ASPECTS OF EARLY DETECTION OF TESTICULAR CANCER AND CANCER-RELATED INFERTILITY

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ASPECTS OF EARLY DETECTION OF TESTICULAR CANCER AND CANCER-RELATED INFERTILITY

ASPECTEN VAN VROEGE OPSPORING VAN ZAADBALKANKER EN KANKER GERELATEERDE SUBFERTILITEIT

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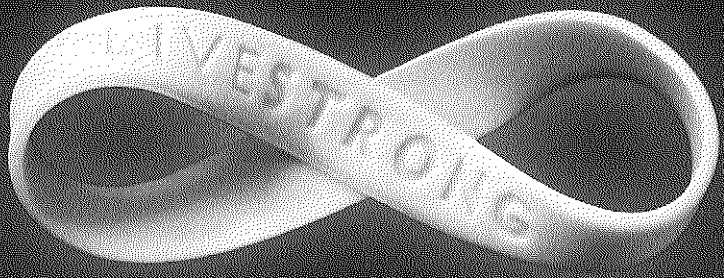
CONTENTS

Chapter 1

Introduction



INTRODUCTION

Infertility is referred to by the World Health Organisation (WHO) as the inability of a couple to achieve pregnancy within one year of regular unprotected intercourse[1]. About 15% of couples who do not achieve pregnancy within 1 year seek medical treatment for infertility. Eventually, 5% of them remain childless against their wishes.

Infertility affects both men and women. In 50% of involuntarily childless couples, a male infertility associated factor is identified together with abnormal semen parameters. Several factors can be responsible for impaired male fertility: congenital or acquired urogenital abnormalities, urogenital tract infections, increased scrotal temperature (such as that caused by varicocele), endocrine disturbances, genetic abnormalities, immunological factors and testicular deficiency (1). Nonetheless, in 30-40% of men with abnormal semen parameters no cause can be found, which is then named idiopathic male infertility.

Testicular development and its bearing on the development of testicular cancer

To permit normal spermatogenesis later in life, it is important for the male gonads that testicular development is not hampered by internal or external factors. To ensure the transmission of genetic information through the spermatozoa, specific germ cell precursors are set aside during early embryogenesis. Known as primordial germ cells, these cells have unique characteristics, such as an capacity to suppress the induction of differentiation, a commitment to either the male or the female lineage, and a capacity to generate the highly specialized daughter cells that, after fertilization, regain an activated differentiation program. The latter means that they can form all embryonic and extra-embryonic tissues, including the germ-cell population [2]. In other words, they are in fact really the totipotent stem-cell population of the body, and represent the circle of life.

The development of the testes starts in the fifth week of life, when the primordial germ cells migrate to the genital ridge; once there, they are termed gonocytes. Eventually, these gonocytes differentiate to type A spermatogonia, which will form spermatozoa after puberty (spermatogenesis). During this differentiation, the germ cells are characterized by several markers, including alkaline phosphatase, c-KIT, OCT3/4, VASA, and NANOG.

If gonadal development is disturbed, it can lead to the so-called testicular dysgenesis syndrome. This was first described by Skakkebaek *et al.*, who states that the development of the testis is disturbed by several internal and external factors [3] that may cause Leydig-cell and Sertoli-cell dysfunction, and may thereby disturb the differentiation of the gonocytes and derivatives. This can create various problems, such as minor spermatogenic disorders, cryptorchidism, hypospadia, infertility and even testicular germ cell cancer.

Testicular cancer, i.e. germ-cell tumors, is the most common cancer in men in their 20s and 30s. Unfortunately, its incidence is rising [4]. In the Netherlands, about 600 patients per year

(2007) are diagnosed with a testicular germ-cell tumor (TGCT), a number that is expected to be more than 700 patients in 2010 (KWF data 2005).

The first sign of such a testicular cancer is often a painless enlargement of the testicle, In about 60% of the patients the tumour has already metastasized to the lymph nodes and sometimes to other organs when first diagnosed. Carcinoma *In Situ* (CIS) is currently accepted as the precursor of all TGCTs , i.e. the seminomas and nonseminomas, in adolescent and young adult males [5, 6]. In many respects, CIS cells resemble PGCs/gonocytes: both have erased genomic imprinting and a similar morphology, and both express the same immunohistochemical markers, such as OCT3/4, PLAP, AP-2γ, and c-KIT [7, 8]. CIS cells probably result from delayed or blocked differentiation of embryonic germ cells. Patients harbour these pluripotent CIS cells in their testicle, which, after puberty, eventually progress into a TGCT [3].

In its pre-invasive stage, CIS can be detected by testicular biopsy, but its inconsistent presentation, which is characterized by a heterogeneous distribution, can result in false-negative diagnosis in some cases [9]. Because a testicular biopsy is also an invasive procedure that brings additional risks, it cannot be used as a screening tool in large populations. One alternative method of detecting CIS is by detecting exfoliated tumor cells in semen, but so far this has not been very successful [10].

Because the detection and treatment of CIS can prevent the development of an invasive cancer, it clearly has major advantages, not least because it spares patients from treatment protocols which are potentially detrimental to the gonads.

Cancer and fertility

Before gonadotoxic treatment is initiated, adult male cancer patients are often referred for semen cryopreservation of a semen sample. Sometimes, the pre-treatment semen quality is impaired due to disease related factors hampering semen cryopreservation. Sperm banking can be a difficult issue to discuss with patients who have recently been diagnosed with cancer, especially in adolescents and pre-pubertal boys, who might not be able to produce a semen sample. Sometimes, in pubertal boys spermatogenesis may not yet have started and it is difficult to predict this based on clinical parameters. This poses a difficult problem for clinicians who encounter pediatric cancer patients.

The recovery of spermatogenesis after gonadotoxic treatments can take several years, and still no serum markers are available to predict whether this will happen. If azoospermia persists after cancer treatment it may be possible to induce pregnancy with cryopreserved semen and assisted-reproduction techniques. The value of sperm banking before cancer treatment is expressed by the total number of men who remain infertile after treatment and the number of live births using this cryopreserved semen after surviving cancer. There is a definite need to discuss semen cryopreservation with all male cancer patients prior to cancer treatment.

As this introduction shows, fertility and cancer are intertwined in many ways. Both are the subject of this thesis.

Chapter 2

Aim and outline of the thesis

AIM OF THE THESIS

The general aim of the work presented in this thesis is to illustrate the relationship between gonadal function and cancer. In doing so, we first concentrate on the increased risk of developing testicular germ cell tumours (TGCT) in infertile men. Next we discuss the best method to evaluate testicular biopsies for detecting Carcinoma in Situ (CIS) of the testis, the precursor of testicular cancer. Furthermore, we elaborate on a non-invasive detection of testicular cancer using immunohistochemistry to detect exfoliated CIS cells in semen. We end this thesis with evaluating the detrimental effects of childhood cancer treatment on gonadal function and present the significance of performing pre-treatment semen cryopreservation in all cancer patients.

OUTLINE OF THE THESIS

Chapter 3 describes a specific subgroup encountered in the subfertile population. It is shown by several large studies that patients who have an impaired fertility are at risk for developping testicular cancer [8]. The risk of developing TGCT in male patients who visit an andrologic clinic is approximately 1%. This is one of the reasons why a scrotal ultrasound is mandatory in the evaluation of the infertile male. Subgroups in the infertile patient population have a higher risk on TGCT. Recently, De gouveia de Brazoa *et al.* found that infertile men with bilateral testicular microliths on scrotal sonopgrahy had 20% risk of CIS[9]. Infertile men, especially those with additional signs of testicular dysgenesis such as testicular microlithiasis (TM), might therefore be a group that might benefit from a screening protocol designed to detect testicular cancer or the precursor lesion. However, standard follow-up schedules for this group are lacking making it difficult to clearly investigate the role of these microliths in the testicular dysgenesis syndrome and the risk for TGCT. We propose an active approach including performing testicular biopsies to detect CIS in the testes for men diagnosed with TM.

In chapter 4 we present the pitfalls encountered in CIS diagnostics due to the random distribution in the testis. CIS is virtually always found in the parenchyma adjacent to TGCTs in orchidectomy specimens, and it is reported that in testicular biopsies, without an invasive tumour, CIS is missed in approximately 0.5 percent [6, 10]. Dieckmann et al. proposed to take a two-sited surgical biopsies from a single testis which improves the diagnostic yield with 18% [11]. Application of immunohistochemistry using specific antibodies can furthermore increase the overall sensitivity and specificity of diagnosing CIS in testicular biopsies. Currently, OCT3/4 is in our opinion the best marker in TGCT diagnostics. One of the biggest advantages is that 100% of the cells of CIS, seminoma and embryonal carcinoma show a nuclear staining [12-14]. In chapter 5 we evaluated the additional diagnostic value of OCT3/4 immunohistochemistry in detecting CIS in testicular biopsies. In chapter 6 we describe an other method to detect

CIS using a non-inavsive method. We describe a pilot study in which we evaluate if OCT3/4 immunohistochemistry could be used to detect exfoliated CIS cells in semen.

In the second part of the thesis we focuss on cancer related infertility. In chapter 7 we describe the semen quality of cancer patients who were referred for semen cryopreservation. In our study we evaluate which patients are at risk for decreased pre-treatment semen quality. In young pre-pubertal or pubertal boys, semen cryopreservation is a difficult issue to discuss. Not all boys have masturbated at this age or feel comfortable talking about masturbation and in some spermatogenesis is still absent. In chapter 8 we describe a group of pubertal cancer patients who are referred for semen cryopreservation. We evaluate which markers could be useful to predict a successful semen cryopreservation attempt. Successful semen cryopreservation offers these young boys a change for offspring later in life. To assess the value of banking sperm the number of men who use their banked semen should be assessed as well as the number of life births after IVF. In chapter 9 we describe a large cohort of cancer patients who banked their semen and evaluate which of them used their semen. We furthermore evaluated the outcome of the fertility treatments in which the banked semen was used. In the last two chapters 10 + 11 we elaborate on the detrimental effects of chemotherapy and radiotherapy given during childhood cancer treatment. We describe a large cohort of patients who were treated for childhood cancer with a very long follow-up. These data give more insight into which patients are at increased risk for post-treatment gonadotoxicity.

Chapter 3

Testicular microlithiasis and Carcinoma *in situ*. Overview and proposed clinical guideline

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SUMMARY

Testicular microlithiasis (TM) has been associated with testicular germ cell tumors (TGCTs) in adolescents and adults and with its precursor carcinoma *in situ* (CIS). A clear definition of TM and the need for further diagnostics and follow-up is lacking. We reviewed the literature of TM and its association with TGCT/CIS and current follow-up advises and propose a management approach based on associated risk factors for TGCT. In the literature, a wide variance of TM incidence is reported in different patient populations. A consensus concerning the malignant potential of TM has not been reached. In addition, a clear definition on TM is lacking. Although a correlation between TM and TGCT or CIS is found, precise management and follow-up schedules are absent. We suggest that all hyperechogenic foci smaller than 3 mm without shadowing should be named TM irrespective of their number. In addition, we suggest a management scheme for physicians encountering TM in daily practice. Our algorithm suggests taking a testicular biopsy in a selected patient population with at least one additional risk factor for TGCT. A long-term active follow-up schedule, including ultrasonography and physical examinations, is not indicated in the remaining patients with TM.

INTRODUCTION

Testicular microlithiasis (TM) is an incidental finding during ultrasonographic investigation of the testis. These TM appear as 1- to 3-mm-sized multiple foci within the parenchyma of the testis [15, 16] (Figure 1). This observation is infrequent but it has generated interest as an informative parameter to identify males at increased risk for Carcinoma *In Situ* (CIS) of the testis and testicular germ cell tumors (TGCT) during adolescence and adulthood, i.e. seminomas and nonseminomas. Although most clinicians classify hyperechogenic lesions detected on scrotal ultrasound investigation as TM, a precise definition has not been accepted yet. In essence TM is a benign condition and has been found together with several urological conditions, such as infertilty [17-19] , cryptorchidism [20-22], testicular torsion [23, 24], testicular atrophy [25],

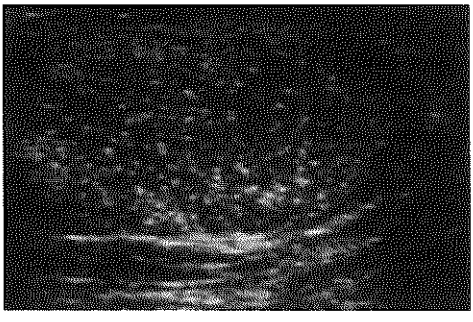

Fig 1: Landscape testicular microlithiasis in a small atrophic inhomogeneous testis.

Klinefelter's syndrome [25, 26] and and disorders of sex development [25]. The association with CIS [9, 15, 27, 28] and TGCTs [27, 29, 30] is also well documented; TM is found in 74% of men with a TGCT [31]. In contrast, it can also be observed in 1.5 to 5.6% of the general population and can therefore be regarded as not testicular cancer specific [18, 32]. The observation and its potential association with TGCT raise concerns among urologists and radiologists but a consensus on follow-up schedules and interventions to rule out malignancy in these patients is lacking. With this overview, we propose a clear definition of TM and give a guideline for clinicians on how to manage these patients.

Incidence

TM is being recognized with increasing frequency because of high resolution of modern ultrasound machines. With transducer frequencies of 7-13 MHz., scrotal ultrasonography is able to identify structures smaller than 1 mm, which leads to a more accurate view of the testicular parenchyma and a higher detection rate of intrascrotal abnormalities. The prevalence numbers of TM in studies of large cohorts of men show a wide range. This may be explained by the difference in the selection of men in these studies. In healthy men, for example, the prevalence varies between 1.5 to 5.6%, compared to 0.8 % to 20.0% in infertile populations (Table 1). TM

Table 1: TM prevalence in general populations and subfertile populations

Authors	*Total Patients*	*TGCT Risk factor*	*TM (n)*	*Prevalence %*	*Criteria*	*MHZ*	*Malignancies (% of men with TM)*
Von Eckardstein et al., 2001	198	None	3	1.5	>5	7.5	1 (33%)
Peterson et al., 2001	1504	None	84	5.6	>5	7-10	0
Total general Population	1702	None	87	5.1%			1 (1%)
Kessaris et al., 1994	150	S	2	1.3		10	0
Aizenstein et al., 1998	180	S	5	2.8	>5	7	0
Pierik et al., 1999	1372	S	12	0.9		7-10	7 (58%)
Von Eckardstein et al., 2001	1399	S	32	2.3	>5	7-10	3 CIS (9%)
Schrey et al., 2001	1030	S	8	0.8	>10	-	1
Turchi et al., 2001	250	S	2	0.8		-	0
Thomas et al., 2001	159	S	10	6.1	>5	7	0
de Gouveia Brazao et al., 2004	263	S	53 (30 (BTM)	20		7.5-12	6 CIS (11%)
Sakamoto et al., 2006	545	S	30	5.5		5-7.5	0
Mazzilli et al. , 2005	283	S	13	4.6		5.5-12	0
Total subfertile population	5899	S	139	2.4			17 (4%)

S: subfertile population, TM: testicular microlithiasis, BTM: Bilateral testicular microlithiasis.

was found in 139 out of 5899 infertile men indicating an average prevalence of around 2.4% in these patients (Table 1). In populations referred for scrotal complaints, a variety in incidence is reported ranging from 0.6 to 4.1% (Table 2). A comparison among most studies is difficult because of the absence of a clear definition of TM as well as the use of different ultrasound frequencies. In addition, the populations used vary widely and for the largest part describe the presence of TM in men with palpable abnormalities of the testis suggestive for testicular cancer. Furthermore, most studies are retrospective and therefore suffer from biases inherent to retrospective studies. A clear incidence number is therefore difficult to give and mainly depends on the population screened.

Definition and Etiology

The exact etiology of TM is unclear. It is suggested that these calcified concretions within the lumen of seminiferous tubules originate from sloughing of degenerated intra-tubular cells and failure of the Sertoli cells to phagocyte the debris [33-35]. Raman spectroscopic mapping demonstrated that testicular microliths were located within the seminiferous tubule and consisted of hydroxyapetite [36]. Although calcifications in a tumor containing testis also revealed high levels of hydroxyapetite, caution should be taken to name these microlithiasis since their origin and pathogenesis are not completely clear [36, 37].

Table 2: Prevalence of TM and association with malignancy in referred patients.

Authors	*Total Patients*	*TM*	*Prevalence %*	*Frequency MHZ*	*Malignancy in men with TM*	*Odds ratio*
Ganem et al., 1999	1100	22	2.0	5-10	8/22 (36.4%)	
Hobart et al., 1992	1710	11	0.6	5-10	5/11 (54.4%)	
Otite et al., 2001	3026	54 (criteria>5)	1.8	7	16/54 (29.6%)	13.2x
Derogee et al., 2001	1535	63 (criteria >3)	4.1	7 and 10	30/63 (47.6)	
Skyrme et al., 2000	2215	34	1.4	7.5	5/34 (14.7%)	
Cast et al., 2000	4819	33 (criteria>5)	0.68	7.7 and 5-10	7/33 (21.2%)	21.6x
Middleton et al., 2002	1079	195	18.1	>7.5	12/195 (6.1%)	
Miller et al., 2007	3279	67	2.0	= or >10	5/67 (7.5%)	
Bach et al., 2001	528	48 (criteria >5)	9.0	7	13/48 (27.1%)	
Sakamoto et al., 2006	969	46	4.7	5 + 7.5	8/46 (17.4%)	
Ringdahl et al., 2004	160	12	7.5	-	4/12 (33.3%)	
Bach et al., 2003	156	23	14.7	7	5/23 (21.7%)	
Pourbagher et al. 2004	5263	40 (BTM)	0.76	7.5	4/40 (10%)	
Kosan et al. 2008	179	21	10.6	10	3/21 (14.3%)	
Total	26018	669	2.5		125 (18.7%)	

BTM= bilateral testicular microlithiasis

TM is classified by testicular ultrasonography as hyper-echogenic lesions between 1 and 3 mm in diameter without shadowing. TM usually has a diffuse pattern but number and distribution are variable [38] . A sub-classification in Classic or Limited TM has been suggested according to the presence of respectively more or less than five microliths per view. A recent study in 1079 patients by Middleton *et al.* showed that this classification is arbitrary in the context of malignant transformation. Their study showed no difference in risk of a co-existing tumor between patient with a classic TM pattern and patients with only few microliths per testis [30]. Sanli et al. recently also demonstrated that a grading of classic TM according to the number of microliths is not mandatory as this does not predict TGCT development [39].

In our opinion, all hyperechogenic foci on testicular ultrasound smaller than 3 mm without shadowing should be named TM, independent of the number of TM per view or in the total testis. These microliths should be located in the testicular parenchyma and not in a TGCT. Furthermore, factors potentially disturbing the normal testicular parenchyma such as testicular tumors, testicular trauma or surgical procedures should also be clearly noted as these might indicate a different etiology.

Risk of CIS of the testis in men with TM

The incidence of TGCTs, i.e. seminomas and nonseminomas, has increased during the last decades [40]. It is currently accepted that CIS is the precursor, which represents a primordial germ cell or gonocyte [2, 41]. Early detection of CIS or organ confined TGCT improves prognosis and prevents the need for orchidectomy and chemotherapy and/or radiotherapy [42]. It is relevant to realize that CIS is in fact an asymptomatic condition and TM might be the only clue to its presence. Kang *et al.* found that the microscopic prevalence of TM among 463 testicular biopsies was higher in biopsies with CIS 39% compared to 2% in biopsies without CIS [43]. Therefore, TM might indeed be useful to define a high risk group in whom CIS is more frequent [44]. TM is commonly associated with CIS in case reports and small studies [9, 15, 27, 45, 46]. Linke *et al.* performed a post mortem study on presumably healthy males with no known testicular complaints and found a prevalence of CIS in 6 out of 1388 patients (0.43%), of whom two (33.3%) had TM [47]. Poubargher *et al.* studied 36 patients with bilateral TM and did not detect any newly formed tumors during the median 34 months follow-up. Follow-up included ultrasound and physical examinations at 6-month intervals. Although no tumors developed, these examinations do not exclude CIS and the follow-up period of this study might be too short as only 50% of the patients with CIS will develop a TGCT in 5 year [48]. In a more recent study of De Gouveia-Brazoa *et al.* the risk of CIS in a infertile population was 20% in the presence of bilateral TM compared to 0.5% in the patients without TM[9]. A recent article by Sanli et al. also suggested a higher risk of developing a TGCT in men with bilateral TM [39].

In patients with a TGCT the risk of CIS or tumor in the contralateral testis is reported to be around 5 percent. If the contralateral testis contains TM, this risk will increase to 22-78% (odds ratio 12.0 to 16.8) [49, 50]. Bach *et al.* performed a retrospective study in 156 patients with a TGCT. In this population, 23 patients were diagnosed with contralateral TM of whom 4 patients (17.3%) were diagnosed with testicular malignancy in that testis compared to only 2 in the remaining 133 patients without TM (1.5%)[50]. Holms *et al.* showed the presence of contralateral tumor in seven out of nine men (77.7%) with contralateral TM compared to only three out 30 (10%) patients when the contralateral testis appeared normal on ultrasound evaluation[49]. However, the absence of TM does not safeguards patients from CIS as nine out of the 25 patients with CIS (36%) from these two studies had no sonographic abnormalities [49, 50]. Based on these studies, it is clear that TM and CIS can be associated but CIS can occur without TM. Most likely, TM is a sign of the testicular dysgenesis syndrome (TDS), a condition that is associated with CIS [51]. Therefore, patients with TM should be examined for other criteria for TDS such as testicular atrophy, testicular maldescent, history of contralateral tumor, hypospadias, low or absent sperm count or inhomogeneous ultrasound appearance [52, 53]. These findings might provide additional evidence for the presence of CIS and therefore the need for testicular biopsy.

Risk of TGCT in men with TM

Although, the association between TM and TGCTs is well documented, there is no convincing evidence to suggest that TM is solely a pre-malignant condition. In the literature, the association between TM and TGCTs ranges from 6.1 % to 54.4 % in a referred population (Table 2). These patients were referred for scrotal ultrasound for a variety of scrotal complaints including testicular torsion, painless lumps, hydrocele, testicular pain, epididymitis and subfertility. Ikinger *et al.* found calcifications in 74% of the testis containing malignancies, using a mammography technique. In that study, there was a slightly higher incidence of TM in non-seminoma patients (87%) than in seminoma patients (60%). Only few case studies report on the actual development of a TGCT after TM was identified [54-56]. The tumor in those studies developed between 6 months and 11 years after discovering the TM. The relative increase in risk of developing a tumor in men with TM in a referred population varied from 13.2 to 21.6 fold (Table 2) [57, 58]. These two studies describe the presence of TM besides an invasive TGCT and not just the pre-malignant CIS stage. These calcifications might be secondary to the presence of the tumor [37]. Treatment of these patients is directed towards treating the suspected tumor and not influenced by the TM. DeCastro *et al.* recently published a 5 year follow-up study of asymptomatic men with TM and found a odds ratio of developing a testicular tumor of 317 compared to men with no TM [56]. Coffey et al recently demonstrated that TM is more frequent in male relatives of men with TGCT, which suggests a familial risk factor for TGCT [59].

FOLLOW-UP

A large survey under British Urologists showed that one-third of them performed follow-up of men with TM [60]. Follow-up should result in early detection of testicular malignancies. In general, most authors describe a follow-up scheme consisting of self-examination in combination with regular physical examination by an urologist and scrotal ultrasound especially if additional risk factors are present. The regimes used differ widely in interval and method of follow-up and are not conclusive about length of follow-up (Table 3).

The corner stone of any follow-up scheme for patients with TM should be self-examination of the testis. Self-examination will aid in early detection of TGCT. Huyghe *et al.* showed that in nonseminoma patients diagnostic delay is associated with worse stage and survival [61]. The physician should therefore raise the patient's awareness so that clinical delay is minimal.

We provide a follow-up schedule with suggestions for men diagnosed with TM, without a testicular lesion, based on the current literature (Figure 2). If TM is diagnosed in combination with other intra-testicular abnormalities suspicious for a TGCT the physician should make his treatment decision on the suspicious lesion. The presence of TM adjacent to the lesion only increases the change of malignancy [62].

Table 3: Overview of studies describing a follow-up schedule for men with TM

Study	*Follow-up indicated*	*Self examination (Interval)*	*Physical examination physician*	*Ultrasound (Interval)*	*Serum markers (Interval)*	*Biopsy*	*Length of follow-up*
Zastrow et al 2005	+ all patients	+ Regular	-	-	?	+/- [A]	?
Dagash et al. 2007	+/- [B]	+ Monthly	-	+/- [B] (Annual)	?	?	?
Sakamoto et al 2006	+/- [C]	?	-	+ (Regular)	?	?	?
Rashid et al. 2004	+	+ Monthly	+ Yearly by primary care taker	+/- [D] Annual + Physical examination urologist	-	?	?
Leenen et al 2002	+ Children	?	+	+ Annual	-	-	Until peak incidence
Bennet et al. 2001	+/- [E]	?	-	+/- Periodic [E]	-	-	?
Otite et al. 2001	+	+	-	+ 12-18 Months	+/- [F]	+/- [F]	20-45 year
Ganem et al. 2000	+ [G]	+	+	+ Annual	-	-	?
Miller et al. 1996	+ [H]	+	+	+ 6-12 Months	-	-	?
Skyrme et al.2000	+	+	+ Biannually	+ Biannually	-	-	?
Pourbagher et al. 2005	+	+ Annual	+	+ Annual	-		-

+/- Done if risk factors are there, - Not suggested, + Suggested, ? Not precisely described

[a]: Additional pathological ultrasound or clinical findings, focal or unilateral TM, existence of a contralateral tumor or infertility with cryptorchisme and atrophic testes

[B]: cryptorchidism, infertility, testicular atrophy, testicular asymmetry or previous history of testicular cancer

[C]: infertile men with bilateral TM or men with unilateral TGCT and TM in the contralateral testis

[d]: Cryptorchidism, Atrophy, Infertility, Intratubular germ cell neoplasia, Gonadal dysgenesis, Contralateral testicular tumor, Exogenous estrogen administration

[e]:Only in case of a contralateral tumor

[F]:Previous history of testicular malignancy or the presence of equivocal findings

[G]:Recommends extensive evaluation including chest X-ray, computerized tomography and testicular biopsy especially in men with a history of testicular cancer and testicular microlithiasis.

[H]:Initial CT abdomen + thorax if no testicular tumor is present

Regular scrotal ultrasonography is often advocated. Using ultrasonography the physician is able to detect non-palpable masses. This might lead to early diagnosis but will mostly not prevent orchidectomy. Furthermore, it remains to be proven that screening testicles for TGCTs with ultrasonography results in better survival rates. In addition, CIS itself is not detectable with

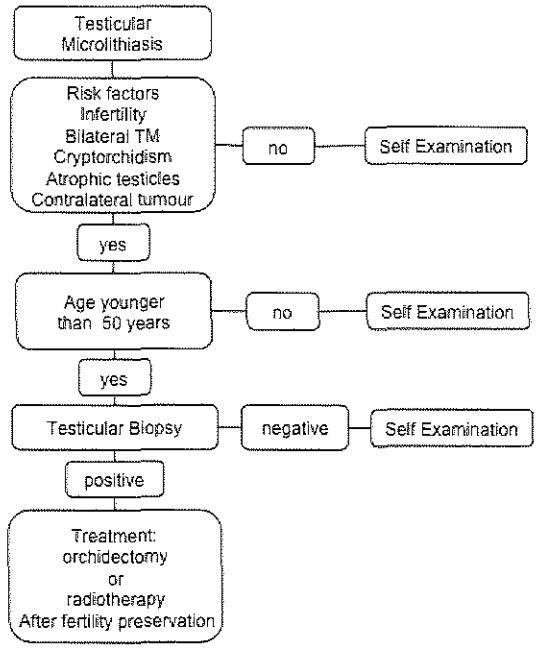

Fig 2. Follow-up scheme for patients with TM without a concomitant testicular tumor.

ultrasonography. Finding the precursor CIS lesion is the only way to improve patient outcome, especially related to retention of hormonal function and thereby quality of life. It is debatable if annual testicular ultrasonography plays a role in the follow-up of TM as a survival benefit has not yet been proven. It might be an alternative for patients with TM with a relative high risk who refuse or are unable to undergo a testicular biopsy. However, until the role of regular scrotal ultrasound in patients with TM regarding survival and cost-effectiveness is investigated this should not be regarded as standard care.

WHEN IS A TESTICULAR BIOPSY INDICATED?

A testicular biopsy is currently the gold standard in CIS diagnosis. However, it is not necessary to perform this invasive procedure in all males with TM. The majority of men with TM will never develop a TGCT [32, 56]. Some patients with TM are at a higher risk for CIS or testicular malignancy, thus follow-up schedules should be focused on this group. Performing a testicular biopsy will accurately diagnose CIS with a false-negative risk of 0.5% [61]. We propose a follow-up protocol in which testicular biopsies should be considered in patients with TM with an increased risk of CIS or TGCT (Fig 2). These include men with infertility and bilateral TM, atrophic testes, undescended testes and men with a history of TGCT and contralateral TM. In adult men with a history of undescended testis the risk of CIS is approximately 2 to

4% [63]. If in these testicles TM is present the risk increases to approximately 10 percent [21]. Men with infertility or abnormal semen analysis have a increased risk on developing a TGCT and this should be taken into account when managing TM in this patient population [8, 64]. In infertile men, the prevalence of TM varies from 0.8 to 20 percent (Table 1). The number of reported malignancies (n=16, 0.3%) in the infertility group might be too low as active follow-up has not been performed and none of the patients had testicular biopsies performed. This could lead to an underestimation of the CIS prevalence in this selected population. Raman *et al.* compared the incidence of TGCTs in 3800 infertile males and compared this with the general population. They found a 20-fold greater incidence of tumors in subfertile males [64]. In men with a TGCT, the risk of CIS or cancer in the contralateral testis is also increased. Harland *et al* performed a retrospective study in 186 testis cancer patients in whom contralateral biopsies were performed [52]. They found that patients with small contralateral testis had a 20% prevalence of CIS in the contralateral testis. Furthermore, they found that men presenting before the age of 30 had a 34% prevalence of CIS. Dieckmann *et al* found a 18% risk of CIS in men under the age of forty whom have a TGCT and a contralateral atrophic testicle [65]. If a testicular biopsy is performed, it should contain at least 30 tubules to allow the pathologist to evaluate a representative part of the testicle. Berthelsen *et al.* proposed a 3x3x3mm biopsy as an accurate biopsy size to detect CIS with considerable security if at least 10% of the tubules contain CIS [66]. Dieckmann *et al.* proposed to take a two-sided biopsy which increased the diagnostic yield with 18% [65]. The discordant result in this study predominantly occurred in normal sized testis and not in the atrophic testis. Performing a two-sided biopsy in the latter group should therefore be done reluctantly as this might lead to severe testicular damage and dysfunction. We strongly recommend the use of immunohistochemistry for the evaluation of testicular biopsies [67]. It is well known that CIS is difficult to identify in testicular biopsies. Immunohistochemistry will provide an extra diagnostic yield in diagnosing CIS. In a recent study, we confirmed earlier reports and found an extra diagnostic yield of 20% in identifying CIS in biopsies form infertile males with immunohistochemistry using OCT3/4, PLAP and c-KIT [68, 69]. OCT3/4 is currently the best marker for CIS, seminomas and embryonal carcinomas [13]. If a testicular biopsy is performed and CIS or TGCT is not present, no active follow-up by an urologist is recommended, because the change of a false-negative result is minimal if performed and evaluated correctly.

