patients in study III, according to diagnosis.

Cancer treatment

Treatment data were obtained from patient files and in the posttreatment analyses patients were divided into four treatment categories:

• Adjuvant chemotherapy (ACT), 1-2 cycles of cisplatin-based chemotherapy in NSGCT stage 1

• Intense chemotherapy (CT), either ≥3 cycles of BEP, or other multidrug regimen

• Radiotherapy (RT), all but one adjuvant radiotherapy for SGCT, stage I

• Chemotherapy (CT) + RT

Controls

In paper III an age-matched control material was available, namely semen from 137 male partners to consecutively recruited pregnant women at the Maternity Ward at Malmö University Hospital.

Semen analyses

In papers I-II semen was analyzed according to WHO guidelines 1999 (WHO 1999). In paper III semen analyses were performed during a time interval of 15 years according to the prevailing guidelines, since 1999 according to WHO guidelines 1999 (WHO 1999).

Analysis of sperm DNA integrity

In all three papers on sperm DNA integrity the Sperm Chromatin Structure Assay, SCSA, was applied. Since SCSA is an indirect indicator of sperm DNA damage, the results of SCSA were validated in paper II by applying the TUNEL assay, which directly reflects the presence of DNA strand breaks.

TUNEL

TUNEL specifically detects DNA strand breaks. An enzymatic reaction, driven by terminal deoxynucletidyl transferase (TdT), incorporates fluorescently labeled nucleotides to free 3´-OH termini at single- or double strand breaks. Negative controls were prepared by omitting TdT from the reaction mixture and positive controls were prepared by adding DNAse, inducing DNA strand breaks. TUNEL positive cells will be detected as green fluorescent and TUNEL negative, i.e. cells without strand breaks, as red fluorescent (Erenpreiss et al., 2004). TUNEL can be applied in either light microscopy or by flow cytometry (FCM). In the FCM a threshold value on a relative fluorescent scale is set and TUNEL positivity is defined as the fraction of sperm above the threshold value. An advantage of FCM is that it allows rapid analysis of a large number of cells, 10 000 sperm, compared to 2-300 cells normally analyzed in bright field microscopy. Furthermore, FCM is shown to be a more precise and objective way in detecting TUNEL positivity (Perreault et al., 2003). In somatic cells TUNEL positivity is thought to reflect apoptosis. However, Sakkas et al found that when analyzing sperm, TUNEL positivity and the expression of apoptotic markers, such as Fas and p53, did not always exist in unison and the origin of sperm DNA strand breaks detected by TUNEL remains unclear (Sakkas et al., 2002). TUNEL has shown to correlate well with both the Comet and SCSA

assays and to the fertilizing ability of sperm. However, in contrast to SCSA, no clear clinical cut-off values have been defined (Erenpreiss et al., 2004; Erenpreiss et al., 2006).

By repeated assessments of control semen samples an inter-assay variability of < 7 % was found in our laboratory.

SCSA

SCSA is a flow cytometric method, allowing the rapid and standardized analysis of 5000 sperm per semen sample. The SCSA analysis is based on the phenomenon that sperm with defect chromatin, such as the presence of strand breaks, will denaturate when exposed to an acid-detergent, whereas sperm with intact DNA will not. By adding acridine orange denaturated, single stranded DNA shifts from green to red fluorescence, whereas intact, double stranded DNA remains green. The extent of DNA denaturability is expressed as the DNA Fragmentation Index (DFI), being the ratio of red to total (red plus green) fluorescence intensity. The DFI value is calculated for each sperm. Plotted on a histogram different populations of the semen sample are defined: the fraction of sperm with no detectable DNA damage, *the main population*, the fraction of high DNA stainable cells (immature spermatozoa), *HDS*, and the fraction of sperm with higher red fluorescence, falling outside the main population, representing the *abnormal sperm*, with detectable fragmented DNA, *DFI*, see figure 4 (Evenson et al., 2002; Spano et al., 2000).

The flow cytometric data were analyzed using dedicated soft ware (List View, Phoenix flow Systems, San Diego, CA (papers I-III) or SCSAsoft; SCSA Diagnostics, Brookings, SD, USA (paper III)). No difference between the two software systems was observed when parallel data analyses were performed at our laboratory (unpublished). Computer gates were used to determine the proportion of spermatozoa with increased levels of red and green fluorescence.

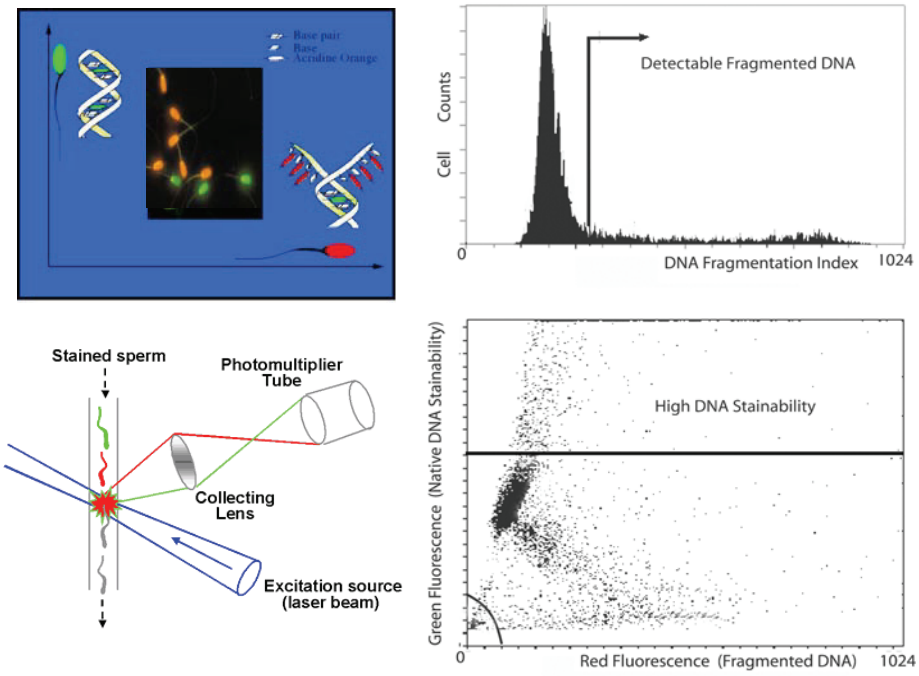

Figure 4. The principles of flow cytometry and the Sperm Chromatin structure assay.

For the flow-cytometer set-up and calibration, a reference sample from a semen donor was used. The interassay variability of DFI was found to be 4.5 % in our laboratory. Furthermore, in an external quality control based on >180 samples both a high correlation (r = 0.8) and an absolute variation in DFI of not more than 1% in average, was found comparing our laboratory with a control laboratory (Giwercman et al., 2003).

Paper IV

The aims in paper IV were to determine if pregnancy outcomes and malformation rates were affected by whether the father had or had not a history of cancer at the time of conception and by mode of conception, natural, IVF or ICSI.

Study population

The study was based on Danish and Swedish civil registration systems, in which each person is assigned a unique personal identification number, thereby enabling a population-based study including nearly all children born in 1994-2005. By linkage of several national registries each child and their parents were identified as well as information on paternal history of cancer, mode of conception, and birth outcomes. Through the national medical birth registries, detailed information on mother and child including birth characteristics was available. By linkage to the national cancer registries the fathers with a history of cancer at the time of conception were found. All diagnoses being reported to the national cancer registries were included. Furthermore, information regarding mode of conception, natural (NC), or assisted reproduction, IVF or ICSI, was retrieved. The national registries used, both Danish and Swedish, have all shown very high coverage (Andersen et al., 1999; Knudsen & Olsen 1998; Källén 1987; Källén et al., 2005; National Board of Health and Welfare 2004; National Board of Health and Welfare 2007; Pedersen et al., 2006; StatisticsSweden 2005; Storm et al., 1997).

All children, born in Denmark or Sweden in 1994-2005, were classified according to the exposures of interest in our study, namely whether the father had a history of cancer, defined as being diagnosed ≥ 1 year prior to the child's birth and mode of conception (NC, IVF or ICSI), making the following categories (see figure 5);

- Children conceived naturally by fathers with no history of cancer
- Children conceived naturally by fathers with a history of malignant disease
- Children conceived by IVF or ICSI by fathers with no history of cancer
- Children conceived by IVF or ICSI by fathers with a history of cancer

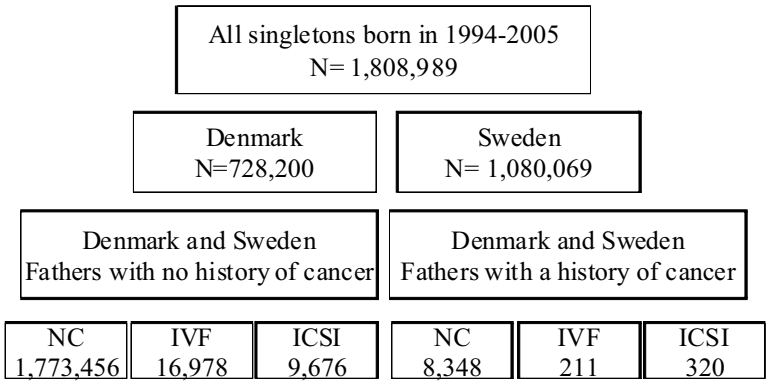

Figure 5. Children in study IV, according to country of origin, parental history of cancer and mode of conception (NC = natural conception)

In the analyses only singletons were included, since multiple births, which is vastly overrepresented in children conceived by ART, have an adverse effect on pregnancy outcome. In addition, the male cancer survivors were divided into seven groups, based on the classification of their diagnosis according to the ICD-7 classification. Treatment data were not available, but specific diagnostic groups can be expected to, in a large extent, have received a certain treatment. Patients treated for a hematological malignancy most probably have received chemotherapy. Furthermore, a subgroup in the Swedish material, assumed to have received radiotherapy, was identified. In Sweden, standard treatment of SGCT stage I was in the current study period abdominal irradiation. In the Swedish material there were 480 fathers diagnosed SGCT of which 80-85 % can be assumed to be stage 1 (Chung & Warde 2006) and consequently treated with radiotherapy. MCS were also divided into childhood or adulthood cancer, diagnosed before or after the age of 18 years.

The semen from MCS used for ART can either be banked pretreatment sperm or fresh posttreatment semen. The information on semen source was generally not available in the registries, but was available for the majority of Swedish IVF/ICSI children, and approximately 2/3 of the used semen was collected posttreatment.

Outcomes

Perinatal outcome data included gestational length, birth weight and small for gestational age (SGA).

The rate of detected congenital malformations depends on the length of the follow-up (Zhu et al., 2005). In our study malformations registered due to hospitalizations up to the end of 2005 were included. When analyzing the outcome malformations, we first looked on all malformations. We, thereafter, excluded some mild or variably registered conditions, and the remaining, mostly major, malformations, were classified as "weeded" malformations (Källén et al., 2005). The excluded malformations were preauricular appendix, patent ductus arteriosus at preterm birth (<37 weeks), single umbilical artery, undescended testicle, congenital hip (sub)luxation, and minor skin malformations (mainly naevi).

