SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Fertility of patients with testicular cancer: genetic, epigenetic and functional examinations on sperm

by Zsuzsanna Molnár, MD

Supervisor: Attila Jakab, MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

Debrecen, 2014

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By Zsuzsanna Molnár MD

Supervisor: Attila Jakab MD, PhD

Doctoral School of Clinical Medicine, University of Debrecen

Head of the **Examination Committee**:
Prof. Zoltán Hernádi MD, PhD, DSc
Members of the Examination Committee:
Zsuzsanna Bereczky MD, PhD
Péter Riesz MD, PhD

The Examination takes place at the library of Department of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen
10:00 a.m. 7th November, 2014

Head of the **Defense Committee**:	Prof. Zoltán Hernádi MD, DSc
Reviewers:	Zsolt Kopa MD, PhD
	András Penyige MD, PhD
Members of the Defense Committee:	Zsuzsanna Bereczky MD, PhD
	Péter Riesz MD, PhD

The PhD Defense takes place at the Lecture Hall of Building "A", Department of Internal Medicine, Faculty of Medicine, University of Debrecen
12:00 a.m. 7th November, 2014

1. Introduction

Recent advances in diagnosing and treating young patients with cancer have greatly improved their long time survival. Testicular cancer is the most common solid tumour in reproductive aged males. An increased incidence of testicular cancer has been observed over the last decades. With increasing numbers of cancer survivors, quality of life issues are receiving increased attention. Cancer treatment whether surgical, radiological, or pharmacologic, can have severe and adverse long-term iatrogenic effects on male fertility. Fertility preservation has become an important part in the counselling with cancer patients. Cryopreservation of spermatozoa is a viable fertility preservation option for men before gonadotoxic chemo- and/or radiotherapy.

Before commencing anticancer treatment, cryopreservation may be suggested to preserve fertility but there are no data regarding the risk of genetic aberrations in these sperms. Larger studies examine the effect of anticancer treatment. Fertility in TC patients has not been extensively evaluated. Although the classical semen analysis is the gold standard for male fertility assessment as it provides quantitative information on the semen status of men, it is also important to investigate the qualitative characteristics suggestive of sperm damage in men with testicular cancer. It is also not clear whether the cancer itself or the triggering factors of cancer per se induce changes in genomic integrity of the spermatozoon.

In the present study we describe the clinical significance, aetiology, grouping, treatment modalities and the prognosis of testicular cancer. Furthermore, we discuss the linkage between infertility and testicular cancer, which may have a common aetiology, and consists of the direct effect of tumour tissue and anticancer treatment on spermatogenesis.

In the present thesis we discuss the fertility preservation's method and practical issues. With performing descriptive and experimental methods we evaluate the

spermatogenesis of men with testicular cancer. We also made comparison between the cancer patients', healthy men's and infertile, oligozoospermic men's results. This evaluation may help us to understand the underlying processes in the pathogenesis of low sperm concentration in men with testicular cancer.

2. Literature review

2.1. Testicular cancer

Testicular cancer (TC) is a relatively rare tumour type accounting for approximately 1% of all male cancers globally. However, testicular cancer has a very distinctive age distribution and in many developed countries it is the most commonly diagnosed malignancy among men aged between 15–40 years. In recent decades, the incidence of testicular cancer has been increasing, with a doubling observed since the 1960s in many Western countries. Northern Europe has high age-standardized incidence rates of 7.8 and 6.7 per 100,000 men, respectively. Despite these observed trends in incidence and geographical variations, few hypotheses exist to explain them.

According to the data of Hungarian National Cancer Register 645 new testicular cancer cases were discovered in 2012 in Hungary. The 59% (378/645) of the patients were less than 40 years old, and there was a peak in the incidence between 30 and 40 years.

Although the cause of TC is unknown, the following risk factors are under consideration: The high occurrence rate in developed countries reflects the role of environmental carcinogenic factors. In patients with cryptorchidism, the risk of developing TC is 8-10%. Men with infertility (low sperm concentration, poor motility of spermatozoa and a high proportion of morphological abnormal spermatozoa) are nearly 3 times more likely to develop subsequent TC. ITGCN has been found in 0.4–1.1% of men undergoing testicular biopsy because of infertility.