DISCUSSION

TM is associated with an increased risk of CIS and TGCT. However, this condition should not be considered premalignant, but it is rather a representation of rapid cell turnover resulting in microlithiasis as shown in a study with 131 specimens collected after orchiedectomy [17, 19]. The overall incidence in subfertile populations lies around the 2.4 % (Table 1), this underscores

that not all patients with TM will develop a TGCT as the risk for TGCT in subfertile males lies around the 1%. This fits well in the testicular dysgenesis syndrome theory (TDS), as the phenotype of this syndrome ranges from infertility to TGCT, and although TM is a sign of TDS not all will develop a TGCT. The relative higher prevalence number in the general population might be explained by the study design of the mentioned studies. In contrast to most studies, Peterson *et al.* conducted a prospective study in which the detection of TM was one of their main goals. The other studies are mostly retrospective studies based on reports, which can lead to lower prevalence numbers.

Although TM is associated with testicular dysfunction the amount of microliths is not a reliable indicator for the risk of tumor development [30, 39]. A difficult clinical situation occurs if only one microlith is found. However, if other risk factors are present a testicular biopsy can be performed. Because prospective data about the risk of CIS or TGCT in this specific subgroup is lacking we cannot make evidence based recommendation. The clinician should therefore make a decision based on the risk factors present. At least the patient should be informed about the risks and self-examination should be taught.

The role of TM in patients with testicular masses is in our opinion not of major importance as this will not aid in making a diagnosis of the tumor. However, it plays a crucial role in predicting formation of TGCTs and the presence of CIS in the contralateral testis [49, 50]. Unilateral TM in contralateral testis after orchidectomy must be considered as a significant signal to consider contralateral biopsy. It is clear that men with TM have an increased risk of developing a TGCT. However, most men with isolated TM will not develop a TGCT [9, 70].

The corner stone of any follow-up scheme should be self-examination of the testicle. Although the benefit of self-examination remains unclear, it at least makes the patient aware of potential symptoms of a TGCT. Hughe *et al.* recently showed a correlation between diagnostic delay and stage and survival [61]. Ninety-one percent of these patients had scrotal complaints of painless swelling, a change in testicular consistency or scrotal pain. This means that all TM patients should be made aware of the symptoms accompanying testicular cancer and advised to perform self-examination. Doing so men will discover testicular lumps earlier and potential diagnostic delay may be brought down.

Many follow-up schedules are described using scrotal ultrasonography. It is calculated that the costs of performing follow-up with ultrasound evaluation in all men with TM lies around the 18-billion dollars per year [32]. Furthermore, it is debatable that performing regular scrotal ultrasound will have any positive effect on mortality from TGCT as this reaches almost 90 percent with the current treatment modalities. Therefore, routine scrotal US should not be used for follow-up in men with TM until prospective studies have proven a significant benefit. We believe that in patients with TM and signs of TDS a testicular biopsy should be considered. We are aware of the limitations of an open testicular biopsy, as false negative biopsies could occur due to the non-uniform distribution of CIS [37, 67]. We strongly advocate the use of OCT3/4 immunohistochemistry in all testicular biopsies.

CONCLUSION

A wide variety in prevalence of TM is described in different populations due to different sample size, composition, US and screening criteria. TM consists of a large heterogeneous group and must be considered a benign condition. It is seen in various benign and malignant processes. Still, its association with TGCT is remarkably, and may be a very useful tool when screening for TGCTs, especially in its pre-invasive stage, in specific risk groups. In view of its association with cancer, regular self-examination in all patients and testicular biopsy in a selected high-risk group is advocated. Testicular biopsies should be considered if multiple factors representing TDS, such as infertility, atrophic testes, undescended testes, are present.

Chapter 4

Heterogeneous distribution of ITGCNU in an adult testis; consequences for biopsy-based diagnosis.

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ABSTRACT

Carcinoma in Situ (CIS) of the testis, also referred to as Intratubular Germ Cell Neoplasia Unclassified (ITGCNU) is currently accepted as the common precursor for all malignant germ cell tumors of adolescents and adults that is, the seminomatous and nonseminoma cancers. These pre-invasive cells have specific cellular characteristics, which can be used for early diagnosis, routinely done by morphological analysis, sometimes supported by immunohistochemistry, of tissue obtained by an open surgical biopsy. False-negative biopsy results can occur mostly due to either non-random distribution of ITGCNU within the testis, misdiagnosis or suboptimal tissue treatment and analysis. In this article, we demonstrate the potential pitfalls in the diagnosis of ITGCNU. The results support the use of the highly specific and sensitive immunohistochemical marker OCT3/4 for the diagnosis of ITGCNU and provide evidence for the non-random distribution of ITGCNU, which is a significant limitation in the diagnosis of this preinvasive lesion.

INTRODUCTION

Testicular germ cell tumors (TGCTs) of adolescents and adults are the most frequent malignancies in Caucasian males aged 20 to 34 years [42]. The disease specific survival of TGCTs of men diagnosed in Europe from 1990–1994 was 97% at 1 year and 93% at 5 years, the highest survival rate for any malignant tumor in men [71]. Despite this high cure rate, the annual increase in incidence of 2-5% is a major concern [72]. Twenty-three percent of the men presenting with a TGCT have metastases and need radio- or chemotherapy with potentially serious side effects, such as infertility [73].

TGCTs, including seminomas and non-seminomas, have carcinoma in situ (CIS), also known as intratubular germ cell neoplasia unclassified (ITGCNU), as common precursor. ITGCNU develops into invasive malignancy in 70% of the cases in 7 years and presumably in all patients over a longer period of time [4]. ITGCNU can be successfully treated with local radiotherapy or orchidectomy. Early treatment prevents progression from this lesion to an invasive TGCT and thereby cures the patient [4]. This avoids adjuvant chemotherapy or radiotherapy to the retroperitoneum, and the risk of development of refractory disease. Therefore, efforts should be made to diagnose TGCT at the pre-invasive stage. ITGCNU is routinely diagnosed by histological analysis of tissue obtained by a surgical biopsy [66]. The identification of ITGCNU in these testicular biopsies is based on morphology aided by immunohistochemical staining for a number of specific markers, of which OCT3/4 [13, 74] is highly sensitive and specific [13]. The percentage of false-negative biopsies is estimated as low as 0.5%. This is supposedly due to the equal distribution of ITGCNU throughout the testis in the majority of patients [6]. However, a growing number of studies and case reports show that ITGCNU may also be present as a focal lesion in the testis and therefore might result in a false-negative conclusion [6].

In this article, we report a patient with infertility and bilateral testicular microlithiasis, in whom ITGCNU and intratubular seminoma was initially missed in a surgical biopsy specimen fixed in buffered formalin, based on morphological criteria alone. However, histological review, and application of immunohistochemistry for OCT3/4 showed the presence in the original biopsy sample. Remarkably, subsequent bilateral open surgical biopsies were devoid of ITGCNU. Based on the original observation based on OCT3/4 immunohistochemistry orchidectomy was performed. A careful pathological investigation of the removed testis confirmed the heterogeneous distribution of ITGCNU.

CASE REPORT

A 30-year-old patient with primary infertility was referred to the Erasmus University Medical Center Rotterdam (Erasmus MC). The referring urologist had 6 months earlier performed bilateral open surgical testicular biopsies, which were originally both classified as disorganized

spermatogenesis with a Johnson score of 8 and no signs of malignancy. Endocrinological and clinical evaluation at the Erasmus MC revealed hypergonadotrophic hypogonadism with atrophic testes, with a FSH level of 64.1U/L (ref 2.0-7.0), LH of 27.2 U/L (ref 1.5-8.0), testosterone of 7.6 nmol/l (ref 10-30) and inhibin-B level of 0 ng/l (ref 150-400). Semen analysis showed azoospermia with a pH of 7.7 and a seminal volume of 6 ml.

Scrotal ultrasound was performed to measure testicular size, signs of epididymal obstruction and of testicular dysgenesis. It revealed bilateral small testes with a volume of only 4 cm^3 and several clustered microliths on both sides. No signs of obstruction were noticed. The patient had no history of cryptorchidism. The levels of the serum tumor markers β-human chorion gonadothropin, α-fetoprotein and lactate dehydrogenase were normal. Because bilateral microlithiasis is a risk factor for ITGCNU in males visiting the fertility clinic, as demonstrated by us previously [9], the slides of the original testicular biopsies were revised, and immunohistological staining for OCT3/4 was done. The biopsy of the right testicle showed few tubules with the typical histology of ITGCNU: a string of large cells with clear cytoplasm and a round nucleus with conspicuous nucleoli located on the basement membrane and bordered by Sertoli cells at the luminal side. In some tubules similar tumor cells completely filled the lumen accompanied by few lymphocytes, thus rendering the picture typical for intratubular seminoma. In the surrounding stromal tissue a mild lymphocyte infiltrate was present. The diagnosis of ITGCNU and intratubular seminoma was confirmed by nuclear staining of the tumor cells for OCT3/4 (Figure 1). The quality of the biopsy from the left testis was insufficient for diagnosis.

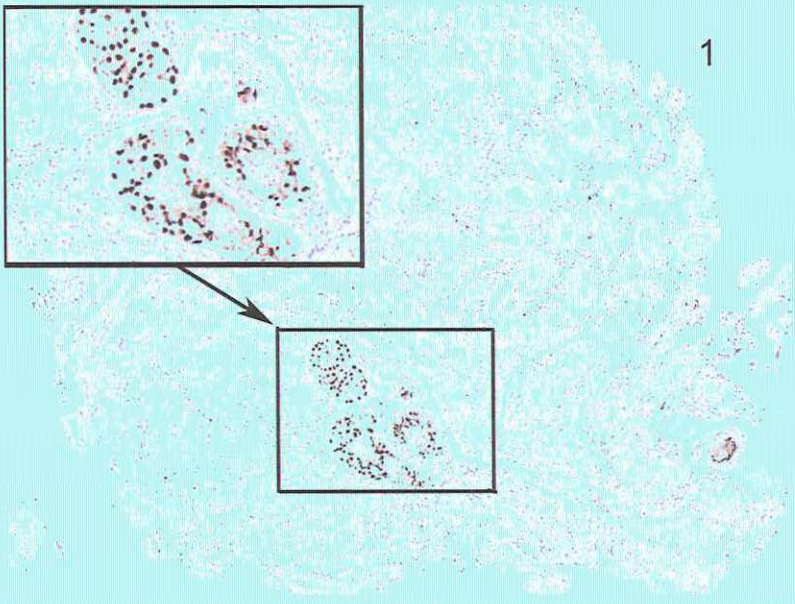

Figure 1. Histology of the open surgical biopsy of the right testis stained immunohistochemically for OCT3/4, demonstrating nuclear staining intratubular seminoma (1.25 x). (Inset) Higher power of intratubular seminoma (40 x).

Seven month after the initial biopsy repeated ultrasonography at our Hospital, one month after the first consultation, showed a new hypo-echoic lesion with a diameter of 3.0 mm x 1.5 mm in the left testicle. Because of the insufficient material of the first biopsy and the newly observed hypo-echoic lesion it was decided to perform a second bilateral open surgical testicular biopsy. Both biopsies showed strongly impaired spermatogenesis but no signs of ITGCNU, intratubular seminoma or an invasive TGCT, neither by morphological criteria nor by immunohistochemistry for OCT3/4. Because of the presence of ITGCNU and intratubular seminoma as demonstrated in the first biopsy, it was decided to perform an inguinal orchidectomy of the right testicle. Because no signs of malignancy were noted in the left testicle in the last biopsy while the first was not informative, it was left in situ. The orchidectomy specimen showed an atrophic testicle with fibrosed tubules. In seven out of the 11 sections taken from different parts of the testis (see Figure 2), ITGCNU was present and some tubules showed intact spermatogenesis. In one section indeed a small invasive seminoma was identified. The focal distribution of ITGCNU is this patient explains the negative finding in the second random open surgical biopsy. After orchidectomy the ITGCNU cells were microdissected for evaluation of the presence of the codon 816 c-KIT mutation, known to be associated with bilateral tumors [75]. No mutation was detected. Computer tomographic scanning of the thorax and abdomen showed no metastases. Because the tumor is therefore a clinical stage I seminoma, the patient was treated with retroperitoneal irradiation. The left testicle will be checked with regular interval using ultrasonography.

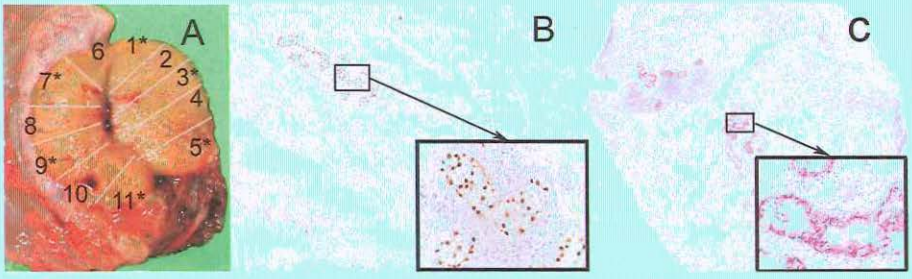

Figure 2. A) Testis parenchyma with normal aspect with 11 marked areas which represent the formalin fixed (*) and snap frozen tissue samples. B) area 1*; Oct 3/4 staining (2.5x). Inset (40 x). C) area 4. Direct alkaline phosphatase staining (1.25x). Inset (40x). Both sections demonstrate the presence of CIS/ITGCNU.

DISCUSSION

This case report illustrates a number of clinically relevant issues. First, it shows that the diagnosis of ITGCNU (and intratubular seminoma) can be missed by a general pathologist on a testicular biopsy fixed in buffered formalin by morphological criteria alone. Second, the re-analysis based on immunohistochemistry is informative to prevent underdiagnosis of ITGCNU. At our institute,

OCT3/4 has proven to be the most robust marker for this purpose. The availability of monoclonal antibodies, proven to be specific and giving reproducible results, allows application of this analysis in daily pathological clinical practice. Third, and possibly most important from a clinical perspective, ITGCNU can be very unevenly distributed in a testis, which may result in a negative finding using a randomly taken testicular open biopsy. Last, this article again underscores our earlier observation that bilateral microlithiasis in infertile males is informative in selecting males with an increased risk for the presence of ITGCNU. In contrast to our initial study, this patient had no history of cryptorchidism, indicating that bilateral microlithiasis is of value in this context even without the clinical observation of undescended testis. Because it is not yet possible to detect ITGCNU with imaging techniques or serological methods, an open surgical biopsy of 3 mm is currently the standard approach for diagnosis, reported with a small risk of false-negative sampling. As demonstrated here, diagnosis of ITGCNU or intratubular seminoma, may be difficult on standard (formalin-fixed, Hematoxyline-Eosine stained) histological slides for a general pathologist not experienced with the possible false negative findings on biopsies. Based on these observations, we recommend that when a testicular biopsy is taken from subfertile men to screen for the presence of ITGCNU, immunohistochemistry for OCT3/4 must be performed. This is even more highly recommended if the patient presents with bilateral microlithiasis. In the literature it is claimed that a small number of ITGCNU cases are missed due to technical or judgment errors, while the majority are due to focal, uneven distribution of ITGCNU within the testicle [6]. Both reasons were combined in the patient presented here. ITGCNU was missed in the second random biopsy of an atrophic testicle measuring a volume of only 4 cm^3 because it may be focal even in the presence of an invasive tumor, in particular in the case of seminoma. A plausible explanation is that the host response elicited by the seminoma may eventually eradicate most of the ITGCNU in the adjacent parenchyma. This is supported by the observation that testicular parenchyma adjacent to a non-seminoma usually shows a more evenly distribution of ITGCNU [10]. Kliesch et al showed that in one quarter of the men who underwent two biopsies of the same testis ITGCNU was found in only one biopsy and thus could easily have been missed if only one biopsy had been performed [76]. A number of conditions are known to predispose to ITGCNU, which are summarized in Table 1. In this perspective it might be useful to take more than one biopsy from each testicle in patients with specific

Table 1: Risk factors (6, 9)

Contralateral tumor	RR	24.8-27.6
History of undescended testes	RR	3.5-17.1
Family history of TGCT	RR	2.15-12.3
Subfertility	RR	1.6-10.0
Bilateral microlithiasis	RR	2.0-20.0
Atrophic testes	RR	2.7-12.7
Ambiquous genitals	CR	25 %

RR= relative risk CR= cumulative risk

risk factors for ITGCNU to improve diagnostic accuracy. If however, no ITGCNU is diagnosed, all patients should at least be advised to perform self-investigation.

CONCLUSION

The case reported is a further example of the non-random distribution of ITGCNU. It further emphasizes the need for specific immunohistochemical staining, using the OCT3/4 marker, on testicular biopsies to improve ITGCNU diagnostics.

Chapter 5

Evaluation of testicular biopsies for Carcinoma in Situ: Immunohistochemistry is mandatory.

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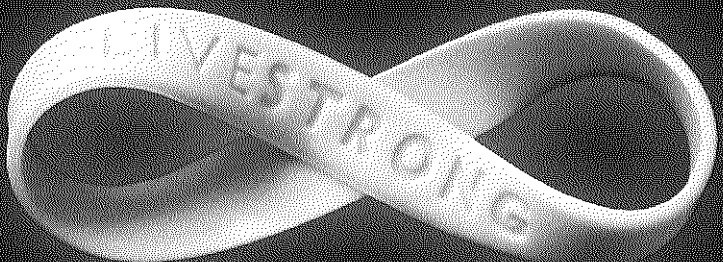

ABSTRACT

Carcinoma *in situ* (CIS) is the common precursor of all type II testicular germ cell tumors (TGCTs), i.e. seminomas and non-seminomas, which can be diagnosed using a surgical biopsy. The objective of this study was to investigate the additional value of immunohistochemistry for the diagnosis of CIS in assessing testicular biopsies taken in the context of infertility. A series of 21 infertile patients were retrieved from the Dutch pathological database (PALGA), being diagnosed with an invasive TGCT, while a matched previously obtained testicular biopsy was diagnosed as non-malignant. From 20 patients, both the invasive tumors as well as the biopsies were revised using morphology and immunohistochemistry for OCT3/4, placental-like alkaline phosphatase and c-KIT, all known established markers for CIS. The presence of CIS or invasive malignancies was scored. There are no interventions. Morphological criteria alone allowed an experienced pathologist in TGCTs to diagnose CIS in five and an invasive tumor in two cases (total n = 7, 35%). Application of immunohistochemistry resulted in the identification of an additional four cases of CIS (total n = 11, 55%, additional value of 20%). The initial correct diagnosis of CIS could have prevented a second gonadectomy in four patients (20%). This study, for the first time, really shows that time of progression from CIS to seminoma is longer than to non-seminoma. Our study demonstrates that immunohistochemistry should be performed for the diagnosis of CIS of the testis on single biopsies obtained because of infertility, resulting in an extra diagnostic yield of at least 20%. Application of this protocol will allow early diagnosis, and therefore prevent any adverse anti-cancer treatment sequelae including gonadectomy, and requiring life long androgen supplementation in some patients.

INTRODUCTION

Carcinoma *In Situ* (CIS), also referred to as Intratubular Germ Cell Neoplasia Unclassified (ITGCNU) is currently accepted as the precursor of all testicular germ cell tumors in adolescents and young adults (TGCTs) , i.e., the seminomas and non-seminomas, which are also referred to as type II TGCTs [5]. CIS represents the malignant counterpart of a primordial germ cell/ gonocyte, which develops during intra-uterine development. In spite of this early initiation, the invasive TGCT manifests itself only after puberty. This means that there is a time window for early diagnosis of this precursor of type II TGCTs. Patients diagnosed with CIS can be cured by orchidectomy or by a low dose of testicular irradiation [79, 80] In the majority of cases testicular irradiation does not interfere with testicular hormonal function [81].

Individuals at risk for type II TGCTs, like those with infertility, may undergo testicular biopsies performed for determination of spermatogenic status. Patients also have biopsy performed specifically for the diagnosis of CIS, including patients with a contralateral TGCT, bilateral testicular microlithiasis, hypoechoic lesions found on testicular ultrasound and cryptorchidism [79, 82]. Although CIS is nearly always found in testicular parenchyma adjacent to an invasive TGCT in orchidectomy specimens, it is reported that in testicular biopsies that do not contain an invasive tumor, CIS is missed in about 0.5 percent [10, 65].

Besides false negative diagnosis due to a heterogeneous distribution [83, 84], CIS can be missed using standard haematoxylin and eosin (H & E) stained sections. The recent identification of highly specific and sensitive markers has significantly increased the detection rate of CIS. OCT3/4 (as known as POU5F1) is a good example in this context [12, 13]. OCT3/4 is present in CIS, seminoma and embryonal carcinoma, showing a consistent and strong nuclear staining. In contrast, no OCT3/4 staining is found in normal adult testis, which makes it specific for the diagnosis of the pre-invasive stage of all TGCTs and false positive results do not occur. [10].

To investigate if immunohistochemistry has additional value over standard morphological examination to diagnose CIS in testicular biopsies, even for an experienced pathologist, we performed a unique study in patients with a type II TGCT, who previously had a testicular biopsy performed in the same testis for infertility or testicular microlithiasis. These biopsies were initially diagnosed as not malignant, and re-evaluated by an expert pathologist and complemented with immunohistochemical staining. We also explored the economical benefits of the implementation of OCT3/4 immunohistochemistry.

MATERIAL AND METHODS

Patient selection

Since 1971 a national pathology database (PALGA) has covered all (n = 70) academic and non-academic pathology departments in the Netherlands, registering histopathology and

cytopathology results. We performed a PALGA database search to identify patients who underwent a testicular biopsy and developed a TGCT later in life. This initial screening resulted in 121 patients: pathology reports of the biopsies revealed twelve cases with CIS (10%), 88 invasive TGCTs (73%; 55 seminomas and 33 nonseminomas) and 21 cases without malignancy fulfilling our criteria. From the ten pathology departments involved, we were able to collect the original paraffin blocks from 20 of these 21 patients (95%), and the original slides from 13 (65%). One patient for whom no pathological samples could be retrieved was excluded from the calculations. The indications for performing a testicular biopsy were recorded if this had been entered in the pathological evaluation. Because of ethical restrictions related to privacy protection to the use of data derived from PALGA, it was not possible to obtain additional patient information, other than that mentioned in the pathology report.

Immunohistochemistry & evaluation

Three micron thick tissue sections were cut of all the retrieved blocks and stained with Haematoxylin and Eosin (H & E) and evaluated by a pathologist experienced in germ cell tumor pathology (J.W.O.). In addition, parallel sections were stained using immunohistochemistry for OCT3/4, as well as c-KIT and placental-like alkaline phosphatase (PLAP), other established markers for CIS, as previously described [10]. To assess if differences in staining pattern of these markers we compared the immunohistochemically stained slides. The biopsy samples were blinded prior to evaluation, and the presence of CIS was scored first on the H & E stained sections and subsequently on the stained slides. CIS was morphologically defined as the presence of large atypical intratubular cells with large hyperchromatic nuclei containing several prominent nucleoli. Moreover, CIS containing seminiferous tubules mostly show thickened basal laminae and reduced luminal size.

According to the recommendations of Holstein and Lauke, who stated that a reliable diagnosis could be made if more than 30 tubules were present in the biopsy, we counted the total number of seminiferous tubules to determine whether a reliable diagnosis could be made [85]. The orchidectomy specimens were diagnosed according to the 2004 WHO classification for Testicular Germ Cell Tumors [86].

Costs

An exploratory cost-effectiveness analysis was performed to assess the costs and effects of performing immunohistochemistry (i.e., OCT3/4) on testicular biopsies in daily practice. The diagnosis of CIS based on a testicular biopsy obtained in the context of infertility and subsequent treatment will prevent development of an invasive type II TGCT, potentially leading to cost reductions regarding testicular cancer treatment. The costs of the three main scenarios involved in the treatment of patients with type II TGCTs were included. The first consists of orchidectomy with an additional 5-year follow-up; the second of orchidectomy combined with abdominal irradiation, and the third of orchidectomy followed by cisplatin-based standard

chemotherapy. Literature analyses revealed that these treatment modalities are equally divided among the type II TGCT patients [87]. The related costs were obtained from our university hospital. These costs were compared with the costs related to treatment of CIS by local testicular irradiation. The number of preventable invasive type II TGCTs was calculated based on identified CIS on biopsies performed for infertility in our institution during the last five years. From this cohort the number of preventable cancers was estimated from the extra diagnostic yield of immunohistochemistry.

RESULTS

In 21 patients, initial morphological investigation of an H & E stained histological section of the testicular biopsy performed in a routine pathology department revealed no CIS or an invasive type II TGCT. However, all 21 individuals developed an invasive testicular cancer later in life, as confirmed by orchidectomy specimen evaluation. The biopsies were performed between 1981 and 2003, of which the clinical indication is summarized in Table 1. The mean age at time of biopsy was 29.1 years (range 16 to 36 years), 33.2 years (range 19 to 44 years) at time of orchidectomy, and the mean timespan between the original biopsy and the orchidectomy was 52.4 months (range 9.3 to 156.5 months). Orchidectomy analysis demonstrated 13 seminomas, six nonseminomas and two CIS only. The time to clinical presentation from CIS to an invasive tumor was 26 months in nonseminomas (median, range 9-78), 47 months for seminomas (median, range 15-157), and 54 months for the patients with CIS-only in the orchidectomy specimen (range 47-61,). In spite of the limited number of cases the difference in time from CIS to clinical presentation is of interest (Fig.1A,B).

The original slides used for initial diagnosis were obtained from 13 cases and the original paraffin blocks from20 patients (for one patient no pathological material was available and was therefore not included in the study). Because one block lacked residual material, the original slides were obtained and one was immunohistochemically re-stained for OCT3/4. In line with the original diagnosis of the orchidectomy specimen, re-evaluation showed 13 seminomas, six nonseminomas, one CIS-only and one burned out tumor with CIS in the adjacent parenchyma (Table 1). Four patients had a history of unilateral TGCT, and in three of them, a biopsy has been performed because of infertility.

Re-evaluation on the basis of morphological examination using well defined chracateristics (see materials and methods section) of the original slides or newly cut slides showed that malignant cells were present in seven out of the 20 patients (35%), that is., five CIS (one of them with a previous TGCT), and two invasive seminomas. Two other biopsies, were recognized as suspicious for the presence of CIS, although it could not be diagnosed with certainty. Immunohistochemistry was performed before coming to a definitive diagnosis (Fig. 2). In addition to the seven cases diagnosed on H & E morphology alone, immunohistochemistry for OCT3/4,

Table 1: Patient characteristics with primarily pathological evaluation and revision results.

Case no.	Age at biopsy (years)	History	Fixative	Age at orchidectomy	1st biopsy result	Revision H&E	Discordances on H&E	OCT3/4	Discordances between H&E and OCT3/4 at revision	Orchidectomy result
1	36	azoospermia	F	38	bilateral JS 8-9	no CIS *	not discordant	negative	not discordant	left, seminoma
2	32	bilateral orchidopexy azoospermia	B	39	right hypospermatogenesis, some SCOS tubules, Leydig cell hyperplasia	no CIS	not discordant	negative	not discordant	right, embryonal carcinoma with teratoma
3	31	orchidopexy, Infertility, oligospermia	B	32	right SCOS with some tubules containing hypospermatogenesis JS 2-3	CIS	discordant	positive	not discordant	right, seminoma
4	35	azoospermia	B	44	bilateral hypospermatogenesis	no CIS	not discordant	negative	not discordant	right, seminoma
5	28	right orchidopexy , azoospermia	B	39	bilateral JS 9-10	no CIS	not discordant	negative	not discordant	right, seminoma
6	27	infertility	F	39	bilateral JS 3	CIS left + right	discordant	bilateral positive	not discordant	right, seminoma
7	30	orchidectomy right, chemotherapy BEP + RPLKD, azoospermia	B+F	33	3 biopsies performed all three showed a maturation arrest	First: no CIS Second: no CIS Third: no CIS	Second +third discordant	first negative, second positive, third positive	discordant	left, seminoma
8	33	-	F	34	bilateral JS 9-10	no CIS *	not discordant	Left positive	discordant	left: seminoma
9	28	azoospermia	F	34	bilateral SCOS, severe spermatogenesis arrest	Right, micro invasive seminoma	discordant	Right positive	not discordant	right, seminoma
10	34	orchidopexy, azoospermia	B	37	bilateral oligospermia, atrophy, Leydig cell hypoplasia	no CIS	not discordant	negative	not discordant	right, immature teratoma

11	29	bilateral testicular microlithiasis	B	29	bilateral JS 10	no CIS *	not discordant	negative	not discordant	left, seminoma
12	28	orchidectomy right TGCT, infertility	F	31	left, hypospermatogenesis, leydig cell hyperplasia	CIS	discordant	positive	not discordant	left, seminoma
13	24	TGCT nonseminoma right 4 year earlier, azoospermia	F	28	left: 30 % SCOS, hypospermatogenesis, some tubules contain spermatozoa	suspect	not discordant	positive	discordant	left, seminoma
14	34	azoospermia	F	38	left JS 9	no CIS	not discordant	negative	not discordant	left, seminoma
15	28	-	F	29	right, fibrosis, necrosis	no CIS *	not discordant	negative	not discordant	right, mixed GCT EC+ yolk sac tumor
16	31	infertility	F	34	bilateral JS 7	CIS	discordant	left negative, right positive	not discordant	right, seminoma.
17	31	azoospermia, nomal gonadotrophins	F	36	left, Maturation arrest	CIS	discordant	positive	not discordant	left, CIS only
18	29	Infection	.	30	right, infection, suspicious for sarcoidosis	no material obtained	.	-	.	right, nonseminoma
19	29	-No remaining material in paraffin block.	B	33	right, JS2 areas with JS 6-7	no Cis	not discordant	negative	not discordant	right, burned out seminoma + CIS
20	16	right TGCT, teratoma,	F	19	left JS 10	suspect	not discordant	positive	discordant	left, nonseminoma
21	21	-	F	21	Left, reactive connective tissue, no testis in this biopsy	nonseminoma	discordant	positive	not discordant	left, nonseminoma + embryonal carcinoma

JS= Johnsen score ; SCOS= Sertoli cell only syndrome ; BEP= Bleomycin/Etoposide/Cisplatin ; RPLND=Retroperitoneal Lymph Node Dissection ; F=formalin, B= Bouin's fixative; * No sufficient biopsy material, i.e., at least 30 seminiferous tubules present, was available.

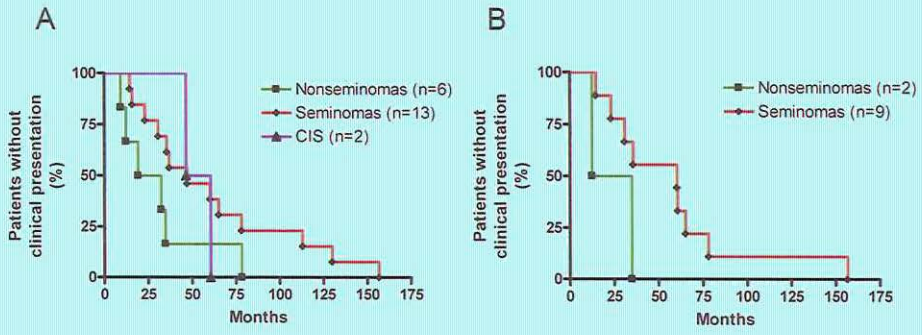

Figure 1: Kaplan Meier curves showing time from biopsy to clinical presentation. A) Time to clinical presentation in the complete group (n=21). B) Time to progression in the patient in whom during the re-evaluation malignancy was found (n=11). In one patient the orchidectomy specimen showed CIS only, and this patient was included in the seminoma group.