	Mode of conception			
Paternal characteristics	**Natural**	**IVF**	**ICSI**	**Total**
	8348	211	320	8879
Mean age at cancer diagnosis	26.2	28.7	28.3	
Timing of cancer				
Childhood and adolescence	1346	13	20	1379
Adulthood	7002	198	300	7500
Child birth < 2 years after diagnosis	784	11	20	815
Child birth > 2 years after diagnosis	7564	200	300	8064
Diagnosis				
Respiratory, digestive and urogenital tracts	1129	21	15	1165
Testicular cancer	2313	69	173	2555
Skin malignancy	1731	40	20	1791
Tumors of the eye and CNS	1084	15	10	1109
Bone and soft tissue tumors	442	3	8	453
Malignancies of blood and lymphatic system	1238	54	86	1378
Other diagnoses	411	9	8	428

Table 6. Paternal characteristics for children born ≥ 1 year after a cancer diagnosis in their father

Statistical analysis

Papers I-III

Papers I-II

To obtain sufficient numbers of individuals, the results of samples collected at T_{12} and T_{24} were combined into one time window T_{12-24} and samples collected at T_{36} and T_{60} were combined into T_{36-60}. If two samples were delivered by a patient in one time window, the mean value was used in the analysis. The data were treated in a cross-sectional manner (Mann-Whitney U test). SO (post-surgery) values were compared to controls in regard to both DFI and TUNEL (paper II). For each therapy group comparisons between SO and the three different time windows were made. Furthermore in paper II, Spearman's rho was calculated to correlate the results of SCSA and TUNEL.

Paper III

The pretreatment samples were divided in three equally large groups according to storage time; less than 3 years, 3-6 years and more than 6 years of storage.

A T-test was used for intra-group comparisons.

When comparing the different diagnose groups: TGCC, Hodgkin's lymphoma and "other cancer" to controls, univariate linear regression models were applied and age was included as a confounder.

For the intra-individual comparison of pre- and posttreatment samples the Wilcoxon test for paired data was used due to the relatively small patient numbers in the treatment categories. If more than one posttreatment was delivered, the sample delivered after the longest follow up time was used.

Papers I-III

DFI $\geq$ 30% was suggested to indicate strongly reduced in vivo fertility (Bungum et al., 2007). In a joint analysis of papers I-III the proportion of patients having pretreatment DFI values $\geq$ 30% was calculated for each diagnose group, and the odds ratios for such high DFI were calculated for each diagnose and compared to controls of paper III by means of binary logistic regression analysis.

Paper IV

Risk ratio (RR) assessments were calculated by use of log-linear models. The outcomes weight for gestational age (≤ 10th percentile; > 10th percentile), gestational age (<37 weeks; ≥ 37 weeks) and birth weight (<2500 g; ≥ 2500 g) all malformations (+/-) and weeded malformations (+/-) were dichotomized.

Primarily, the impact of paternal cancer (+/-) on the above mentioned outcomes was assessed in univariate models. Separate analyses were performed for Denmark and Sweden, as well as for the combined Danish and Swedish data. For outcomes that in both countries, separately and in a consistent manner, were associated with paternal cancer, a multivariate analysis was performed. The multivariate models included paternal history of cancer, mode of conception as well as the following confounders, all of which previously were shown to affect birth outcomes; calendar-year of birth (1-year classes), maternal age (5-year classes), parity (1, 2, 3+), maternal smoking (yes/no) (Andersen et al., 2008; Clausson et al., 1998; Hollier et al., 2000; Zhu et al., 2005) and weight for gestational age and country of origin (Denmark or Sweden).

The multivariate analyses were performed for all MCS, childhood (<18 years of age at diagnosis) and adulthood cancer, timing of child birth in relation to diagnosis (≤2 years or >2years after diagnosis) and for the specific categories of diagnoses. Furthermore, a multivariate analysis comparing childhood and adulthood cancer, using adulthood as a reference, was done.

In order to investigate whether the use of IVF or ICSI increased the risk of low birth weight, preterm birth, small for gestational age or malformations in children of MCS, an interaction analysis (+/- paternal cancer X mode of conception) was performed for those outcomes which turned out statistically significant in the multivariate analysis. In order to increase the statistical power, IVF and ICSI were merged into one group if similar trends were found in the separate analyses for those two treatment methods.

Results and General Discussion

The results of paper I will not be discussed separately. Paper II is a continuation of paper I, with a larger patient material and with the addition of TUNEL as a method of assessing sperm DNA integrity. The results of paper I were confirmed in paper II and these will be discussed as one entity, as the findings of the TGCC study.

In paper II sperm DNA integrity was assessed with both TUNEL and SCSA. Although only a moderate correlation between the two assays was found, Spearman's rho = 0.41 ($p = 0.01$), the general conclusions reached with the two assays were very much alike both in terms of pretreatment DFI in patients as compared to controls and the effects of treatment and follow-up time. The following discussion is therefore based on the results of the SCSA analysis.

Sperm DNA integrity and cancer disease

The effect of cancer disease per se on sperm DNA integrity was investigated in papers I-III.

In the TGCC study pretreatment DFI was investigated in 25 samples, nine from stage I patients treated with orchiectomy only and 16 samples from men delivering samples for SCSA analysis prior to the initiation of therapy. Pretreatment DFI did not differ significantly from controls, the median DFI being 12% in patients vs. 11 % in the military conscripts. This finding was in conflict to other studies (Fossa et al., 1997; Gandini et al., 2000; Kobayashi et al., 2001; O'Donovan 2005; Spermon et al., 2006). The selection of control group might contribute to the diverging results. In the TGCC study an unselected group of men was used, whereas most of the other studies had proven fertile men as controls. Since fertility is associated with low DFI values, the use of proven fertile men might imply lower DFI values in such a control group than in the general population. In common for all these

studies, including ours, was the relatively small patient groups, the largest, by Gandini et al, comprised of 58 patients (Gandini et al., 2000).

The discrepancy between ours and others findings was one of the reasons for the initiation of study III in which sperm DNA integrity was assessed in cryopreserved pretreatment semen from 121 cancer patients.

In studies I and II semen was handled for the purpose of SCSA, which did not include the standard cryopreservation in liquid nitrogen. As discussed earlier, such procedure of cryopreservation might have a negative impact on sperm DNA (de Paula et al., 2006; Spano et al., 1999; Thomson et al., 2008) and paper III allowed investigating the possible impact of the length of cryopreservation. In paper III DFI was significantly elevated in both TGCC and Hodgkin patients (mean DFI 17.5% and 16.5% respectively) compared to controls (mean DFI % 11.5). The same trend was seen in 19 patients with "other cancer", however without reaching the level of statistical significance, mean DFI being 15.2% (see table 7). Furthermore, no effect of the length of cryopreservation was observed. Since DFI increases with age, as discussed earlier, in study III another control material was used, allowing adjustment for age. In paper III proven fertile men were used as controls and as argued before, this could imply lower DFI values than among the general population. However, mean DFI % in this group was at the same level as found in a population-based group of age-matched Norwegian men (Romerius, unpublished data).

In a joint analysis of the TGCC study and paper III the risk of having DFI $\geq$ 30%, indicative of strongly reduced in vivo fertility (Bungum et al., 2007), was calculated for each diagnose group. TGCC patients in this analysis comprised of 109 men, 25 pretreatment samples from the TGCC study and 84 cryopreserved samples from paper III, and 13/109 had DFI $\geq$ 30% compared to 0/18 of Hodgkin patients and 5/137 of controls. In contrast to other diagnoses, TGCC was associated with a significantly increased risk of having DFI $\geq$ 30% compared to controls (OR 3.6 95 CI 1.1-13).

Diagnosis	*Non-age adjusted*		*Age adjusted*	
	Mean difference %	95 %CI	Mean difference %	95% CI
Germ Cell Cancer	6.0	3.2-8.8	7.7	4.9-10
Hodgkin's lymphoma	5.0	-0.1-10	7.0	2.0 - 12
Other cancer	3.7	-1.3 – 0.1	3.6	-1.2- 8.4
Controls	ref	-	ref	-

Table 7. Difference in sperm DNA Fragmentation Index between different categories of cancer patients and fertile controls, crude as well as age-adjusted.

The level of sperm DNA impairment in cryopreserved semen from cancer patients has a clinical relevance. Semen is cryostored for future use and clinically the most important issue is the quality of the sperm at the time it is used. Paper III demonstrated that cancer disease per se can be associated with impaired sperm DNA quality, even though it cannot be excluded that the procedure of cryopreservation contributes to the raise in DFI. However, in our lab, when performing SCSA on cryopreserved and fresh semen from 30 men with semen of different qualities, no negative impact of cryopreservation was seen.

It is plausible to assume that the etiology of the observed sperm DNA damage differs with diagnosis. It is well-established that TGCC patients suffer from reduced fertility, supported by the increased risk of having DFI values indicative of in vivo infertility. This risk was not observed in Hodgkin patients, further supporting the possibility of different origins of the impaired DNA integrity seen in the two patient categories.

According to the theory of testicular dysgenesis syndrome, the association between TGCC and other male urogenital disorders, such as poor semen quality, hypospadia and cryptorchidism, is due to disruption of gonadal development in fetal life (Skakkebaek et al., 2001). Such a general disturbance of testicular development may also include the impairment of sperm DNA, which has been shown in men with hypospadia and cryptorchidism (Smith et al., 2007). The gonadal impairment, including defect sperm chromatin, observed in HL probably relates to systemic disease

rather than to testicular dysfunction. Rueffer et al found that poor semen quality in Hodgkin patients was associated with fever, high sedimentation rate and advanced stages of the disease, factors known to correlate with increased cytokine activity and systemic oxidative stress (Rueffer et al., 2001). In our study we were unable to correlate DFI to such parameters, but it seems reasonable that the elevated DFI is due to systemic oxidative stress, previously speculated to cause sperm DNA injury (Tremellen 2008).

Even though the increase in DFI % seen in semen from patients with other diagnoses than GCC or HL did not reach statistical significance, a proportion of these men had an increase, and in a clinical perspective, our results indicate a risk of sperm DNA impairment in all cancer patients, regardless of diagnosis.

Oncological treatment and sperm DNA integrity

In papers I-III we have analyzed the effect of oncological treatment, radio- and chemotherapy on sperm chromatin quality.

In the TGCC study testicular cancer patients delivered semen before treatment and at fixed time intervals after treatment. Using pretreatment DFI as default values, (SO, n =25), a cross-sectional analysis of the impact of therapy and follow-up time on DFI was done in the TGCC study, the results being illustrated in figure 6. We found no negative withstanding effects of treatment. One or two cycles of adjuvant cisplatin-based chemotherapy had no impact on DFI. More intense chemotherapy induced temporary azoospermia in the majority of patients. When spermatogenesis was resumed, DFI was significantly lower than in pretreatment samples. In 17 semen samples delivered 3-5 years after treatment, sperm concentration had returned to pretreatment values whereas DFI was significantly lower compared to SO (median DFI 9.1 vs. 12%, $p = 0.02$). Since the decrease in DFI was observed after the resumption of spermatogenesis and remained throughout the observation time of five years, the effect must be on a stem cell, spermatogonia level. A possibility is that spermatogonia giving rise to

spermatozoa with DNA strand breaks have defect DNA repair mechanisms and that these stem cells therefore are more susceptible to chemotherapy, hence being sorted out.

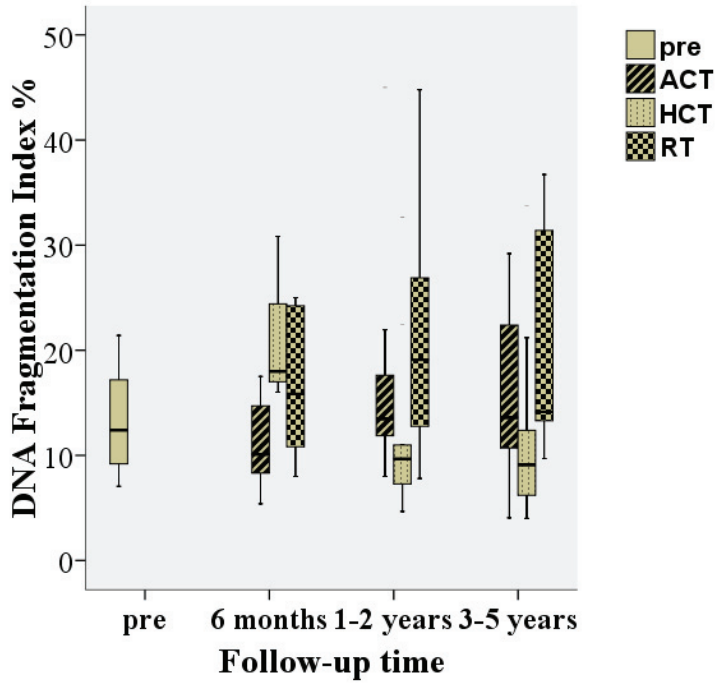

Figure 6. The effect of treatment on DFI in TGCC patients according to follow-up time (Bars indicate median values. Boxes represent interquartile intervals and whiskers represent 95 % confidence intervals).