A previous history of TC is the strongest risk factor for TC. Approximately 1-2% of TC patients will develop a second primary TC contralaterally. ITGCN can be found in the contralateral testicle of TC patients in approximately 5 per cent of cases, which corresponds with the expected frequency of metachronous TC. ITCGN cells are widely accepted as a precursor of TC (both seminoma and non-seminoma). First-degree relatives have a higher risk of developing TC than the general population, although the incidence is low. About 2% of TC patients report having an affected relative31 Brothers are at particularly high risk, with a relative risk of 8–10. Among sons of affected men, 2-6-fold increases in TC have been reported.

Testicular cancer has one of the highest cure rates of all cancers with an average five year survival rate of 95%. If the cancer has not spread outside the testicle, the 5-year survival is 99% while if it has grown into nearby structures or has spread to nearby lymph nodes, the rate is 96% and if it has spread to organs or lymph nodes away from the testicles, the 5-year survival is around 74%.

2.2. Fertility after anticancer treatment

Rates of permanent infertility and compromised fertility after cancer treatment vary and depend on many factors. The effects of chemotherapy and radiation therapy depend on the drug or size/location of the radiation field, dose, dose-intensity, method of administration (oral versus intravenous), disease, age, sex, and pretreatment fertility of the patient. Infertility can result from the disease itself, anatomic problems (eg, retrograde ejaculation or anejaculation), primary or secondary hormonal insufficiency, or more frequently, from damage or depletion of the germinal stem cells.

2.3. Cryopreservation

Cryopreservation is the storage of biological material at -196°C at which biochemical processes of cell metabolism are slowed or interrupted. Storage of sperm is available and it have to be offered before potentially sterilising chemotherapy or radiotherapy or before surgery that might interfere with fertility.

The cryopreservation techniques currently used are not yet optimal because damage occurs to cells during cryopreservation and prolonged storage. Most damage occurs during freezing and thawing. Sperm morphology, motility and vitality decrease significantly after thawing. Furthermore, it has been reported that cryopreservation does not affect then DNA integrity, the frequency of chromosomal abnormalities or alter the sex ratio or cause epigenetic changes in human sperm. The success rate of ART procedures using banked semen from cancer patients is 33–56%.

2.4. Spermatogenesis and sperm biomarkers

Spermatogenesis is the process in which spermatozoa are produced from male primordial germ cells by way of mitosis and meiosis. The initial cells in this pathway are spermatogonias, which yield primary spermatocytes by mitosis. The primary spermatocyte divides meiotically into two secondary spermatocytes; each secondary spermatocyte then completes meiosis as it divides into two spermatids. These develop into mature sper, this is called spermigenesis.

In the field of Andrology the ultimate goal was the development of objective sperm biomarlers that would predict sperm fertilizing potential, independently from sperm concentration and motility in the semen. The creatine kinase activity reflects arrested sperm development at the level of cytoplasmatic retention. The pregnancy outcome was found to be correlated with sperm creatine kinase levels; wheras sperm concentration and motility were non-contributory. The next biomarker was the sperm chromatin assessment using aniline blue staining. Aniline blue staines persistent histones in sperm nucleus. Increased level of persistent histones indicates a break in histone protamine change process, which significantly affects DNA integrity. There is more than 80% similarity in the sperm biomarkers examination results, indicating that once sperm undergoes a developmental arrest, this even structurally and functionally affects multiple cytoplasmic and nuclear attributes within the same sperm.

2.5. Objectives

The aims of this study were the followings

1. Evaluation of the spermiogram of patients with testicular cancer before anticancer treatment, and to comapare the results with other malignancies occuing in the reproductive age.

2. To elucidate the question of fertility after anticancer treatment for testicular cancer we performed a survey among patients who underwent sperm cryopreservation procedure in our department. A structured questionnaire was designed to collect data on demography, anticancer treatment, and histological type of cancer, family planning intentions and fertility prior to and after treatment.

3. Genetic analysis of semen from normozoospermic, inertile, oligozoospermic men and patients with testicular cancer before any adjuvant therapy. The frequency of numerical choromosome abnormalities was estimated performing multicolour FISH examination.