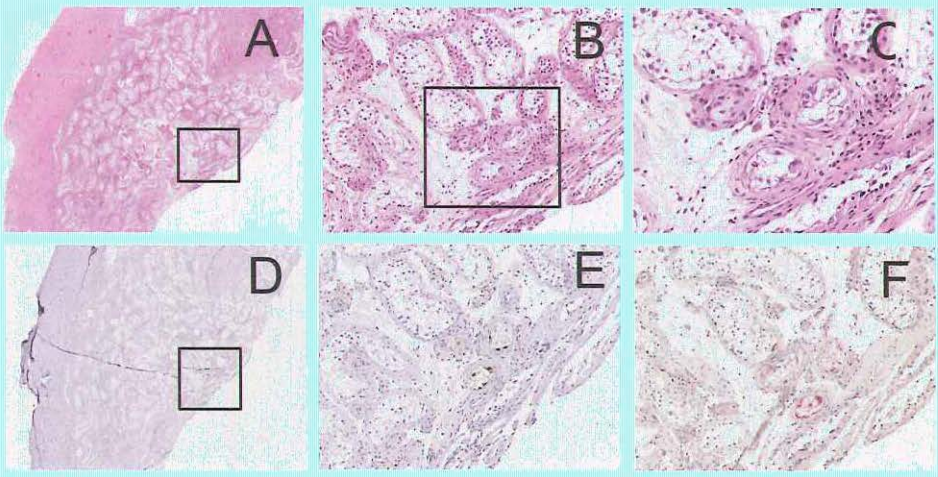

Figure 2. A) Histology of an open surgical biopsy of the testis stained with H & E (25X) (patient no. 7, Table 1). It was identified as suspected, but not proven to contain CIS; B) Higher power of suspected lesion (H & E 100x); C) Higher power of suspected lesion (H & E 400x).
D) Histology of the same biopsy stained immunohistochemically positive for OCT3/4 (25X). The region with positive cell is indicted in the square; E) Higher power image of suspected lesion (OCT3/4, 200x); Only three tubules contained OCT3/4 positive cells (Brown); F) PLAP immunohistochemistry (200x) of the same region, confirming the presence of CIS cells (Red).

PLAP and c-KIT identified four more cases of CIS (in total: 11 out of 20, 55%). These included the two cases identified as suspected for CIS (Figure 3). Four men had a history of TGCT in the contralateral testicle. In all 13 cases, no discrepancies were seen between the diagnoses made on the original slides and the diagnoses made based on the newly cut slides from the matched paraffin block.

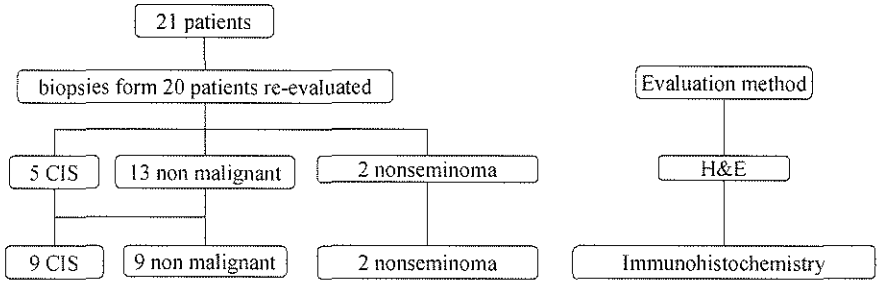

Figure 3: Flowdiagram showing biopsy outcome according to type of evaluation.

For unknown reasons one patient (no. 7, Table 1) had undergone three successive biopsies within a period of 18 months. On the basis of the H & E stained sections no CIS was identified, both originally and upon review, but immunohistochemistry demonstrated CIS in the last two biopsies, although not recognized before. In four cases (patients no. 1, 8, 11, and 15, Table 1) who all underwent bilateral biopsies, the biopsy samples were negative for CIS on H & E evaluation and contained less than 30 tubules. Therefore, these biopsies are in principle not eligible for diagnosis, because of the risk of false negative findings. However, immunohistochemical staining revealed CIS in one patient (no. 8). Immunohistochemistry was thus able to correctly diagnose CIS in 10 out of the 16 biopsies in which a reliable diagnosis could be made.

Overall, Bouin fixed biopsy specimen showed a weaker nuclear OCT3/4 staining than formalin-fixated specimens, which was not found for c-KIT and PLAP. In spite of this weaker staining intensity, no CIS was missed as compared to the c-KIT and PLAP staining.

During the last 5 years within our institute a total of 158 testicular biopsies in the context of infertility were taken, of which 10 (6.3%) contained CIS using immunohistochemistry for OCT3/4. Based on the additional value of OCT3/4 immunohistochemistry for the diagnosis of CIS (see above, i.e., 20%), evaluation of only H & E stained tissue sections would have under diagnosed two cases. The costs for the three treatment regimens for type II TGCTs and follow-up are summarized in table 2. The extra costs of performing OCT3/4 immunohistochemistry on a single section was estimated as Euro 13, resulting in Euro 2.054 for all 158 biopsies. Therefore,

Table 2: Estimated cost of TGCT treatment including 5-year followup, not including long-term effects

	Costs (Euro)
Orchidectomy	4683
Orchidectomy with radiotherapy	9639
Orchidectomy with chemotherapy	15244
Average costs TGCT treatment	9855
CIS treatment (Local irradiation)	7149
Costs immunohistochemistry	1027
Average cist CIS diagnosis + treatment	8176

TGCT, testicular germ cell tumor; CIS, carcinoma in situ

the average extra cost per OCT3/4-based screened CIS diagnosed patient was Euro 1.027. The costs per patient for early diagnosis and treatment of CIS is than Euro 8.176, which is still Euro 1.679 lower than the average costs related to treatment of invasive cancer.

DISCUSSION

This study describes the additional value of immunohistochemistry for diagnosis of CIS in testicular biopsies in a specific cohort of men with infertility. The findings support the earlier epidemiologically based conclusion that CIS will always progress to an invasive tumor, and that no spontaneous regression occurs [41]. Diagnosis of CIS is therefore an absolute indication for treatment. This study demonstrates that evaluation of standard H & E stained sections of testicular biopsy material results in underdiagnosis of CIS, even by a specialized pathologist, and that the additional use of immunohistochemistry is mandatory.

In 1983, placental-like alkaline phosphatase (PLAP) was the first marker used in the detection of seminomas. Since then, other markers such as c-KIT, AP_2-gamma and OCT3/4 have followed [10, 88-90]. Currently, OCT3/4 is the most robust marker in TGCT diagnostics and 100% of the CIS, seminoma and embryonal carcinoma cells show a strong nuclear staining. No false positive results have been reported, and based on the biological function of OCT3/4 this is unlikely to occur [12, 13]. This is in contrast to the application of PLAP, which is positive in 93-98%, and the finding of only focal c-KIT positive staining patterns in CIS [14, 91].

Our study is based on a revision of testicular biopsies of 20 patients who developed a TGCT after an initial CIS-negative biopsy. Because the development of a clinically manifest tumor is the only method to prove that a biopsy is false-negative, follow-up data are crucial to accurately define the extra diagnostic yield of immunohistochemistry of biopsy specimens. We have shown that immunohistochemistry has at least a 20 percent extra diagnostic yield in this population and therefore seems mandatory for any pathological laboratory. This percentage might even be higher when biopsies are adequately taken as in 10 out of 16 of the biopsies with more than 30 seminiferous tubules malignancy was found. It is known that the peak incidence in age of nonseminomas is lower than that of seminomas. We describe, although in a small patient population, a trend in difference in time to clinical presentation from CIS to seminomas and CIS to nonseminomas, which is seen in daily practice but is difficult to prospectively demonstrate (Figure 2).

Immunohistochemical staining results are dependent on tissue fixation and standardized protocols. Our study found a weaker signal using the OCT3/4 marker in Bouin-fixated tissue than in the specimens fixed in formalin. This is in contrast to a previous study, in which no effect of the fixative on OCT3/4 immunohistochemistry was found, in case of limited fixation time [13]. Although the time of fixation could not be retrieved from the PALGA database, the weaker signal is most likely explained by the long Bouin's fixation of the samples included in the study

described in this paper. Guidelines on the use of fixatives for testicular biopsies are contradictory on the use of Bouin's fixative or formalin [79, 92]. Stieve's fixation might be an alternative, although no such samples were included in this study. However, we observed previously no significant changes with standardized fixatives of different origin for the detection of OCT3/4 by immunohistochemistry [10].

CIS was thought to be evenly distributed throughout the testis: a one single open surgical biopsy of at least 3 mm in diameter (with more than 30 tubules), would be sufficient for diagnosis [85]. However, CIS can also be non-randomly distributed throughout the testis or the parenchyma can be damaged during surgery which might result in false negative biopsy results [83, 93], possibly limited by the use of two-sided biopsies [65]. In our cohort biopsies from four patients contained fewer than 30 tubules and a reliable diagnosis could not be made. However, OCT3/4 immunohistochemistry showed the presence of CIS in one of these biopsies. The reasons for the 5 remaining negative biopsies out of the series of 20 cases (25%) are unclear. This percentage is higher that in published series (0·5%) [65] showing the representative value of a testicular biopsy to diagnose CIS. Potential explanations might be the selected group of patients for this study (selection bias), or indeed a heterogeneous distribution of CIS. The cases in which the biopsies lacked CIS were not the same patients who had undergone therapy for a previous TGCT. Furthermore we show here that an experienced pathologist is able to find more abnormalities, which advocates a centralized evaluation of testicular biopsies. This might be explained by the low incidence of TGCTs, which might result in a reduction of pathologist awareness.

Disseminated TGCTs need treatment containing irradiation or chemotherapy, which may have severe long-term consequences. Moreover, chemotherapy will not always eradicate CIS in the contra-lateral testis, resulting is possible development of a contralateral cancer, leading to complete castration [94]. CIS is curable in almost 100% of the patients using a low dose of irradiation of the testis, although rare exceptions are reported [79]. Testicular irradiation eradicates all germ cells, both normal and malignant, but the effect on Leydig cell function is limited [95]. The choice between irradiation and orchidectomy for the treatment of CIS depends on the patient's choice and concomitant factors as fertility preservation and pre-treatment hypogonadism. In our study, four patients with a history of TGCT developed a contralateral tumor after a false-negative biopsy. In all cases, review of the initial biopsy showed the presence of CIS. Treatment of CIS in these four patients with local radiation could have prevented the second orchidectomy, definitive infertility and life-long treatment with androgens. At least one of these four patients was also treated with chemotherapy, confirming that receiving chemotherapy (bleomycin/etoposide/cisplatin) does not always prevent against the development of a contralateral cancer.

Recent studies have shown a prevalence of CIS in infertile men of 1- 5% [4, 96, 97]. We therefore believe that for infertile men who are subjected to testicular biopsies for the evaluation of spermatogenesis not only the quality and quantity of spermatogenesis should be routinely

checked but the presence of CIS should also be investigated. It is the responsibility of the referring physician to accurately judge the risk for CIS, and to inform the pathologist of the risk factors in a particular patient. Subsequently, it is the responsibility of the pathologist to apply the best diagnostic procedure, preferentially including immunohistochemical staining.

The cost-effectiveness analysis suggested a cost reduction from the use of OCT3/4 immunohistochemistry of approximately Euro 1.500 per additionally found patient. Although not at all complete, it illustrates the potential health care profit when OCT3/4 is used routinely on all testicular biopsies in daily practice, especially in the context of infertility.

The design of this study has some limitations, including absence of clinical data related to treatment modality, survival and quality of life. This prevents a firm conclusion on the benefit of diagnosing CIS in the initial biopsy. However, it can be concluded that the implementation of immunohistochemistry is most-likely cost effective, and improves quality of patient care, and should therefore be used as standard diagnostic practice.

Chapter 6

Non-invasive detection of testicular Carcinoma in Situ in semen using OCT3/4.

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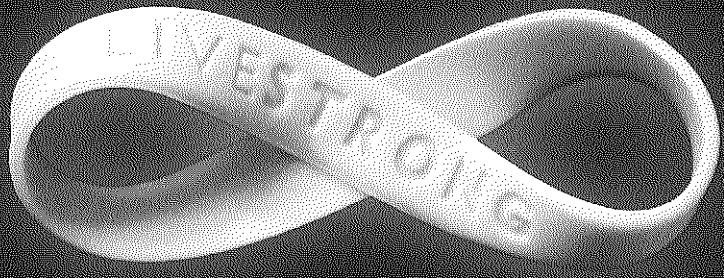
European Urology. 2008 Jul;54(1):153-8.



ABSTRACT

Objective: Carcinoma in situ (CIS) is accepted as the precursor of the germ cell tumors of the adult testis. CIS cells are located within the seminiferous tubules and can be exfoliated into semen. We performed a study to detect CIS cells in semen using the highly specific immunohistochemical marker OCT3/4, potentially a method for noninvasive diagnosis.

Material and methods: In 2006, 41 men at risk for CIS of the testis were found eligible for this study. Indications for inclusions were a suspicious lesion on scrotal ultrasound investigation (n = 14), patients on surveil- lance after a history of a testicular tumor (n = 14), and 13 patients with bilateral testicular microlithiasis (TM).

Results: Three of the13 men (23%)whounderwenttesticularbiopsiesfor bilateral TM were histologically diagnosed with CIS (two bilateral), and their semen showed OCT3/4-positive cells in all cases. Twelve of the 14 patients (86%) with a solid mass were diagnosed with a TGCT with adjacent CIS in the parenchyma, and in 9 cases (75%) OCT3/4-positive cells were present in the semen. No OCT3/4-positive cells were found in patients with biopsies who did not show any evidence of malignancy.

Conclusion: This study demonstrates that OCT3/4-positive cells can be found in semen from the majority of patients with CIS. The observations indicate that there is probably a time window in which the CIS cells are exfoliated, which gives an opportunity for early detection of CIS cells in semen of men at risk for TGCT.

INTRODUCTION

Testicular germ cell tumors of adults (TGCTs), seminomas and nonseminomas (also known as type II GCTs)[98], account for 1-2% of all malignancies in men. Although the disease is uncommon an annual rise in incidence is observed in most Western countries [40, 72, 98, 99]. In the Netherlands a 5% annual increase was found between 1990 and 2005 [100]. Carcinoma in situ (CIS) is accepted as the precursor of TGCT [5, 101]. CIS cells originate from primordial stem cells or gonocytes that escape normal development at an early point during intrauterine development [102]. These CIS cells start to proliferate during puberty, presumably after a raise in sex hormone levels. Patients with CIS of the testis will develop testicular cancer within 5 years in 50% and probably all patients will develop testicular cancer ultimately [4]. CIS is frequently found in the adjacent parenchyma of TGCTs [10]. CIS cells are located inside the seminiferous tubules at the basal membrane, in the niche of the spermatogonia, but they can leave their original location and spill over into the lumen. In addition, CIS cells can show a pagetoid spread to neighbouring tubules, the rete testis [103] and even epididymus (see Figure 1).

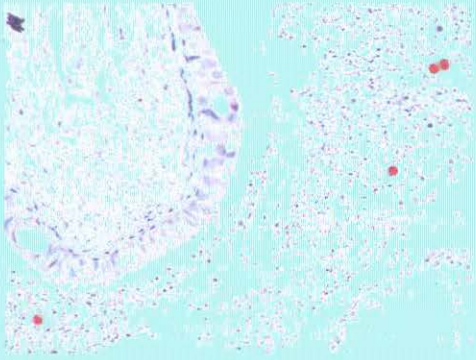


Fig 1: Post orchidectomy specimen showing distinct OCT3/4 positive cells (stained red) in the epididymal lumen.

In view of this, it is plausible that CIS cells are exfoliated in semen, as are spermatozoa. CIS is present in the testis long before a tumor develops and may be used for screening purposes in men at risk for TGCT, specifically in men with fertility problems (0.6%), testicular microlithiasis (20%) and men who were treated for TGCT, and those at risk for a contralateral tumor (5%)[9, 63, 77]. Type II TGCTs are highly sensitive to treatment, which is also true for CIS [4, 104]. This allows early local treatment, preserving hormonal function in most cases[81]. Ever since it was established that CIS is the precursor of TGCTs efforts have been made to detect CIS cells in semen. The use of semen for detection of neoplastic cells in patients with testicular cancer was already suggested by Czaplicki in 1987 [105] and Giwercman in 1988 [106]. Different methods such as Fluorescent in situ hybridization [107], immunohistochemistry using Ap-2 gamma and PLAP [7, 108, 109] and immunohistochemistry with magnetic beads using the M2A antibody

[110] proved to be unsuccessful or too laborious. In contrast to the markers mentioned above OCT3/4 is a very robust nuclear marker that has proven to be an absolute and specific marker for CIS, seminoma and embryonal carcinoma in testicular tissue and is now used as a standard marker in diagnosing CIS and TGCTs [12, 13, 74] (see Figure 2A). Our goal of this pilot study was to develop a reliable staining method. Moreover, we tried to evaluate the use of the OCT3/4 marker for early detection of CIS cells in semen of patients with known risk factors for CIS or TGCT.

Fig 2 (A): Seminiferous tubule with Carcinoma *In Situ* stained with the OCT3/4 marker (Brown).(B+C): Microscopic slides of semen showing spermatozoa and OCT3/4 positive cells from 2 patients diagnosed with a TGCT.

PATIENTS AND METHODS

Patient selection

The Institutional Ethics Committee approved this study and all participants gave written informed consent (code MEC-2005-282).

In this pilot study we selected, based on ultrasound evaluation, patients who were at risk of harboring CIS in their testicles. Between January 2006 and January 2007 a total of 42 patients and 15 controls were included (see Table 1 for group characteristics). Fourteen patients were diagnosed with a testicular mass or ultrasound detected lesion suspect for malignancy; all except 1 underwent unilateral inguinal orchidectomy. This latter patient underwent an open testicular biopsy with frozen section analysis. No malignancy was found on frozen section, and therefore no orchidectomy was performed. All orchidectomy patients were counseled for semen cryopreservation and were asked to donate a semen sample for this study. In six of the 14 orchidectomy patients we were able to obtain a complete semen sample before orchidectomy. Eight out of the 14 orchidectomy patients only donated a residual part of at least 0,3 ml after the majority was cryopreserved for future fertility treatments. Also, patients who were diagnosed with testicular microcalcifications (TM), during analysis for male infertility, candidates for testicular biopsies were asked to participate. A total of 13 men with bilateral TM and bilateral testicular biopsy were included and all donated a complete semen sample. Fourteen post-orchidectomy patients were included. These patients were either diagnosed with a marker relapse (n=12) during their active surveillance protocol or were informed about

Table I: Oct3/4 staining results listed by diagnosis.

Group	Number	Diagnosis	Mean age at time of diagnosis	OCT3/4 positive staining
Suspect for having a TGCT	14	4 seminoma[a]	33.8	2/4 (50%)
		4 nonseminomas[a]	23.7	4/4 (100%)
		2 combined tumors[a]	26.6	2/2 (100%)
		1 Leydig cell tumor	26.0	0
		2 testis containing CIS with a burned out tumor	28.9	1/2 (50%)
		1 Sertoli cell only	28.0	0
Post -orchidectomy	14	11 nonseminomas	26.3	0
		3 combined tumors	24.8	0
Bilateral testicular microcalcifications	13	3 CIS (2 bilateral)	34.4	3 (100%)
		10 No malignancy	34.0	0
Control group	15	Not available	-	0
Total	56	15 CIS containing testis		12/15 (80%)

[a] All testicular germ cell tumors had CIS in the adjacent parenchyma

this study by their physician who requested participation (n=2). None of these patients had testicular abnormalities on clinical examination or ultrasound. Moreover, 15 normospermic patients from the Andrology clinic with no known risk factors for TGCT were used as negative controls. All semen samples were produced by masturbation.

Methods

The testicular biopsies and the orchidectomy specimens were examined using standard protocols for the detection of TGCT and CIS. The semen samples were aloud to liquefy after production and thereafter dissolved in 10% phosphate buffered formalin for one hour. In one patient who underwent a bilateral orchidectomy the left testicle showed a spermatocele, which content was aspirated and used for the detection of OCT3/4 positive cells. At least 0,3 ml semen was obtained if the patient participated. After fixation the samples were centrifuged for 20 minutes with 1600 G where after the pellet is resuspended in phosphate buffered saline and vibrated, using an automatic shaker, to make a single cell solution. Cytospins of this suspension were made on a strong adhesive microscope slide (Starfrost©) and were dried overnight. Immunohistochemistry with monoclonal anti-OCT3/4 (POU5F1, Santa Cruz sc-10, sc-s279) antibodies was performed on the formalin fixed semen samples as described earlier[13]. After the first 15 patients we modified our last staining step by converting from a 3-diaminobenzidine Tetrahydrochloride (DAB, brown) to a 3-amino-9-ethylcarbazole (AEC, red) reaction to increase the contrast between the OCT3/4 positive cells and the spermatozoa and lower the background staining. No differences in cell morphology were seen between these techniques. As a positive control microscope slides were used with OCT3/4 positive cells from an established TGCT cell line (NT2)[111]. The microscope slides were blinded and evaluated separately by two individu-

als and were scored positive if a distinct nuclear staining was seen in large cells with large nuclei and clearly recognizable cytoplasm. Doubtful staining was scored negative.

RESULTS

Three out of the 13 men (23%) who underwent testicular biopsies for TM were diagnosed with CIS, which in 2 of the cases was bilateral. The 14 orchidectomy specimens showed a seminoma in 4 patients (of which 1 was bilateral), nonseminoma in 4 patients, a combined tumor (containing both a seminoma and a nonseminoma component in a single tumor) in 2 patients. In 2 testicles CIS was found next to a burned out tumor. In one patient who had a suspicious intratesticular hypoechogenic lesion an open testicular biopsy was performed and immediately stained with a direct alkaline phosphatase staining method [112]. Histopathological evaluation of this sample showed a Sertoli cell only (SCO) pattern. No CIS or malignant component was found and therefore no orchidectomy was performed. One orchidectomy specimen showed a Leydig cell tumor.

All orchidectomy specimens containing a TGCT showed CIS in the tissue adjacent to the tumor. In 12 out of the 15 patients (80%) harboring CIS at the time of semen donation OCT3/4 positive cells were found in the semen sample (Table I). Two out of the three negative patients were diagnosed with a seminoma. OCT3/4 positive cells were seen with distinct CIS morphology as shown in Figure 2B+C.

The number of OCT3/4 positive cells ranged from 1 to >10 per sample. No correlation was found between the extensiveness of CIS in the testis or ejaculate volume and number of exfoliated CIS cells. In patients with bilateral CIS who donated at least two total ejaculates OCT3/4 positive cells could be found in most successive samples. Patient characteristics, and a comparison between the OCT3/4 antibody staining of semen and the histopathological findings are shown in Table 1.

In the patient with the bilateral seminoma only OCT3/4 positive cells could be found in the aspirated spermatocele and not in the semen sample. No OCT3/4 positive cells were found in the patients who had no histological proven abnormalities of the testis nor did we find OCT3/4 positive cells in the semen samples from the post orchidectomy group. Mean age of the three patients in which no OCT3/4 positive cell were found in the semen although proven TGCT was higher compared to the patients in which OCT3/4 positive cells were seen, respectively 35.8 versus 28.4 (p=0.067). Mean age in the seminoma patients with or without OCT3/4 positive cells were respectively 26.8 versus 40.9 years (P=0.12).

DISCUSSION

CIS is an asymptomatic condition that can be found in testicular biopsies in infertile patient with risk factors for TGCT or in patients with abnormal findings on ultrasound examination [4]. A testicular biopsy is still the gold standard in diagnosing CIS with a false-negative percentage of 0.5%, and recently it is even suggested to take a two-site biopsy to increase the diagnostic yield [11]. Although it is a very sensitive diagnostic test, it is reported that CIS cells are not always randomly distributed, explaining false negative test-results in some cases [93]. Since the discovery of exfoliated CIS cells in semen in 1988 [106], a great deal of effort has been put in finding a method to detect these cells in patients at risk for TGCTs [110, 113]. A non-invasive detection method would be helpful to a certain extend [13].

In this study we were able to detect OCT3/4 positive cells in semen from all CIS bearing patients. This specific patient group may benefit from early detection.

Although other studies as well as our own study have shown that CIS cells can be detected in semen there are still a number of pitfalls. Malignant cells from the invasive tumor are not likely to be exfoliated due to the predominant intact architecture of the seminiferous tubules. The primary location of the CIS cells beneath the tight junctions of the Sertoli cells may prevent spread in the first stage before proliferation has started. There is probably a time window between the early stage of CIS and the time the tumor obstructs the tubules in which the CIS cells are being exfoliated. Also the impact of the change in environment on cell morphology of CIS cells passing through the male genital tract may prevent proper detection. In the third place, with this technique the natural surrounding, in which the CIS cells are easily recognizable, is lacking making a positive identification more difficult and totally dependent of immunohistochemistry and cell morphology. In our study all patients showed recognizable CIS cells stained with the OCT3/4 marker (Figure 2B+C). The use of immunohistochemistry to detect the CIS cells, although it is highly specific, should be performed using a standardized method. Expertise to perform these staining techniques accurately to avoid false negative results is, therefore, of major importance. Moreover, the specificity of the antibodies used must be confirmed, and a standardized method for immunohistochemical detection must be applied, including proper positive and negative controls. Hoei-Hansen et al reported a diagnostic rate for the detection of CIS cells in semen from men diagnosed with CIS only of the testis, using AP_2-gamma of 50% and 0% for OCT3/4 [7]. They were however not able to compare these techniques as they have not stained the same slides for AP_2-gamma as well as OCT3/4. Also possible sub-optimal immunohistochemical detection of OCT3/4 might be related to the published limitation. They also described three false-positive results were seminal fluid showed borderline AP_2-gamma stained cells. A subsequently performed testicular biopsy failed to confirm this diagnosis. We did not encounter any false-positive result using the OCT3/4 marker. Although we did not have pathological examination of the controls, no abnormalities were seen on ultrasound or clinical investigation, making the change of an existing TGCT very small [114]. All three cases,

in which no OCT3/4 cells were found in the ejaculate, showed severe fibrotic tubules potentially hampering exfoliation of CIS cells. The incidence of pagetoid spread of CIS cells in the rete testis differs between patients with seminoma and non-seminoma, with less pagetoid CIS involvement in the seminoma group [103]. Patients with a seminoma often have an extensive host response, less CIS and more atrophic tubules [10]. This lymphocytic infiltration may even result in a complete eradication of CIS in seminoma patients [10, 115]. Seminoma patients are in general older than non-seminoma patients and the risk of any of these factors inhibiting exfoliation is increased. In our study semen from two patients (50%) with seminomas did not show any OCT3/4 positive cells in comparison in all patients with non seminomas OCT3/4 positive cells were seen. A similar tendency was seen in the study of Hoei-Hansen et al in which they saw a significant difference in positive cells exfoliated between seminoma (17,4%) and non-seminoma patients (56,6%) [7]. Age (and histology of the tumor) might therefore play an important role in the exfoliation of CIS cells, with a higher change of finding these cells in patients at young age.

The purpose of this study was to try to detect CIS cells in semen especially to diagnose CIS before an overt tumor has developed. Although TGCT is a highly curable disease the potential long-term side effects of the treatment protocols used are numerous and severe. Long-term side effects will occur in approximately 20-30% and consist mainly of nephrotoxicity, ototoxicity, neurotoxicity and gonadal damage [116]. CIS can be treated with orchidectomy or local radiotherapy with a curability rate reaching 100%, thus preventing potential hazardous chemotherapy in case CIS is left untreated and a testicular tumor develops. In this study two patients had bilateral CIS and were advised to undergo bilateral radiation therapy. However, in case of infertility couples may choose to have artificial reproductive techniques performed before radiation treatment is performed. The third patient with unilateral CIS underwent an inguinal orchidectomy to prevent the potential scatter radiation on the contra lateral testicle in case of radiation therapy.

The combination of the peak-incidence of TGCTs, the long-term presence and exfoliation of CIS cells and well known risk factors may provide a setting in which screening could be useful. Also the relative high incidence (5%) of patients with TGCT occurring in the contralateral testis underscores the need for close follow-up and early detection in this specific group [117]. Patients visiting a fertility clinic have a 20-fold greater incidence of testicular germ cell cancer than fertile men and CIS is found in approximately 0.6% and could therefore be a population who could benefit from screening [64] . In addition, infertile males with bilateral TM, who have approximately a 20 percent chance of harboring CIS, might specifically profit from this technique [9]. Due to the relative low incidence, the high number of patients at risk and the overall good survival in case of TGCT this test should be easy to use, cheap, highly specific and sensitive to make it cost-effectiveness. We are aware that the numbers in our study are relative small en the results presented here are therefore preliminary. Future research will focus on a more specific enrichment of the CIS cells, integrating an automatic screening tool for the detection

of OCT3/4 positive cells, increasing the numbers to substantiate our results and make a cost-benefit analysis and perhaps finding treatments that eradicate CIS while preserving remaining spermatogenesis.

Chapter 7

Gonadal dysfunction in male cancer patients before cytotoxic treatment

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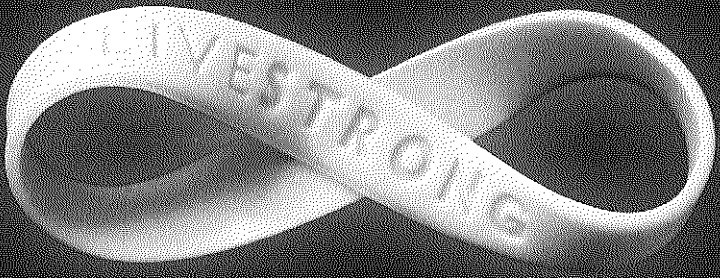

ABSTRACT

Male patients diagnosed with cancer are often referred for semen cryopreserva- tion before gonadotoxic treatment but often have low semen quality. The aim of this study was to evaluate which type of cancer affects gonadal function and proposes a risk factor for low pre-treatment semen quality. Between January 1983 and August 2006, 764 male cancer patients were referred for semen cryo- preservation prior to chemotherapy and radiotherapy. We compared semen characteristics and reproductive hormones between different groups of cancer patients. In addition, we evaluated the role of tumour markers in patients with testicular germ-cell tumours (TGCT) on fertility. Abnormal semen parameters were found in 489 men (64%) before cancer treatment. Patients with TGCT and extragonadal germ-cell tumours had significantly lower sperm concentra- tions and inhibin B levels than all other patient groups. No semen could be banked in 93 patients (12.2%). Eight hundred and thirty-nine of 927 (90%) produced semen samples were adequate for cryopreservation. Inhibin B in all groups showed to be the best predictor of semen quality. Although pre-treat- ment raised tumour markers were associated with a decrease in inhibin B and increased follicle stimulating hormone, both predictive for low semen quality; no direct linear association could be found between raised beta-HCG, alfa-feto-protein and semen quality. Only 1/3 of cancer patients had normal semen parameters prior to cancer treatment. Patients with TGCT and extragonadal GCT have the highest risk for impaired semen quality and gonadal dysfunction at the time of semen cryopreservation.

INTRODUCTION

Survival rates of young male cancer patients have improved substantially during the last decades because of to improved anti-cancer treatment modalities and early detection. Post-treatment quality of life has become an important issue in the management of men with, testicular cancer, Hodgkin's lymphoma and leukemia, the commonest malignancies in patients of reproductive age. Therapy for these malignancies may consists of aggressive chemotherapy and radiotherapy and is accompanied by unwanted side effects, such as infertility and sexual problems. Especially the potential sterilizing effects of these treatment regimes are of major concern for young patients. This depends on the type and amount of cytotoxic agents used. The only established method to secure the potential reproductive capacity is cryopreservation of sperm[118]. If the patient wants to father a child after surviving cancer and gonadotoxic treatment has resulted in spermatogenic failure, banked semen can be used for artificial reproductive techniques (ART), usually in vitro fertilization (IVF) and intra cytoplasmatic sperm injection (ICSI). The number of pregnancies obtained with IVF and ICSI using cryopreserved semen average around 24% for IVF and 33%for ICSI [118, 119]. About 10% of the patients will eventually use their frozen semen [118]. Since the introduction of ICSI, even low numbers of motile spermatozoa may be sufficient for successful treatment and should be cryopreserved. It may be important to know in advance which cancer patients are at risk for impaired pre-treatment semen quality because defective spermatozoa are more vulnerable to the freezing process and this may negatively influence the outcome of ART in the future. So far, clinical diagnosis itself has not shown to be a predictor of the deleterious effects of the cryopreservation process on sperm quality [120]. In male cancer patients systemic and local effects of the cancer can influence the pre-treatment fertility, although the precise mechanism has not yet been totally clarified [121]. In this study, we evaluated semen parameters and reproductive hormones from male cancer patients referred for semen cryopreservation at our fertility clinic. Furthermore, we assessed if symptoms associated with advanced disease such as fever, night sweats, fatique and weight loss influences sperm quality. With this study, we aim to assess the potential fertility impairing effect of different types of cancer before cancer treatment and evaluate which cancer patients are at the highest risk of having diminished gonadal function prior to therapy.