Adjuvant abdominal radiotherapy (RT) induced temporary sperm DNA damage. In the 19 patients assessed 1-2 years after treatment DFI was significantly elevated compared to SO (median DFI 19 vs. 12%, p= 0.03), whereas median DFI in the 14 men delivering semen 3-5 years after treatment was at the level of pretreatment DFI.

In paper III a longitudinal analysis of cryopreserved pre- vs. posttreatment DFI on an individual basis was performed in the 58/71 patients who had delivered posttreatment samples which allowed SCSA analysis. With a median time after treatment of 3 years, no change of sperm chromatin quality was observed (median DFI_{pre} 13.5 % vs. DFI_{post} 12.6 %). In contrast to

the TGCC study, we found no effect of chemotherapy on DFI (median DFI_{pre} 13.5 vs. DFI_{post} 12.4 %). However, whereas HCT patients in the TGCC study all had received cisplatin-based treatment, chemotherapy treatment was not as uniform in paper III, including different cancer diagnoses. As previously discussed, the effect on germ cells varies with different chemotherapeutic drugs (see table 3) which might explain the diverging results. This possibility is supported by the fact that Spermon et al found a decrease of the proportion of sperm with defect DNA after chemotherapy in a study on TGCC patients (Spermon et al., 2006) whereas in a smaller study, including a variety of malignant diagnoses, no such decrease was observed (O'Donovan 2005).

Birth outcomes in the offspring

Paper IV is included as a manuscript and additional results, not available in the manuscript, were incorporated in the thesis.

In Paper IV the question at issue was whether male cancer survivors (MCS), especially those conceiving with IVF/ICSI, are at an increased risk of having offspring with adverse birth outcomes. In a Danish-Swedish register study, including 1.8 million singleton children, we have investigated the impact of paternal cancer and mode of conception on the risk of preterm delivery, low birthweight, small for gestational age and congenital malformations (see table 8). In total 8,879 singleton children were fathered by MCS, defined as being diagnosed with cancer $\geq$ 1 year before child birth. A total of 511 children, fathered by MCS were conceived by IVF/ICSI. As shown previously (Hansen et al., 2002; Källén et al., 2005; Sutcliffe & Ludwig 2007; Zhu et al., 2006), an association between IVF and ICSI and increase in adverse birth outcomes, both in terms of gestational age, birthweight and malformation rate was found. Paternal history of cancer did not influence gestational age or birthweight, but a moderately increased risk of having a malformed child was found for MCS, with a risk ratio of 1.17 (95 % CI 1.05-1.31). The distribution of type of malformation did not differ between the different

groups of exposure. The risk was somewhat higher for childhood cancer survivors (RR 1.44, 95 % CI 1.10-1.89) than for fathers being diagnosed after the age of 18 (RR 1.14, 95 % CI 1.01-1.30), but there was no statistically significant difference between the two groups (RR 1.26, 95 % CI 0.93, 1.70) (table 9).

Paternal cancer ≥ 1 year before child's birth								
No			*Yes*			*Total*		
NC	**IVF**	**ICSI**	**NC**	**IVF**	**ICSI**	**NC**	**IVF**	**ICSI**
1,773,456	16,978	9,676	8,348	211	320	1,781,804	17,189	9,996
Mean birth weight (g)								
3,554	3,429	3,462	3,583	3,465	3,433	3,554	3,429	3,460
SGA (%)								
9.1	12	12	8.6	10	10	9.1	12	12
All malformations (%)								
4.4	5.4	5.5	4.8	8.1	4.4	4.4	5.4	5.5
Weeded malformations (%)								
3.2	3.7	3.9	3.7	6.2	3.4	3.2	3.7	3.9

Table 8. Birth outcomes according to paternal history of cancer and according to mode of conception, natural (NC), IVF or ICSI (SGA = small for gestational age)

The impact of the timing of conception after diagnosis was analyzed, comparing malformation rates in the 815 children born within 2 year after the father's cancer diagnosis with those born later and no difference was observed.

Mode of conception did not modify the risk among MCS of having an infant with a weeded malformation (p-value for interaction 0.77), i.e. IVF/ICSI did not constitute a particular risk for the offspring of MCS.

Apart from a recent study on 487 first-born children to male cancer survivors, demonstrating an increase in malformation rate similar to the level found by us (OR 1.5) (Magelssen et al., 2008), the observed increased malformation rate in our study is in contrast to previous studies (Boice et al., 2003; Byrne et al., 1998; Green et al., 2003b; Hawkins 1991; Meistrich & Byrne 2002). However these studies have been smaller cohort studies, mainly focusing on childhood cancer survivors, the largest being on 1329 children and mostly focusing on the effect of oncological treatment (Meistrich & Byrne 2002), (see table 2).

Diagnosis	Denmark cases / total (%)	Sweden cases / total (%)	Total cases / total (%)	RR adjusted 95 % CI
No paternal Cancer	23637/724195 3.3 %	34321/1075195 3.2 %	57958/1800110 **3.2 %**	Ref
Paternal cancer all diagnoses	149/4005 3.7 %	181/4874 3.7 %	330/8879 **3.7 %**	**1.17** (1.05,1.31)
Childhood and adolescence	21/505 4.2 %	38/874 4.3 %	59/1379 4.3 %	1.44 (1.10,1.89)
Adulthood	128/3500 3.7 %	143/4000 3.6 %	271/7500 3.6 %	1.14 (1.01,1.30)
Birth ≤ 2 years post diagnosis	17/374 4.5 %	17/441 3.9 %	34/815 4.2 %	1.38 (0.97,1.96)
Birth > 2 years post diagnosis	132/ 3,631 3.6 %	164/ 4,433 3.7 %	296/ 8064 3.7 %	1.17 (1.03,1.32)
Respiratory, digestive urogenital tract	21 / 407 5.2 %	25 / 758 3.3 %	46 / 1165 3.9 %	1.26 (0.94,1.69)
Testicular cancer	36 / 1447 2.5 %	41 / 1108 3.7 %	77 / 2555 3.0 %	0.98 (0.78,1.22)
Skin malignancy	42 / 889 4.7 %	38 / 902 4.2 %	80 / 1791 4.5 %	1.37 (1.09,1.72)
Tumors of eye and CNS	16 / 477 3.3 %	31 / 632 4.9 %	47 / 1109 4.2 %	1.38 (1.04,1.84)
Bone and soft tissue tumors	3 / 127 2.4 %	5 / 326 1.5 %	8 / 453 1.8 %	0.53 (0.26,1.11)
Haematological Malignancies	28 / 562 5.0 %	26 / 816 3.2%	54 / 1378 3.9 %	1.21 (0.92,1.59)
Other diagnoses	3 / 96 3.1 %	15 / 332 4.5 %	18 / 428 4.2 %	1.31 (0.83,2.09)

Table 9. Prevalence and risk ratios of weeded congenital malformations in children fathered by men with or without a history of cancer, and according to type of cancer

This study is, so far, the largest one dealing with birth outcomes in children of men treated for cancer but it has some limitations. Apart from some known genetic and environmental (maternal exposure) causes, the etiology of congenital malformations is to a large extent unknown (Brent 2004). In a study on cancer survivors, the potential effect of treatment is an obvious matter to investigate. Since such information was unavailable, malformation rates were analyzed according to the cancer diagnoses, thereby attempting to identify categories likely to have received a certain treatment. Men treated for haematological malignancies were presumed to have received chemotherapy, and a large proportion of Swedish men diagnosed with SGCT were presumed to have received abdominal radiotherapy.

In none of these two groups a further increase in malformation rate was found, indicating that the increased malformation rate is rather due to the disease *per se* than to its treatment. This is further supported by the fact that the increased malformation risk was seen also for skin malignancies, a category of diagnoses for which standard care is surgical excision only. Thus, even though we cannot exclude that the observed increase in malformation rate is due to treatment-induced sperm DNA damage, the modest increase could also be due to a constitutional genetic instability, contributing to both the risk of developing cancer in younger age and having a child with a birth defect. This hypothesis is partially supported by Zhu et al, investigating whether parents of children with congenital malformations had an increased risk of developing cancer after birth of the child. In general, no such risk was found, however parents to children with cleft lip/palate had a higher risk of developing cancer (Zhu et al., 2002).

In a clinical perspective the modest increase seen, with a malformation rate of 3.7 %, should be interpreted with caution. On an individual basis the findings must be considered as reassuring, especially since IVF and ICSI do not seem to constitute a particular risk for these men. On the other hand, our study raises several questions needed to be addressed, such as the possibly increased risk for childhood vs. adult cancer survivors.

Furthermore, by gaining access to treatment data, the potential contributions of cancer disease per se and different treatment modalities can be further elucidated.

General Conclusions

In my thesis fertility and possible implications for the offspring has been investigated for male cancer survivors. In papers I-III sperm DNA integrity was analyzed in relation to both cancer disease *per se* and its treatment. Although the conclusions of papers I and II in certain matters differ from the results in paper III, the main findings are in concordance. Sperm chromatin quality is impaired in cryopreserved semen from cancer patients, but treatment does not cause any further, persisting DNA damage.

However, although the increase in DFI pretreatment in general was moderate, testicular cancer patients are at an increased risk of having severe DNA damage with subsequent reduced fertility. But, since ICSI is the method of choice in case of male infertility, especially in cases with impaired DNA integrity, and data on ICSI children, as discussed earlier, are fairly reassuring, our data do not discourage from the use of cryopreserved semen.

The fact that we found no persisting negative effect of treatment on sperm DNA integrity is supported by several other studies, including reports on sperm aneuploidy rates in men treated for TGCC, HL and NHL (Robbins et al., 1997; Tempest et al., 2008; Thomas et al., 2004) and smaller studies on sperm DNA integrity in childhood cancer survivors (Thomson et al., 2002; van Beek et al., 2007) and TGCC patients (Spermon et al., 2006). Thus, in terms of sperm DNA, there are no indications that cancer treatment poses a risk for the offspring. Furthermore, even though treatment data was not available in paper IV, we have no indications that the moderate increase in malformation rate observed was due to cancer therapy. However, it should be kept in mind that sperm mutagenicity probably is drug specific and that the knowledge, especially in terms of severer DNA damage such as chromosomal abnormalities, is still limited. Another issue not fully addressed is the potential risks of conceiving during or shortly after the cessation of oncological treatment, a risk strongly suggested by numerous animal studies. In paper IV the risk of malformations was not higher among

children conceived shortly after the father's diagnosis than in children born > 2 years after diagnosis. However, since treatment temporarily reduces fertility, the men conceiving shortly after diagnosis were probably less intensely treated. This is supported by the fact that men with skin malignancies, most often treated with surgery only, comprised 30 % of the fathers with children born 1 to 2 years after diagnosis.

The primary aim of study IV was to investigate the risks of ART in male cancer survivors. In the 511 ART children fathered by men with a history of cancer we found no such increased risk. This observation supports the results of papers I-III, that sperm in cancer patients in general do not carry extensive DNA damage.