4. Epigenetic examination of semen from normozoospermic, inertile, oligozoospermic men and patients with testicular cancer before any adjuvant therapy. We detected residual histones with the help of aniline-blues stainig method.

5. Functional testing of semen from normozoospermic, inertile, oligozoospermic men and patients with testicular cancer before any adjuvant therapy. Hialuronian binding assay was used to detect the fertilisation potential.

6. Correlation analysis was performed between the examined parameters in cancer and non-cancer patient group.

7. Assasing the result originating from the studied groups, analysing the differences in spermatogenesis and fertilisation potential to make clinical consequencies.

3. Patients and methods

3.1. Evaluation of semen quality in men with testicular cancer before therapy

We studied 119 patients with testicular cancer (TC group, n=68), Hodgkin's disease (HD group, n=37) and non-Hodgkin-disease (NHD, n=14) who cryobanked sperm before chemo- or radiotherapy in the University of Debrecen, Hungary, Medical and Health Science Centre, Department of Obstetrics and Gynecology, Andrology and Cryopreservation Laboratory in a 10 year period. All TC patients were evaluated within 1 month after orchidectomy. Sperm parameters (ejaculate volume, sperm concentration, total sperm count, forward motility) were evaluated according to WHO.

3.2. Fertility of men with testicular cancer after therapy

We retrospectively assessed the database in the University of Debrecen, Hungary, Medical and Health Science Centre, Department of Obstetrics and Gynecology, Andrology and Cryopreservation Laboratory searching for all male testicular cancer (TC) patients who were referred to the institute to cryopreserve semen before starting chemo- or radiotherapy during a period of 11 years, a total number of 86 male patients. Written informed consent was obtained from all patients after that they were asked to complete our questionnaire. We collected data on age, marital status, family history, type of the surgical procedure, hystological type of the cancer, the type of anticancer treatment and the couples' obstetric history, diseases of the children conceived before and after the diagnosis of TC and whether cryopreserved semen was used. Data were analysed and matched to the patients' sperm parameters and to the TC histological types. During the 11-year follow-up 86 patients were referred to our institute for semen cryopreservation after surgery for testicular cancer and before starting chemo- or radiotherapy. The questionnaire was completed and returned by 59 (68.6%) of the 86 patients.

3.3. Sperm concentration, hyaluronic acid binding capacity, aneuploidy and persistent histones in testicular cancer

3.3.1 Patients

Patients with TC

We examined semen samples of 28 postorchidectomy patients with TC, who were referred for semen cryopreservation before the initiation of any adjuvant radio- or chemotherapy, to the Andrology Laboratory of the Department of Obstetrics and Gynecology, Medical and Health Science Center, University of Debrecen.

Infertile, oligozoospermic (IO) men

Semen samples of 20 age-matched infertile men referred for semen analysis to the Andrology Laboratory were also studied.

Nomrozoospermic (NZ) men

Twenty healthy age-matched sperm donors with normal sperm characteristics were also studied.

3.3.2 Methods

Semen analysis

Semen specimen was collected after a requested abstinence of 2–4 days. Semen analysis was performed manually according to the World Health Organization guidelines.

Fluorescence in situ hybridization (FISH)

The sperm FISH was performed using three FISH probes: alpha-satellite sequence-specific centromeric probes for chromosome 17 (D17Z1, SpectrumAqua), X (DXZ1, SpectrumOrange) and Y (DYZ1, SpectrumGreen) (Abbott/Vysis, Des Plaines, IL, USA). The nuclei were counterstained with 4′ –6′ diamidino-2-phenylindole (DAPI)

(Abbott/Vysis). The sperm FISH analysis was carried out by scoring a minimum of 5000 sperm heads. The overall hybridization efficiency was >98%. Nuclei that overlapped or displayed no signal due to hybridization failure were omitted from the scoring. A spermatozoon was considered disomic when it showed two fluorescence signals of the same color, comparable in size and brightness in the same focal plane, positioned inside the sperm head and at least one signal apart. Scoring was performed using a Zeiss Axioplan2 (Carl Zeiss, Jena, Germany) fluorescence microscope, and the images were captured and analyzed by ISIS software (MetasystemsMetasystems, Althussheim, Germany). We chose chromosome 17 as its aneuploidy frequency matches the average aneuploidy of all autosomal chromosomes (i.e. 0.12%). The sperm disomy frequencies and estimated numerical chromosome aberrations were calculated as described previously by Egozcue et al.