MATERIAL AND METHODS

Between January 1983 and August 2006, 764 male cancer patients were referred to the Andrology clinic for semen cryopreservation. All patients were referred before receiving any gonadotoxic treatment. From all patients diagnosis was reported and a short history was taken concerning potential fertility impairing problems. Symptoms associated with advanced disease including weight loss, recent episodes of fever, night sweats or fatigue were recorded.

Semen was produced by masturbation in a private room and if desired visual erotic stimulation was available. Abstinence time was not standard noted in our patients. After collection of the semen sample volume, total sperm count ($x10^6$), sperm concentration ($x10^6$/mL), progressive sperm motility (%) and morphology (% normal forms) were evaluated according to the guidelines of the World health Organization (WHO) laboratory manual for the examination of human semen valid at time of evaluation [122]. The protocols for sperm concentration and motility measurement are comparable for the different manuals. Reference values for sperm concentration are more than 20 $x10^6$/ml and for progressive motility more than 50% [122]. Semen samples were found suitable for cryopreservation if motile spermatozoa were present. A second semen collection and cryopreservation procedure was offered if time was available before cytotoxic treatment was started. Peripheral blood samples were obtained as part of the diagnostic procedures for analysis of reproductive hormones, including luteinizing hormone (LH), follicle stimulating hormone (FSH), inhibin B and testosterone [123]. All serum samples were collected within 3 months prior to the date of semen cryopreservation and before inva-

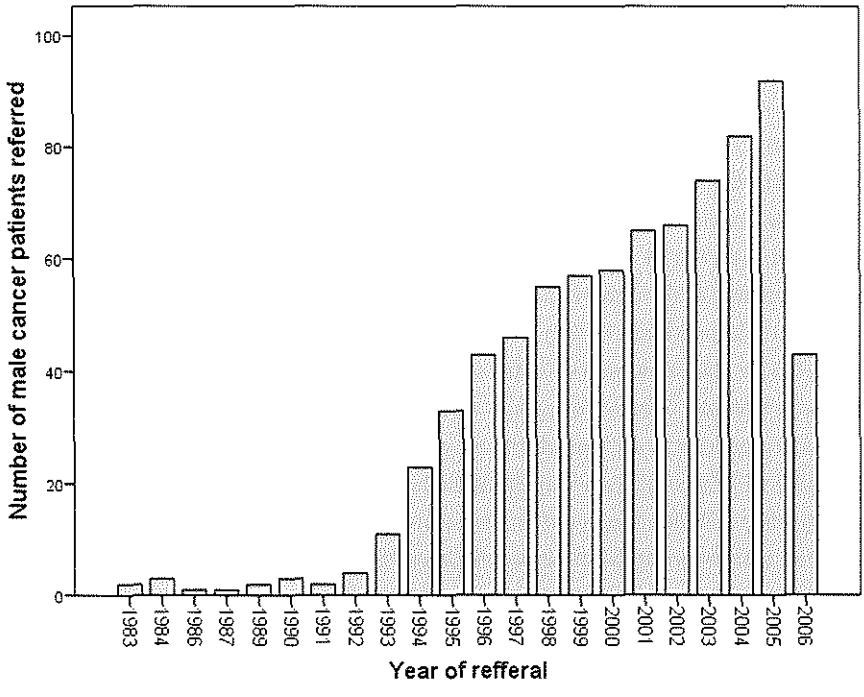

Fig 1: Number of men referred for semen cryopreservation according to year of referral.

sive gonadotoxic anticancer treatment. Reference values were for LH 3-10 U/l, for FSH 3-8 U/l, for Inhibin B 150-400 ng/l and for testosterone 10-30 nmol/l [124]. In the patients diagnosed

Table 1: Patient characteristics according to diagnosis. All numbers are given in median and range.

	Total number of patients Endocrinological data available in ()	Age (Years)	Volume (ml)	Sperm Concentration (10^6/ml)*	Total number (10^6)	Sperm Motility (%)*	Conc >20 million (%)	Azoospermia samples (%)	Inhibin B (ngl/l)	FSH (U/l)	LH (U/l)	Testosterone (nmol/l)
Hematological malignancies	96 (22)	26.3 14.6-51.6	2.5 (0-9)	18 (0-323)	34.3 (0-581)	34 (0-81)	34	25	205 33-304	3.1 0.4-31.7	4.7 1.8-9.6	13.5 4.5-24.9
Brain tumors	21 (7)	28.9 14.4-50.9	2.2 (0.2-7)	48.5 (0-749)	126 (0-1282)	50 (5-89)	61	13	91* 72-224	3.2 1-12.4	2.3 0.1-3.7	10.5 2.2-13.9
Carcinoma's	35 (14)	35.6 15.3-56.9	3.4 (0.2-15)	39 (0-135)	80 (0-1080	40 (0-66)	48	15	156 64-308	3.7 0.3 10.2	3.8 1.8-11.4	16.3 10.9-21.1
Extragonadal germ cell tumors	17 (7)	26.9 14.9-40.9	1.8 (1-5)	5.6* (0-36)	12.3 (0-94)	23 (0-61)	10	25	102* 33-236	2.5 0-10.0	2.3 1.2-8.2	14.1 5.0-42.6
Hodgkin lymphoma	173 (67)	25.2 (13.8-44.1)	2.0 (0-11)	20 (0-243)	35 (0-899)	39 (0-71)	38	23	153 61-324	3.5 1.5-18.2	3.5 0.8-11.1	16.5 3.4-32.0
Non Hodgkin Lymphoma's	92 (38)	29.4 (15.7-51.2)	2.4 (0-8)	33 (0-387)	64 (0-968)	30 (0-66)	41	24	162 73-332	3.9 0.7-19.5	3.7 1.2-10.7	14.1 6.0-27.6
Sarcoma's	38 (9)	22.3 (13.9-38.3)	1.8 (0.1-7)	25 (0-260)	28.8 (0-1058)	37 (0-69)	41	20	148 69-351	2.2 0.6-13.7	2.9 1.0-5.2	12.7 6.9-26.8
TGCT	292 (149)	27.1 (14.6-45.7)	2.6 (0.2-9)	9.9* (0-308)	25.5 (0-766)	41 (0-83)	28	18.6	73* 1-312.0	5.0 0-128.0	3.2 0.1-23.8	16.1 0.6-63.5
Total	764 (313)	26.9 (13.8-56.9)	2.3 (0-15)	17 (0-749)	35 (0-1282)	38 (0-89)	35	21	117 (1-351)	3.9 (0-128)	3.5 (0.1-23.8)	15.0 (0.6-63.5)

* groups in which Inhibin B levels were significantly lower than all other groups without * ($P<0.05$).

with TGCT levels of beta-HCG (ref 0-1.9 IU/l), alfa-fetoprotein (AFP, ref 0-9microg/l) and Lactacte dehydrogenase (LDH, ref 0-449 U/l) were measured.

For statistical analysis, SPSS 16.0 was used (SPSS inc, Chicago, Ill): p-values lower than 0.05 were considered significant. Values are depicted as median to correct for the distorting effect of outliers in combination with the minimum and maximum level. Non-parametric analyses were used if the distribution was not normal and the Mann-Whitney-U-test was used to compare different groups. Correlations were calculated using the spearman's coefficient, due to the non-normal distribution of the hormone values.

RESULTS

Seven hundred sixty-four male patients had a median age of 26.9 years, range 13.8-56.9. The number of patients referred substantially increased during the last years (Fig 1). The majority of patients were diagnosed with a testicular germ cell tumor (n=292, 38%) or Hodgkin lymphoma (n=173, 23%, Table 1). Semen characteristics for the complete group were as follows; median spermatozoa concentration $20x10^6$/ml (range 0-749), total sperm count $39.6x10^6$ (range 0-1282), sperm volume 2.4 ml (range 0.1-10.8) and progressive motility 39.0% (range 0-80). Semen characteristics and hormone evaluation per diagnoses are listed in table I. Patients with testicular germ cell tumors (TGCT) and extragonadal-germ-cell tumors (extragonadal GCT) showed a significant lower sperm concentration than the other patients ($p<0.05$). Only 330 (36%), of the 914 semen samples, showed sperm concentrations in the normal range (above $20x10^6$/ml). In the men diagnosed with TGCT and extragonadal GCT only 28 and 10 percent respectively had normal sperm concentrations.

Seventy-four patients (9.7%) provided 78 semen samples, in which no spermatozoa could be found (n=61) or no motile spermatozoa were found (n=13). In these patients cryopreservation was not possible. A total number of 19 patients (2.5%) failed to produce a semen sample due to severe illness or stress. The 671 patients who succeeded to provide a semen sample produced 839 semen samples.

Seventy-four patients were diagnosed with an azoospermia of which 38.5 percent were diagnosed with a TGCT compared to 15.4 percent with Hodgkin Lymphoma (HL) 15.4 percent with non-Hodgkin-Lymphoma (NHL) and 14.1 percent with a hematological malignancy. Interestingly, only one out of the 17 patients with an extragonadal germ-cell tumor was diagnosed with an azoospermia.

In 313 patients a hormonal evaluation and semen preservation was performed, in 262 men (84%) this was done at the same day of semen cryopreservation. Median serum levels for the complete cohort were: FSH 3.9 U/l, Inhibin B 117 ng/l, LH 3.5 U/l and testosterone 15 nmol/l (Table 1). One hundred ninety-four (61%) patients had Inhibin B levels below normal, 65 patients (21%) had an increased FSH level. Inhibin B was the only marker, that significantly correlated

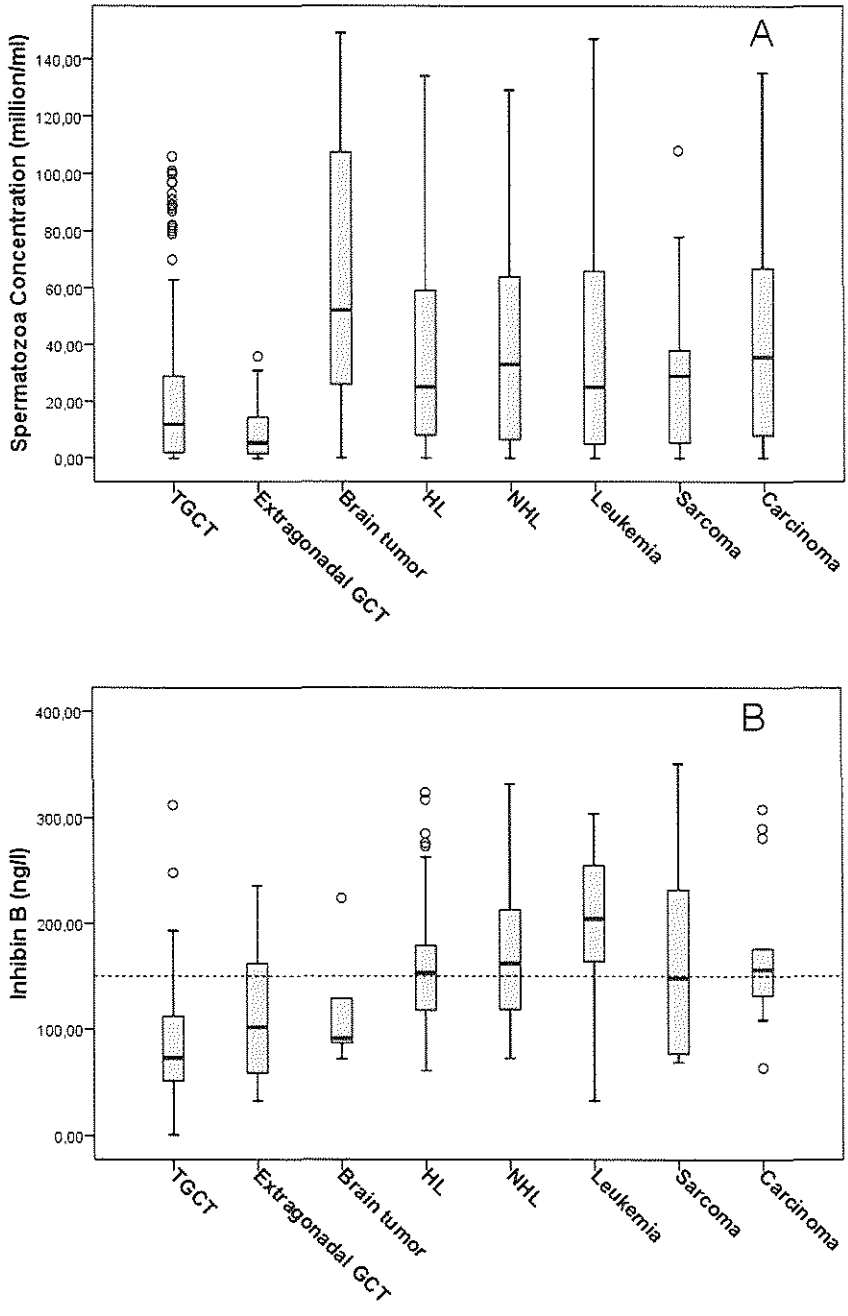

Fig 2:A) Spermatozoa concentration according to diagnosis. B)Inhibin B levels according to diagnosis. Horizontal dotted line represents low border of normal for Inhibin B (150ng/l, Pierik et al 2003)

Table 2: Tumour markers and fertility markers according to known or unknown pre-orchidectomy tumour markers.

Group	Inhibin B (ng/l)	FSH (U/l)	LH (U/l)	Testosterone (nmol/l)	Semen volume (ml)	Sperm Concentration ($x10^6$/ml)	Sperm motility (% progressive)
TGCT patients with known pre-orchidectomy tumour markers	74 (1-312)	3.2 (0-51.8)	3.0 (0.1-23.8)	17.8 (1-63.5)	2.9 (0.5-8.0)	13.5 (0-124)	42 (0-73)
TGCT patients with unknown pre-orchidectomy tumour markers	73 (8-248)	7.3 (0-128)	3.4 (0.1-17.5)	15.1 (0.6-33.5)	2.4 (0.4-7.2)	21 (0.2-308)	42 (8-82)
Correlation of pre-orchidectomy tumour markers							
Alfa-fetoprotein (AFP)	-0.25	-0.44**	-0.36**	0.37**	0.22	-0.16	-0.23
Beta-HCG	-0.52**	-0.73**	-0.19	0.62**	0.15	0.03	-0.07
Lactacte Dehydrogenase (LDH)	-0.29*	-019	-0.02	0.27	0.13	-0.01	-0.02
* P<0.05 ** P<0.01	Values **are** depicted as median and lower and upper range. Correlations are depicted between the pre-orchidectomy tumour markers and fertility markers.						

with pre-treatment sperm concentration (r=0.428, p=0.00001), for FSH these values were non-significant (r=-0.105, p=0.072). In 85 patients with spermatozoa concentrations below 10 million/ml only 33 (38%) had abnormal FSH levels compared to 63 (74%) with decreased Inhibin B levels. Patients diagnosed with TGCTs, extragonadal GCTs and brain tumors showed significant lower median levels of Inhibin B compared to patients diagnosed with HL, NHL, leukemia and carcinomas (Fig 2). FSH was not statistically different between patient groups. In TGCT patients in whom inhibin B was measured, 119 out of 127 men (94%) had subnormal levels (<150ng/l). In the patients diagnosed with an extragonadal GCT no differences in fertility markers were seen between the patients diagnosed with intracranial GCTs and the patients diagnosed with retroperitoneal or mediastinal GCTs.

Of the men diagnosed with TGCTs 73 had a nonseminoma (49%), 52 a seminoma (35%), 22 a mixed germ cell tumor (15%) and two CIS only (1%). In 128 TGCT patients we were able to collect tumor markers and in 68 patients we were able to collect both pre-orchidectomy tumor marker and reproductive hormones (Table 2). Although high beta-HCG levels were correlated with a decrease in Inhibin B which is usually predictive for low semen quality, no correlation was found between beta-HCG and semen quality (Table 2).

In 267 patients accompanying symptoms were noted at time of semen cryopreservation (SCP); 49 had febrile episodes before SCP, 48 stated to have night sweats, 61 complained of fatigue and 56 had weight loss. Although, no correlation was found between these symptoms and sperm concentration caution should be taken to interpretate these findings as these symptoms were only noted in a small cohort. However, out of 20 patients in whom one of these

symptoms was documented and no sperm could be cryopreserved eight patients (40%) had febrile episodes compared to 38 out 242 (16%) in whom motile spermatozoa were seen. The same trend was seen for fatigue, weight-loss and night sweats.

DISCUSSION

We present a large study, which clearly demonstrates that most male cancer patients referred for semen cryopreservation are at increased risk for a diminished sperm quality. Almost two-third of all semen samples were abnormal indicating that patients with cancer are at risk for an impaired semen quality. In approximately 11 percent of the males we were unable to cryopreserve semen before gonadotoxic therapy due to absence of motile spermatozoa or failure to produce and 64% of the semen samples were abnormal according to the reference values of the WHO guidelines for semen analysis. Only a minority of the men could not produce an ejaculate due to severe illness and stress. In these patients, the use of a penile vibrator or an electro-ejaculation under general anesthesia could be considered. However, the low semen quality in male cancer patients should not withhold physicians to refer patients with cancer for SCP since in approximately 90 percent semen could be preserved. Fortu- nately, we also demonstrate a large increase in the num- ber of men referred in this time-period. As cancer diagnoses did not increase proportionally, the increase in referral can be attributed to an increase in SCP awareness of the medical oncologists and probably also of the patients. The success of new assisted reproduction techniques, such as intracytoplasmic sperm injection, probably plays a major role in the referral pattern as only few motile spermatozoa are necessary for ICSI and success rates are reasonable good [118].

We have measured reproductive hormones, including Inhibin B, and demonstrated a correlation with low semen quality in patients before cancer treatment. Combining semen analysis and hormonal evaluation, patients with TGCTs and extragonadal GCTs demonstrated significantly decreased gonadal function as compared to the other patient groups. In contrast, to the significant differences in Inhibin B no significant difference in FSH levels was seen between TGCT patients and the patients diagnosed with other malignancies. Petersen already showed that TGCT patients had lower Inhibin B levels than healthy controls but failed to show a difference between TGCT patients and HL patients in a smaller cohort [125]. Vigersky et al. found a pituitary dysfunction in 11 HL patients, but we could not confirm these observations in a much larger cohort of HL patients[126].

Patients with TGCTs without pre-orchidectomy raised tumor markers were still diagnosed with lower levels of Inhibin B than the other patient groups. Jacobsen showed that declined pre-orchidectomy sperm quality can recover after orchidectomy[127]. However, if an increased FSH level was found before orchidectomy no recovery was noted. This indicates that not only the endocrine effect of the testicular tumor itself but also a pre-existing gonadal dysfunction

is present. An elevated FSH also indicates that the contralateral testicle has a diminished spermatogenesis. This was already histological demonstrated in testicular biopsies by Hoei-hansen et al.: they found one or more signs of TDS in as much as 25.2% of the contralateral testicles in men with TGCTs [45].

In our study no significant differences in FSH levels were found between the patients diagnosed with germ cell tumors and those diagnosed with other forms of cancer in contrast with Inhibin B levels which were significantly lower in the TGCT group. Inhibin B might be a better marker to detect signs of testicular dysgenesis than FSH in male cancer patients.

Jacobsen et al recently showed in a large cohort study of 32.442 Danish men that low semen quality is a risk factor for extragonadal-GCTs [8]. We can confirm these observations by clearly showing an impaired gonadal function in this group. Patients diagnosed with extragonadal-GCTs had lower sperm concentrations and lower Inhibin B levels than those patients with other malignancies as seen in the TGCT group. TGCTs and extragonadal-GCTs probably arise from the same primordial germ cell (PGC), which, if normally differentiated, provides the basis for normal spermatogenesis. A problem in normal differentiation might be the basis of extra-gonadal GCTs, which might also explain the higher chance of diminished spermatogenesis in these patients.

Beta-HCG has a LH-like effect and therefore interferes with pituitary function leading to an increase in testosterone (as shown in table 2) and estrogen levels which both have an inhibitory effect on pituitary function. Although the effect of elevated beta-HCG and AFP might play a partial role in the decline of sperm quality, these elevated markers do not totally explain the decrease in spermatogenesis and impaired semen quality is also found in patients with TGCT without increased tumor markers.

Fever might be one of the causative factors resulting in decreased semen quality. Recently, Carlsen et al. demonstrated a decline in sperm concentration following fever [128]. Although males presenting with fever, night sweats, fatique and weight loss had a slightly higher chance of having azoospermia no statistically significant correlation could be found between these symptoms and neither sperm parameters nor reproductive hormones.

CONCLUSIONS

Semen cryopreservation is possible in approximately 90% of referred men with cancer. A clear decline in sperm quality is seen in most patients. Patients diagnosed with TGCTs and extragonadal-GCTs are at increased risk of severe spermatogenic failure, which indicate a pre-existing gonadal dysfunction. Increased levels of beta-HCG and AFP have an impact on reproductive hormones, but the effect on spermatogenesis seems minimal.

Chapter 8

Semen cryopreservation in pubertal boys before gonadotoxic treatment and the role of endocrinological evaluation in predicting sperm yield.

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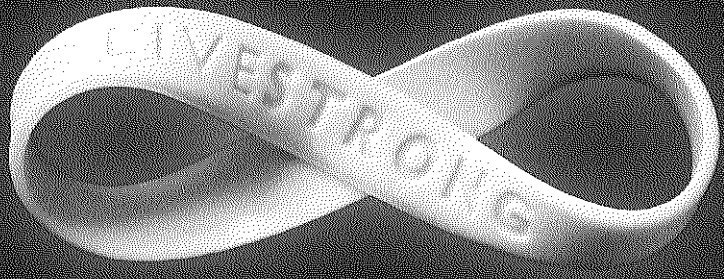

ABSTRACT

Study objective: To evaluate the feasibility of semen cryopreservation (SCP) in pubertal boys before receiving gonadotoxic therapy and to identify which pre-treatment parameters might predict successful cryopreservation.

Design: retrospective data analysis

Setting: Tertiary fertility centre/ Academic Children's hospital

Patients: Between 1995 and 2005, 80 boys (median age 16.6 year, range 13.7-18.9) consulted the outpatient clinic of Andrology for SCP before a potential gonadotoxic treatment

Interventions: We assessed the pre-treatment semen parameters, hormone levels and patients characteristics.

Main outcome measures: Measurement of the number of adolescents able to cryopreserve semen.

Results: Thirteen boys failed to produce semen by masturbation. In 53 boys semen quality was adequate for cryopreservation. In fourteen patients semen analysis did not show motile spermatozoa and therefore SCP could not be performed. Although Inhibin B showed a strong correlation with sperm count ($p<0.01$), no significant difference was found in serum testosterone, inhibin B, LH and FSH levels in the patients with or without successful sperm yield. Moreover, median age was not different between patients with and without a successful sperm yield.

Conclusions: Semen cryopreservation in boys is a feasible method to preserve spermatozoa before gonadotoxic therapy is started and should be offered to all pubertal boys despite their young age. Serum hormone levels do not predict sperm yield.

INTRODUCTION

During the last decades, mainly as a consequence of improved treatment efficacy, the overall number of childhood cancer survivors has increased substantially[129]. Subsequently, long-term sequelae after treatment of pediatric cancer have gained increasing interest. An important potential long term side effect is infertility[130, 131]. Each type of cancer requires specific therapy protocols that may be associated with impairment of gonadal function. Previous studies have shown that patients receiving agents with alkylating properties, such as Procarbazine, Cyclophosphamide and Cisplatin and/or irradiation are at a high risk of fertility problems later in life [132-134]. In general, rapidly dividing spermatogonia are most susceptible for the toxic effects of chemotherapy [133]. Moreover, immunosuppressive agents, used both in cancer and in the treatment of non-malignant systemic diseases, may reduce male fertility [135]. Cryopreservation of spermatozoa is the only established method to enable future assisted reproduction techniques (ART) if patients are rendered infertile after therapy. Currently, sperm banking is routinely offered to adults who are to receive potentially spermatotoxic therapy, whereas pubertal boys may not benefit from sperm banking due to uncertainties about their spermatogenesis and the ability to produce semen. However, cryopreservation of spermatozoa seems to be useful from the onset of sperm production (spermarche). In individual cases, the occurrence of spermarche, an mid-pubertal event, is difficult to predict due to a wide variety in testicular size and secondary sex characteristics [136]. So far only few studies have described semen cryopreservation in adolescents [137-141]. However, none of these studies examined the role of serum hormone levels in predicting sperm yield and successful sperm cryopreservation. We evaluated the feasibility of semen cryopreservation in pubertal boys before receiving gonadotoxic therapy and tried to identify whether serum hormone levels could predict a successful sperm yield.

PATIENTS AND METHODS

We evaluated the results of semen analysis and endocrine serum markers in boys aged 13-18 years who were referred by the department of pediatric oncology for cryopreservation before start of gonadotoxic therapy between December 1995 and December 2005. Information on age at semen analysis, endocrine serum markers, type of disease, patient characteristics, treatment regimes and follow-up data were obtained. Patients who previously received chemotherapy were excluded. Semen cryopreservation was rendered successful if motile spermatozoa could be banked potentially preserving the chance of fatherhood. The study protocol was approved by the hospital medical ethical board and was performed in accordance with the Helsinki agreement of 1975 on human experimentation.

Assessment of semen collection and semen analysis

The pediatric oncologist provided information on the possibility to cryopreserve spermatozoa, before cytotoxic treatment was started. In principle, semen samples were collected by masturbation and alternatively by urine collection after retrograde- or electro-ejaculation. A physician of the Andrology department counseled all patients and if desired, visual erotic stimulation was provided. Patients with a retrograde ejaculation were catheterized and a buffer medium was installed in the bladder for collection of retrograde ejaculated sperm. After masturbation urine was collected and motile spermatozoa, if present, were cryopreserved. After the collection of semen volume, sperm count ($x10^6$), sperm concentration ($x10^6$/mL), progressive sperm motility (%) and morphology (% normal forms) were evaluated according to the guidelines of the WHO laboratory manual for the examination of human semen.[142, 143] Semen samples were defined to be adequate for cryopreservation in any case where motile spermatozoa were found, independent of concentration. A second semen collection and cryopreservation procedure was offered if motile spermatozoa were found in the first semen sample if time was available before cytotoxic treatment was started. All patients, in whom semen could be banked, signed an informed consent form concerning ownership and destination of the banked semen in case of death.

Evaluation of endocrine hormone levels.

Peripheral blood samples were obtained as part of the diagnostic procedures for analysis of serum hormone levels, including luteinizing hormone (LH), follicle stimulating hormone (FSH), inhibin B, testosterone and sex hormone-binding globulin (SHBG) levels. Serum samples were stored at -30° Celsius until analysis. Inhibin B was measured using kits purchased from Serotec Ltd (Oxford, UK). Within-assay and between-assay coefficients of variation (CV) were less than 9%, and less than 15%, respectively. Serum FSH and LH were determined with the Immulite assay (Diagnostic Products Corporation (DPC), Los Angeles, CA, USA). Within-assay and between-assay CV were less than 6% and 9%, and less than 5% and 11% for FSH and LH, respectively. Serum testosterone levels were determined using coated tube radioimmunoassays (DPC). Intra- and interassay variation coefficients were 3% and 4.5%. SHBG was determined using an Immulite assay (within and between-assay < 4% and < 7%).

Statistical analysis

For statistical analysis SPSS 11.5.0 was used (SPSS inc, Chicago, Ill). Correlations were assessed using Pearson correlation. For group differences the independent t-test or the Mann-Whitney U test was used according to the distribution of the variables according to the Kolmogorov-Smirnov test. P values <0.05 were considered significant.

RESULTS

Between 1995 and 2005 a total number of 80 boys (median age 16.6 year, range 13.7-18.9) were referred to the Andrology outpatient clinic for semen cryopreservation prior to a potential fertility impairing treatment. Patient characteristics, sperm concentration, sperm motility, volume and endocrinological evaluation at time of semen cryopreservation are listed by diagnosis in table 1.

Table 1: Age, serum hormone levels and sperm characteristics listed by diagnosis. Values are given in median and standard error of the mean ().

Disease	n	Age	Inhibin B ng/L	FSH U/l	Testosterone nmol/l	LH U/l	Sperm conc (10^6/ml)	Sperm motility (%)	Sperm volume (ml)	Cryopreserved (% of total)
Ewings sarcoma	6	15.7 (0.6)	120.5 (38.3)	5.7 (4.0)	10.6 (3.4)	3.2 (0.9)	9.2 (3.9)	29.0 (5.6)	1.8 (0.8)	4 (66.7%)
Osteosarcoma	2	16.8 (0.9)	257.0	2.1	16.9	1.6	35.5 (2.5)	35.0 (15.0)	0.8 (0.4)	2 (100 %)
Mesenchymal tumour	6	15.6 (0.7)	91.5 (12.1)	5.9 (1.8)	13.4 (1.6)	3.2 (0.7)	2.4 (3.4)	24.5 (7.4)	1.4 (0.3)	4 (66.7%)
Hodgkin's Lymphoma	20	16.3 (0.3)	141.0* (22.3)	3.1 (0.7)	11.7 (1.8)	2.3 (0.5)	12.0 (5.1)	29.5 (3.9)	0.8 (0.2)	14 (70.0%)
NHL	8	17.4 (0.5)	152.0* (26.9)	2.9 (1.2)	9.8 (2.2)	3.4 (0.7)	5.9 (26.0)	20.5 (3.9)	1.0 (0.4)	6 (75.0%)
ALL	7	17.2 (0.5)	171.0* (20.4)	1.2 (1.5)	11.8 (1.3)	3.1 (1.5)	62.0 (60.0)	32.0 (3.7)	1.8 (0.2)	6 (85.8%)
AML	3	17.3 (0.4)	186.0* (19.0)	4.0	12.8 (1.9)	3.8 (1.7)	12.5 (12.5)	33.0 (33.0)	2.3 (0.1)	1 (33.3%)
AA/MDS	2	16.5 (0.5)	159.5 (119.5)	6.0 (3.9)	12.4 (6.1)	3.1 (0.8)	7.8 (5.2)	26.0 (10.5)	3.9 (1.2)	1 (50.0%)
Autoimmune disease	4	17.3 (0.7)	154.0* (14.7)	1.4 (3.5)	6.6 (2.8)	5.2 (3.8)	2.2 (20.8)	2.0 (3.5)	0.8 (0.2)	1 (25.0%)
Testicular germ cell tumour	15	17.0 (0.2)	85.0* (12.0)	0.2 (1.6)	19.5 (3.9)	1.7 (2.5)	12.5 (12.0)	29.0 (5.2)	1.7 (0.3)	11 (73.3%)
Brain tumour	7	15.6 (0.6)	80.0 (29.6)	5.5 (3.0)	5.0 (1.3)	2.2 (0.8)	2.5 (4.8)	7.0 (10.9)	1.0 (0.2)	3 (60.0%)
Others	2	15.9 (0.6)	137.0	5.0	7.4	3.1	0.4	0	1.0	0 (0%)
Total	80	16.9 (0.1)	130.0 (9.6)	2.9 (0.6)	12 (0.1)	2.8 (0.5)	9.1 (6.5	26.0 (2.1)	1.0 (0.1)	53 (66.3%)

NHL = Non Hodgkin lymphoma, ALL =Acute lymphoblastic leukemia, AML = Acute myeloid leukemia, AA = Aplastic anemia, MDS = Myelodysplastic syndrome, Mesenchymal tumor group consist out of 2 rhabdomyosarcoma's, 1 synovial sarcoma, 1 sarcoma like hamangioma and 1 nerve sheet tumor, Autoimmune disease group consists out of 2 wegener disease, 1 systemic lupus erythromatosus and 1 kidney transplant, brain tumors group consists out of 2 germinoma's, 2 pineal tumors, 2 medullobastoma's, 1 pylocytair astrocytoma the other group consists out of 1 carcinoma of the nasophanrynx and 1 desmoplastic round cell tumor . * Inhibin B levels compared to the TGCT patients $P<0.05$.