Whether there is a difference in the risk of transmittance and penetration of defect DNA to the offspring between iatrogenic sperm DNA damage, due to oncological treatment and endogenous DNA damage caused by cancer disease and other health conditions, is unknown. If both pre- and posttreatment sperm are available, our data do not provide a general guidance as to what semen to use for ART. In paper IV we had no possibility of addressing this issue, since the source of semen used for ART, pre- or posttreatment, was insufficiently known.

In terms of sperm DNA integrity, DFI remained unaffected by therapy, i.e. slightly elevated compared to fertile controls apart from the decreased DFI seen after intense CT in TGCC patients. However, since abdominal radiotherapy induced transient sperm chromatin damage and there are several studies indicating that chemotherapy can induce transient severe sperm DNA injury (De Mas et al., 2001; Frias et al., 2003; Martin et al., 1999), time after treatment must be considered when discussing ART with these men. Ideally, the choice of semen source should therefore be determined by an individual evaluation, by comparing pre- and posttreatment sperm quality, if possible including sperm DNA quality.

Future Perspectives

The two major projects of my thesis, the TGCC study and the register study are still ongoing projects.

The TGCC study closed for inclusion in July 2006 and by then more than 300 patients had been included, both from Lund and Stockholm. The work on sperm DNA integrity so far has been an interim analysis on 96 patients. Thus, the TGCC study will, at its final closure in 2011, allow a further detailed analysis of the impact of TGCC and its treatment on sperm DNA integrity. Furthermore, several other analyses have been performed in these patients, including the study of variations, polymorphisms, of genes involved in sexual development and spermatogenesis. A longitudinal analysis of the impact of treatment on sperm DNA integrity, taking genetic variations into account, could therefore improve our knowledge regarding the susceptibility to germ cell toxicants. Such knowledge would help us to understand the great interindividual difference in germ cell toxicity that exists, thereby enabling a more individualized estimation of the individual patient's future fertility.

The Danish-Swedish register study has several issues left to address.

Even though our data do not indicate any specific risks with ART for male cancer survivors, further analyses are required. The work of collecting treatment data on the IVF/ICSI fathers has already begun. Furthermore, the semen source, cryopreserved pretreatment or fresh posttreatment, used for IVF/ICSI procedures, need to be fully known and linked to treatment data in order to fully appreciate the potential risks of ART.

With the current data available we hypothesize that the increased malformation rate among MCS is rather due to a common tendency to develop cancer in young age and to have a malformed child. To further strengthen this hypothesis treatment data need to be collected, at least for a chosen subpopulation of the cancer fathers. Also, the possibly further

increased risk for childhood cancer survivors warrants further investigation. In a first step it thus seems appropriate to collect treatment data for this group of men and correlate treatment to birth outcomes.

The unique properties of the national registries in Denmark and Sweden have enabled the creation of the database used in paper IV, and much further work is planned. We aim to look at later childhood health outcomes, such as childhood cancers, hospitalizations, other specific diseases/illnesses in relation to paternal cancer and mode of conception. Furthermore, to fully address the potential health hazards for the children to cancer survivors, a prolongation of the observation time would be of importance.

Finally, my thesis lacks a gender perspective, even though male fertility is of importance not only for males. By matching the mothers to the national cancer registries, as was performed with the fathers, equivalent analyses to those performed and planned for male cancer survivors can be done for female cancer survivors as well.

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Original publications

I

The Impact of Testicular Carcinoma and its Treatment on Sperm DNA Integrity

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BACKGROUND. In patients with testicular germ cell carcinoma (TGCC), spermatogenesis and fertility are impaired. Intracytoplasmic sperm injection has improved their possibility of fatherhood, but might also impose a risk of transmitting DNA defects to the offspring. The aim of the current study was to evaluate the impact of chemotherapy and irradiation on sperm DNA integrity.

METHODS. The study included 74 patients with TGCC. Semen samples were collected before and at specific time points after patients received therapy. Sperm DNA integrity was assessed by the sperm chromatin structure assay. Controls comprised 278 military conscripts.

RESULTS. There was no significant difference in the fraction of sperm with fragmented DNA (DNA fragmentation index [DFI]) between controls and patients with TGCC before postoperative cancer treatment (11% vs. 13%). Men treated with adjuvant radiotherapy had a transiently (up to 2 years) higher DFI than nontreated patients (18% vs. 13%; $P = 0.03$). Patients who received 1–2 cycles of adjuvant chemotherapy had a significantly lower DFI 6 months after treatment than after 1–2 years (9.1% vs. 13%; $P = 0.004$). Higher doses of chemotherapy among patients resulted in a significantly lower DFI compared with controls (7.3% vs. 11%; $P = 0.028$), which persisted throughout the 5 years of follow-up.

CONCLUSIONS. Postorchiectomy, the DFI in sperm samples from patients with testicular carcinoma was at the level of controls. Radiotherapy caused a transient increase in the proportion of DFI, whereas this value decreased after chemotherapy. The biologic implications of such changes in sperm DNA after cancer therapy need to be elucidated. ***Cancer* 2004;100:1137–44.**

KEYWORDS: testicular germ cell carcinoma, sperm DNA integrity, chemotherapy, irradiation.

Testicular germ cell carcinoma (TGCC) is the most common malignant disease among young men, and the incidence is increasing.[1] In view of the excellent prognosis, with a cure rate surpassing 95%,[2] the clinical challenge of today lies in minimizing the long-term effects of the treatment. Studies have shown that the future possibility of parenthood is of major concern in the constantly growing population of young survivors of cancer.[3]

From the fertility point of view, patients with TGCC represent a particular challenge. Not only is the reproductive function affected by the treatment given (i.e., radiotherapy (RT) and chemotherapy) but TGCC is also known to be associated with male sub/infertility[4] and undescended testis, all of which are considered to be a part of the testicular dysgenesis syndrome.[5] Therefore, until recently, these patients were at high risk of not being able to have offspring because their semen, even before treatment, generally is of poor quality.

The introduction of intracytoplasmic sperm injection (ICSI) has significantly improved the possibility of patients with TGCC to achieve fatherhood. However, there is concern that surpassing the biologic control system with ICSI may increase the risk of transmitting a defective paternal genome to the offspring.

The mutagenic effects of both RT and chemotherapy are well known from animal studies.[6,7] In humans, a number of studies have shown an increase in the number of both autosomal and sex chromosome aneuploidy in sperm samples after irradiation and chemotherapy, but data suggest that these changes are transient.[8–12] Less is known regarding more discrete sperm DNA damage, which theoretically do not interfere with the process of fertilization and might be transmitted to the offspring. Studies of patients with TGCC indicated that sperm DNA might be damaged postorchiectomy, even before irradiation and chemotherapy.[13–15] However, whether irradiation or chemotherapy further affects sperm DNA integrity has not been reported.

We have, therefore, asked the following questions. Is TGCC as such associated with impairment of sperm DNA integrity? Can the latter be deteriorated by chemotherapy or irradiation? Finally, we investigated the persistence of the eventual changes in sperm DNA caused by cancer treatment.

MATERIALS AND METHODS

Materials

The current study is a part of a longitudinal survey of reproductive function in males treated for TGCC. The study was initiated on March 1, 2001. All men with TGCC who were age < 50 years and who were treated < 5 years before inclusion were eligible for the study. Six fixed time points for delivery of semen samples were defined: T_0: after orchiectomy but before chemotherapy or RT; T_6, T_{12}, T_{24}, T_{36} and T_{60}: 6 months, 12 months, 24 months, 36 months, and 60 months after completion of treatment. Patients could enter the study at any time between T_0 and T_{60} and were asked to deliver semen samples at the remaining time points.

Until April 1, 2003, 135 patients eligible for the study had come for control or treatment at the outpatient clinic of the Department of Oncology, Lund University Hospital (Lund, Sweden). Twenty-four patients who did not differ in age or diagnosis from the included patients chose not to participate, whereas 11 patients were excluded due to various reasons (bilateral disease, psychologic and psychiatric reasons, hepatitis C virus infection, or linguistic problems). Of the 100 patients included in the survey, 1 died of progressive disease and 1 was lost during follow-up. Of the remaining 98 men, 24 were excluded from further analysis due to azoospermia ($n = 11$), retrograde ejaculation ($n = 11$), or too low sperm counts to allow the analysis of sperm integrity ($n = 2$). For the remaining 74 men, at least one semen sample was analyzed.

TABLE 1
Clinical Data Regarding 74 Patients with Testicular Carcinoma Included in the Current Study[a]

Characteristics	No adjuvant therapy ($n = 6$)	ACT ($n = 30$)	HCT ($n = 18$)	RT ($n = 20$)	Total ($N = 74$)
Median age (yrs)	25	28	27	36	29
SGCT	0	0	6	20	26
NSGCT	6	30	12	0	48
Clinical stage[b]					
I	6	30	5	20	61
II	0	0	8	0	8
III	0	0	3	0	3
IV	0	0	2	0	2

ACT: one to two cycles of adjuvant chemotherapy; HCT: more than two cycles of chemotherapy; RT: radiotherapy; SGCT: seminomatous germ cell tumors; NSGCT: nonseminomatous germ cell tumors; BEP: bleomycin, etoposide, and cisplatin; EP: etoposide and cisplatin.

[a] Orchiectomy was performed in all patients.

[b] According to the Royal Marsden Hospital staging system for testicular carcinoma,[2] Chemotherapy for NSGCT included the BEP regimen and treatment for SGCT included the EP regimen.

The background characteristics of the study population are given in Table 1. The patients were treated within the SWENOTECA protocol (a Swedish Norwegian Testicular Cancer collaborative cancer care program). Patients with nonseminomatous germ cell tumors (NSGCT) were treated with the BEP regimen (90,000 IU of bleomycin, 500 mg/m^2 of etoposide, and 100 mg/m^2 of cisplatin per cycle, administered during 5 days) or a similar cisplatin-based regimen. Bleomycin was given to a maximum dose of 3×10^5 IU. Patients with seminomatous germ cell tumors (SGCT) who were receiving chemotherapy were treated with EP (BEP minus bleomycin). The interval between chemotherapy cycles was 3 weeks.

Adjuvant RT was given to a total absorbed dose of 25.2 grays (Gy) in 14 fractions to the clinical target volume of the paraaortic and ipsilateral iliac lymph nodes. The dose to the remaining lead-shielded testicle was measured at the start of the treatment. Based on a retrospective calculation of seven randomly selected patients in the study, the total scattered dose to the remaining testicle was estimated not to exceed 0.5 Gy.

The patients were allocated to groups according to the treatment they received. In Group a, patients received surgery only (SO). This group included six patients with Stage I disease (Royal Marsden Hospital staging system[2]) who did not receive adjuvant therapy

and 14 men from Groups b–d who were assessed after orchiectomy, before further treatment was received. Group b included 30 patients with Stage I NSGCT who received 1–2 cycles of adjuvant chemotherapy (ACT): (1 cycle of BEP, $n = 24$; 1 cycle of CVB [etoposide replaced by vinblastin at a dose of 0.3 mg/kg, maximum 22 mg per cycle]), $n = 2$; 2 cycles of CVB, $n = 3$; one cycle of JEB, cisplatin replaced with carboplatin, $n = 1$). Group c was comprised of 18 patients with disseminated disease who received > 2 cycles of chemotherapy (HCT; 3 cycles of BEP, $n = 7$; 4 cycles of BEP, $n = 4$; four cycles of EP, $n = 6$; more intensive chemotherapy, $n = 1$). Finally, Group d was comprised of 20 patients with Stage I SGCT who received adjuvant RT. The control group included 278 military conscripts, the data on whom were published in an earlier study.[16]

Written informed consent was obtained from all men who participated in the study according to protocols approved by the ethical review board of Lund University.