Aniline blue (AB) staining of the sperm head.

The slides were stained by 5% AB solution (Sigma Co., St. Louis, MO, USA) acidified to pH 3.5 with acetic acid for 5 min, and then washed with tap water and allowed to completely air dryat room temperature.

Mature spermatozoa, having completed histone-protamine replacement, stained very lightly with AB (light = mature spermatozoa). The slightly immature spermatozoa were stained more extensively (intermediate = diminished maturity), and immature spermatozoa with substantial degrees of persistent histones were darkly stained (dark = immature spermatozoa).

For each stained smear, 200 spermatozoa were evaluated under a light microscope in oil immersion magnification (100× objectives). Spermatozoa with unstained nuclei were considered normal, while those AB stained (intermediate + dark) considered abnormal.

The results were expressed as percentages of stained and unstained sperm. An ejaculate with a rate of blue-stained sperm ,20% was considered normal.

Sperm hyaluronic acid (HA) binding assay

The semen sample was maintained at room temperature (18–288C) for 30–60 min to allow it to liquefy. The HA-binding test (MidAtlantic Diagnostics, Martlon, NJ, USA) was carried out at room temperature: the sample was mixed and 10 ml was pipetted onto the center of the chamber. The CELL-VU gridded cover slip was placed over the chamber, taking care to avoid air bubble formation. As per the protocol, the chamber was incubated at room temperature for at least 10 min, but not more than 20 minutes; this period proved to be necessary for sperm to bind to HA. The number of bound, motile sperm and total, motile sperm was scored. The ratio of HA-binding motile sperm was calculated as follows: %Bound = 100 × Bound Motile/Total Motile.

3.4. Statistical analysis

Kolmogorov–Smirnov test was used for the evaluation of the normality of the data. Most parameters were non-normally distributed therefore analyses were performed by Mann–Whitney U-test. Spearman's rho was calculated for correlation analysis. A value of P , 0.05 was considered statistically significant. All analyses were performed with the SPSS Statistics software, version 22.0 (IBM Corps., Armonk, NY, USA).

3.5. Ethical approval

Prior to the study, all patients and controls were given detailed information about the aims and procedures of the present investigation and written informed consent was obtained. The author's respective institutional ethics review board approved the study protocol (reference number: DEOEC RKEB/IKEB: 2736/2008 and 2976/2012-EHR).

4. Results

4.1. Evaluation of semen quality in men with testicular cancer before therapy - results

During the examined 10 year period 119 patientes had semen cryopreservation with oncological indication. 57% of cancer patients (n=68) had testicular cancer (TC), their main age was 22,7 years (range:16-42) at the time of the diagnosis. The indication was Hodgkin disease (HD) in 31% of the cases (n=37) , main age was: 24,6 years (range: 17-34). Non-Hodgkis disease (NHD) was the cause of the cryopreservation in 12%-a (n=14), their main age was 28,6 years (range: 17-39). Azoospermia before chemotherapy or irradtion was detected in 13,2% in the TC group, in 10,8% in the HD group and 7,2% in the NHD group. Oligozoopsermia was present in 55,9% (n=38) in the TC, 59,5% (n=22) in the HD and 71,4% (n=10) in the NHD group. The main sperm concentration (without the azoopsermic patients) was 24,9 $x10^6$/ml in the TC, 30,9 $x10^6$/ml in the HD and 37,4 $x10^6$/ml in the NHD group. There was a significant diefference between the TC and NHD group regarding sperm concentration.

4.2. Fertility of men with testicular cancer after therapy -results

The mean age of responders to our survey was 27 years (range: 16–41 years) at the time of cryopreservation and 32 years (range: 21–47 years) at the time of the study. 71% of the patients were under 30 years at the time of the study. At the time of the study 31 couples had reproductive plans. 13 natural and 7 assisted conceptions occurred. Thirteen testicular cancer patients reported natural conception with a mean of 4.4 years after treatment. The total number of pregnancies was 18, out of which 4 ended in miscarriage prior to the 12th week of gestation (22.2%). One of these was a twin pregnancy. There were two unwanted pregnancies which were terminated for social reasons. The successful pregnancies resulted in the birth of 8 girls and 4 boys. Those patients who reported spontaneous pregnancies after treatment for testicular

cancer had a sperm concentration below 20 x 10^6/mL in 7 cases, 15 x 10^6/mL in 3 cases and less than 1 x 10^6/mL in one case at the time of semen cryopreservation. Two birth cases occurred within 1 year of the completion of anticancer treatment. No congenital malformation was reported.