Sixty-seven patients produced a total of 92 samples of which 91 were obtained by masturbation. Forty-seven patients produced only one sample before starting therapy whereas 19 patients produced two and one patient three semen samples. Two patients had retrograde ejaculations and delivered in total three urine samples after masturbation, of which only in one the semen quality was sufficient for cryopreservation. In one patient electro-ejaculation, under general anesthesia, was performed because the patient was unable to produce a semen sample by masturbation due to a tumor related neurological disorder. Thirteen out of 80 patients (16.3%), aged 13.9 to 18.7 years (median 16.7), initially failed to produce semen

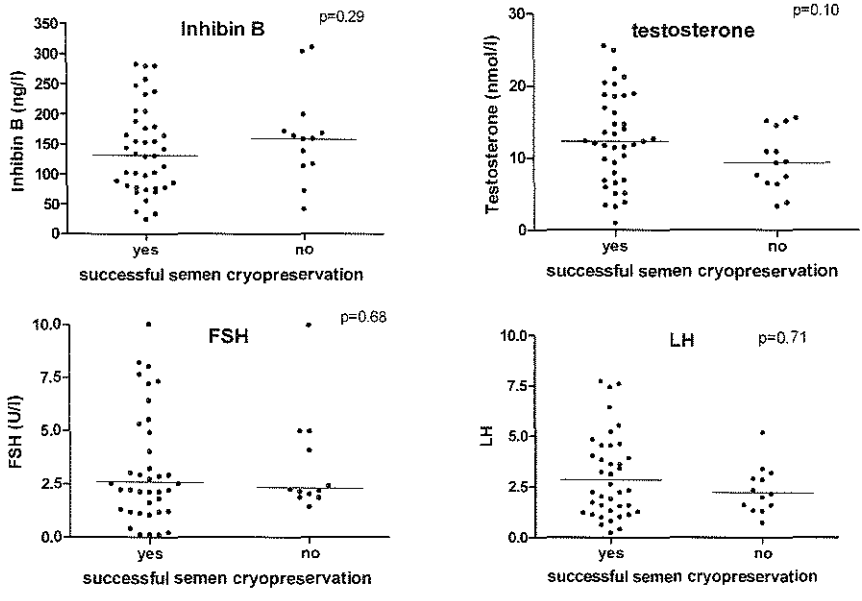

Fig I: Lines indicate median values. Mann-Whitney U test showed no significant difference between the two groups. Semen samples were defined to be adequate if motile spermatozoa were found and successively were banked.

by masturbation. The main reason for not being able to produce was that they were either to ill or tired to produce a sperm sample. Only one patient returned three days after the first failed attempt and successfully produced an adequate semen sample (volume 0.4 ml, sperm concentration 174x10^6/ml and 2% progressive motility). In the other 12 patients time did not allow a second attempt and an electro-ejaculation procedure was not offered. Age at time of masturbation of the patients who were capable or those who were incapable of producing a semen sample was 17.1 versus 16.5 years (NS, p=0.13). Fifty-three patients (66.7%) aged 13.7 to 18.9 year (median 17.0) produced adequate semen samples. These boys produced 71 semen

Table 2: Serum hormone levels and sperm characteristics in different age groups. Values listed as median and standard error of the mean ().

Age group (years)	n	%	Inhibin B ng/l	Testosterone nmol/l	FSH U/l	LH U/l	Sperm concentration 10^6/ml	Sperm motility (%)	Sperm volume (ml)	Production succeeded (%)
13-14	3	3.8	205.0 (30.0)	5.6(0.9)	1.3 (0.3)	1.8 (0.1)	111.0 (41.9)	59.0 (5.8)	1.7 (0.3)	2 (66.7%)
14-15	7	8.8	112.0 (20.0)	4.4 (2.5)	4.5 (1.9)	2.2 (2.1)	7.4 (39.6)	12.5 (5.4)	1.0 (0.2)	6 (85.7%)
15-16	15	18.8	137.0 (27.3)	13.5 (1.5)	2.7 (1.0)	2.6 (0.5)	2.1 (2.8)	13.0 (4.1)	0.9 (0.2)	14 (93.3%)
16-17	19	23.8	102.0 (20.1)	13.3 (1.7)	2.9 (1.3)	2.2 (1.6)	3.6 (3.5)	19.5 (5.8)	1.2 (0.3)	13 (68.4%)
17-18	26	32.5	136.0 (13.6)	11.5(1.7)	2.9 (1.4)	4.0 (1.0)	25.0 (15.4)	27.0 (3.6)	1.0 (0.3)	19 (73.1%)
18-19	10	12.5	113.0 (28.4)	13.3(4.9)	2.3 (1.7)	4.2 (0.7)	9.7 (1.5)	33.0 (4.0)	1.8 (0.3)	9 (90%)
Total	80	100.0	130.0 (9.6)	12.0(1.0)	2.9 (0.6)	2.8 (0.5)	9.1 (6.5)	26.0 (2.1)	1.0 (0.1)	67 (83.7%)

samples, which were subsequently cryopreserved. Fourteen patients (17.5%), aged 14.4 to 17.8 year (median 15.8), produced 21 insufficient semen samples of which 15 (71.4%) showed no spermatozoa at all and 6 (28.6%) showed immotile spermatozoa only.

Endocrinological evaluation at time of cryopreservation was performed in 61 patients. Inhibin B was strongly correlated with sperm concentration ($R=0.360$, $p<0.01$). None of the serum markers levels (Inhibin B, LH, FSH, testosterone) was different between patients with and without adequate sperm yield (Fig I). Because Tanner stage at time of diagnosis was only documented in 16 patients no correlations about semen quality could be made. Testicular volume was not measured at time of diagnosis.

Patients with acute lymphoblastic leukemia (ALL) produced higher sperm concentrations, as compared to patients with solid tumors, brain tumors, Hodgkin lymphoma (HL), acute myeloid leukemia (AML) and autoimmune diseases $p<0.05$ (table1). Testicular germ cell tumor (TGCT) patients had significantly lower levels of inhibin B than patients with HL, ALL, AML and autoimmune diseases (table 1). Median age at diagnosis was not different between the different diagnosis groups (table 1).

Median follow-up time of the complete group was 3.4 years (range 0.8- 14.0) and median age at follow-up was 20.7 years (range 16.2-32.3). Twelve out of the 80 patients (15%) died during this follow-up after a median time spam of 1.3 years (range 0.4-7.2). Of these 12 patients only five had banked semen successfully. Until time of evaluation none of the patients applied for an assisted reproduction technique (ART) with the cryopreserved semen. Ten boys returned to the andrology clinic after a median follow-up time of 1.1 year (range 0.4-2.9) to evaluate their post treatment semen quality. Semen analysis in four of them (2 with sarcoma, 2 with

HL) showed an azoospermia whereas in the others (2 with HL, 1 with AML, 3 with TGCT) motile spermatozoa were found.

DISCUSSION

Our study indicates that semen cryopreservation is feasible to secure the potential to father a child in two-third of these pubertal boys, in the age range from 13 years on. Reproductive potential may not be the major concern for young boys with cancer or a systemic disease. Usually, there is not much time to discuss sperm banking because treatment needs to be started soon after diagnosis. However, cryopreservation is currently the only established method allowing the possibility to produce offspring should they survive their disease and remain infertile after therapy [144]. Therefore, raising the issue of potential infertility and possibility of semen cryopreservation, with the purpose to preserve fertility in later life should be part of the information given by the pediatric oncologist/hematologist to teenagers at diagnosis. Ginsberg et al. showed in a somewhat older adolescent patient population that discussing semen cryopreservation within 1 week of diagnosis was accepted by the majority of patients[141].

In this cohort of boys no semen could be cryopreserved due to failure to masturbate in 13 out of 80 patients and the absence of motile spermatozoa in 14. The cause of pre-treatment oligospermia is not always clear, but a direct tumor effect, congenital predisposition or endocrine disruption may be factors involved [145]. The high level of distress during disease and the stress of producing semen in a hospital may also cause an impairment of semen quality, as well as ejaculatory problems [146, 147]. If it is oncological safe to postpone treatment clinician's should attempt to obtain multiple semen samples to preserve sufficient spermatozoa for future use. The assistance of a trained oncology nurse or nurse-practitioner in explaining the procedure and accompanying the patient to the Andrology clinic, to diminish the stress of talking about masturbation in presence of the parents, should therefore be considered [148]. Edge et al. investigated the level of understanding the need for banking sperm in young boys and found that this level to be high with an average score of 7.2 out of 10 [149].

In our study patients with ALL had significant better sperm concentrations as compared to the other patient groups (fig II). This finding was not reported in other studies reporting pre-treatment semen quality [19, 137, 150]. These differences might be explained by symptoms, such as fever or weight loss, which accompany malignant disease. Also the inter-individual differences in pubertal stage and age can cause differences in sperm quality between these patient groups. Compared to the study of Bahadur et al. we did not find a significant difference in sperm concentration between AML patients as compared to patients with other malignancies. This discordance might be explained by the difference in patient age between these our study and theirs (respectively 17.2 versus 14.3 years. However it should be stressed that in both studies the number of AML patients was small and these results should be confirmed in larger series.

Inhibin B is currently the best marker for spermatogenesis and can be used to assess gonadal function [123, 151]. Although inhibin B levels showed a significantly positive correlation with sperm concentration a cut off point for successful semen cryopreservation could not be found. Although patients with TGCT had significantly lower levels of inhibin B than the patients with other malignancies, the sperm concentrations did not differ significantly. It is not known what could explain the lower pre-treatment levels of Inhibin B. In this cohort it is unknown in what proportion the testicular dysgenesis syndrome, which in severe cases predisposes to TGCT, and in which the gonadal function and fertility is already diminished before diagnosis [51] plays a role.

The youngest boy who produced an adequate semen sample was 13 years of age. Age is not a predictive factor for successful spermatogenesis, but there is a trend towards a relation with successful semen cryopreservation ($p=0.06$). We advocate that all boys from 12-13 years of age, who are about to undergo gonadotoxic treatment, should be offered semen cryopreservation whenever they have at least entered the first phases of pubertal development. The group aged 13 to 14 years had low Testosterone levels but normal to high Inhibin B levels. This can be explained by the fact that Inhibin B levels increase after Tanner stage G1P1 and testosterone levels start to increase after Tanner stage G2P2 [152]. These boys might therefore just have entered the early phases of pubertal development.

Sexual activity should be discussed before referring boys for cryopreservation to assess the possibility of collection of semen through masturbation. If the patient is unable or sexually immature or when attempts to produce semen by masturbation have failed other options should be discussed[153]. Vibratory stimulation and electroejaculation (EEJ), under total anesthesia, have proven to be a successful technique to collect semen in patients with ejaculation disorders due to various etiologies [153]. Two studies reported the use of penile vibratory stimulation or EEJ in adolescents before anti cancer therapy [153, 154]. So far, a total of 8 pediatric patients have been reported in which semen could be collected which contained motile spermatozoa and in which cryopreservation was feasible. EEJ seems to be an adequate option if masturbation fails. Recently, it became clear from questionnaires that the motivation from parents and patients for this procedure in these circumstances is very high. [155]. Recently, we performed our first electro-ejaculation in a 13 year old boy whit Hodgkin Lymphoma, who was not sexually mature enough to masturbate. We obtained motile spermatozoa and were able to freeze 2 straws. This data was not included in this study. In our study 13 (16.3%) patients were unable to produce semen by masturbation, mainly due to their medical condition. From other studies we know that masturbation failure rates in adolescents is as high as 8.9 –13.9 % which is similar to our results (16.3%) [137, 138, 149]. It should be underscored that the number of boys being unable to produce semen is probably underestimated since a number of patients decline from being referred for semen cryopreservation through masturbation. This indicates that the proportion of boys that might benefit of EEJ may even be higher. The possible role of performing a testicular sperm extraction (TESE) or freezing non-motile spermatozoa still is a point of discussion.

Intra-cytoplasmic sperm injection (ICSI) allows couples to achieve pregnancy even with very low numbers of motile spermatozoa and therefore cryopreservation of any amount of motile spermatozoa is indicated [156]. As ICSI is also being performed with non-motile spermatozoa the clinical availability and outcome of this invasive techniques in these young patients should be investigated[157]. In our clinic the use of banked semen for ART is around the 9 percent (unpublished data). Pregnancy rates after ICSI treatment with cryopreserved semen are reported to be as high as 34.7 percent per cycle [158]. Due to the young age and the relative short follow-up period so far, none of the included patients in this study requested the use of the banked semen for ART as yet. In the near future long term follow up data of ART after cryopreservation will become available in these childhood cancer survivors.

Chapter 9

Use rate and assisted reproduction technologies outcome of cryopreserved semen from 629 cancer patients.

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ABSTRACT

Objective: To assess the utilization rate and ART outcome of cryopreserved semen of cancer patients with an average follow-up of 7 year (range 2-23).
Design: retrospective data analysis.
Setting: University-affiliated Andrology and Reproduction center.
Patients: 629 male cancer patients who were referred for SCP between 1983 and 2004.
Interventions: Review of patient characteristics and ART outcome.
Main outcome measures: Utilization rate and live births using cryopreserved semen.
Results: A total of 749 semen samples from 557 men were preserved. Ninety-one patients died during follow-up and another 29 requested disposal. Forty-two patients requested the use of their banked semen. ART data were available in 37 patients. A total of 101 ART cycles (32 IVF, 53 ICSI, 9 cryo-ET, 7 IUI) were performed resulting in respectively 8, 16, 2 and 1 pregnancies. Pregnancies rates for IVF and ICSI were significantly higher than those for IUI.
Conclusion: So far, 7.5 percent of the cancer survivors have used his banked semen, which led to life births in 49 % of the couples. Semen cryopreservation is a reliable method to preserve the fertility potential and gives couples a reasonable change of achieving parenthood.

INTRODUCTION

Survival rates for patients with malignancies have steadily increased during the last decades, due to the use of multi-drug regimens. These treatment protocols may have deleterious effects on testicular and ovarian function. Male and female cancer patients are at risk of becoming infertile from cancer therapy [132]. The chance for reproduction after chemotherapy is difficult to predict, due to many contributing factors and wide inter-individual variance [145]. The only established method to secure fertility in male cancer patients before gonadotoxic therapy is semen cryopreservation (SCP), which in time can be used for assisted reproduction techniques (ART). Before the "IVF/ intracytoplasmatic sperm injection (ICSI) era", cryopreserved semen was only used for intra-uterine inseminations (IUI) and the first successful human pregnancy using banked semen was achieved in 1953 [159]. However, because of the deleterious effects of the freezing and thawing of the semen, the post-thaw quality was often not good enough for IUI [160]. The introduction of new sophisticated ART during the last decades such as in vitro fertilization (IVF) and ICSI overcame these severe sperm concentration and motility problems [156, 161]. These techniques provided patients, who banked semen and were infertile after treatment, with a reasonable chance of conception [162].

Although the total number of patients referred to our clinic for sperm banking is steadily increasing, the awareness of most oncologists of this subject is still insufficient. Several reports described the lacking knowledge of oncologists about assisted reproduction techniques [163-165]. The success rates of IVF and ICSI treatments using cryopreserved semen are currently almost as high as using fresh semen [166, 167]. The use rate of cryopreserved semen differs widely between the different studies. Only few studies reported on the utilization rate of cryopreserved semen in combination with ART outcome of large oncological patient cohorts using these new techniques (Table 1). In this retrospective study, we report the use rates of cryopreserved semen and subsequent ART outcome from a large cohort of 629 male cancer patients presenting for SCP in a university Hospital. With this study, we intend to inform medical specialists about the utilization and success rates and convince them to discuss SCP with all of their patients that are at risk of becoming infertile.

MATERIAL AND METHODS

We evaluated patient characteristics, type of cancer, use of the cryopreserved semen and ART outcome of all male cancer patients, who banked semen between 1983 and December 2004 at the Erasmus Medical Center, Rotterdam, the Netherlands. From a total of 907 patients counseled for SCP, 629 were referred for sperm banking prior to receiving a potential gonadotoxic therapy. After producing their semen sample, all male patients were counseled by a physician of the department of Andrology. Semen samples were cryopreserved if motile spermatozoa were

table 1: Overview of recent studies describing use rate of cryopreserved semen and outcome.

	Total number of patients who banked semen	Discontinuation	Disease group	Duration of the program	Number of patients who used their banked semen (percentage)	IUi Cycli (pregnancies)	IVF Cycli (pregnancies)	ICSI Ccyli (pregnancies)	Frozen embryo transfer (pregnancies)	Number of pregnancies with ART	Percentage of couples achieving parenthood	Live births
Revel et al. 2005. (Revel et al 2005)	-	-	Diverse	1-18 year + Ivf treatment between 1999-2002	21	-	-	62	18	26	12/21 (57.0%)	23 (5 twins)
Chung et al. 2004 2. (Chung et al 2004)	164 92 prior to therapy	37	Diverse	10 years 1993-2003	6/164 (3.7%)	10 (0) 3 ICi	5 (1)	3 (1)	-	2	2/6 (33.3%)	2
Magelsen et al. 2005 (Magelssen et al 2005)	422	-	TGCT	1983-2002	29/422 (6.9%)	-	-	-	-	16	14/29 (48.3%)	15 1 twice pregant 1 triple pragnacies
Ragni et al 2003. (Ragni et al 2003)	686	124	Diverse	1986-2001	36/686 (5.2%)	40 (3)	6 (0)	42 (11)	-	14	12/28 (43%) 8 lost to follow-up	12 2 ongoing
Mesequer et al. 2006. (Meseguer et al 2006)	184	16	Diverse	1991-2004	30/184 (16.3%)	5 (1)	-	25 (14)	5 (1)	16	12/30 (40.0%)	12 3 ongoing
Agarwal et al. 2004. (Agarwal et al 2004)	316	-	Diverse	1982-2001	29/316 (9.2%)	42 (3)	26 (6)	19 (7)	-	15	11/29 (37.9%)	19 3 twins 2 triplets
Kelleher et al. 2001. (Kelleher et al 2001)	833	191	Diverse	1980-2002	64/833 (7.7%)	35 (11)	22 (6)	22 (12)	-	20	29 (45.3%)	39
Lass et al. 2001. (Lass et al 2001)	306	?	Diverse	11 years	11/306 (3.6%)	12 (3)	14 (2)	6 (4)	7 (0)	9	8/11 (72.3)	9 1 Twin
Our results	557	120	Diverse	1983-2004	42/557 (7.5%)	7 (1)	32 (8)	53 (16)	9 (2)	27	18/37 (46.8%)	25 3 twins 2ongoing

ICI= Intracervical inseminations; TGCT= Testicular germ cell tumors

found. Simultaneously, type of disease and a short history was noted. When a gonadotoxic treatment could be delayed, patients were advised to return for subsequent donations. Patients were advised to return to our andrology clinic at least 6 months after treatment to evaluate sperm production. Spermatogenesis recovery was scored positive if motile spermatozoa were seen in a post treatment sperm analysis. If patients were at least two years in remission and proven to be infertile sperm could be used for ART in our fertility clinic.

Cryopreservation

Semen was obtained by masturbation and was evaluated according to the WHO guidelines for semen analysis valid at that time [142]. The ejaculate was cryopreserved if motile spermatozoa were seen independent of concentration. After diluting the semen sample with cryoprotectant (Orange Medical, Tilburg, the Netherlands) the samples were cooled and stored in aliquots in liquid nitrogen vapour. All patients signed an informed consent form concerning ownership and destination of the banked semen in case of death.

Assisted reproduction techniques

Couples requesting fertility treatment were evaluated regarding a nationally approved fertility protocol, in which menstrual cycle abnormalities and tubal patency were included for the woman. Depending on the amount and quality of the semen cryopreserved, IUI, IVF or IVF-ICSI was considered. Beside the semen amount available, regular criteria were used (female age, cycle abnormality, tubal patency) to decide which form of ART was appropriate.

Intrauterine inseminations were performed in the natural menstrual cycle. Detection of ovulation was established by a LH-urine test (Ovulady, Clindia bv., Leusden, The Netherlands). A single insemination was performed the day after a positive test.

IVF, whether or not combined with ICSI, was initiated using a standard long agonist suppression protocol with 150 IU recombinant follicle-stimulating hormone (FSH; Gonal-F, Serono Benelux bv, Amsterdam, The Netherlands or Puregon, NV. Organon, Oss, The Netherlands), after Gonadotropin Releasing Hormone (GnRH)-agonist (Decapeptyl, Ferring bv, Hoofddorp, The Netherlands) down-regulation. When at least 3 follicles > 16 mm were present, 10.000 IU of human chorionic gonadotropin (hCG, Pregnyl, Organon, Oss, The Netherlands) was injected subcutaneously. Ovum pick-up was performed 36 hours later. Embryos were transferred 3 days after oocyte pick-up. Luteal phase support was started on the day of oocyte pick up.

Statistical analysis

Data concerning the partner, oocyt retrieval, oocyt implantation and pregnancy outcome were recovered from the electronic database from the department of Reproduction Medicine. Patient and sperm characteristics are expressed as mean and standard error of the mean (SEM) or median and range if there was a non normal distribution. Statistical analysis was performed using the statistical package for the social sciences (SPSS 11.5, Chicago).

RESULTS

Six-hundred and twenty-nine patients were referred for sperm banking before gonadotoxic therapy. Referrals greatly increased after 1993 from 15 patients to more than 80 per year in 2004. Median age was 27 years (range 14-57 years). The median follow-up time after semen cryopreservation was 7 years (range 2 - 24 years), at which the median age was 34 years (range 18-66 years). Patients were diagnosed with testicular germ cell tumors (TGCT, n=236), Hodgkin lymphomas (HL, n=143), non Hodgkin lymphomas (NHL, n=81), sarcomas (n=31), carcinomas (n=28), acute myeloid leukemias (AML, N=26), acute lymphoid leukemias (ALL, n=30), brain tumors (n=18) , chronic lymphoid leukemia (n=4), chronic myeloid leukemia (n=10), hematological malignancies other than mentioned above (n=11), extragonadal germ cell tumors (n=8) one melanoma and two schwannomas. A total of 749 semen samples were produced ranging from 1-5 samples per patient. Seventeen patients (2.7%) were unable to produce a semen sample and in 55 (8.7%) patients the semen sample provided, did not contain motile spermatozoa and therefore was not suitable for cryopreservation. We were able to preserve semen in 557 patients (88.6%). Semen characteristics at time of semen cryopreservation for the complete group were; median concentration 17.5 million/ml (range 0-749 million/ml); median progressive motility 38.5 % (range 0-89%); median volume 2.4 ml (range 0 - 10.8 ml). Men diagnosed with testicular cancer or extragonadal germ cell tumors had significantly less spermatozoa per semen sample compared to other patients ($p<0.01$, table 2).

Table 2. Semen characteristics by diagnosis

	Age at time of SCP	Volume (ml)	Concentration (10^6/ml)	Progressive motility (%)
Hematological malignancies	23,9 (8.6)	2,2 (1.7)	17,0 (53.0)	34 (20)
Brain tumors	28,9 (10.1)	2,2 (1.8)	42,0 (181.4)	60 (27)
Carcinoma's	36,0 (11.2)	2,9 (1.9)	42,0 (38.8)	39 (20)
Extragonadal germ cell tumors	30,3 (6.4)	2,4 (1.3)	13,0 (13.8)	17 (25)
Hodgkin lymphoma's	25,5 (6.2)	2,0 (1.7)	20,0 (44.6)	37 (20)
Non Hodgkin lymphoma's	28,4 (7.8)	2,5 (1.7)	36,0 (64.5)	32 (16)
Sarcoma's	22,3 (6.6)	1,9 (1.4)	30,0 (58.2)	39 (16)
Testicular germ cell tumors	27,3 (5.5)	2,6 (1.7)	9,0 (34.4)*	41 (20)
Total	26,8 (7.4)	2,3 (1.7)	17,0 (56.4)	38 (20)

* $p<0.01$
Values are median (sd).

Use rate

Out of the total group of 557 men with cryopreserved semen 218 (39%) returned for semen analysis after cancer treatment. Motile spermatozoa were found in 155 (71.1%) out of the 218 men, of which 20 men reported a spontaneous pregnancy. Data on post treatment spontaneous pregnancies was not structurally obtained from the complete group. During follow-up 91 (14.3%) patients died from recurrent or persistent malignancy or therapy related complications

without using their banked semen. The median time span between cryopreservation and death was 1.3 years (range 0-14 years, median 1.3). Twenty-nine patients requested disposal of their cryopreserved semen. Within this group eight did so, after returning for semen analysis, which showed motile spermatozoa in seven and azoospermia in one. Three out of the 29 men reported a spontaneous pregnancy.

Of the 557 patients who banked semen 42 (7.5%) patients requested the use of the banked sperm after a mean time of 57 months (range 15-130 months). Post treatment semen analysis showed an azoospermia in all 42 men. Nine patients requested to take their banked semen to another fertility clinic of which five were lost to follow-up.

Assisted reproduction outcome

The remaining 37 patients all underwent ART, of which, 33 patients were treated in our hospital. The mean age of the female partners at time of the assisted reproduction technique was 32 years (range 21-40 years). These 37 couples had a total of 101 ART cycles; 7 IUI cycles, which resulted in one pregnancy (14.3%); 32 IVF cycles, resulting in 8 pregnancies (25.0%), 53 cycles of ICSI, resulting in 16 pregnancies (30.1%) and 9 cryopreserved embryo transfers (ET) resulting in 2 pregnancies (22.2%; table 3). A total of 25 children were born: 19 singletons, 3 twins. Two couples are currently awaiting a child. Mean age between the females who did or did not achieve a pregnancy was not significantly different, respectively 32.1 versus 32.3 years (p=0.86).

Table 3. ART characteristics per treatment.

Treatment		IVF	ICSI	Frozen ET	IUI
No. of cycles		32	53	9	7
Age female years		30.5 ± 3..9	34.0±4.1	28.1±4.5	
No. of oocytes (mean, range)		342 (10.7, 1-26)	395 (7.6, 0-25)	-	-
No. 2-pronuclear oocytes (mean fertilization rate)		128 (42.4%)	236 (57.8%)	-	-
No. fertilisation failures (cycles)		9 (28.1%)	5 (9.4%)	3 (33.3%)	-
Embryo transfers	0	10	6	3	
	1	6	20	2	-
	2	16	27	4	
(Mean ±sd)		1.2 ±0.9	1.4 ± 0.7	1.1 ± 0.9	
No. transferred embryos		37	74	10	-
No. clinical pregnancies		8/32 (25.0%)	16/53 (30.1%)	2 (22.2%)	1 (14.3%)
No. Live births	(total)	8	15	1	1
	Singletons	8	9	1	1
	Twins	0	3	0	0
	EUG	0	1	0	0
	Early abortion	0	1	1	0
Active Pregnancies		0	2	0	0
Pregnancies per number of embryo's transferred	1	3 (60%)	5 (25%)	0 (0%)	
	2	5 (31%)	11 (40.7%)	2 (50%)	

All eight pregnancies resulting from IVF treatments, four cycles in which two embryos were transferred, were singletons. Three out of the 27 ICSI cycles, in which 2 embryos were transferred, resulted in twin pregnancies. One ICSI treatment resulted in an ectopic pregnancy. Eighteen out of the 37 partners achieved at least one live birth with the use of cryopreserved semen and two patients are currently awaiting a child. Thus, resulting in a success percentage, with two couples currently awaiting a child, of at least 54%. Four patients had two successive pregnancies using banked semen. In a total of 20 cycles (11 IVF, six ICSI and three cryotransfers) we were unable to transfer an embryo. In only one case this was due to the absence of motile spermatozoa after thawing. The other failures were either due to fertilisation failures, ovarian stimulation failures or bacterial infection of the culture dish. Three cryopreserved embryos did not survive freezing and were not transferred after thawing. Thirty-three patients out of the 37 patients still have semen banked, which can be used for future treatment. Mean storage time between the couples who did achieve pregnancy and those who did not was respectively 4.7 and 4.8 years ($p=0.87$). No correlation was found between the storage time and pregnancy rate.

DISCUSSION

This study reports on the utilization rate of cryopreserved semen and ART outcome of a large cohort of cancer patients from one semen bank in an university Hospital. To our knowledge this is one of the largest studies and the longest follow-up reported on the use and outcome of cryopreserved semen. Few single centre studies have reported on utilization rates as well as ART outcome using sophisticated techniques, such as IVF and ICSI, using cryopreserved semen (See table 1). Schmidt et al. recently reported the ART outcome of a large cohort of men with malignant disease, however, they also included men in which spermatogenesis was recovered and ART was performed using fresh semen[158].

In our opinion the usefulness of sperm banking is primarily measured by analyzing the number of children born and number of males achieving fatherhood using the banked semen. These are in essence the main goals for which men bank their semen. During the last years new assisted reproduction techniques have substantially increased chances of becoming father using cryopreserved semen [156, 175, 176]. Male cancer patients might have an impaired pre-treatment semen quality but this certainly should not rule out sperm banking, since with ICSI only a few motile spermatozoa are needed [162, 177]. Our study reports on an average success rate of achieving parenthood using cryopreserved semen of at least 54% (Table 1). This success rate differs widely between studies ranging from 33 to 73 percent (Table 1). Failure to achieve a pregnancy using the cryopreserved semen after treatment is a devastating outcome and must therefore be discussed prior to the SCP procedure. Patients have to be informed that post-thaw quality cannot be predicted and usually 50-75% of the spermatozoa will not survive freezing. In our study longer storage time did not correlate with lower pregnancy rates. Secondly the

female partner may have fertility problems leading to a poor response when stimulated. Freezing of spermatozoa may also affect fertilization potential. In our study fertilization failures were more often seen in IVF cycles. Two females did not respond to the hormonal treatments, which resulted in an early discontinuation of IVF procedure.

Post-cancer quality of life studies revealed male infertility as one of the most devastating long-term side effects of anticancer therapy [132]. As cancer survival has increased largely during the last decades, oncologists are more aware now of the long-term quality of life. It is our responsibility to inform the oncologist about the new possibilities in fertility treatment and relative good pregnancy outcome using the cryopreserved semen. In the past patients with low quality semen were often rejected from SCP due to disappointing results of IUI and IVF [178]. In our clinic each year more male cancer patients are referred for elective sperm cryopreservation. During the last decade we have actively informed the oncologists about semen cryopreservation using the region wide Comprehensive Cancer Centre Rotterdam (CCHR). This is a knowledge centre, which maintains an extensive network and fulfils a coordinating function within the field of oncology in the southwestern part of the Netherlands.