Semen Analysis

Fresh sperm samples were collected in plastic jars and analyzed within 1 hour. Sperm concentration was assessed according to the 1999 World Health Organization guidelines.[17] An aliquot of the semen sample was stored at −80 °C for the subsequent flow cytometric sperm chromatin structure assay (SCSA) analysis.

Sperm Chromatin Structure Assay

The SCSA analysis is based on the phenomenon that chromatin with abundant double DNA breaks has a tendency to denaturate when exposed to acid-detergent, whereas normal chromatin remains stable. Acridin Orange (AO) stains the native double-stranded DNA and the single-stranded nucleic acids. In excitation of blue light, the intact DNA emits green fluorescence, whereas the denatured DNA emits red fluorescence.[18] The extent of DNA denaturation is expressed as the DNA fragmentation index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity. DFI expresses the percentage of cells containing denatured DNA.[19] Five thousand cells were analyzed by FACSort (Becton Dickinson, San Jose, CA). The analysis was performed as previously described[16,19] with minor adjustment of the time of incubation with AO, which lasted ≥ 3 minutes. This procedure has been checked by an external quality control procedure[20] against another laboratory (ENEA, Rome), strictly adhering to the protocol developed by Evenson et al.[19] A comparison of the DFI measurement procedures performed in the two laboratories revealed a high level of correlation (r) ($r = 0.8$; $P < 0.0005$). The mean ratio between the Rome and Malmö values was 1.01 (standard deviation [SD] = 1.05). The DFI was calculated using List View software (Phoenix Flow Systems, San Diego, CA).

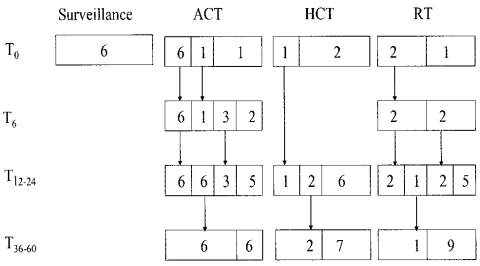

FIGURE 1. Flow chart illustrating 106 semen samples, delivered by 74 patients with transitional germ cell carcinoma at different time points. T_0: before postoperative cancer treatment; T_6 ,T_{12-24} ,T_{36-60} : 6 months, 12–24 months, and 36–60 months after completion of treatment. The patients are divided according to treatment surveillance. ACT: patients received one to two cycles of adjuvant chemotherapy; HCT: patients received more than two cycles of chemotherapy; RT: patients treated with radiotherapy. The arrows connect samples delivered by the same patients.

Statistical Analysis

Statistical analysis was performed using SPSS 11.0 software (SPSS, Chicago, IL). The results of samples collected at T_{12} and T_{24} were combined into one category (T_{12-24}) to obtain sufficient numbers of individuals in each group. The same was done for the observations at T_{36} and T_{60} (T_{36-60}). Finally, a joint category of samples delivered between 6 months and 5 years after treatment was created to compare the posttreatment DNA integrity between the different groups and in relation to the controls. If any patient delivered more than one sample in any of the joined time intervals, the mean value was included in the analysis.

The group descriptive values were expressed as medians and ranges. The data were mainly treated in a cross-sectional manner (Mann–Whitney U test). There were 10 intergroup comparisons of the posttreatment DFI values for the different therapy forms. Furthermore, when comparing the DFI values at different time points for each of the three therapy forms, 18 comparisons were performed.

For patients who received one to two doses of chemotherapy, a sufficient number of patients were followed (Fig. 1) with repeated semen samples to allow a longitudinal analysis (Friedman's test). Spearman's rho was calculated to assess the correlation between the DFI value and the sperm concentration

TABLE 2
DFI in Patients with Testicular Carcinoma in Relation to Treatment

Characteristics	No. of patients	Median DFI (range)	Control group	SO	ACT	HCT	RT
Control group	278	11 (1.7–62)		NS	NS	$P = 0.028$	$P = 0.001$
SO	20	13 (4.8–40)	NS		NS	$P = 0.018$	$P = 0.033$
ACT	29	11 (5.4–29)	NS	NS		$P = 0.015$	$P = 0.008$
HCT	16	7.3 (4.0–21)	$P = 0.028$	$P = 0.018$	$P = 0.015$		$P < 0.0005$
RT	19	18 (9.5–64)	$P = 0.001$	$P = 0.033$	$P = 0.008$	$P < 0.0005$	

DFI: DNA fragmentation index; ACT: one to two cycles of adjuvant chemotherapy; HCT: more than two cycles of chemotherapy; RT: radiotherapy; NS: not significant; SO: surgery only.

TABLE 3
DFI and Sperm Concentration in Relation to Treatment and Follow-Up Time in Patients with TGCC[a]

Characteristics	Control group	SO	ACT			HCT			RT		
		T_{0-60}	T_6	T_{12-24}	T_{36-60}	T_6	T_{12-24}	T_{36-60}	T_6	T_{12-24}	T_{36-60}
No. of patients	278	20	12	20	12	0	9	9	4	10	10
Median sperm concentration (10^6/mL) (range)	53 (0–390)	23[e] (0.6–94)	14 (3–32)	27 (0.8–77)	31 (3.8–100)	—	3.9 (0.9–58)	23 (5.9–92)	3.0[f] (0.1–13)	12 (4–63)	27 (0.9–210)
Percent oligozoospermia (95% CI)	16 (12–21)	40 (19–64)	75 (40–93)	30 (12–54)	25 (5.5–57)	—	89 (52–100)	44 (14–79)	100 (40–100)	80 (44–97)	30 (6.7–65)
Median DFI (range)	11 (1.7–62)	13 (5.3–40)	9.1[b] (5.4–17)	13[b] (5.9–35)	14 (7.6–29)	—	8.1 (4.1–27)	7.3[d] (4.0–21)	18 (8.0–58)	18[c] (9.5–39)	13 (5.4–64)

DFI: DNA fragmentation index; TGCC: testicular germ cell carcinoma; SO: surgery only; ACT: one to two cycles of adjuvant chemotherapy; HCT: more than two cycles of chemotherapy; RT: radiotherapy; CI: confidence interval.

[a] Oligozoospermia is defined as sperm concentration < 20 million per milliliter.

[b] $P = 0.004$ for comparison between T_6 and T_{12-24}.

[c] $P = 0.049$ for comparison to surgery only.

[d] $P = 0.049$ for comparison to surgery only.

[e] $P < 0.005$ for comparison to controls.

[f] $P = 0.029$ for comparison to surgery only.

as well as the age of the men. The Fisher exact test was used to make an intergroup comparison of the proportion of men with DFI ≥ 27%. This level was previously suggested to be associated with an increased risk of early embryonal loss.[21]

RESULTS

The results of the current study are presented in Tables 2 and 3 and Figure 2.

Sperm Chromatin Structure Assay

The DFI value in the SO group (n=20) did not differ in comparison to controls (n = 278): 13% and 11% (P = 0.35), respectively. When pooling the data from all posttreatment samples, RT patients (n = 19) showed a higher DFI than both chemotherapy groups: RT versus ACT, 18% versus 11% (P = 0.008); and RT versus HCT, 18% versus 7.3% ($P < 0.0005$). The RT group was also found to have significantly higher DFI values than the SO group (P = 0.03) and the controls (P = 0.001). Thirty-two % of patients receiving RT had a DFI value ≥ 27%; compared with 8 % of the controls (P = 0.005). The DFI level in the RT group was, in relation to the SO group and the controls, normalized after 3–5 years of follow-up.

A time-dependent difference was observed in the cross-sectional analysis of the ACT patients. The DFI at T_6 was significantly lower than at T_{12-24}, the median values being 9.1% versus 13% (P = 0.004). It was also lower than at T_0 (the median values being 9.1% vs. 13%) and at T_{36-60} (the median values being 9.1% vs. 14%). The differences did not reach the level of statistical significance (P = 0.08 and 0.06, respectively). In the same treatment group, six patients were followed longitudinally from T_0 to T_{12}. A significant variation (P = 0.03) with a decrease at T_6 (8.7% vs. 12% at T_0 and

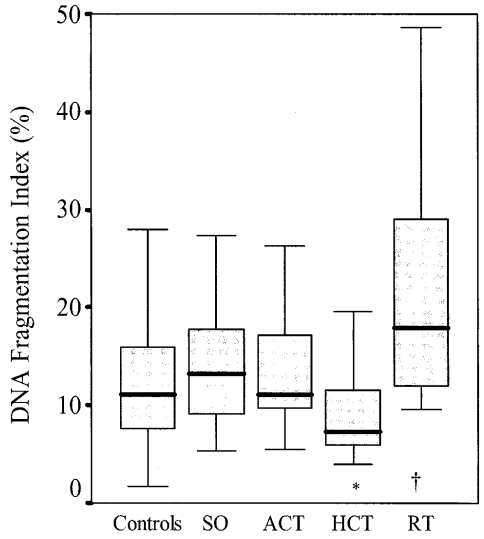

FIGURE 2. Box and whisker plot showing DNA fragmentation index in controls and different categories of patients treated for testicular carcinoma. SO: men treated with orchiectomy only; ACT: patients received one to two cycles of adjuvant chemotherapy; HCT: patients received three or more cycles of chemotherapy; RT: radiotherapy. Bars indicate median values. Boxes represent interquartile intervals and whiskers represent the 95% confidence intervals. *HCT < SO, $P = 0.018$, HCT < controls, $P = 0.028$. †RT > SO, $P = 0.033$, RT > controls, $P = 0.001$.

13% at T_{12}) was found. HCT patients ($n = 16$) had a lower DFI than controls (7.3% vs. 11%; P=0.028), SO patients (7.3% vs. 13%; $P = 0.018$), and ACT patients ($n = 29$), 7.3% versus 11% ($P = 0.015$). The decrease in DFI in the HCT group remained over time.

Sperm Concentration

Table 3 shows the values, according to the therapy and follow-up time, as well as the percentage of patients presenting with sperm concentrations $< 20 \times 10^6$/mL. No correlation was found between sperm concentration and DFI or between age and DFI in men who were treated with SO.

DISCUSSION

In a prospective study on sperm chromatin structure in patients with TGCC, the DFI before RT or chemotherapy was equal to that of the military conscripts in the control group. In the first 2 years after adjuvant irradiation, a significant increase in the DFI was found. Chemotherapy caused a decrease in the DFI value. In patients treated with 1–2 cycles of adjuvant chemotherapy, a transient reduction in the DFI value was observed 6 months after treatment. In more heavily treated patients, a permanent decrease in the DFI value was found during the follow-up time of 5 years.

To our knowledge, the current study represents the largest yet to address the issue of sperm DNA integrity in patients with cancer.

For the statistical analysis, many intergroup comparisons were performed. The question arises: Are the statistically significant findings reported in the current study chance findings? For example, 1 of 20 statistical tests is expected to give a P value < 0.05. However, of the 28 tests performed, 10 resulted in a P value below the level of statistical significance, making the possibility of mass significance less probable.

The risk of damage to sperm DNA due to cancer or its treatment is a source of potential worry. An increased risk of genetic aberrations in children fathered by cancer survivors has not been shown.[22,23] However, these reports are often based on rather small series. Furthermore, the majority of large studies represent a follow-up of survivors of pediatric cancer. It can be assumed that the sensitivity to DNA damage of quietly active prepubertal germ cells is less pronounced than in the proliferating postpubertal cells of spermatogenesis. Finally, these surveys were based on conceptions that had occurred naturally. The introduction of ICSI in the treatment of infertility due to low sperm count does suggest a risk of bypassing the biologic selection mechanisms and of transferring genetic damage from the sperm to the embryo.[24]

The finding that patients with TGCC, postorchiectomy and before any further treatment, did not differ from a control group in DFI is in disagreement with previous results. Three studies have reported significantly elevated DFI in comparison to controls.[13–15] However, these studies were based on relatively small sample sizes and used sperm donors or proven fertile men, known to have a superior semen quality, as controls. Because some of the conscripts in the control group might be potentially subfertile, we cannot exclude that the DFI values in the postorchiectomy group are higher than the DFI level of fertile men but correspond to that of the general population of young males.