Altogether 7 patients utilized cryopreserved semen sample for in vitro fertilisation (IVF) (11.9%). The mean time was 4.1 years. The use of the specimens resulted in six pregnancies, from which 4 resulted in live births (2 girls and 2 boys) and 2 miscarriages during the first trimester. No malformation was reported in these pregnancies after IVF.

4.3. Sperm concentration, hyaluronic acid binding capacity, aneuploidy and persistent histones in testicular cancer - results

Investigation of chromosome numerical aberrations by FISH

In the 68 individuals, we evaluated a total of 342 000 spermatozoa, i.e. on average 5030 sperms per patient. The X/Y ratios were close to 1:1 in all groups. Higher sex and 17 chromosome disomy frequencies in the IO group when compared with the NZ and TC groups resulted in significant differences in estimated numerical chromosome aberrations. A significant difference was observed in the frequency of estimated numerical chromosome aberrations between IO and NZ ($P<0.001$), IO and TC ($P< 0.001$), IO and TCO ($P<0.008$) and IO and TCN ($P<0.001$) groups; no significant differences were found between the NZ and TC, TCOand TCN, NZ and TCN, and NZ and TCO groups. Upon comparing the NZ group with the TCO group, we found no significant difference in sex chromosome disomy, chromosome 17 disomy and estimated chromosome aberration frequencies.

AB staining results

We evaluated a total of 15 564 spermatozoa from 68 individuals, i.e. 229 per patient on average. The mean proportion of AB-stained sperms was 16.9% in the NZ group, 45.7% in the IO group, 23.0% in the TC group, 19.9% in the TCN group and 30.7% in

the TCO group. The difference in AB staining was found to be significant between IO and NZ (P <0.001), IO and TC (P<0.001), IO and TCO (P< 0.001), TCO and TCN (P =0.028), NZ and TCO (P=0.005) and IO and TCN (P=0.011) groups. There was no difference between NZ and TC, and NZ and TCN groups.

Sperm HA-binding assay results

The mean sperm HA-binding capacity of the motile spermatozoa was 81.1% in the NZ group, 41.9% in the IO group, 56.9% in the TC group, 60.9% in the TCN and 46.8% in the TCO group. The HA-binding capacity of the NZ men proved to be significantly higher than the IO (P<0.001) and the TC group (P<0.001). The IO group had significantly lower HA-binding capacity than the TC group (P=0.019). The difference was significant between IO and TCO (P =0.007), NZ and TCN (P<0.001) and NZ and TCO (P=0.001) groups, but not between TCO and TCN, and IO and TCN groups.

Histological findings

The TC group was further divided into two subgroups according to the histological subtype of the testicular tumor, seminoma (n=16) and non seminoma (n=12) groups. We did not find any significant differences between the two groups regarding age (30.4 versus 27.1 years; P=0.16), sperm concentration (31.4 versus 22.7 $\times$ 10^6/ml; P = 0.226), X/Y ratio (1.05 versus 1.1; P=0.982), sex chromosome disomy(0.37 versus 0.4; P = 0.214), chromosome 17 disomy (0.07 versus 0.1; P=0.599), total diploidy (0.14 versus 0.2; P=0.19), estimated chromosome aberration frequencies (3.86 versus 3.93; P=0.239), HA-binding capacity (57 versus 57%; P=0.45) and the AB-stained proportion of cells (21 versus 26%; P=0.133).