The use rate in our cohort is comparable with the other large studies. We calculated the use rate with the total number of patients who actively had semen preserved in our sperm bank. Although the use rate of 7.5% may seem low, these men could not have fathered their own child if their sperm had not been banked. If we only include cancer survivors use rate increases to 9.6%. We did not include the post mortem use of cryopreserved semen, which in our clinic sporadically occurred. The use rate will increase in time as some of the men who banked their semen will not be ready to procreate or have a stable relationship in which children are desired. We also have to keep in mind that a number of patients already have fathered a child before gonadotoxic treatment and their wish to undergo assisted reproduction treatment may be low. Studies evaluating the reason of sperm disposal showed that most requests were obtained from men who regained fertility or had improved semen quality after treatment[179]. Furthermore, the patients were not actively followed to assess spontaneous pregnancies. Seventy-one percent of the men returning for semen analysis showed at least some recovery of spermatogenesis and may potentially achieve a spontaneous pregnancy. Men who did achieve a spontaneous pregnancy will most likely not attend our clinic to use the banked semen.

In conclusion: We strongly recommend discussing SCP with all men at risk of becoming infertile after receiving gonadotoxic treatment. Semen cryopreservation is at this moment the only established and reliable method to preserve fertility. Semen can be stored for a reasonable long time without affecting pregnancy rates. The use of cryopreserved semen will lead to successful pregnancies in more than half of the couples.

Chapter 10

Effect of childhood cancer treatment on fertility markers in adult male long-term survivors.

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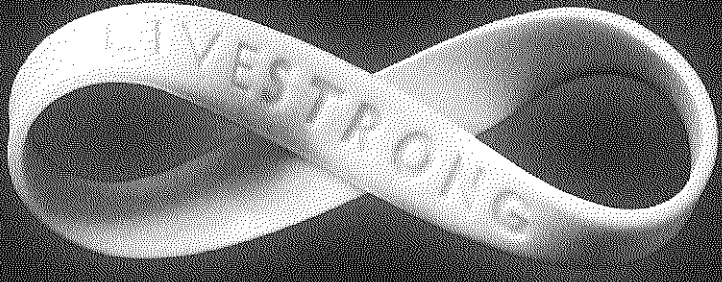

ABSTRACT

Background: Although it is accepted that pediatric-cancer treatment harbors a risk of gonadal damage large cohort studies using up-to-date fertility markers are lacking.

Procedure: The aim of our study was to evaluate the gonadal toxicity of childhood cancer treatment using fertility markers. We included 248 adult male long-term survivors of childhood cancer. Median age at diagnosis: 5 years, median age at follow-up: 24 years, median follow-up time 18 years. We evaluated patient characteristics, treatment modalities, testicular size, endocrinological parameters including Inhibin B, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone.

Results: The median value of Inhibin B in the cancer survivor group was 126 ng/l versus 177 ng/l in the control group ($P<0.001$). In the survivors, 67% had Inhibin B levels below the normal reference value of 150 ng/l compared with 26% in the control group ($p<0.05$). Inhibin B was the most sensitive discriminator between survivors and controls. Significantly decreased Inhibin B levels and increased FSH levels were found in men treated for Hodgkin and non-Hodgkin lymphoma, acute-myeloid leukemia, neuroblastoma and sarcoma as compared to other malignancies. Cumulative dosages of procarbazine and cyclophosphamide were the only independent chemotherapy-related predictors for decrease of Inhibin B levels and increase of FSH. Age at time of treatment did not influence post-treatment Inhibin B or FSH levels.

Conclusions: Severe gonadal impairment is a risk in a considerable subgroup of childhood cancer survivors based on current fertility markers, like inhibin B. Boys receiving gonadotoxic treatment before puberty are not protected from post treatment gonadal dysfunction.

INTRODUCTION

In the past decades survival of childhood cancer improved significantly following optimized treatment modalities [180]. Therefore, the number of survivors of childhood cancer in the general population is estimated to increase [181, 182]. Several studies have shown an impaired gonadal function in adult male survivors of childhood cancer [183, 184]. Post-treatment gonadal damage showed to be depending on the type of used agents and cumulative dosages administered during childhood [185-187]. Usually, studies were based on one type of malignancy and treatment protocol and had limited number of survivors included [185-187]. Age at time of treatment was suggested to give some protection against chemotherapy induced gonadal damage due to quiescent stage of the testis in the pre-pubertal age [188]. At present, semen analysis serves as the gold standard for predicting the reproductive capacity of men in their reproductive age. However, young adults treated for childhood cancer are reluctant to provide a semen sample for analysis. Therefore, reliable serum markers that reflect gonadal function in men may serve as a more convenient screening tool in determining reproductive function in male cancer survivors at least to start with. In recent studies we and others have shown that Inhibin B is the best serum marker for spermatogenesis [123, 124, 151, 189-191]. Inhibin B values after puberty depend on FSH secretion and are associated with sperm concentration and testicular volume not only in healthy individuals but also after chemotherapy and/or radiotherapy [192, 193]. However, studies in large cohorts of adults treated for childhood cancer describing the role of Inhibin B as male fertility marker are not available as yet. The aim of this study was to evaluate gonadal damage in a large single center cohort of male childhood cancer survivors using modern fertility markers.

PATIENT AND METHODS

Between January 2003 and September 2006, 291 male survivors of childhood cancer were recruited to our late effects outpatient clinic. All had been treated in our centre. Of them 30 did not respond and thirteen survivors were not included in the analysis because they did not receive chemotherapy or radiotherapy during their childhood treatment. Written informed consent was obtained from all participants, according to protocols approved by the ethical review board of the Erasmus MC. The distribution by diagnosis is given in Table I. Of the Hodgkin Lymphoma (HL) group, 22 included survivors have been described in a previous larger series [191]. In order to describe the complete cohort of survivors these patients were included in the study.

At the time of the study, all survivors were at least 18 years of age and at least five years after cessation of treatment. Median age at initial diagnosis was 5 years (range 0-15 years). Median age at follow-up was 23 years (range 18-41 years) with a median follow-up time of 18 years (range 5-39 years, Table I). Complete physical examination including measurement of testicular

volume and serum hormone analysis consisting of LH, FSH, testosterone and Inhibin B was performed. Testicular volume was measured by Prader Orchidometer. Sperm analysis performed in a subset of cancer survivors was performed according to the 1999 WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction [142].

The control group consisted of 74 normospermic men, with a sperm concentration >20x10^6/ml and progressive motility >50%, aged median 33.4 years (range 23-51 year). Peripheral blood samples in survivors and controls were taken at time of their visit. The normospermic men were recruited from the fertility clinic. During andrological work-up they were found to be normospermic. The reference values of LH, FSH, Inhibin B and testosterone values for male adults in our institute are 1.5-8.0 U/l, 2.0-7.0 U/l, 150-400 ng/l, 10.0-30.0 nmol/l, respectively. Within-assay and between-assay coefficients of variation (CV) for Inhibin B were 9% and 15%, LH 6% and 9%, FSH 5% and 11%, testosterone 3% and 4.5%, respectively[194]. The cut-off value of 150 ng/l for Inhibin B is taken as it has been shown that this provided the highest sensitivity and specificity in identifying low semen quality[124].

Statistical Analyses: The computer program Statistical Package for Social Science (SPSS inc., Chicago, Illinois, version 11.1) was used for data analysis. Non-parametric analyses were done as a non-normal distribution if the distribution was not normal. P-values <0.05 were considered significant. Survivor characteristics are expressed as median with range.

RESULTS

The range of serum hormone values of the complete survivor group and the control group are depicted in Table I. In general, the survivors had higher FSH and LH levels and lower testosterone and Inhibin B levels than the controls (Tables I and II). Inhibin B was the strongest discriminator between the survivors (median of 123.0 ng/l, range 0-393) and the controls (median 176.5 ng/l, range 60-556; p<0.0001, Fig. 1).

One hundred forty-five of the 221 survivors (65%) in whom Inhibin B was measured had values below 150 ng/l in contrast to 19 (26%) of the 74 controls (Fig. 1). When analysed by type of cancer, survivors of Hodgkin's lymphoma, non-Hodgkins lymphoma, malignant mesenchymal tumors, acute myeloid leukemias, sarcoma's and neuroblastomas showed median Inhibin B levels below 100 ng/l suggestive for severe gonadal dysfunction (Table I, Fig. 2). Survivors of acute lymphoblastic leukemia and renal tumors had relative normal median Inhibin B levels of respectively, 140 ng/l and 146 ng/l (Table I, Fig. 2).

We compared sperm analysis with endocrinological parameters in a subgroup of 21 patients; nine had an azoospermia with a median inhibin B level of median 39 ng/l (range0-79). Two patients were diagnosed with an oligospermia (sperm concentration between 5 and 20 million/ml), sperm concentrations of 13x10^6 and 15x10^6 spermatozoa/ml, and had Inhibin B levels of 67ng/l and 72 ng/l. Five patients who had severe oligo-asthenospermia (sperm concentrations

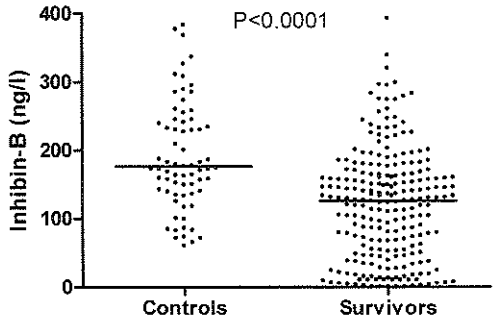

Figure 1: Inhibin B values of controls and childhood cancer survivors. Each dot represents one person. The horizontal lines represent the median values of inhibin B in the normospermic controls (176.5 ng/l) and in the survivors (123.0 ng/l, p<0.0001).

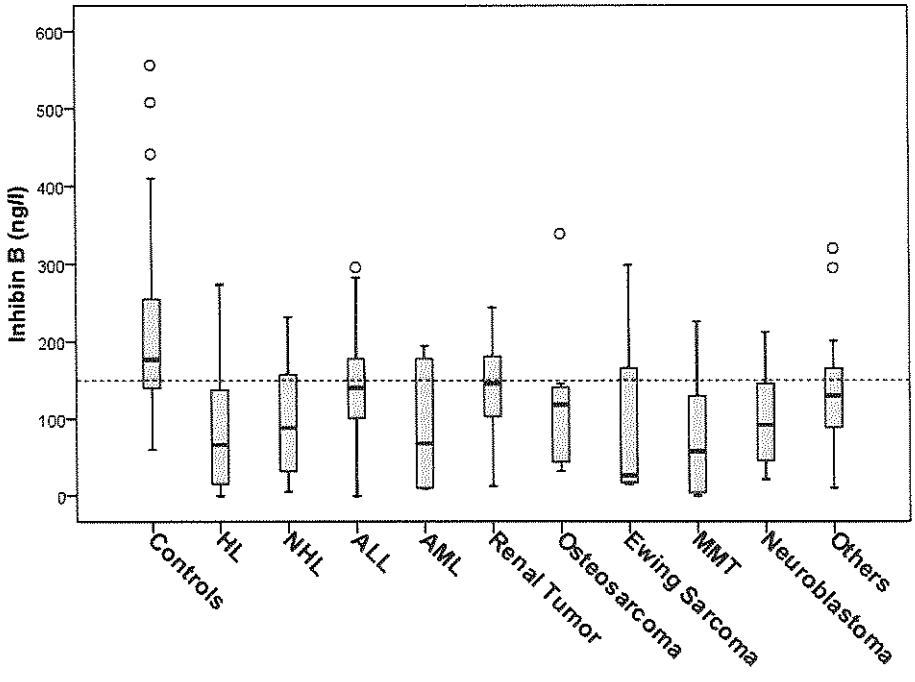

Figure 2: Median Inhibin B values and quartile ranges per diagnosis. The horizontal dotted line represents the lower threshold of normal (150 ng/l)

below 5x10^6/ml) and Inhibin B levels of median 25 ng/l (range 2-39). Two of them had severe oligospermia and with only a few immotile spermatozoa in their semen, with Inhibin B levels of 2 ng/l and 19 ng/l. The five survivors with a normospermia had median Inhibin B levels of ng/l (range 125-234). Inhibin B levels showed a significant correlation with sperm concentration in both survivors ($r = 0.671$, p=0.01) and controls ($r = 0.345$, p=0.03).

Using a linear regression model, treatment determinants for low Inhibin B levels (Inhibin B <150 ng/l) were calculated. Only regimens containing cyclophosphamide or procarbazine but not any other drugs, were independent determinants for low Inhibin B levels in the patients treated with chemotherapy ($p<0.01$). None of the other chemotherapeutic agents was independently associated with altered Inhibin B levels. Cyclophosphamide was part of the treatment protocol in 131 of the 248 survivors with a median dosage of 4.8 gram/m^2 (range 0.25-32 gram/m^2). Inhibin B levels showed a significant negative correlation with total cumulative dosage (TCD) of cyclophosphamide (r=-0.531, $p<0.001$) (Fig. 3). Inhibin B levels were abnormal in all but one survivor receiving a TCD of more than 10 gram/m^2 (Fig. 3).

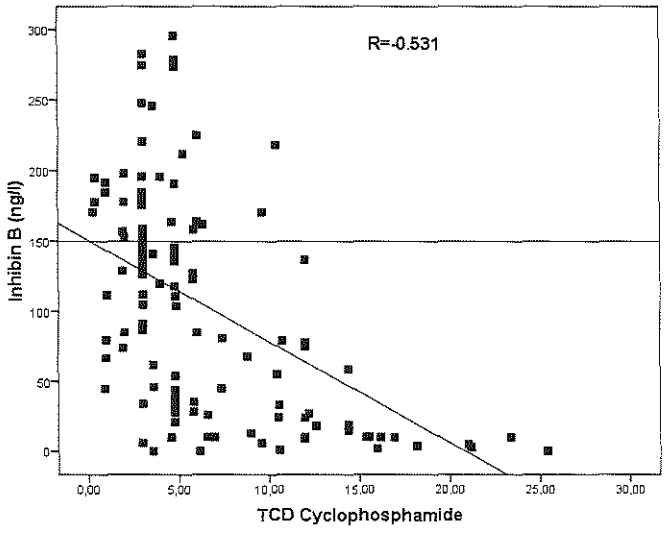

Figure 3: Dose-effect of cyclophosphamide (gram/m^2) on Inhibin B levels. The horizontal line shows the lower threshold of normal for Inhibin B (150 ng/l) the other line shows the linear regression (Rsq =0.201, correlation coefficient R=-0.531, p>0.001)

HL survivors treated with MOPP had lower Inhibin B levels, median 15 ng/l vs 143 ng/l , $p<0.001$) smaller testis volumes , i.e. 12.7 ml versus 17.8 ml ($P<0.01$), higher FSH levels (median 22.6 U/l versus 4.7 U/l, $p<0.001$) and higher LH levels (5.9 U/l versus 2.4 U/l, $p< 0.001$) than the HL survivors who did not receive procarbazine. These values indicate a severe gonadal dysfunction in the 15 HL patients treated with procarbazine.

Age at which cyclophosphamide and procarbazine were administered showed no correlation with Inhibin B levels (r=-0.06, p=0.526 and r=0.182, p=0.516, respectively). Young age at time of cyclophosphamide administration did not show a protective effect on gonadal function whether they received low or high TCD of cyclophosphamide (Fig 4A and 4B). This was even more obvious when we evaluated patients younger and older than 10 years with a relatively low (TCD < 10 gram/m^2) dosage of cyclophosphamide. Median Inhibin B level in the patients

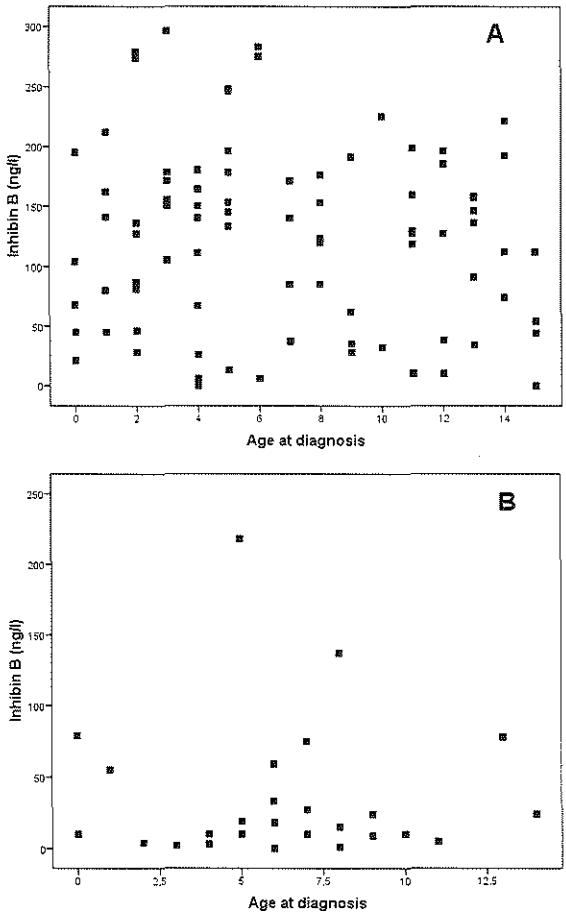

Figure 4: Distribution of Inhibin B in survivors treated with cumulative doses of cyclophosphamide of 0.25 - 10 gram/m^2, correlation coefficient R=-0.097, p=0.371 (A) and 10-30 gram/m^2 correlation coefficient R=-0.052, p=0.80 (B) according to the age at time of treatment.

younger than 10 years (n=58) was 138 ng/l compared to 127 ng/l in the older group (n=29) (p=0.402). Although the groups were smaller, no age-effect was seen in the patients receiving cyclophosphamide in higher concentrations (TCD > 10 gram/m2). The survivors treated before the age of 10 (n=28, inhibin B available in 22) had median Inhibin B level of 21 ng/l (range 0-218) compared to 10 ng/l (range 5-78) in the older group (n=5) (p=0.845).

The 10 survivors (1 HL, 4 AML, 4 ALL, 1 NHL), who received total body irradiation (TBI 7.5 or 12 Gy) as part of myelo-ablative therapy, had extremely low Inhibin B levels (median 10.0, range 0.0-62.0 ng/l). Of those, only two survivors had low testosterone levels (7.6 and 8.6 nmol/l) whereas the others had normal testosterone levels (>=10 nmol/l). No impairment in gonadotrophins was found in the subgroups. Additionally, four survivors (3 ALL, 1 AML) had received testicular irradiation (24 Gy) on both testes. One survivor with a MMT was treated with

pelvic radiotherapy (45 Gy), which included the scrotum. Testicular volume was evaluated in three as one had testicular implants (Inhibin B = 0 ng/l) and in one patient it was unfortunately not documented. These three survivors showed severe impairment of testicular growth (testis volume median 2.0 ml, range 1 to 3 ml). Median Inhibin B level available in the four survivors, in whom the testicles were not removed, was 10 ng/l, range 0-13).

Testicular volume did not differ between the controls and the survivors in the total group nor in one of the subgroups by diagnosis (p=0.391). This is reflected by the fact that only 18 males (7.3%) of the cancer survivors had subnormal levels of testosterone (<10 nmol/l) compared to eight men (10.8%) in the control group. All but three men out the patient group fully completed pubertal development compared to all in the control group.

DISCUSSION

Although several studies have shown the value of modern fertility markers for prediction of spermatogenesis, studies on gonadal function after chemotherapy and irradiation in large series of adult male cancer survivors, using Inhibin B as a novel serum markers are lacking [130, 184, 191, 195, 196]. Therefore, we performed a study in a full single centre cohort of male childhood cancer survivors (n=248).

We found a significant difference in reproductive hormone levels between survivors and controls, of which Inhibin B proved to be a better discriminator than FSH for the assessment of spermatogenesis between the two groups (table II). This was already known in healthy individuals but this study shows that this is also the case in cohorts of male long-term survivors of childhood cancer. We used a cut-off value of 150 ng/l. We acknowledge that this cut-off value is arbitrary and therefore performed the same analyses with a cut-off value of 80 ng/l and also found that cyclophosphamide and procarbazine were the only independent treatment determinants for impaired gonadal function.

Patients with Hodgkin lymphoma, non-Hodgkin lymphoma, malignant mesenchymal tumors, acute myeloid leukemia, Ewing sarcoma and neuroblastoma have a relative high risk of subfertility after treatment. This is in line with the review article by Brougham and Wallace, who divided childhood cancer types into three subgroups according to risk of subfertility [197]. Our study confirms that diagnostic subgroups are indicative for post treatment gonadal function, but mainly explained by the different treatment regimes accompanying these diagnoses.

In our study, cyclophosphamide and procarbazine containing regimens, both compounds with alkylating properties were the most deleterious drugs for the gonadal function. This finding supports earlier studies in which these drugs were associated with severe gonadal dysfunction[198, 199]. Cyclophosphamide showed a negative dose correlation with Inhibin B levels. Almost all survivors who received a cyclophosphamide TCD of 10 gram/m^2 or more were at risk for severe spermatogenic dysfunction. These findings support the earlier observations

in which a dose-dependent effect of cyclophosphamide was found [130, 198]. This dose effect was not found for procarbazine containing regimens, which indicates that this agent might already be gonadotoxic in low concentrations in males. Our study confirms that not only the type of agent used but also the cumulative dosage is important in predicting post-treatment gonadal function[200, 201].

Total body- and testicular irradiation causes severe gonadal dysfunction[202-204], which is illustrated by the fact that 8 out of 10 survivors treated with TBI had almost undetectable Inhibin B levels (<=26 ng/l). In addition, local radiotherapy to the testis was administered in 5 men who all showed extremely low Inhibin B levels. Although semen analyses were not available of these men, the previous published correlations between Inhibin B levels and sperm count indicates that these survivors probably have a severely impaired spermatogenesis [123, 191].

This study illustrates that young age at time of childhood cancer treatment does not prevent post-treatment gonadal damage. Although such protection at very young age has been suggested in the past, our findings confirm those of Thomson et al. who observed severe gonadal dysfunction in male survivors even if treated in pre-pubertal life [196]. Pre-pubertal age has been classically characterized as a quiescent period potentially providing some kind of protection against chemotherapy [188]. However, during this period there is active proliferation of Sertoli and Leydig cells which might explain the damage after cytotoxic treatment [205-208]. Childhood cancer patients and/or their parents should therefore be informed about the risk of infertility after chemotherapy independent of age and pubertal stage. Therefore, sperm preservation should be considered for risk groups as young as possible, and if necessary assisted by artificial procedures like electro-ejaculation methods [154, 194].

The question whether Inhibin B can replace semen analysis as a gold-standard remains unanswered. Although Inhibin B is highly specific, it still is an indirect marker and controversies exist. Sperm analysis remains the gold-standard especially as with modern assisted reproduction techniques, like intra-cytoplasmic sperm injection, only a few normal spermatozoa are needed for conception. Therefore, we propose to encourage survivors to have Inhibin B levels measured after chemotherapy as a first step to assess gonadal damage in order to identify survivors at risk for impaired spermatogenesis.

In the total group treated with chemotherapy, testosterone levels were slightly lower than the controls, however; only 7 % of the survivors had low testosterone values. This might indicate that the Leydig cells are more resistant to gonadotoxic treatment than the germinal cells. The minimal dysfunction of Leydig cells is supported by the observation that all except three survivors, of which two received testicular radiotherapy, fully completed pubertal development, which is mainly dependent on testosterone production. It is conceivable that most boys will complete their pubertal development possibly by an increase in LH levels, which occurred in our cancer survivors i.e. significant higher levels of LH were found in the survivors compared to in the normospermic men. This might indicate that in some survivors (10 percent in this study) a Leydig cell dysfunction exists, which can be compensated by elevated LH levels. This

underscores that the assessment of reproductive capacity in childhood cancer survivors by solely evaluating secondary sexual characteristics or testosterone is insufficient.

This study demonstrates the importance of fertility counselling and pre-treatment semen cryopreservation in male survivors of childhood cancer. Moreover, the results of this study show the ongoing need to consider the risk of gonadal toxicity of certain agents in the design of new protocols for newly diagnosed children with cancer. Inhibin B levels may be helpful to assess the gonadal function. Determination of Inhibin B levels, being the best marker for assessing gonadal function and recovery, is a relative easy and quick non-invasive method to assess gonadal function. Although it can be used to identify patients at risk with impaired reproductive capacity, combination with sperm analysis remains mandatory to evaluate reproductive options when there is an active child wish. Our results emphasize the need for gonadal function assessment and fertility counselling in long-term survivors of childhood cancer.

Table I: Patient characteristics per diagnosis group.

Diagnosis	Nr	Age at diagnosis	Age at visit	Inhibin B	FSH	LH	Testosterone	Testis volume
ALL	88	5.0 (0.3-15.0)	23.0 (18.0-40.0)	140 (0-393)	3.6 (0.5-58.7)	3.5 (0.1-14.9)	15.5 (3.7-27.3)	20.0 (2.0-25.0)
AML	11	3.0 (0.5-14.0)	21.0 (18.0-33.0)	68 (10-195)	3.9 (0.3-15.0)	3.2 (0.1-8.9)	12.8 (8.5-45.1)	15.0 (10.0-20.0)
HL	28	11.0 (2.0-15.0)	24.0 (18.0-41.0)	54 (0-274)	9.2 (1.7-51.0)	3.6 (1.2-10.9)	13.2 (8.8-32.7)	15.0 (8.0-22.5)
NHL	42	8.0 (2.0-14.0)	23.0 (18.0-36.0)	88 (5-232)	5.5 (0.9-31.7)	4.0 (0.9-11.5)	15.5 (7.6-27.9)	15.0 (9.0-22.5)
Renal tumors	27	3.0 (0.7-11.0)	23.0 (18.0-39.0)	146 (13-244)	4.0 (0.5-47.4)	3.3 (0.1-16.3)	14.5 (4.5-28.9)	17.5 (10.0-25.0)
MMT	18	5.5 (1.0-14.0)	25.0 (18.0-36.0)	55 (0-226)	14.4 (0.5-65.8)	4.7 (0.1-12.9)	14.6 (2.8-28.9)	15.0 (4.0-16.5)
Osteosarcoma	9	12.0 (1.0-15.0)	23.0 (18.0-37.0)	146 (38.-339)	6.0 (0.7-17.3)	3.6 (2.0-8.5)	14.8 (9.7-23.2)	20.0 (11.0-25.0)
Ewing Sarcoma	6	8.0 (5.0-15.0)	25.5 (18.0-36.0)	36 (15-299)	8.6 (1.7-15.8)	3.6 (1.1-8.1)	16.1 (9.7-20.2)	16.3 (15.0-20.0)
Neuroblastoma +Ganglioneuroblastoma	11	0.1 (0.1-1.0)	24.0 (18.0-30.0)	79 (21-212)	6.3 (3.3-20.6)	3.9 (1.3-7.5)	15.0 (7.6-20.4)	15.0 (12.0-20.0)
Others	8	1.5 (0.3-9.0)	20.0 (18.0-30.0)	114 (10-295)	3.6 (1.4-21.4)	3.7 (1.4-7.2)	14.4 (12.5-24.7)	22.5 (15-25.0)
Controls	74	-	33.4 (23.3-50.6)	176.5 (60-556)	3.6 (0.6-18.0)	2.7 (0.3-19.2)	16.8 (4.7-27.7)	16.5 (11.0-30.0)

ALL= Acute lymphoblastic leukemia, AML= acute myeloid leukemia, HL= Hodgkin Lymphoma, NHL= Non-Hodgkin lymphoma, MMT= Malignant mesenchymal tumor. Others consist out of 1 embryonal adenocarcinoma, 1 Langerhans histiocytosis, 2 hepatoblastomas, 1 malignant schwannoma, 1 nasopharynx carcinoma, 2 yolk sac tumors, 1 Grawitz tumor. Values given as median and range.

Table 2: Comparison of serum hormone levels between survivors and controls. Values are indicated as median and range. Percentages of abnormal levels for each serum marker and sperm concentration are given.

	Survivors (n=248)	Percentage of abnormal levels	Controls (n=74)	Percentage of abnormal levels	P-value
Inhibin B (ng/l)	123.0 (0-393)[1]	65 %	176.5 (60-556)	25%	< 0.0001
Testosterone (nmol/l)	14.7 (2.8-45.1)[2]	8 %	16.8 (4.7-27.7)	10%	0.05
FSH (U/l)	4.6 (0.3-65.8)[2]	32 %	3.6 (0.6-18.0)	17%	0.01
LH (U/l)	3.6 (0.1-16.3)[2]	10 %	2.7 (0.3-19.2)	2%	<0.0001
Sperm concentration (10^6/ml)	0.01 (0.0-158.0)[3]	76 %	52.5 (21.1-234.0)	0 %	<0.0001
Testicular volume (ml)	17.5 (2-35)[4]	17 %	16.5 (11-30)	14 %	0.391

[1] (n=221) [2] (n=244)
[3](n=21) [4](n=233)

Chapter 11

Cranial irradiation does not result in pituitary-gonadal axis dysfunction in very long-term male survivors of childhood acute lymphoblastic leukemia.

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ABSTRACT

Introduction: One of the risks of childhood cancer treatment is fertility impairment later in life. In the past a large proportion of children with acute lymphoblastic leukemia (ALL) has received cranial irradiation as part of their treatment. The aim of this study was to evaluate whether cranial irradiation negatively affects pituitary regulated gonadal function in male survivors of childhood ALL

Procedure: We examined gonadal function, including Inhibin B, LH, FSH, testosterone, and pituitary axis function by measuring TSH, Free-T4 and IGF-I levels in 89 long-term male survivors of childhood ALL after a median follow-up time of 19 year (range 7-34 years).

Results: Twenty-nine out of 89 male ALL survivors received cranial irradiation. Inhibin, FSH, LH, Testosterone, testicular volume as well as TSH and Free-T4 levels were not different in the cranial irradiated group as compared to the non-irradiated group. IGF-I levels were significantly lower in the cranial irradiated group. Survivors treated with total body irradiation or testicular irradiation had significantly decreased gonadal function based on hormone levels.

Conclusion: These data show that, in contrast to the negative influence on the growth hormone axis, cranial radiotherapy as part of ALL treatment does not have a deleterious long-term effect on the hypothalamic–pituitary-gonadal axis or pituitary-thyroid axis.

INTRODUCTION

Currently, more than 75 % of all pediatric acute lymphoblastic leukemia (ALL) patients survive and reach adulthood [209]. Successful treatment of children with ALL involves administration of multi-agent chemotherapy and includes central nervous system (CNS) prophylaxis. In the past, CNS prophylaxis consisted of cranial irradiation, which has been replaced by intrathecal chemotherapy in most of the current treatment protocols. An increasing cohort of childhood ALL survivors who have been treated with cranial radiotherapy in the past has now reached reproductive age. It has been reported that cranial irradiation can influence the hypothalamic-pituitary-gonadal axis and potentially lead to impaired endocrine function resulting in growth disturbances and impaired fertility[210, 211]. In addition, it has been suggested that young patients treated before 10 year of age are at increased risk for gonadal dysfunction after treatment[212-215].

Earlier fertility studies in pediatric ALL survivors were mainly based on pituitary hormone level measurement or on registration of life birth rates and did not include novel markers like Inhibin B[213]. Inhibin B is produced by the Sertoli cells and is strongly correlated with sperm characteristics and fertility capacity in men and also correlates with spermatogenic status in testicular biopsies [124, 151]. Inhibin B has a negative feedback mechanism with follicle stimulating hormone (FSH) and it has been shown to be a good marker for testicular damage after chemotherapy [195, 216, 217].

In the present study, we evaluated the impact of childhood cranial radiotherapy on pituitary regulated gonadal function in a large single center cohort of male long-term survivors of childhood ALL including novel markers like Inhibin B.