Chemotherapy and irradiation damage DNA. However, to our knowledge, the extent, duration, and the biologic significance of such an effect on sperm chromatin integrity is not known.

The effect of RT on spermatogenesis is dose dependent and can induce transient or permanent sterility.[25] A recent study in mice,[26] in agreement with

other experiments,[27] has shown a dose-dependent increase in DNA damage in testis cells 14 days after irradiation. This, in turn, produced an increased fraction of sperm with DNA strand breaks 45 days after treatment. DNA damage returned to control levels 100 days after irradiation. The overall results showed that DNA damage induced in premeiotic germ cells is detectable in primary spermatocytes and is still present in mature spermatozoa. In the current study, which confirms the high radiosensitivity of differentiating spermatogonia, two methods were used to evaluate DNA damage. SCSA and COMET gave comparable results, emphasizing the correlation between DNA fragmentation and susceptibility of sperm chromatin to denaturation. Other animal studies have shown that pregnancies resulting from mating with an irradiated male are associated with impaired fetal development, pregnancy loss, abnormal somatic development, and tumor induction in the fetus.[7] Genetic instability was detected in children whose fathers previously had been exposed to irradiation,[28] but no mutagenic effect in humans, either in regards to an increased malformation rate or to tumor induction, was detected.[23,29] However, given the strong evidence from animal studies, the conclusions of human studies should be evaluated with caution.

The DFI was found to be significantly increased in men who received irradiation. The effect is probably due to a scattered radiation of the remaining testicle, which was retrospectively estimated not to be > 0.5 Gy in seven randomly selected patients. The biologic implications of the moderate increase in DFI level are not known. In the current study, the median value of DFI of 18% 2 years after irradiation is far below the 30–40% previously reported as the threshold of infertility in vivo.[30] However, sperm concentrations below the level of normozoospermia were found in all men 6 months after irradiation and in 80% of the patients 1–2 years after treatment. This means that a great proportion of such patients are possible candidates for ICSI. More than 30% of patients receiving RT had DFI values $\geq$ 27%. This level of DFI is associated with an increased risk of an early embryonic loss.[21] Furthermore, the increased median DFI might be a general indicator of more profound sperm DNA damage.

The potential risk of using sperm samples with postirradiation DNA damage for the most advanced techniques of assisted reproduction is stressed by results of studies using the human sperm-hamster oocyte technique. In such models, the fertilization ability remained despite radiation-induced sperm DNA damage. The risk of transmitting defective DNA to the offspring is apparent.[31]

Only men with SGCT received irradiation. These men were somewhat older than the men with NSGCT. However, in men who received orchiectomy only, we found no association between the age of the patient and the DFI. Furthermore, a normalization of DFI levels 3–5 years postirradiation was observed. Therefore, age is not a probable explanation for the higher level of sperm DNA strand breaks in the RT group.

The effect on sperm DNA of many chemotherapeutic drugs has been studied in animals.[6,31] Cisplatin, etoposide, and bleomycin have all been shown to induce damage to sperm DNA, acting at different stages of the germ cell cycle.[6] However, it is difficult to translate these findings to the clinical situation. Interspecies diversity in regard to mutagenic effects of chemical compounds can exist.[32] Furthermore, the animal studies were based on monotherapy and used higher doses of drugs than used for humans. These studies also had a follow-up time of days to weeks, not months to years, as applied in the current study. Human-based experience is rather scarce and has mostly been based on numeric chromosomal aberrations as assessed by either fluorescence in situ hybridization (FISH) methods or the human sperm-hamster oocyte test.[9] The FISH studies indicate a transient effect on the ploidy of sperm cells, with chromosome abnormalities normalized within 1–2 years.[10–12,33] However, with the hamster oocyte technique, more permanent chromosomal aberrations were observed,[9] although the studies are few, and based on a very limited number of patients.

The finding of a decrease in DFI after chemotherapy is somewhat surprising. Available literature does not offer an obvious explanation. The suppression of apoptosis and subsequent accumulation of genetically damaged cells has been suggested as one possible mechanism behind cancer treatment-induced sperm DNA damage.[7] Cisplatin has been shown to increase germ cell apoptosis.[34] Spermatogenic cells with abnormal DNA might be more susceptible to such an effect, thus being eliminated when exposed to cisplatin. The extent and rapidity of postchemotherapy regeneration of spermatogenesis are dose dependent.[35] This might explain the transient decrease in DFI after low-dose chemotherapy and a more prolonged effect after more intense cytotoxic treatment.

The finding that chemotherapy was not associated with an increase in the level of DFI damage is in accordance with a recent study on survivors of pediatric cancer that also addressed the issue of sperm DNA integrity.[36] In the current study, using the transferase-mediated dUTP nick-end labelling (TUNEL) method, no increase in indices of sperm DNA damage was found in cancer survivors compared with controls. Conversely, using the single cell microgel elec-

trophoresis (COMET) assay, Chatterjee et al.[37] found a transient increase in the proportion of sperm samples with abnormal DNA after treatment for chronic lymphatic leukemia. However, that study was based on one patient only and a different cytotoxic drug, fludarabine, was administered. It cannot be excluded that different mixtures of chemotherapeutic agents may have diverging effects on sperm DNA.

By using SCSA for evaluation of sperm integrity, we were able to evaluate a large number of controls and patients receiving different cancer treatments. DFI is an indirect indicator of DNA damage[19] but has shown to be closely correlated to the results of methods (i.e., COMET and TUNEL) giving a more direct measurement of DNA strand breaks.[38,39]

In the current study, no increase in sperm DNA injury was observed in patients with TGCC before treatment, irradiation, or chemotherapy. This indicates that the use of cryopreserved sperm, which is collected before treatment, constitutes no risk of transmitting defect DNA. We also found that scattered irradiation appeared to transiently impair sperm DNA integrity, which normalized 3–5 years after treatment. These observations should be taken into consideration when discussing the safety of ICSI and the use of pretreatment versus posttreatment sperm samples from men who have been exposed to RT. Finally, a decrease in the DFI was found in patients treated with chemotherapy. It remains to be elucidated whether this finding represents a real postchemotherapy removal of an abnormal germ cell subpopulation or whether the ejaculated sperm might be associated with other types of DNA damage not detectable by the SCSA method. In addition, our results call for further investigations of sperm DNA in patients with cancer using other methods of assessment of sperm DNA damage and including patients receiving other types of cancer therapy.

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Sperm DNA integrity in testicular cancer patients

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BACKGROUND: We evaluated the impact of testicular germ cell cancer (TGCC), its treatment and length of follow-up on sperm DNA integrity. METHODS: In 96 TGCC patients, semen was collected at specific intervals until 5 years after treatment. Sperm DNA integrity was assessed by the sperm chromatin structure assay (SCSA, *n* = 193) and by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL, *n* = 159) assay. Results were expressed as DNA fragmentation index (DFI). Controls comprised of 278 military conscripts. RESULTS: Post-surgery testicular cancer (TC) patients did not differ from controls. Compared with pretreatment values, radiotherapy induced a transient increase in $SCSA_{DFI}$ (medians: 12 versus 19%; *P* = 0.03), normalizing after 3–5 years. One year or more after therapy, 5/13 (38%) of normozoospermic, irradiated patients had $SCSA_{DFI}$ >27% compared with 7% of normozoospermic controls (*P* = 0.002). More than two cycles of chemotherapy decreased DFI 3–5 years post-therapy (median $SCSA_{DFI}$: 12 versus 9.1%, *P* = 0.02; median $TUNEL_{DFI}$: 11 versus 7.5%, *P* = 0.03). CONCLUSION: Irradiation increases sperm DNA damage 1–2 years after treatment, and 38% of irradiated patients with normozoospermia had high (>27%) DNA damage, which may affect the sperm-fertilizing ability. TC *per se* is not associated with an increase of DFI, and DFI is reduced by three or more cycles of chemotherapy.

Key words: chemotherapy/radiotherapy/SCSA/sperm DNA/testicular cancer/TUNEL

Introduction

Testicular germ cell cancer (TGCC) is the most frequent malignant disease in young men. With adequate treatment, 90–95% of the patients are cured (Schmoll *et al*., 2004). The excellent cure rate puts focus on long-term effects of the treatment, such as fertility preservation.

Sperm concentration is negatively affected by cancer treatment, the extent depending on both type of therapy and dosage (Eberhard *et al*., 2004). However, with standard TGCC treatment, the impairment is transient and pretreatment values are regained within 5 years.

Cancer treatment is potentially mutagenic, and animal studies have shown sperm DNA injury following exposure to chemotherapy or radiotherapy (Witt and Bishop, 1996; Brinkworth, 2000). Less is known regarding the effect of cancer treatment on human spermatozoa. An increased proportion of aneuploid spermatozoa following chemotherapy were reported, but the effect seems to be transient (Martin *et al*., 1997; Frias *et al*., 2003; Thomas *et al*., 2004). However, it cannot be excluded that more discrete changes in the sperm genome can be more persistent. Such phenomenon could have serious implications. Despite the recovery of spermatogenesis, the fertilizing capacity of the spermatozoa might be seriously impaired. Furthermore, new powerful assisted reproduction techniques (ART) such as intracytoplasmic sperm injection (ICSI) reduce the demands of sperm quality in terms of concentration, motility, morphology and DNA integrity (Morris, 2002). A potential worry with ICSI is that by surpassing normal biological control mechanisms in fertilization, there is a risk of transmitting defect paternal DNA to the offspring. Fertility in TGCC patients is reduced even before treatment (Petersen *et al*., 1998). Hence, these patients can be expected to benefit from ICSI, thereby being at risk of transmitting therapy-induced DNA damage to the offspring.

Studies have shown that the fertilizing capacity of the spermatozoa is also dependent on the integrity of their DNA (Larson *et al*., 2001; Carrell *et al*., 2003; Bungum *et al*., 2004). Several methods for assessment of sperm DNA breaks exist, and the sperm chromatin structure assay (SCSA) is the method mostly used for clinical purposes. Studies indicated a serious impairment of fertilization *in vivo* when the SCSA DNA fragmentation index (DFI) exceeds the level of 27–30% (Larson *et al*., 2001; Bungum *et al*., 2004). Nevertheless, SCSA has been regarded as an indirect method for the assesment of sperm DNA fragmentation as it relies on the assumption that DNA denaturability mirrors the presence of DNA strand breaks. There are other more direct tests available to measure the level

of sperm DNA breaks, such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL).

We have previously reported preliminary results on the effect of different TGCC treatment modalities on sperm DNA integrity, as assessed by SCSA (Stahl *et al*., 2004). Radiotherapy induced a transient increase of $SCSA_{DFI}$, the values normalizing after 3–5 years. Chemotherapy induced a decrease in $SCSA_{DFI}$ which, with more than two cycles of chemotherapy treatment, was observed throughout the 5 years of follow-up. In the present study, we performed both SCSA and TUNEL analysis on an extended number of ejaculates to detect the level of sperm DNA fragmentation with two independent sperm DNA integrity approaches.

Our aim was to investigate cancer therapy-induced changes in sperm DNA integrity to improve the prediction of fertility in TGCC patients. In addition, we aimed at assessing the potential risk of using sperm from men treated for cancer for ART.