Correlation analyses between sperm concentration, HA-binding capacity, estimated numerical chromosome aberrations and AB staining

For correlation analysis, the IO and NZ men together constituted a control group. Correlation analysiswas performed for the different parameters in the above-defined control group and the TC group. In the control group, a statistically significant (P< 0.001) correlation was found between sperm concentration and HA-binding capacity

(Spearman's rho = 0.842), estimated chromosomal aberrations (Spearman's rho =-0,642) and AB-stained proportion of cells (Spearman's rho=-0,678); estimated numerical chromosome aberrations and HA-binding capacity (Spearman's rho =-0,876) and AB-stained cells (Spearman's rho =0,559); HA-binding capacity and AB-stained proportion of cells (Spearman's rho=-0,811). In the TC group, we found a significant correlation only between the sperm concentration and estimated chromosomal aberrations (Spearman's rho =-0,642). No other significant correlations were observed between the other parameters examined.

5. Discussion

To the best of our knowledge, this is the largest study to date which has investigated the pretreatment semen parameters of patients with TC, using not only the conventional test (sperm concentration), but also evaluating the sperm integrity at multiple levels, i.e. membrane remodelling (HA-binding assay), nuclear condensation (AB staining) and numerical chromosome aberrations (FISH). Furthermore, to evaluate the cancer effect on semen quality, we compared the semen characteristics of patients with TC to IO men and NZ men. In addition, we examined the correlation of numerical chromosomal aberrations, and functional and structural characteristics of semen in the non-cancer (NZ and IO men) and TC group. We also analyzed whether the histological subtype of TC influenced spermiogenesis. The present study did not reveal any significant difference regarding X/Y ratio, disomy of sex chromosomes and chromosome 17, diploidy and estimated numerical chromosome aberrations between the NZ andTCgroups, although sperm concentration, HBcapacity andABstaining results were significantly lower in TC group. Further, aneuploidy rate and AB staining results for oligozoospermic TC patients did not differ from those of NZ men. Significant difference between the TCO and TCN groups was found only in sperm concentration and AB staining. In every examined parameter, there was a significantly

lower value in the IO group when compared with patients with TC. Even the TCO group had significantly better results than the IO group. In the control group of men without cancer (NZ + IO), significant correlation was found between sperm concentration and aneuploidy rate, AB staining and HA binding; the HA-binding capacity was related to the aneuploidy rate and the AB staining. There was a significant, but weaker correlation between aneuploidy and AB staining, and this may well be explained by a previous report that the degree of condensation affects the efficacy of hybridization at FISH, as reported in a double probing study**.** Ourcorrelation findings support a previous study, and perhaps confirm that in men without cancer sperm concentration, aneuploidy frequency, nuclear condensation and fertilization potential detected by HA-binding capacity are related characteristics of the semen. In contrast, in the TC group, with an exception of a significant correlation between sperm concentration and estimated numerical chromosome aberration, no correlations were found between the other studied parameters. This finding may suggest that sperm concentration per se cannot predict the quality of the sperm of the patients with TC. This assumption definitely deserves further investigation. Recently, it has been hypothesized that acommon defect is implicated in the pathogenesis ofTCand decreased spermatogenesis (e.g. testicular dysgenesis syndrome). A possible explanation for the differences in the sperm concentration found between the TC and the NZ groups may be the intratesticular (tumor generated local inflammation, elevation of inflammatory markers and other factors, local tissue trauma due to the orchidectomy) and environmental (stress of treatment) factors to which the TC patients are exposed. As a result, the TC patients' quantitative aberrations do not necessarily go hand-in-hand with qualitative abnormalities, e.g. sperm aneuploidy and integrity. Furthermore, it has been reported that in testicular regions closer to the cancer site, spermatogenesis is affected more severely when compared with more distant parts of the testis. In our study cohort, although significantly lower than the NZ group, the mean sperm concentration (27.7×10^6/ml) in the TC group was above theWorld