Patients and Methods

Patients

Out of a total of 500 survivors of childhood cancer 99 registered adult male survivors of childhood ALL, diagnosed and treated in our hospital between 1973 and 1998, were identified. Seven survivors refused participation and three were lost to follow-up. All 89 survivors who participated in this cross-sectional study were in continuous complete remission and at least five years after completion of therapy. Written informed consent was obtained from all participants, according to protocols approved by the ethical review board of the Erasmus MC. General fertility characteristics of these survivors have been reported previously[217]. The present study focuses on the differences of endocrine late effects between patients treated with or without cranial radiotherapy. Median age at time of diagnosis was five years (range 0-15 years) and median age at follow-up was 25 years (range 18-40 years), with a median follow-up time of 19 year (range 7-34 years). Data concerning treatment protocols, disease and patient characteristics were retrieved from the medical records. All 89 survivors were treated according to

the national ALL protocols at time of diagnosis, including the ALL-2,3,5,6,7,8,9 protocols of the Dutch Childhood Oncology Group (DCOG)[218-224]. Thirteen survivors had been diagnosed with a relapse and were treated accordingly. Twenty-five male survivors (28%) received cranial irradiation with a median cranial irradiation dosage of 25 Gy (range 15-30 Gy) (Fig 1). Twenty patients were treated with 25 Gy, 1 with 30 Gy, 1 with15 Gy, 1 with 20.4 and 2 with 18 Gy. None of the survivors had been treated with cranio-spinal irradiation.

The survivors who received total body irradiation (TBI), testicular irradiation, mediastinal irradiation or orbital irradiation with or without cranial radiotherapy were evaluated separately (n=9). The patient with orbital irradiation and the patient with mediastinal irradiation were excluded from the main analysis of endocrine side-effects.

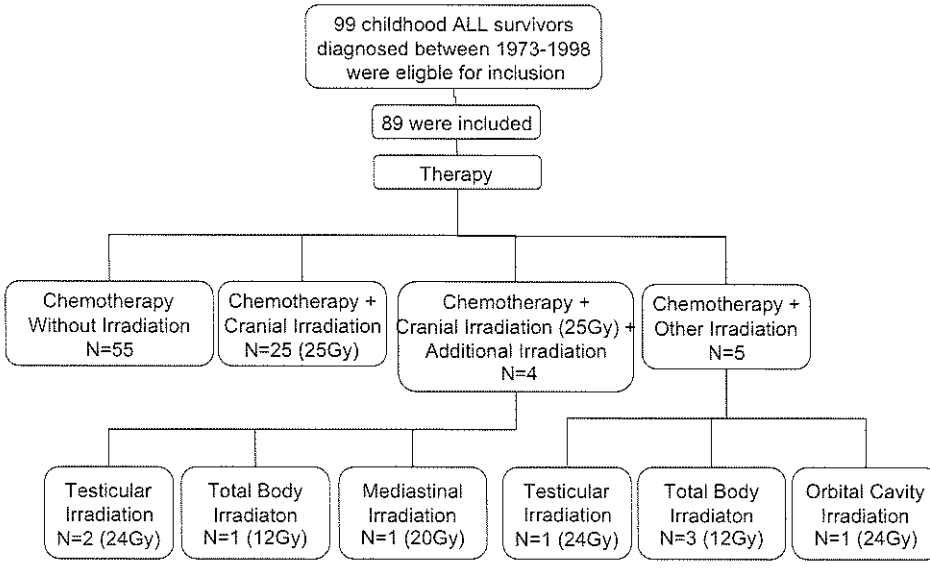


Fig 1: Flowdiagram showing the radiotherapy and chemotherapy modalities of the treated ALL patients. Median irradiation dosages are given between brackets.

Endocrinological evaluation

A complete physical examination and serum hormone analysis from peripheral blood samples were taken at time of the visit at our outpatient clinic for childhood cancer survivorsthe. Samples were obtained by vena puncture, processed within 2 hours after withdrawal and stored at -21°C. Gonadal function was evaluated by Inhibin-B, Luteinizing hormone (LH), Follicle-stimulating Hormone (FSH), testosterone and Sex hormone binding globulin (SHBG). In addition, thyroid-stimulating hormone (TSH), Free T4 (FT4) and Insulin-like Growth Factor (IGF-I) were also measured to determine the broader effect of cranial irradiation on the pituitary axis. Levels of IGF-I between the subgroups were compared with reference values by using

SDS scores (Z scores), which express the difference between the measurement of an individual and the median value of the reference population [225]. The thresholds for normal values of hormone levels are indicated in Table 1.

Statistics

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL). Differences between treatment groups were tested using the student t-test and correlations were tested using Pearson's correlation. P values are all measured in the *two-way* classification with $p<0.05$ considered statistically significant.

RESULTS

Hormone levels from the pituitary-gonadal axis and survivor characteristics were available for all survivors with the exception of one missing value of Inhibin B (Table 1). 41 Forty-one of the male survivors (47%) had decreased (<150ng/l) levels of Inhibin B of which 19 had Inhibin B levels below 100ng/l. Inhibin B levels strongly correlated with FSH ($r=-0.559$, $p<0.00001$), LH ($R=-0.423$, $p<0.0001$) and testicular volume ($r=0.505$, $p<0.00001$).

Neither the cumulative dosage of cyclophosphamide nor any of the other chemotherapeutic agents were significantly different between the survivors with low or normal Inhibin B levels independent of the threshold level of 150ng/l or 100ng/l. FSH and LH levels were increased in respectively 14 (16%) and 8 (9%) patients. Of the survivors who had both an increase in FSH and LH, 3 patients were treated with testis irradiation and 4 with total body irradiation. Only 3 patients were diagnosed with low levels of testosterone of which 1 was treated with testicular irradiation (9 nmol/l) whereas two received chemotherapy only (both 7.7 nmol/l). All patients (n=7) treated with total body or testicular irradiation showed signs of testicular damage illustrated by high levels of LH, FSH and low levels of Inhibin B (Fig 2). Age at time of treatment did not correlate with levels of Inhibin B, FSH, LH, or testosterone in the long-term survivors.

The additional negative impact of cranial irradiation on testicular function is minimal as no differences in Inhibin B, FSH, LH and testosterone levels were found between survivors treated with chemotherapy in combination with cranial irradiation (n=25) and survivors treated with chemotherapy alone (n=55) (Table 1). Median testicular volumes in both groups were not different (median 20 ml, range 10-25, p=0.54). In addition, no difference in pituitary or testicular hormone levels were found between those patients treated with ≥25 Gy (n=21) and those treated with <25 Gy (n=4).

TSH and FT4 levels were available in 38 survivors, of whom all except one had FT4 levels within the normal range. TSH levels were normal in all except two survivors of which one received chemotherapy only and one chemotherapy with TBI. Both had borderline abnormal TSH levels (0.36 and 0.37 mU/l) but normal FT4 levels. No difference in TSH and FT4 levels were

Table 1: Hormone levels according to treatment modality. The two patients treated with orbital irradiation and mediastinal irradiation are not shown. Normal range is given for each hormone. Values given as median and range. Z-scores are given as mean and standard deviation.

Table 1	Complete group	No radiotherapy	Cranial irradiation (med 25 Gy, range15-30)	P-value¥	Total body irradiation	P-value¥	Testicular irradiation	P-value¥
Number of patients	87	55	25		4		3	
Age at diagnosis (yrs)	5.0 (0-15)	6.0 (1-15)	4.2 (0.1-14)	0.02	4 (4-12)	0.70	5 (2-5)	0.27
Age at follow-up (yrs)	25 (18-40)	24 (18-34)	29 (23-40)	<0.0001	25 (22-35)	0.25	30 (21-33)	0.11
Follow-up time (yrs)	19 (7-34)	18 (7-24)	26 (16-34)	<0.0001	18 (15-31)	0.13	25 (16-31)	0.01
Inhibin-B (150-400 ng/l)	155 (0-393)	155.5 (67-392) [a]	177.0 (35-393)	0.85	15 (10-20)	<0.0001	10 (0-10)	<0.0001
Testosterone (10.0-30.0 nmol/l)	16.2 (7.7-29.4)	16.6 (7.7-29.4)	16.4 (11.1-20.9)	0.21	12.2 (11.8-16.0)	0.09	11.9 (9.0-17.0)	0.14
SHBG(10-70 nmol/l)	28.9 (10.6-67.7)	26.2 (10.6-51.4)	31.6 (15.1-51.3)	0.03	22.8 (15.0-40.2)	0.80	47.8 (42.7-67.7)	<0.0001
LH (1.5-8.0 U/l)	4.0 (0.1-18.9)	3.4 (0.1-8.1)	3.1 (1.3-5.3)	0.38	10.8 (7.7-13.9)	<0.0001	13.9 (8.2-18.9)	0.08
FSH (2.0-7.0 U/l)	4.0 (1.1-58.7)	3.4 (1.3-14.1)	4.3 (1.1-15.7)	0.14	23.7 (15.9-31.4)	0.01	48.2 (42.8-58.7)	0.01
Testicular volume (>15ml)	20 (2-25)	20.0 (13-25)	20.0 (10-25)	0.79	12.5 (4-13)*	0.02	2.5 (2-3)*	<0.0001
Free T4 (11-25 pmol/l)	14.8 (11.8-22.7)	15.1 (11.6-30.1)[b]	15.0 (11.8-22.7)[c]	0.69	12.5 (12.3-12.6)	0.06	14.8 [j]	0.79
TSH (0.4-4.3 mU/l)	1.3 (0.36-3.43)	1.14 (0.37-2.48)[d]	1.2 (0.46-2.76)[e]	0.42	1.9 (0.36-3.43)	0.19	0.79 [j]	0.61
Height (cm)	178 (163-192)	182 (165-192)	175 (163-189)	0.006	169 (167-184)	0.035	170 (165-186)	0.13
Body Mass Index	24.1 (17.9-39.6)	23.9 (17.9-39.6)	25.4 (19.2-33.8)	0.04	19.5 (18-24.2)	0.06	20.7 (19.6-21.4)	0.13
IGF-I (15-47 nmol/l)	24.3 (6.5-54.2)	27.4 (13.1-54.2)[h]	17.3 (12.8-47.5)[i]	0.003	20.6 (11.5-32.3)	0.17	7.8 (6.5-23.0)	0.02
IGF-I (z-scores)	-0.49 (1.3)	-0.11 (1.1)	-0.82 (1.1)	0.02	-0.82 (1.3)	0.24	-2.2 (1.6)	0.01

¥ p-values as compared to the non-irradiated group

[a] n=54, [b] n=10, [c] n=21, [d] n=11, [e] n=21, [f] n=1, [g] n=21, [h] n=38, [i] n=21, [j] n=1

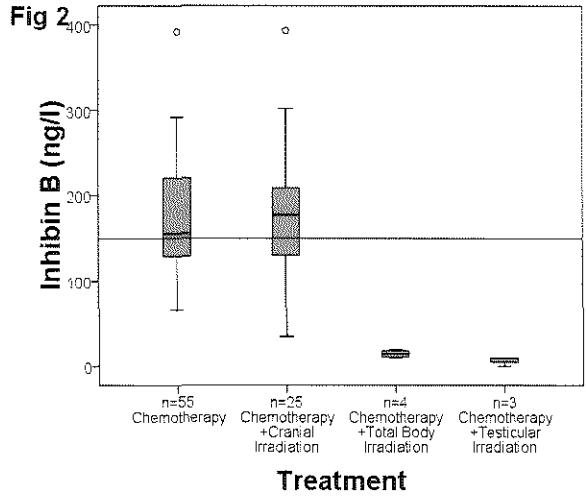


Fig 2: Median Inhibin B values and quartile ranges according to treatment and location of irradiation. One patient in the TBI group and two survivors in the testicular radiated group also received cranial irradiation. The horizontal line represents the lower threshold level for Inhibin B (150ng/l).

found between the patients treated with or without cranial irradiation. The patient treated with orbital, mediastinal or testicular irradiation were also excluded from this analysis.

Of the patients treated with or without cranial irradiation IGF-I was measured in respectively, 38 and 21 survivors. Males who received cranial irradiation had significantly lower IGF-I levels (p= 0.003), lower Z-scores (p=0.02) , shorter stature (p=0.006) and a higher body mass index (p=0.04) than those treated with chemotherapy alone (Table 1). Total body height was significantly lower in the cranial irradiated and TBI group compared to the non-irradiated group (table 2). The survivors treated with TBI had a significant higher BMI compared to the non-irradiated group (Table 1).

Table 2 nog n

Discussion

This is the first study that evaluated the long-term effect of cranial irradiation in very long term male survivors of childhood ALL on fertility using novel gonadal markers. We found no deleterious effect of cranial irradiation on gonadal function thereby providing important information for b adult survivors of childhood ALL treated in the past, as well for the small subgroup of ALL patients that still benefits from cranial irradiation in current treatment protocols [218, 226].

Our study does not confirm the results reported by Siimes *et al.* They reported an increased risk on testicular damage in a group of 41 childhood ALL survivors treated with cranial irradiation by measuring LH, FSH, testosterone testicular size and pubertal stage. In their study 17 out of 41 survivors were irradiated with a median dose of 20-24 Gy. In the current study, we show in a larger single center cohort (n=80, of which 25 subjects received cranial irradiation), with a

longer follow-up time (19 years), including novel fertility markers, that cranial irradiation does not determine the risk of gonadal damage.

Moreover we studied the differential influence of cranial irradiation on gonadal the thyroid and growth hormone axes, which indicates that the negative effect of cranial irradiation on growth hormone production, which has previously been reported in other studies, is selective[211, 227]. In addition, it suggests that impairment of height in the cranial irradiated group is due to growth hormone deficiency, as illustrated by low IGF1 levels, and not by thyroid hormone depletion, although we realize that growth hormone function tests would be required to confirm these results in these individual survivors[211, 228, 229].

Interestingly, the study by Siimes *et al* reported a difference in testicular volume between cranial irradiated and non-irradiated cases. Our study in a larger cohort and with a longer follow-up time, shows no difference in testosterone, Inhibin B values nor testicular sizes between the survivors treated with or without cranial irradiation. It is feasible that an interobserver variability in the study by Siimes *et al.* may have played a role, as survivors were evaluated in 4 different cancer centers. In our study we had the opportunity to determine testicle size in all survivors by one and the same clinician.

Quigley *et al.* reported elevated gonadotrophin levels in childhood ALL patients treated with 24Gy cranial radiotherapy and chemotherapy and confirmed gonadal damage by testicular biopsies showing an absence of germ cells[210]. Their analysis was performed immediately after cessation of treatment and not repeated after a long-term follow-up. This suggests that recovery of gonadal function takes occurs[230].

Another method to study male fertility after childhood ALL is to investigate the number of life births in male and female ALL survivors and compare this with an age matched control group, which was done by a Norwegian group[214]. Only four out 131 male adult ALL survivors fathered children of which three received cranial irradiation. The conclusion of their study was that their data were probably incomplete and therefore statements concerning the detrimental effect of cranial irradiation as component of ALL treatment on male gonadal function could not be made. In our study we did not include semen analysis or life births as end points. However, it is questionable whether proven fatherhood can be used as a marker for gonadal damage after chemotherapy, as achieving a pregnancy is not solely dependent of sperm quality. Comparing gonadal markers is more suitable for the assessment of treatment induced male gonadotoxicity, as reflected by strongly correlated Inhibin B and FSH values [195].

Interviews are another way to get insight in to fertitlity rate in childhood cancer survivors. Byrne et al performed telephone interviews and assessed life births in 213 men treated for ALL before age 18 on protocols of the Children's Cancer Group and compared these results with 145 male siblings. They found a decreased number of life births in childhood ALL survivors treated with cranial radiotherapy before the age of 10 years at diagnosis, their fertility was only 9% (RF 0.09, 95% CI 0.01–0.82) compared to controls [213]. However, this study did not include female

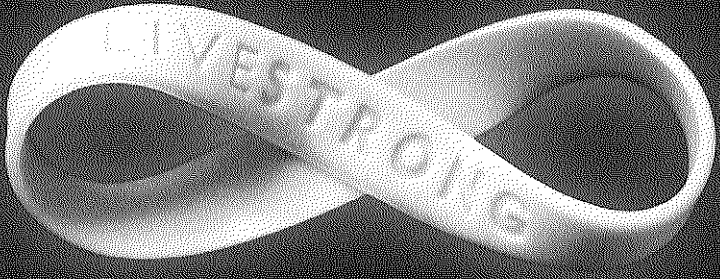
fertility information as a determinator for a couples fertility potential. Therefore life births do not accurately reflect male gonadal function.

In our complete ALL survivor group cancer younger age at time of treatment was not associated with a higher risk of impaired gonadal function. It is not possible to conclude whether cranial irradiation after the age of 10 years induces a lower risk for pituitary and gonadal damage from our analysis as the group of survivors who were treated after the age of 10 was too small.

Our study suggests that previously applied treatment protocols for ALL are not highly gonadotoxic. Except for cyclophosphamide, most chemotherapeutic agents administered in ALL treatment have a relatively mild gonadotoxicity, which is underscored by the current study [217]. In our cohort, the total cumulative dosage of cyclophosphamide did not exceed 10mg/m^2, which is considered to be the threshold level for its gonadotoxic effect. This made it possible to evaluate the effect of cranial radiotherapy as independent variable[217]. Furthermore this study shows that it remains important to refer all patients for semen cryopreservation before treatment[231].

Chapter 12

General Discussion



GENERAL DISCUSSION

Since the discovery of Carcinoma in Situ as the precursor of testicular germ cell cancer, research on the origin and possible causes of testicular maldevelopment has increased. The hypothesis that several diseases of the male reproductive system share a common environmental cause was first postulated in the early 1990s, with reports on endocrine disruption causing genital malformations and reproductive disorders in animal wildlife. Parallel to these observations temporal trends in testicular cancer and other male reproductive disorders were observed in humans [235]. More recently, it was reported that men with prenatal exposure to estrogen diethylstilbestrol (DES) or phtalates had an excess risk for undescended testis, hypospadia and testicular cancer [236].

Development of CIS seems dependent on the micro-environment created by Sertoli cells in the testis and by Granulosa cells, being ther female counterpart of Sertoli cells, in the dysgenetic gonad. These Sertoli cells are also involved in the development of TM. It has been suggested that these calcified concretions within the lumen of seminiferous tubules originate from sloughing of degenerated intra-tubular cells and from failure of the Sertoli cells to phagocyte the debris [36-38]. The occurrence of TM in patients who are at risk for developing testicular cancer stresses both the importance of the Sertoli cell function for correct development of the testis, and the role of the Sertoli cell malfunction in TDS. Future research should focus on potential disrupters of Sertoli-cell development and function during the neonatal period.

Althogh the role of Anti-Mullerian Hormone (AMH) levels during postnatal gonadal maturation and after puberty remains vague, it was recently suggested that they are involved in germ-cell development, and also that they affect the differentiation and function of Leydig cells [237, 238]. Their role of AMH as a potential marker for testicular dysgenesis should therefore be further investigated.

As stated in this thesis, we recommend an active approach should be taken in men with TM and additional risk factors for TGCT. This approach should include testicular biopsies. The physical and social impacts of such an active approach should be explored in a prospective study. To evaluate the testis, regular ultrasonography is advised in most of the follow-up protocols of TM. Indicators for TDS and CIS of the testis are gonadal dysfunction and ultrasonic features as TM, an inhomogenous parenchyma of the testis and testicular atrophy [47].

In the United States the economic burden of evaluating and following men with TM when they are at risk for testicular cancer was estimated by Peterson et al. to be greater than $18 billion and therefore not cost-effective [35].It might therefore be better to perform highly accurate testicular biopsies in men with TM and associated risk factors for TGCT. Stoehr et al. showed recently that the only benefit of scrotal ultrasonography lies in the detection of asynchronous tumors, not of CIS[239]. The use of other imaging methods such as CT-scanning or MRI to evaluate the testicular parenchyma is also not likely to help the detection of CIS, which these imaging techniques cannot detect.

If CIS is detected before it progresses to an invasive tumour stage and subsequentely treated this may result in a significant reduction of TGCT and the consequences of its treayment [5]. It is therefore important to identify high risk-groups for TGCT in whom diagnostic testicular biopsies can be performed, as this invasive procedure is unsuitable for large population screening programs. This will probably include men with cryptorchidism, gonadal dysfunction (impaired fertility and hypogonadism) and ultrasonographic features such as TM. A prospective case finding study should be performed to evaluate this early detection program of CIS.

We have shown that the detection of CIS in testicular biopsies is hampered by various factors which can be partially overcome if immunohistochemistry is performed on all testicular biopsies. As shown in chapter 5, few CIS cells can be present within the biopsy specimen. Recently, OCT3/4, a reliable new diagnostic marker has been added to the palette of markers. Although it has proven to be highly specific and sensitive, it is not yet used widely. In a specific highly selected population, we demonstrated that immunohistochemistry can produce an additional diagnostic yield of 20 percent in detecting malignant disease. While the additional value of performing immunohistochemistry in a non-selected population has yet to be examined, its advantages in detecting CIS or TGCTs are unlikely to differ.

Schulze et al. performed histological analyses on Testicular Sperm Extraction (TESE) material and found CIS in 0.7% of the biopsies [240]. As the testicular biopsies were not stained with specific CIS or TGCT markers, the actual prevalence of CIS in the above mentioned study might even be higher. We therefore stress that in all testicular biopsies for TESE and for assessing spermatogenic status should use immunohistochemistry should be used specifically to test for the presence of CIS. Also, two-sited biopsy has already been shown to produce an additional diagnostic yield of 18% [13]. If CIS is highly suspected, a two-sited biopsy should therefore be performed in all testicular biopsies in addition to immunohistochemistry.

We demonstrated that even without immunohistochemistry, an experienced pathologist is able to find more cancer in testicular biopsies. This means that testicular biopsies should ideally be evaluated in a referral centre by an experienced pathologist. This will reduce the number of false-negative diagnoses and improve diagnostic outcome. As this thesis has already pointed out, there are obviously many advantages to detecting CIS before it has advanced to an invasive cancer. Although many advantages detecting CIS does not eliminate the need to perform an orchidectomy or to irradiate both the testis in men diagnosed with CIS. This will result in sterility and hypogonadism.

It is becoming increasingly clear that CIS originates from primordial germ cells/gonocytes. Gene-mapping studies of CIS cells showed that CIS is not a malignant cell in a classic sense, but is an arrested polyploid gonocyte: oncogenes are not overrepresented, while tumor suppressor genes are not underrepresented [238]. However, it is still uncertain what triggers the CIS cell to evolve into an intratubular seminoma or embryonal carcinoma, and, as often occurs after puberty, eventually into an invasive cancer. If this transition is understood, it might be possible

to develop treatment options that can prevent CIS from developing and progressing to an invasive cancer.

In this thesis we have shown that CIS can also be detected in testicular biopsies, but another opportunity for detecting it is presented by new non-invasive techniques that use exfoliated CIS cells in semen. Potentially, this non-invasive detection method may provide an extra diagnostic tool for detecting CIS in patients at risk, or even for the male population as a whole in a screening-based setting. While many methods of detecting exfoliated CIS cells have been tried with discouraging results [107, 110, 113, 239], Hoei-Hansen and coworkers were recently the first to diagnose CIS in an infertile patient by detecting exfoliated CIS cells [108]. We confirmed the strength of the method using a novel method, which detects CIS before the patient presents with symptoms.

Unlike many other diseases, such as bladder carcinoma or prostate cancer, screening for TGCT is based on the detection of the pre-invasive stage, and thus on preventing the development of malignancy. In 2006, 605 young males in the Netherlands were diagnosed with a TGCT, most of them of a reproductive age. The incidence of TGCT in males aged 15-34 was 12 per 100,000 (Dutch Cancer Society/Koningin Wilhelmina Fonds KWF data). Due to an annual increase in TGCT of approximately 5%, it has been calculated that 728 young males in the Netherlands will be diagnosed with a TGCT in 2010. Because the overall 5-year survival is approximately 95%, most men will survive [87]. This also means that they will live with the consequences of the treatment of TGCT for a substantial part of their lives [240].

The detection and treatment of CIS would prevent many of the sequelae seen when a patient is treated for TGCT. Potentially, this new non-invasive method may provide clinicians with an extra diagnostic tool. Although it should still be tested in larger populations, the idea of exfoliating CIS cells offers new diagnostic opportunities. We acknowledge that a screening programme with the current non-invasive test is not yet aplicable in general practise. The current test should first be validated in a large multi-centre study, and the method of detecting CIS cells in semen must be optimised before it can be used on a large scale. Such studies should also show whether all young men should be screened, or that screening should be performed only in high-risk populations. However TGCT proves to be a disease which is an seriuos candidate for developing a screening strategy.

FERTILITY AND GONADAL FUNCTION BEFORE AND AFTER CYTOTOXIC TREATMENT.

Male spermatogenesis is a complicated process that starts with the division of stem cells and ends with the formation of mature spermatozoa. Many factors can influence male fertility, including the presence of malignant disease [241]. In 2005, 40,684 males in the Netherlands were diagnosed with cancer, 869 of whom were in the 15-34 age group (Dutch Cancer Society

data). The impact of being diagnosed with cancer in this specific age group, when most men are in their reproductive period, is very high. While most of them are concerned about their fertility after treatment, they are not aware of the possibility of impaired fertility before cancer treatment has started. We showed that fertility was reduced in two-thirds of the male cancer patients at diagnosis, which is far more than in a general population.

The etiology of diminished spermatogenesis before treatment is probably multifactorial. Disease-related factors may be involved, such as endocrine disturbances, stress and an increased catabolic state that can accompany cancer, especially in an advanced stage [121, 145, 162]. These factors are difficult to overcome in order to optimize pre-treatment semen quality.

In a study of young cancer patients, we showed that men with TGCT have a lower semen quality and lower Inhibin B levels compared to patients diagnosed with other malignancies. In contrast to men with testicular dysgenesis syndrome, patients first diagnosed with germ-cell tumors often showed to have normal FSH levels. This may be explained by the negative effect on FSH secretion of beta-HCG, which is produced by some testicular tumors.

On the basis of the 20-year prevalence period, the number of cancer survivors in the Netherlands is expected to increase from 366,000 in 2000 to 692,000 to 2015 (Dutch Cancer Society data). This means that the number of cancer survivors will almost have doubled over a 15-year period, producing an annual increase in prevalence of almost 6% (Dutch Cancer Society data). Although, due to the use of new treatment protocols, children diagnosed with cancer nowedays have a very good chance of surviving their disease, most treatment protocols unfortunately have long-term sequalae. Approximately two-third of all childhood cancer survivors will suffer from adverse treatment-induced side effects, one-third of which will are severe, and sometimes even life threatening [242]. Because childhood cancer therapy continues to be refined, the study of late effects is also an ongoing process.

In this thesis, we have focused on fertility and endocrine disturbances caused by treatment-induced toxicity. One of the most commonly used alkylating agents in paediatric cancer treatment is cyclophosphamide, which has a dose-dependent effect of induced infertility. Patients receiving a total cumulative dose of more than 10 grams/m^2 are at risk of severe gonadal damage. We found no similar dose-dependent effect in patients treated with procarbazine. However, all these patients had very low Inhibin B levels, suggesting severe gonadal dysfunction independent of the total cumulative dose of the drug. We conclude that, procarbazine is extremely mutagenic to germ cells, and causes irreversible damage [243].

Although the total study group consists of 261 survivors, we acknowledge that our results may have been influenced by the wide diversity of different cancers and treatment protocols. Even so, this is the largest single-centre long-term follow-up study to date. Ideally, future studies may come to more definitive conclusions, although, treatment protocols are constantly changing, making it difficult to obtain large patient populations with comparable treatment protocols. This problem could partially be overcome by the introduction of a nation-wide database, which has already been initiated by the Dutch Childhood Cancer Workgroup.

In our study, the age at time of treatment did not correlate with post-treatment gonadal function. Previous studies claimed that, due to the lack of activity, pre-pubertal testes have a lower risk of gonadal damage due to cancer treatment [212]. The pre-pubertal period, however, is not a quiescent time for the testis[188], and it seems that chemotherapy before puberty also can impair gonadal function. Besides its harmful effect on the spermatogonial stem cells, chemotherapy in childhood can also damage Sertoli cells and Leydig cells, which proliferate in this period [244]. A decline in Sertoli cell numbers affects spermatogenesis later in life. Because pre-pubertal boys are also at risk for impaired fertility later in life, options to preserve fertility in these boys should be developed.

There are different methods of measuring the detrimental effect of cancer treatment protocols on gonadal function: by evaluating the total number of live births after cancer treatment, and by measuring reproductive hormones and semen quality, which reflect gonadal function. In our opinion, live births rates are not a good marker of gonadal damage after gonadotoxic treatment.This also depends on the fecundity of the partner. Even, if the patient is diagnosed with a reduced sperm quality, he may still be able to induce pregnancy in a fertile partner.

We found that most cancer survivors were reluctant to provide a semen sample for analysis and used serum Inhibin B as an alternative method of determing gonadal damage after gonadotoxic treatment [124]. However, while low Inhibin B indicates that spermatogenesis is impaired, it does not indicate the extent of impairment. A clear cut-off value for the presence of spermatozoa is not found. Fertility markers are not a good substitute for semen analyses in the assessment of future treatment options for ART.

The direct gonadotoxic effect of radiation and chemotherapy treatment can also influence gonadal function by disrupting the hypothalamic-pituitary-gonadal axis. We investigated the specific effect on gonadal function of cranial radiotherapy in children with Acute Lymphoblastic Leukaemia (ALL). It has been reported that a radiation dose of>18 Gy can disrupt the hypothalamic-pituitary-gonadal axis, potentially resulting in impaired endocrine function and growth disturbances[210, 211].

It has been suggested that the future fertility of these children may be impaired, and that low age at time of treatment (<10 years) is a risk factor for post-treatment gonadal dysfunction [212-215]. However, in a cohort of 89 male childhood ALL survivors with a very long follow-up, we found no deleterious effect of cranial irradiation additional to the gonadotoxic effect of chemotherapy on pituitary-gonadal function. Although the treatment protocols differed slightly, these findings are reassuring with regard to the fertility of men who have received cranial irradiation for ALL. Although it was not the primary objective, our study demonstrated an impact of cranial radiotherapy on the growth hormone and thyroid axis. This emphasizes the need for long-term follow-up in these patients. To ensure timely diagnosis and early hormone replacement therapy, regular testing is recommended.

The risk of infertility after treatment is a major concern for cancer survivors [245, 246]. The only established method for preserving fertility is semen cryopreservation (SCP). Surprisingly,

despite the fact that that it is a simple procedure, Schover et al. demonstrated that only 27% of men in a cohort in the United States diagnosed with cancer was referred for semen cryopreservation [245]. Lack of information was usually the reason that sperm banking in these patients was not performed.

For the various reasons described in this thesis , not many young boys with cancer are referred for sperm banking, despite the fact, as we have demonstrated, that it is successful in the majority of pubertal boys. Although it may be a difficult subject to discuss, young boys in whom spermatogenesis may have started should be informed about the possibility of cryopreserving their sperm. Edge et al. investigated the level of understanding the need for banking sperm in young boys and found it to be high, with an average score of 7.2 out of 10 [149]. Unlike our study, they also found a significant difference in successful sperm banking between younger and older patients.

Edge et al. also clearly demonstrated that male patients who had less understanding of sperm banking and who had more difficulty discussing sperm banking were at risk for unsuccessful sperm banking. Similarly, Schover et al. demonstrated that men who chose to bank sperm scored significantly higher on knowledge tests concerning cancer-related infertility than those who did not bank semen [245]. These findings suggest that discussing sperm banking is of utmost importance, and should be performed by an experienced physician or nurse practitioner.