Patients and methods

Patients

This study is a part of a longitudinal survey of reproductive function in males treated for TGCC, initiated in 2001. All men with TGCC, below the age of 50, diagnosed <5 years before inclusion, were eligible for the study. Fixed time intervals for delivery of semen samples were defined: T_0—after orchidectomy but before further therapy; T_6, T_{12}, T_{24}, T_{36} and T_{60}—6 to 60 months, respectively, after completed treatment. Patients entered the study at any time between T_0 and T_{60} and delivered samples at the remaining intervals.

In November 2004, 178 eligible patients had passed through the Department of Oncology, Lund University Hospital, Lund, Sweden. Twenty-five patients, not differing from the included patients in the distribution of age, histological subtype or tumor stage, refused to participate, whereas 25 patients were excluded for various reasons (bilateral disease, psychological, psychiatric reasons, hepatitis C or linguistic problems). Of the 128 patients included in the survey, one died, and four were lost during follow-up. Of the remaining 123 men, 27 were excluded from sperm analysis because of azoospermia ($n = 14$), retrograde ejaculation ($n = 11$) and development of contralateral disease after inclusion ($n = 2$). In the remaining 96 men, at least one semen sample was analysed.

All men participated with written informed consent according to protocols approved by the ethical review board of Lund University.

For SCSA analysis, 278 military conscripts, with a mean age of 18 years, served as controls. Data on the conscripts have been published previously (Richthoff *et al*., 2002). Twenty-four of these men were randomly selected as controls for TUNEL analysis.

Cancer treatment

The patients were treated according to the SWENOTECA protocol (Klepp *et al*., 1997; Laguna *et al*., 2001), the Swedish-Norwegian Testicular Cancer collaborative cancer care program (www.ocsyd.lu.se). Patients with nonseminomatous germ cell cancer (NSGCT) were treated with the BEP regimen (bleomycin 90 000 IU; days 1, 8, 15, to a maximum dose of 3×10^5 IU; etoposide 500 mg/m^2 and cisplatin 100 mg/m^2 per cycle, both given days 1–5, with a 3-week interval) or a similar regimen. Patients with seminomatous germ cell cancer (SGCT) were treated with EP (BEP minus bleomycin) or BEP.

The adjuvant radiotherapy (RT) was administered to a total absorbed dose of 25.2 Gy in 14 fractions to the clinical target volume of infradiaphragmal para-aortic and ipsilateral iliac lymph nodes. The dose to the remaining, lead-shielded testicle was measured at the start of the treatment. On the basis of a retrospective calculation of seven randomly selected patients in the study, the total dose to the remaining testicle was estimated not to exceed 0.5 Gy (range 0.04–0.43).

The patients were allocated into groups according to treatment given (for patient characteristics, see Table I):

(i) Surgery only (SO); nine patients with stage 1 disease, receiving no adjuvant therapy and 16 men from groups ii–iv assessed after orchidectomy, before further treatment.

(ii) Thirty-three patients with NSGCT, clinical stage (CS) I [according to the Royal Marsden Hospital staging system (Dearnaley *et al*., 2001)], receiving 1–2 cycles of adjuvant chemotherapy (ACT): [one BEP, $n = 27$; one CVB (etoposide replaced by vinblastin 0.3 mg/kg, maximum 22 mg/cycle), $n = 2$; two CVB, $n = 3$; two JEB, cisplatin replaced with carboplatin, $n = 1$].

(iii) Twenty-three patients with disseminated disease receiving more than two cycles of chemotherapy (CT) (HCT): (three BEP, $n = 8$; four BEP, $n = 10$; four EP, $n = 4$; more intensive CT, $n = 1$).

(iv) Twenty-nine patients with SGCT, CS I, receiving RT.

(v) Two patients with disseminated disease receiving both RT and CT.

In a number of semen samples, the biological material was too sparse to allow both TUNEL and SCSA analyses. A total of 193 samples from 95 of the 96 patients were analysed with SCSA, and 159 samples from 90 of the 96 patients were analysed by TUNEL (Figure 1).

Semen analysis

Fresh semen samples were collected, and within an hour post-ejaculation, sperm concentration was assessed according to WHO guidelines 1999 (World Health Organization, 1999), and the results in both TGCC patients and controls were in accordance with previous reports.

An aliquot was stored at –80°C for the subsequent SCSA and TUNEL analysis.

SCSA

The SCSA analysis is based on the phenomenon that chromatin with abundant DNA strand breaks has a tendency to denaturate when exposed to acid detergent, whereas normal chromatin remains stable. Acridine Orange stains the native double-stranded DNA and the single-stranded nucleic acids, and in excitation of blue light, the intact DNA emits green fluorescence, whereas the denaturated DNA emits red fluorescence. The extent of DNA denaturability is expressed as the DFI, being the ratio of red to total (red plus green) fluorescence intensity. DFI hereby expresses the proportion of cells containing denaturated DNA (Evenson *et al*., 2002). Five thousand cells were analysed by FACSort (Becton Dickinson, San Jose, CA, USA). The analysis was performed as previously described (Stahl *et al*., 2004). An intra-laboratory coefficient of variation (CV) of 4.5% was found.

TUNEL

The TUNEL assay quantifies the incorporation of fluorescently labelled dUTP at breaks in double-stranded DNA, utilizing a reaction catalysed by terminal deoxynucleotidyl transferase. TUNEL positivity in somatic cells reflects apoptosis, but the origin of sperm DNA strand breaks detected by TUNEL remains unclear (Sakkas *et al*., 2002; Perreault *et al*., 2003). The TUNEL analysis was performed according to the manufacturer's (Roche Diagnostics GmbH, Manheim, Germany)

Table I. Clinical data regarding the 96 testicular germ cell cancer patients included in the present study

Treatment	No adjuvant therapy ($n = 9$)	ACT ($n = 33$)	More than 2 cycles of chemotherapy ($n = 23$)	Radiotherapy ($n = 29$)	Chemotherapy and radiotherapy ($n = 2$)	Total ($n = 96$)
Median age (range)	29 (20–41)	29 (16–42)	28 (20–48)	36 (21–47)	48 (46–49)	30 (16–49)
NSGCT	8	33	18			59
SGCT	1		5	29	2	37
Stage I	9	33	6[a]	29	2[a]	79
Stage II			10			10
Stage III			3			3
Stage IV			4			4

ACT, 1–2 cycles of cisplatin-based chemotherapy; NSGCT, non-seminomatous germ cell cancer; SGCT, seminomatous germ cell cancer.
Stage refers to the clinical stage according to the Royal Marsden Hospital Staging System (14).
[a]Presenting with more advanced disease after initial staging.

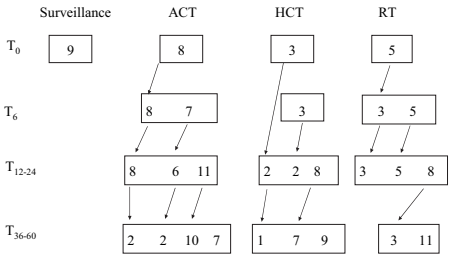

Figure 1. Flow chart illustrating distribution of the 193 semen samples, delivered by 96 TGCC patients at different time-points; T_0: before post-operative cancer treatment; T_6, $T_{12–24}$, $T_{36–60}$: 6, 12–24 and 36–60 months, respectively, post-treatment. The patients were divided according to treatment; surveillance; ACT, 1–2 cycles of chemotherapy; HCT, more than two cycles of chemotherapy; RT, radiotherapy. The arrows connect samples delivered by the same patient. Semen samples were assessed by either SCSA or TUNEL or both. If two samples were delivered by a patient in one time category, $T_{12–24}$ or $T_{36–60}$, it is presented as one sample in the figure.

protocol with slight modifications as previously described (Erenpreiss *et al*., 2004). A total of 10 000 events were accumulated for each measurement and analysed by FACSort (Becton Dickinson) flow cytometer; the same was used for SCSA. The intra-laboratory CV of 8.6% was found.

Statistical analysis

Statistical analysis was performed using the SPSS 11.0 software (SPSS, Chicago, IL, USA). To obtain sufficient numbers of individuals, the results of samples collected at T_{12} and T_{24} were combined into one time category $T_{12–24,}$ and samples collected at T_{36} and T_{60} were combined into $T_{36–60}$. If two samples were delivered by a patient in one time category, the mean value was used in the analysis. The group descriptive values were expressed as medians and ranges. The data were treated in a cross-sectional manner (Mann–Whitney *U*-test). SO (post-surgery) values were compared with those of controls in regard to both DFI and TUNEL. For each therapy, group comparisons between SO and the three different time categories were made, a total of nine comparisons for DFI and TUNEL, respectively.

The proportion of men at $T_{12} – T_{60}$ with $SCSA_{DFI}$ ≥27% was calculated, and for normozoospermic (sperm concentration $\geq 20 \times 10^6$/ml) men, the odds ratio for such high $SCSA_{DFI}$, as compared with controls, was calculated for each therapy group by means of binary logistic regression analysis. If one patient had delivered more than one ejaculate, the first sample was used for analysis.

Spearman's rho was calculated to correlate the results of SCSA and TUNEL.

$P < 0.05$ was considered statistically significant.

Results

Surgery only

Semen from TGCC patients before post-surgery treatment, SO patients, did not differ from that of controls in regard to sperm DNA integrity (Tables II and III and Figures 2 and 3).

Chemotherapy

ACT

Patients receiving ACT did not differ from SO at any time-point, neither in $SCSA_{DFI}$ nor in $TUNEL_{DFI}$ (Tables II and III and Figures 2 and 3).

HCT

Between 1 and 2 years after therapy, $TUNEL_{DFI}$ was significantly lower than in the SO group. $SCSA_{DFI}$ at $T_{12–24}$ was also lower compared to SO, but without reaching statistical significance.

Between 3 and 5 years after therapy, both $TUNEL_{DFI}$ and $SCSA_{DFI}$ were significantly lower than in the SO group (Tables II and III and Figures 2 and 3).

RT

At $T_{12–24}$, $SCSA_{DFI}$ was significantly higher in patients receiving RT than in SO. $TUNEL_{DFI}$ at $T_{12–24}$ was also higher compared with SO, without reaching statistical significance. At $T_{36–60}$, neither $SCSA_{DFI}$ nor $TUNEL_{DFI}$ differed between RT and SO (Tables II and III and Figures 2 and 3).

The data for patients receiving combined therapy were too few to analyse.

Proportion of patients with $SCSA_{DFI}$ >27%

Among the normozoospermic controls, 6.9% had $SCSA_{DFI}$ ≥27%, and in normozoospermic TGCC patients treated by

Table II. $TUNEL_{DFI}$ and sperm concentration in testicular germ cell cancer patients according to treatment and follow-up time

	Control group	SO	ACT			HCT			RT		
		$T_{0–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$
Patients (*n*)	24	19	12	22	19	3	10	17	4	13	13
Median sperm concentration (10^6/ml) (range)	57 (13–320)	21[a] (2.6–86)	21 (5.3–38)	31 (1.0–66)	32 (2.5–104)	0.6 (0.5–1.4)	7.1 (3.5–58)	23 (5.4–92)	4.2 (3.1–13)	25 (5.5–112)	39 (7.5–174)
$TUNEL_{DFI}$ median (range)	11 (2.5–31)	11 (2.7–28)	9.1 (1.7–23)	9.6 (4.1–30)	11 (6.3–22)	3.1 (2.9–17)	7.5[b] (1.2–19)	7.5[c] (4.1–27)	18 (4.5–52)	18 (2.4–44)	11 (4.5–33)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.
[a]$P = 0.001$ in comparison to controls.
[b]$P = 0.03$ in comparison to SO.
[c]$P = 0.02$ in comparison to SO.