Semen analysis showed that 14 pubertal patients from our cohort of 80 adolescents had no motile spermatozoa and the samples in question were not cryopreserved, as our clinic performs in-vitro fertilization only with motile spermatozoa. Perhaps non-motile spermatozoa should be frozen, as they might be used for future assisted-reproduction techniques. Although immature spermatids and secondary spermatocytes are currently used for Intracytoplasmic Sperm Injection (ICSI), success rates are still very low [247-249]. In TESE, non-motile testicular spermatozoa are sometimes used, which sometimes results in fertilization. However, the fertilization rate is lower than compared to the rate when motile testicular spermatozoa are injected [250]. Successful pregnancies have also occurred with the use of ICSI in men with immotile cilia syndrome in whom only non-motile spermatozoa were present [251]. These data suggest that we should consider altering our pre-freeze requirements. If non-motile spermatozoa are cryopreserved, patients should be properly informed of the limited results that have been achieved with them. It should also be noted that these cells are currently not used for assisted reproduction techniques in the Netherlands and that techniques using fresh non-motile spermatozoa are associated with a very low chance of pregnancy.

Due to the absence of spermatozoa or of motile spermatozoa, semen cryopreservation was not feasible in 14 patients in our cohort of 80 pubertal boys – a specific but diverse group in whom pre-treatment endocrinological evaluation does not predict sperm yield. This makes it difficult to identify patients who might benefit from semen cryopreservation. We nonetheless advocate that all boys with cancer who have reached Tanner stage G2 should be referred for SCP.

In boys who are unable to produce a semen sample, the use of electro-ejaculation techniques should also be considered. This minimally invasive procedure can be performed in combination with other procedures for which general anaesthesia is required, such as bone-marrow biopsies or port-a-cath placements. Electro-ejaculation in children is still not widely used. Hovay et al. published results on its successful use in a group of six adolescents aged 18 +/- 3 [153]. Large studies of the use and success rates of electro-ejaculation in pubertal boys with cancer are not yet available. Since attempts to produce semen may cause anxiety, distress and failure to produce sperm, there may be good arguments for making electro-ejaculation the standard procedure for all young boys referred for SCP. These topics should be evaluated in a prospective study.

In boys in whom spermarche has not yet started, there are no good options for securing future fertility. As the inability to become a father in the future can have a high impact on a patient's psychological wellbeing in later adulthood, further research should focus on alternative methods for preserving fertility in prepubertal children [155]. Fertility-preservation techniques might focus on culturing and transplanting spermatogonial stem cells, which can be harvested from testicular biopsies. Spermatogonial Stem Cell Transplantation (SSCT) was first introduced in a rodent model by Brinster and Avarbock [252], when insertion of a germ-cell suspension into an infertile rodent resulted in the restoration of the spermatogenic process.

The use of testicular allografting has also been investigated. Mice-donor testicular tissue was retrieved from cloned mice and transplanted into the testes of recipient nude mice. After three months, the donor testicular tissue had grown into the host testicle and colonized the seminferous tubules, in some cases inducing spermatogenesis [253]. As testicular tissue should be harvested for these techniques, a surgical procedure is needed in young cancer patients. Although this is an invasive procedure, Van den Berg et al. demonstrated that the majority of parents accept the use of biopsy to collect spermatogonial stem cells [155].

Van den Berg et al. also reported that hemicastration was accepted by as many as one-third of the parents of childhood-cancer patients. Although the idea is promising, this technique has some major drawbacks. The major limitation of autologous reimplantation is the re-introduction of the malignant disease [254], especially in haematological cancers. Most cancers in childhood are capable of penetrating the testicular tissue, increasing the risk of reintroduction [255]. This might be overcome by selecting the germ cells by fluorescence-activated cell-sorting. While Fujita et al. had promising results in transplanting testicular tissue from leukemic mice to recipient mice without inducing leukemia [256], it should be mentioned that recurrence can be caused by as few as five reintroduced leukemia cells [257]. The sorting method should therefore be 100% secure. Other options such as in-vitro maturation of spermatogonia or xenografting should also be investigated.

Although cryopreservation causes sperm quality to decline by approximately 50%, the introduction over recent decades of sophisticated new assisted-reproduction techniques such as *in-vitro* fertilization (IVF) and intra cytopalsmatic sperm injection (ICSI) have given men a fair chance of future paternity [156, 161]. Currently, the success rates of IVF and ICSI treatments

using cryopreserved semen are almost as high as those using fresh semen [167, 258]. We demonstrated that approximately 7.5% of all referred men and approximately 10% of survivors , all of whom had been rendered infertile by treatment , used their cryopreserved semen. Using this semen, 49% of couples achieved a pregnancy and life birth.

Our data also illustrates the relatively low success rate of Intra Uterine Insemination (IUI) using cryopreserved semen over IVF. The use of cryopreserved semen for IUI should thus be discouraged in certain cases, especially those in which few straws of semen can be stored. With the new ICSI technique, thawing one straw of banked sperm is often sufficient for IVF treatment. This allows the couple to undergo several treatments if necessary. Although IVF treatment is more invasive than IUI, the higher success rate and the lower quantity of motile sperm required favour the use of cryopreserved semen for IVF. Physicians treating male cancer patients should thus be encouraged to discuss the topic of semen cryopreservation with all of these patients.

Chapter 13

Conclusions and further research

CONCLUSIONS

This thesis clearly demonstrates that the infertile patient is at risk for developing testicular cancer especially if he is diagnosed with TM. As illustrated in this thesis we advocate an active approach in men with TM and risk factors for TGCT, which includes performing testicular biopsies. The health- and social impact of such an active approach should be further investigated. Future research should also focus on the aetiology behind these microliths: the role of the Sertoli cell and potential disruptors of gonadal development in the origin of TM should be examined. Also efforts should be made to evaluate to which hormonal disruptors pregnant women are exposed to during early pregnancy. In this stage, male gonadal development is at increased risk.

CIS is sometimes difficult to diagnose in testicular biopsies. Efforts have been made to decrease the number of false negative results for CIS. This thesis demonstrates a reliable method to evaluate testicular biopsies. On all testicular biopsies immunohistochemistry using specific markers, such as OCT3/4, should be performed. This will increase the diagnostic yield of testicular biopsies and decreases the number of false negative results.

We demonstrate that CIS cells are exfoliated in semen which can de visualized using OCT3/4. These findings give new insights for a screening programme for men at risk for testicular cancer which might result in higher cure rates and less treatment related sequelae. Further research should focus on examining novel non-invasive diagnostic methods. Potential diagnostic markers could possibly be found in the semen of TGCT patients. If these diagnostic markers are reliable a screening programme for testicular cancer is possible and should be investigated in a prospective multicenter study.

Adult male cancer patients are often referred for semen cryopreservation before gonadotoxic treatment to secure their future fertility potential. Unfortunately pre-treatment sperm quality can already be impaired before cancer treatment has started. We demonstrate that only 7.5% will use their cryopreserved semen which is successful in 50% of the cases. This thesis presents pre-treatment sperm quality in male cancer patients and illustrates that especially patients with TGCTs and extra-gonadal GCTs are at increased risk for diminished pre-treatment fertility.

We demonstrate that treatment protocols for childhood cancer which contains procarbazine, high doses of cyclophosphamide, testicular and total body irradiation can cause severe post treatment gonadal damage independent on the age at which this treatment is given. Patients treated with cranial irradiation did not have pituitary-gonadal-axis dysfunction, which offers some hope regarding fertility for childhood ALL survivors. This thesis also clearly illustrates that semen cryopreservation is feasible in pubertal boys with cancer and should therefore be discussed with the patient and his parents. Nor age at time of diagnosis or fertility markers can predict successful semen cryopreservation in this specific group of patients.

Chapter 14

Summary

SUMMARY

Approximately 15% of all couples are affected by subfertility defined as the inability to conceive after one year of unprotected intercourse. In approximately 20 percent of the couples with fertility problems, a disturbance in male reproductive capacity is the sole cause. In the introduction, an update is given on the interaction between testicular development, infertility and testicular cancer.

Testicular development is an early embryonic process and disturbances, either through internal or external factors, may have major consequences resulting in the so-called testicular dysgenesis syndrome. This syndrome has a variety of clinical symptoms ranging from minor spermatogenic disturbances, cryptorchidism, hypospadia, infertility and even testicular cancer. It is shown by several large studies that patients who have an impaired fertility are at increased risk for the development of a testicular germ cell tumour (TGCT). Testicular cancer is the most common cancer of young men in their 20s and 30s and the incidence of testicular cancer is growing. Carcinoma In Situ (CIS) is currently accepted as the precursor of all TGCTs of adolescents and young adults (TGCTs), i.e., the seminomas and nonseminomas. CIS develops in the testis after birth and will progress to cancer after puberty. This offers an opportunity for screening as outlined in the introduction. The second part in the introduction elaborates on the detrimental effects of cancer and its treatment on gonadal function.

In Chapter 4 we discuss the relevance of Testicular microlithiasis (TM). TM has been associated with testicular germ cell tumors in adolescents and adults (TGCTs) and with its precursor carcinoma in situ (CIS). A clear definition and a consensus concerning the malignant potential of TM and the need for further diagnostics and follow-up are lacking. We reviewed the literature of TM and its association with TGCT/CIS and current follow-up advises. A wide variety in prevalence of TM is described in different populations due to different sample size, composition, ultrasound and screening criteria. TM consists of a large heterogeneous group and must be considered in essence a benign condition. Still, its association with TGCT is remarkable, and may be very useful when screening for TGCTs, especially in its pre-invasive stage, in specific risk groups. In view of its association with cancer, we propose to perform testicular biopsies in selected high-risk groups consisting of those males with additional risk factors for TGCT development.

In Chapter 5 and 6 we describe the importance of proper evaluation of testicular biopsies. As mentioned CIS can be found in the testis before a testicular tumour has developed. The gold-standard for detecting CIS is to perform a random surgical biopsy as CIS is often spread throughout the testis. However false-negative biopsy results do occur as shown in Chapter 5. We show that there are multiple causes for false-negative biopsy results including non-heterogeneous distribution and the presence of only few malignant cells in the complete biopsy. We demonstrated that in a selected patient population of men whom were diagnosed with an invasive TGCT, while a matched previously obtained testicular biopsy was diagnosed

as non-malignant, immunohistochemistry was able to prevent 20% false negative diagnosis. Furthermore, we performed a simplified calculation to calculate the cost-effectiveness of performing immunohistochemistry on all testicular biopsies. In these two chapters evidence is provided that the implementation of immunohistochemistry is most-likely cost effective, and improves quality of patient care, and should therefore be used as standard diagnostic practice.

Two of the major characteristics of CIS including the presence of CIS before tumor development and secondly the low number of false negative biopsy results make CIS a target for early detection and treatment. However, a testicular biopsy is an invasive procedure not suitable for large scale screening. In chapter 7 we elaborate on an alternative method to detect CIS. CIS cells lie in the semiferous tubules and are exfoliated with the spermatozoa. We performed a pilot study in which we were able to detect these exfoliated cells in patients with CIS and TGCTS using immunohistochemical markers. These findings advocate a large multicenter research to evaluate the specificity and sensitivity of this test and should evaluate the potential for a screening program for TGCTs. This potential screening program could than be used in selected high risk populations such as infertile men or men with undescended testes.

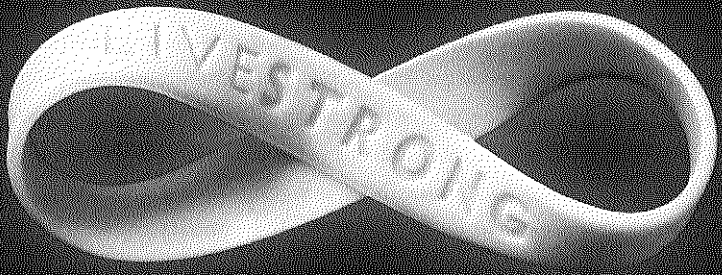
In chapter 8 we analyzed semen and endocrinological hormone profiles of a large cohort of male cancer patients presenting for semen cryopreservation. Male cancer patients are referred for semen cryopreservation as the upcoming treatment is often gonadotoxic. We show an increase in referral and demonstrate that approximately 90% of all men are able to secure their fertility potential by freezing motile spermatozoa. Furthermore, we demonstrate that patients diagnosed with TGCTs or extragonadal germ cell tumors have a more impaired gonadal function than those diagnosed with other forms of cancer. This is in line with the testicular dysgenesis syndrome in which an impairment of testicular development leads to a variety of problems as described above.

In chapter 9 we describe the success rate of semen cryopreservation in young pubertal boys. During the last decades, mainly as a consequence of improved treatment efficacy, the overall number of childhood cancer survivors has increased substantially. As a result the number of young boys referred is also steadily increasing. Semen cryopreservation in this specific subgroup is a stressful procedure. We analyzed if hormone profile could predict sperm quality in this diverse group. Unfortunately no predictors were found which could indicate successful sperm yield. However, we advocate that young boys diagnosed with cancer should be referred to a sperm banking clinic to assess if semen cryopreservation is possible. If the patient is unable or unwilling to masturbate electro-ejaculation could be an option to obtain motile spermatozoa. These spermatozoa can than be preserved until the patient has reached adulthood and wants to use his semen to conceive. To determine the usefulness of semen cryorpreservation we have evaluated the total number of men who used their cryopreserved semen. Approximately 10% of the cancer survivors have claimed their semen for an assisted reproduction technique (ART). A total of 101 ART cycles were performed resulting in 25 life births. In fifty % of the men

who used their cryopreserved semen at least one ART resulted in a life birth. These numbers illustrate the importance of semen cryopreservation in male cancer patients.

To assess which patient groups are at increased risk of gonadal damage after cancer treatment we analyzed long-term survivors of childhood cancer. In chapter 10 we determined pituitary and gonadal hormones including Inhibin B, which is a good marker to assess spermatogenesis, to assess their gonadal function. Specific risk groups were found including those receiving either procarbazine or high dosages of cyclophosphamide. Furthermore patients treated with total body irradiation were at increased risk for gonadal dysfunction. We demonstrated that young age at time of cryopreservation did not protect the gonads from the detrimental effects of chemotherapy. With this data we are able to inform young patients concerning their risk on gonadal dysfunction and might indicate which patients might benefit from more aggressive fertility preserving techniques. In chapter 11 we focus on patients treated for childhood Acute Lymfoblastic Leukaemia (ALL) to assess if cranial irradiation has an additional detrimental effect on fertility in ALL treatment regimens. Therefore we compared two cohorts of childhood ALL survivors of which one was treated with cranial irradiation. We demonstrated that cranial irradiation (median 24 Gy) had an effect on pituitary function including the growth hormone axis but did not have an additional effect on gonadal function.

Samenvatting



SAMENVATTING

Verminderde vruchtbaarheid (subfertiliteit) wordt gedefinieerd als het uitblijven van een zwangerschap binnen 1 jaar bij onbeschermde geslachtsgemeenschap. Bij ongeveer 15% van alle paren is er nog geen zwangerschap opgetreden na 1 jaar en zij worden beschouwd als subfertiel. In ongeveer 20 procent van deze paren is een stoornis van de mannelijke vruchtbaarheid de enige oorzaak. Een ontwikkelingsstoornis van de zaadballen (testes) is een belangrijke oorzaak van mannelijk onvruchtbaarheid. In de inleiding van dit proefschrift wordt de relatie tussen een ontwikkelingsstoornis van de zaadballen en mannelijke onvruchtbaarheid besproken, evenals de relatie met zaadbalkanker. De ontwikkeling van de testes is een vroeg embryonaal proces en verstoringen, hetzij via interne of externe factoren, kunnen grote gevolgen hebben die uiteindelijk kunnen leiden tot het zogenaamd "testicular dysgenesis" syndroom. Dit syndroom heeft meerdere klinische uitingen, variërend van een gestoorde spermaproductie, indalingstoornissen van de testes (cryptorchisme), afwijkende ontwikkeling van de plasbuis (hypospadie), onvruchtbaarheid en zelfs zaadbalkanker. In verschillende grote studies is aangetoond dat mannen met een verminderde vruchtbaarheid een verhoogd risico hebben op de ontwikkeling van kiemceltumor van de zaadbal. Zaadbalkanker is de meest voorkomende kwaadaardige aandoening bij jonge mannen in hun 20^{e} en 30^{e} levensjaar. De incidentie van zaadbalkanker neemt jaarlijks toe, de oorzaak hiervan is nog onbekend.

Carcinoma In Situ (CIS) is het voorloperstadium van zaadbalkanker. CIS kan ontstaan in dysgenetische testes en is dan al aanwezig na de geboorte. Uiteindelijk zal CIS uitgroeien tot zaadbalkanker na de puberteit, als hormonen de groei van de zaadballen stimuleren. De aanwezigheid van CIS biedt een kans voor vroegdetectie van zaadbalkanker, zoals geschetst in de inleiding van dit proefschrift.

Na de eerste 2 inleidende hoofdstukken beschrijft hoofdstuk 3 van dit proefschrift de relevantie van kleine verkalkingen in de zaadballen, de zogenaamde "Testicular Microlithiasis" (TM) voor de diagnostiek van zaadbalkanker. Uit diverse studies komt naar voren dat TM geassocieerd is met CIS en kiemceltumoren van de testes (TGCTs). Een duidelijke definitie van TM ontbreekt nog in de literatuur. Ook bestaat er geen consensus over de relatie tussen kiemceltumoren en TM en de noodzaak van verdere diagnostiek en follow-up. Een overzicht van de literatuur die de associatie tussen TM en TGCT /CIS onderzocht hebben wordt verricht. Vervolgens worden adviezen geformuleerd over de manier waarop mannen die gediagnosticeerd zijn met TM verder onderzocht en gecontroleerd moeten worden. Een grote verscheidenheid in de prevalentie van TM is beschreven wat mede te wijten is aan het bestaan van verschillende steekproefgrootte, gebruik van verschillende echografische apparatuur en selectiecriteria voor kandidaten in de verschillende studies. Onze conclusie is dat TM moet worden beschouwd als een in essentie goedaardige aandoening, maar dat er een associatie is met zaadbalkanker , vooral bij mannen met risicofactoren op zaadbalkanker. TM zou een zeer nuttig hulpmiddel

kunnen zijn bij het vroegtijdig ontdekken van zaadbalkanker, in een fase dat er nog geen sprake is van CIS. In verband met de verhoogde kans op zaadbalkanker bij mannen met TM , wordt voorgesteld om weefselbiopsiën te nemen van beide testes in mannen met tevens een verhoogd risico op zaadbalkanker. Dit wordt in hoofdstuk 4 verder geillustreerd middels een case report waarin enkele valkuilen van het nemen en beoordelen van een testis biopsie besproken worden. Hoofdstuk 5 en 6 beschrijven het belang van een correcte evaluatie van testisbiopsiën. Zoals vermeld kan CIS in de testis worden gevonden voordat een tumor zich heeft ontwikkeld. Voor het stellen van de diagnose CIS is het nemen van een chirurgisch weefselbiopsie van de testis nodig. Deze biopsie kan op een relatief willekeurige plaats genomen worden omdat CIS meestal verspreid voorkomt in de aangedane testis. Echter fout-negatieve resultaten doen zich voor, zoals ook wordt aangegeven in hoofdstuk 4. Een niet heterogene verspreiding van CIS in de testis en de aanwezigheid van slechts enkele kwaadaardige cellen in het volledige biopsie weefsel zijn enkele van de in dit proefschrift aangetoonde oorzaken. Immunohistochemie was, in een geselecteerde patiëntenpopulatie van mannen die werden gediagnosticeerd met een invasieve vorm van TGCT, in 20% van de gevallen in staat om fout-negatieve uitslagen te voorkomen. Dit terwijl een eerder verkregen testisbiopsie als niet-kwaadaardig was afgegeven. Tevens werd een berekening verricht van de kosteneffectiviteit van de uitvoering van immunohistochemie op alle testis biopsiën. In deze twee hoofdstukken wordt bewijs geleverd dat de uitvoering van immunohistochemie kosteneffectief is, de kwaliteit van de patiëntenzorg verbetert, en daarom altijd verricht zou moeten worden. Twee belangrijke kenmerken van CIS, zoals de aanwezigheid van CIS vóór de ontwikkeling van zaadbalkanker en het lage aantal fout-negatieve biopsiën geeft de mogelijkheid voor vroegtijdige opsporing van zaadbalkanker. Het verkrijgen van testes weefsel voor onderzoek is echter een invasieve procedure en daarmee ongeschikt voor grootschalige screeningsdoeleinden. In hoofdstuk 7 wordt een alternatieve methode beschreven om CIS te detecteren. CIS cellen liggen in de zaadbuizen van de testis en worden uitgescheiden met het sperma tijdens een zaadlozing. In een studie werd door ons aangetoond dat deze cellen bij patiënten met CIS en zaadbalkanker in het sperma met behulp van immunohistochemische markers opgespoord kan worden. Deze bevinding pleit voor een groter onderzoek om de specificiteit en sensitiviteit van deze diagnostische test te evalueren. Deze test zou mogelijk in de toekomst gebruikt kunnen worden om zaadbal- kanker vroegtijdig op te sporen in een geselecteerde groep mannen met een hoog risico, zoals onvruchtbare mannen of mannen met niet ingedaalde testes. Hoofdstuk 8 beschrijft een groot cohort van mannelijke patiënten met verschillende vormen van kanker. Deze mannen werden, voorafgaand aan hun behandeling, verwezen voor het invriezen van semen (cryopreservatie). Zij worden doorgaans verwezen omdat een behandeling met chemotherapie of bestraling de functie van de testes kunnen verminderen. In deze studie blijkt dat bij ongeveer 90% van alle mannen het sperma van voldoende kwaliteit is om in te vriezen. Bovendien blijkt dat patiënten met zaadbalkanker een verminderde testisfunctie hebben vergeleken met mannen die met andere vormen van kanker zijn gediagnosticeerd.

Dit ondersteunt de "testiculaire dysgenesie" hypothese waarbij een stoornis van testiculaire ontwikkeling leidt tot diverse problemen, waaronder onvruchtbaarheid en zaadbalkanker.

Hoofdstuk 9 beschrijft het nut van cryopreservatie van sperma bij jongens vanaf de puberteit, die gediagnosticeerd worden met een kwaadaardige ziekte. Gedurende de laatste decennia zijn de overlevingskansen voor deze jongens sterk toegenomen, vooral door een verbeterde werkzaamheid van de behandelingen. Hierdoor neemt het aantal jongens dat wordt verwezen voor cryopreservatie ook toe. Cryopreservatie kan om verschillende redenen voor deze jongens een stressvolle procedure zijn en het lukt niet altijd om sperma te produceren. In onze studie werden geen indicatoren gevonden die het succes van semen cryopreservatie kunnen voorspellen, zoals het stadium van de ontwikkeling van de geslachtsorganen of de hormoonspiegels. Dit betekend dat alle jonge jongens die gediagnosticeerd worden met kanker en waarvan aangenomen kan worden dat de spermaproductie begonnen is, verwezen moeten worden naar een semenbank om te beoordelen of cryopreservatie mogelijk is. Als de patiënt niet in staat is te masturberen of hier bezwaar tegen heeft, zou elektro-ejaculatie een optie kunnen zijn om zaadcellen te verkrijgen: in narcose wordt dan een zaadlozingsreflex opgewekt door stimulatie van de betreffende zenuw. Deze zaadcellen moeten bewaard worden totdat de patiënt volwassen is en zijn semen wil gebruiken voor voortplanting. Om het nut van cryopreservatie van semen vast te stellen is het gebruik hiervan geëvalueerd. Ongeveer 10% van de mannen die hun ziekte heeft overleeft maakt gebruik van het ingevroren semen voor een geassisteerde voortplantingstechniek. Een totaal van 101 cycli van geassisteerde voortplanting werd uitgevoerd en dit resulteerde in 25 kinderen. Deze cijfers illustreren het belang voor mannen van het invriezen van sperma voorafgaande aan een vruchtbaarheids beperkende behandeling van kanker.

In hoofdstuk 10 wordt getracht antwoord te geven op de vraag welke groepen patiënten een verhoogd risico lopen op schade aan de testes door een behandeling van kanker op kinderleeftijd. Hiervoor werden hypofysaire en gonadale hormonen bepaald, waaronder inhibine B wat een goede marker is voor de productie van zaadcellen. De kinderen die het chemotherapeuticum procarbazine of hoge doseringen cyclofosfamide hadden gekregen danwel behandeld waren met totale lichaamsbestraling hadden een hoog risico op ernstige testes schade. Het krijgen van chemotherapie op jonge leeftijd leeftijd, waarin ogenschijnlijk de testes in een latente periode lijken te verkeren, beschermt niet tegen de schadelijke effecten van chemotherapie. Met deze gegevens kunnen de ouders van kinderen geïnformeerd worden over hun risico op testis schade als gevolg van de behandeling: deze is afhankelijk van het gebruikte geneesmiddel en de totale dosis die nodig is om het kind te genezen van kanker. Hiermee kunnen patiënten geselecteerd worden die baat zouden kunnen hebben bij meer invasieve technieken voor het behoud van de vruchtbaarheid.

In hoofdstuk 11 wordt een onderzoek verricht naar de geslachtshormonen van patiënten die als kind behandeld werden voor Acute Lymfoblastische Leukemie (ALL). Hierin wordt onderzocht of een bestraling van de hersenen een extra negatief effect heeft op de vruchtbaarheid

bij de andere behandelingen voor ALL. Gekeken is naar een mogelijke verstoring van de hypofysefunctie. Daartoe werden de kinderen vergeleken die behandeld waren zonder of met hersenbestraling. Hieruit blijkt dat de bestraling (mediaan 24 Gy) inderdaad een effect had op de hypofysefunctie, inclusief de groeihormoon-as, maar geen additioneel effect had op de functie van de testis.

Dit proefschrift had als doel de relatie tussen de ontwikkeling van de testes, zaadbalkanker en verminderde vruchtbaarheid weer te geven. Hiervoor zijn diverse studies verricht waarin aangetoond werd dat het hebben van testiculaire microlithias, wat een teken is van een ontwikkelingsstoornis van de testis, een risico factor is voor het krijgen vaan zaadbal kanker. Tevens werd aangetoond dat de voorloper van zaadbalkanker op te sporen is via een niet invasieve methode wat wellicht in de toekomst de mogelijkheid geeft tot preventief onderzoek. Hierna werd toegelicht dat het opslaan van semen voorafgaand aan schadelijke chemotherapie belangrijk is bij zowel adolescenten als volwassen mannen. Dit omdat uit dit onderzoek bleek dat chemotherapie op kinderleeftijd zeer schadelijk kan zijn voor de nog ontwikkelende testes.

Chapter 15

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Chapter 16

Dankwoord

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Niels

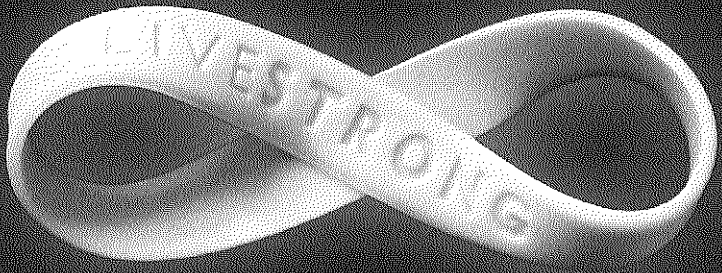
Hoofdstuk 17

Appendices

List of publications

Portfolio

Curriculum Vitae



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Testicular microlithiasis and Carcinoma in situ: Overview and proposed clinical guideline.
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Sperm DNA integrity in cancer patients before and after treatment.
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Final reproductive outcome of high-dose sex steroid treated tall boys
A.E.J. Hendriks, N.J. van Casteren, W.P.A. Boellaard, J.C. Romijn, F.H. de Jong, A.M. Boot, and S.L.S. Drop
Accepted in Journal of clinical endocrinology and metabolism

PORTFOLIO

Name PhD student: Niels Van Casteren		
	Year	Workload (Hours/ECTS)
Erasmus MC Department: Urology section Andrology PhD period: january 2006 - December 2008 Promotor(s):Prof.Dr. C. H.Bangma, Prof. Dr. L.H.J. Looijenga		
Supervisor: Dr. G.R. Dohle		
General academic skills - Biomedical English Writing and Communication	2008	3 ECTS
IIn-depth courses (e.g. Research school, Medical Training)		
National conferences Spreker externe refereeravond Urologie te Rotterdam Title: Maligne cellen in semen van mannen met een hoog risico op CIS	February 2006	2 ECTS
Spreker Interne refereer avond: Title: Prognostische waarde van semenanalyse voor de kans op zwangerschap: een literatuuroverzicht	September 2007	1 ECTS
Najaarsvergadering NVU Podium session: Immunohistochemie is noodzakelijk voor het accuraat diagnosticeren van CIS in de testis.	October 2007	1 ECTS
Tour de L'europe, Rotterdam Podium session: Early detection of germ cell cancer.	March 2009	1 ECTS
International conferences European Society for Andrological Urology (ESAU) te Amsterdam Podium session: Early detection of CIS in semen. Poster: Semencryopreservation in pubertal boys	October 2006	2 ECTS
6th workshop on testicular cancer and CIS in Kopenhagen Poster presentation: Early detection of CIS in semen.	November 2006	2 ECTS
AUA te Anaheim, USA Podium session Title: Evaluation of the use of cryopreserved semen and ART outcome of 629 male cancer patients. Poster presentation: Testicular function in 261 adult male survivors of childhood cancer.	May 2007	2 ECTS
EAU Milan Poster presentation: Immunohistochemistry on testicular biopsies for Carcinoma *in Situ* diagnosis: OCT3/4 as mandatory marker. (Winner best poster price of that session)	March 2009	2 ECTS
13th Congress of the European Hematology Association Poster presentation: Gonadal function in 248 male survivors of childhood cancer	June 2008	2 ECTS
3rd Münster Symposium on Late Effects after Tumour Therapy in Childhood and Adolescence Podium session : Effect of childhood cancer treatment on fertility markers in adult male long-term survivors. Podium session : CNS irradiation does not result in pituary-gonadal axis dysfunction in long-term male survivors of childhood ALL.	February 2009	2 ECTS

Annual SIOP congress Soa Paulo. International society of pediatric oncology. Winner best poster price: Cranial radiotherapy does not result in pituitary-gonadal dysfunction in long term male survivors of childhood acute lymphoblastic leukaemia.	October 2009	2 ECTS
Seminars and workshops		
Didactic skills		
Other - European Andrology academy. Two year training and exam clinical andrology.	November 2008	10 ECTS

2. Teaching activities

	Year	Workload (Hours/ECTS)
Lecturing Speaker European society human reproduction & embryology (ESHRE) course for semen analysis Title: Sperm motility Title: Sperm antibodies	2005-2007	6 ECTS
Supervising practicals and excursions Supervising medical students (Practica + VO) Supervising Interns.	2006-2008 2007	2 ECTS 1 ECTS

CURRICULUM VITAE

Niels Jacobus van Casteren was born on May 19th 1980 in a small town named Overasselt in the east of the Netherlands. After graduating from the Kandisnky college in Nijmegen in 1998, he departed to the "big" city of Rotterdam to start his medical education at the Erasmus University. After his graduation in May 2005 he worked as a M.D. at the department of Urology at the Erasmus MC. It was there where he came in contact with Gert Dohle and Rob Weber, both physicians with a strong interest in Andrology. Niels started his PhD. research in January 2006 at the department of Urology, section Andrology. During this period, he was trained in clinical Andrology according to the European Andrology Association (EAA) guidelines. In November 2008, he performed his examination in clinical Andrology in Rome at the congress of the European Academy of Andrology.

The research presented in this thesis has been done in close collaboration with Leendert Looijenga and the LEPO group at the department of experimental Pathology at the Josephine Nefkens Institute and with Marry van den Heuvel-Eibrink at the department of Pediatric Oncology/Hematology at the Erasmus MC-Sophia children's hospital.

In May 2008 he applied for the Urologic traineeship and in January 2009 he started his residency in general Surgery at the Maasstad hospital in Rotterdam under supervision of Dr. E. van der Harst. After two years he will perform his urologic residency at Erasmus MC and the last two years will be spent at Sint Franciscus Gasthuis in Rotterdam.

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