Table III. $SCSA_{DFI}$ and sperm concentration in testicular germ cell cancer patients according to treatment and follow-up time

	Control group	SO	ACT			HCT			RT		
		$T_{0–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$	T_6	$T_{12–24}$	$T_{36–60}$
Patients (*n*)	278	25	15	24	21	3	12	17	8	16	14
Median sperm concentration (10^6/ml) (range)	52 (0.5–390)	23[a] (0.6–86)	15 (3.0–38)	29 (1.0–66)	30 (2.5–104)	0.6 (0.5–1.4)	7.8 (3.5–58)	23 (5.4–92)	2.7 (0.1–13)	14 (0.7–112)	34 (7.5–174)
$SCSA_{DFI}$ median(range)	11 (1.7–62)	12 (7.1–72)	10 (5.4–28)	13 (8.0–45)	14 (4.1–29)	18 (16–31)	9.7[b] (4.7–33)	9.1[c] (4.0–34)	16 (8.0–47)	19[d] (7.8–45)	14 (9.7–64)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SCSA, sperm chromatin structure assay; SO, surgery only; SO, surgery only.
[a]$P < 0.001$ in comparison to controls.
[b]$P = 0.09$ in comparison to SO.
[c]$P = 0.02$ in comparison to SO.
[d]$P = 0.03$ in comparison to SO.

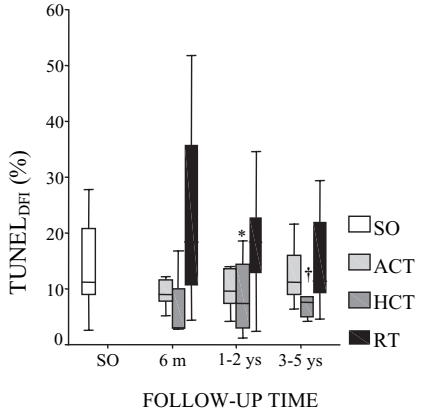

Figure 2. Box and whisker plot showing $TUNEL_{DFI}$ in TGCC patients related to treatment and follow-up time. Bars indicate median values. Boxes represent interquartile intervals and whiskers represent 95% confidence intervals. ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only. *HCT $T_{12–24}$ < SO, $P = 0.03$; †HCT $T_{36–60}$ < SO, $P = 0.02$.

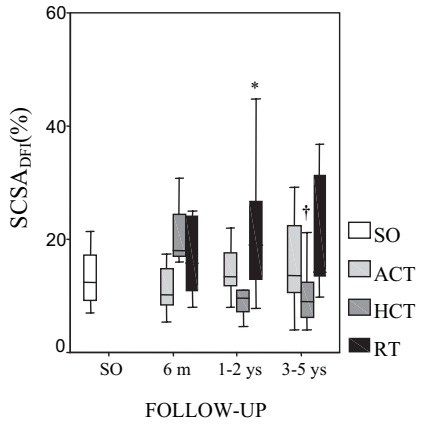

Figure 3. Box and whisker plot showing $SCSA_{DFI}$ in TGCC patients related to treatment and follow-up time. ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SO, surgery only. Bars indicate median values. Boxes represent interquartile intervals and whiskers represent 95% confidence intervals. *RT $T_{12–24}$ > SO, $P = 0.03$; †HCT $T_{36–60}$ < SO, $P = 0.02$.

RT, this proportion was 38%. The odds ratio for $SCSA_{DFI}$ ≥27 was significantly increased only in the RT group (Table IV).

Table IV. Number of men with $SCSA_{DFI}$ >27% in relation to total number of subjects investigated

	Proportion of men with normozoospermia (%)	Odds ratio in comparison with controls (95% CI)
Controls	16/233 (6.9)	Reference
ACT	3/24 (12)	1.9 (0.5–7.2)
HCT	0/7 (0)	Not done
RT	5/13[a] (38)	8.5 (2.5–29)

ACT, 1–2 cycles of cisplatin-based chemotherapy; HCT, more than two cycles of chemotherapy; RT, adjuvant radiotherapy; SCSA, sperm chromatin structure assay; SO, surgery only.
For the controls and the patients, the calculations were only made for those having normozoospermia (sperm concentration ≥20 × 10^6/ml). Odds ratios (95% CI) between the different treatment groups and controls are given.
[a]$P = 0.002$ in comparison to controls.

SCSA–TUNEL correlation

$SCSA_{DFI}$ and $TUNEL_{DFI}$ correlated significantly (Spearman's rho = 0.41; $P = 0.01$).

Discussion

In the present prospective study, sperm DNA integrity was investigated in 96 TGCC patients and correlated with treatment and time of follow-up. TGCC patients, before post-surgical treatment, had no increase of defective sperm DNA compared with controls. Adjuvant abdominal radiotherapy induced a transient increase in the proportion of sperm with DNA strand breaks. The normozoospermic RT patients had 8.5 times increased odds ratio for $SCSA_{DFI}$ ≥27% compared with controls, indicating a therapy-induced decrease of fertility *in vivo* despite normal sperm concentration.

Three or more courses of chemotherapy induced a permanent decrease of DFI.

This is the largest study on sperm DNA integrity in cancer patients, and two methods—SCSA and TUNEL—were applied. In samples analysed with both methods, a moderate correlation between the results of the two analyses was found. However, when comparing different treatment groups, identical trends were found regardless of method.

Sperm DNA integrity has been receiving increasing attention. Discrete DNA injuries have been demonstrated in human spermatozoa and shown to affect the fertilization ability *in vivo*, and possibly even *in vitro*, regardless of standard semen parameters (Larson *et al*., 2000; Bungum *et al*., 2004). Furthermore, it is known that defective paternal genome can be transmitted to the offspring (Cram *et al*., 2000), but the significance of iatrogenic DNA damage, induced by cancer treatment, is unknown. Large follow-up studies on the offspring of cancer survivors have not shown any adverse effects of cancer treatment. However, these studies were based on children born after natural conception (Blatt, 1999; Meistrich and Byrne, 2002), and there is a fear that the ICSI procedure, surpassing the biological control system of natural conception, imposes a risk of transmitting defect DNA.

In contrast to our study results, previous studies indicated that TGCC *per se* was associated with impairment of sperm

DNA integrity. Three studies used proven fertile men as controls (Fosså *et al*., 1997; Kobayashi *et al*., 2001; O'Donovan, 2005), and because fertility is associated with a low level of sperm DNA damage, our approach of using a group of unselected males as controls might explain the divergent results. Finally, Gandini *et al*. (2000), comparing sperm DNA integrity, assessed with TUNEL, in an unselected healthy population with Mb Hodgkin and TGCC patients found a statistically significant higher $TUNEL_{DFI}$ in both cancer groups. However, when comparing the Gandini study with ours, the differing conclusions are explained with the difference in controls ($TUNEL_{DFI}$ 2.5 versus 11%) and not in cancer patients. In the present study, the median $TUNEL_{DFI}$ in controls was at the same level as their median $SCSA_{DFI}$. The finding of the same DFI in SO patients and in controls cannot be explained by age difference between the two groups. TGCC men were older than controls, which should rather lead to a higher DFI among these men because this sperm characteristic is known to increase by age (Spano *et al*., 1998).

In clinical terms, a normal level of sperm DNA damage before therapy in TGCC patients indicates that the use of cryopreserved semen constitutes no increased risk of transmitting damaged DNA compared with non-TGCC ICSI-candidates.

The genetic risks of paternal exposure to irradiation are unclear. Whereas animal models have demonstrated both the transmission of radiotherapy-induced genetic damage and a subsequent increase in both early embryonic loss and malformations (Brinkworth, 2000), the potential hazards for humans are less evident. Following oncological treatment, no such risk has been detected (Meistrich and Byrne, 2002; Tawn *et al*., 2005). Observations from the Chernobyl accident are inconsistent regarding a possible increase in the rate of malformations or genetic diseases, but data suggested an increase in germline mutations (Dubrova, 2003).

Our study demonstrated the significant sensitivity of spermatozoa to radiotherapy. An estimated total dose of <0.5 Gy in 14 fractions was enough to induce long-standing, although not permanent, sperm DNA damage. The proportion of spermatozoa with DNA strand breaks was significantly higher in RT patients the first 2 years after therapy. One year or more after RT, patients with a normal sperm count had 8.5 times higher odds ratio of $SCSA_{DFI}$ ≥27% compared with controls, which may indicate *in vivo* infertility, which cannot be foreseen by judging the sperm concentration only. Although there are still some controversies regarding the impact of high DFI on fertility, the available data indicate that $SCSA_{DFI}$ ≥27% reduces the probability of *in vivo* fertilization, either by natural conception or by intrauterine insemination, almost to zero with ICSI being the most efficient way of achieving pregnancy (Bungum *et al*., 2004).

The median age of the RT patients was 36 years, when even a transient reduction of fertility can have consequences in terms of fatherhood. If these men present with infertility despite normal semen parameters, sperm DNA integrity should be assessed, and use of cryopreserved sperm might be indicated in cases of high level of sperm DNA damage.

The decrease in $SCSA_{DFI}$ following chemotherapy observed in our previous report (Stahl *et al*., 2004) was confirmed with the TUNEL analysis. This is inconsistent with several animal models in which chemotherapy induced sperm gene mutations and transmittable chromosome aberrations (Marchetti *et al*., 2001; Hales *et al*., 2005). Both cisplatin and etoposide were shown to induce sperm DNA injury and germ cell apoptosis (Sjoblom *et al*., 1998; Zhang *et al*., 2001; Cherry *et al*., 2004; Stumpp *et al*., 2004). However, mouse models and the human clinical setting differ in many aspects. Experimental studies are mostly monotherapeutical, using either a high single dosage or chronic low-dosage exposition (Sjoblom *et al*., 1998; Brinkworth, 2000; Hales *et al*., 2005). Most importantly, animal studies focus on the acute gonadotoxicity, which at least in humans differs completely from the long-term effects.

Few studies on humans address the issue of sperm chromatin in relation to anticancer therapy. The induction of sperm aneuploidy has been described after both radiotherapy and chemotherapy, but no permanent changes have been described (Martin *et al*., 1997; De Mas *et al*., 2001; Frias *et al*., 2003). Thomas *et al*. (2004) reported the absence of increase in sperm aneuploidy rates after anticancer therapy in 14 TGCC and 14 lymphoma patients, investigated between 7 months and 7 years after treatment with RT, CT or both.

The effect of cancer therapy on sperm DNA integrity is even less studied. A study on non-azoospermic adult childhood cancer survivors, compared with 66 proven fertile men, found no significant difference regarding sperm DNA integrity using the TUNEL assay (Thomson *et al*., 2002).

The decrease in the proportion of spermatozoa with DNA breaks seemed to occur after cessation of the spermatogenic arrest caused by CT. We, therefore, suggest that this effect may be exerted via the spermatogonial stem cells. One can hypothesize that spermatozoa with DNA strand breaks arise from stem cells with defective DNA repair mechanisms, which thereby make them more vulnerable to chemotherapy. Cisplatin was previously shown to increase apoptosis of germ cells (Cherry *et al*., 2004) and may add to the elimination of spermatogonia with DNA breaks. Whether sperm DNA is affected in the same way by other chemotherapy combinations remains to be investigated. The clinical application of our findings is restricted to patients receiving bleomycin, etoposide and cisplatin, for whom our study results indicated no increased risk in using post-therapy sperm for *in vitro* fertilization, including ICSI, and that normal fertility can be expected in those achieving full recovery of sperm count. However, semen cryopreservation should be performed before treatment, because neither the full extent of the therapy nor the degree of sperm recovery for the individual patient can be foreseen at the initiation of treatment.

In conclusion, irradiation induced an increase in the number of sperms with DNA damage, lasting for at least 1–2 years post-therapy, whereas more than two cycles of chemotherapy reduced the proportion of sperms with impaired DNA integrity. Further studies need to be performed for other patient groups, for example, those being treated for cancer in childhood and adolescence, because other treatment regimens as well as age at treatment might influence the effect on sperm DNA integrity.

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