Health Organization, (2010) reference value. The French National study published data on 1158 men with TC. Here the diagnosis of azoospermia and the percentage of severe oligozoospermia were independent of tumor histological type and disease stage. However, sperm concentration and total sperm number were significantly lower at Stage III independent of histological type. This suggests that the tumor size may influence spermatogenesis (Rives et al., 2012). The only established method to preserve fertility in male cancer patients before gonadotoxic anticancer treatment is semen cryopreservation, which may be used in assisted reproduction techniques (ART). Our results suggest that semen samples dedicated for cryopreservation from TC patients, before the initiation of chemotherapy or irradiation, do not carry an elevated risk for numerical chromosome aberrations, even if the sample is oligozoospermic. Furthermore, we observed that the IO and NZ samples of patients with TC did not differ qualitatively. This may explain the 33–56% success rate of ART procedures using banked semen from cancer patients. Additionally, it has been reported that cryopreservation does not affect the frequency of chromosomal abnormalities or alter the sex ratio in human sperm. One previous study compared sperm aneuploidy, using multi-color FISH, in five patients with TC before treatment with healthy NZ donors but did not enumerate the estimated numerical chromosomal aberrations. Our study, on the other hand, not only recruited a larger number of patients with TC before treatment (n= 28), but also compared them with a NZ and an IO group. Although it is unclear whether malignancy per se can adversely affect the genomic integrity of sperm, our findings support a previous study where no significant difference in sperm DNA fragmentation was found upon comparing 98 men diagnosed with cancer and healthy sperm donors. In our study, the HA-binding capacity was decreased in the TC group compared with the NZ group. Furthermore, in the patient group the HA-binding capacity did not correlate with sperm concentration. As such, it may be speculated that sperm function and fertilization potential cannot be estimated by sperm concentration in cancer patients. Additionally, the low sperm concentration

and HA-binding capacity in TC patients may not necessarily suggest a higher aneuploidy frequency and a decreased nuclear condensation. We found that AB-stained cell proportion was not significantly different between the NZ and TC groups, but was significantly higher in the IO group, when compared with the other two groups. It is known that a higher and lower proportion of AB-stained sperms are associated with recurrent pregnancy loss and fertility, respectively. A recent study examined human sperm vacuoles and found that their presence is associated with failure of chromatin condensation as detected by AB staining. It has been demonstrated that mature sperms that undergo plasma membrane remodeling and are able to bind to solid state HA present no persistent histones or increased frequency of chromosomal aneuploidies.

Limitations of our study include lack of data on confounders influencing sperm characteristics such as smoking, occupational or environmental hazards. Co-morbidities and other andrological conditions were not studied.

This is the first study to demonstrate that sperm qualitative characteristics in cancer therapy-naı¨ve oligozoospermic TC patients differ significantly from those in IO men and do not differ from those in NZ men. Our results definitively need to be validated in further studies of similar groups of patients and in patients with other types of tumor where cryopreservation is advisable.

5.1. New statements

1. Sperm concenration is lower and azoopsermic patinets rate is higher in the testicular cancer compared to Hodgkin disease and Non Hodgkin disease group. This is correspond with literature data.
2. At the time of the study 31 couples had reproductive plans. 13 natural and 7 assisted conceptions occurred. Thirteen testicular cancer patients reported natural conception with a mean of 4.4 years after treatment. The total number of pregnancies was 18, out of which 4 ended in miscarriage prior to the 12th week of

gestation (22.2%). Those patients who reported spontaneous pregnancies after treatment for testicular cancer had a sperm concentration below 20 x 10^6/mL in 7 cases, 15 x 10^6/mL in 3 cases and less than 1 x 10^6/mL in one case at the time of semen cryopreservation. Altogether 7 patients utilized cryopreserved semen sample for in vitro fertilisation (IVF) (11.9%). The mean time was 4.1 years. The use of the specimens resulted in six pregnancies, from which 4 resulted in live births and 2 miscarriages during the first trimester.

3. A significant difference was observed in the frequency of estimated numerical chromosome aberrations between infertile and testicular cancer group, the infertile and oéigozoospermic tasticular cancer patients; no significant differences were found between the normozoospermic and testicular cancer groups. Upon comparing the normozoospermic group with the oligozoospermic testicular cancer group, we found no significant difference in sex chromosome disomy, chromosome 17 disomy and estimated chromosome aberration frequencies.

4. The difference in aniline-blue staining was found to be significant between infertile and normozoospermic, infertile and testicular cancer, infertile and oligozoospermic testicular cancer group. There was no difference between normozoospermic and testicular cancer men.

5. The HA-binding capacity of the normozoospermic men proved to be significantly higher than the infertile and the testicular cancer patientes. The infertile group had significantly lower HA-binding capacity than the testicular cancer group. The difference was significant between infertile and oligozoospermic testicular cancer, but not between infertile and normozoospermic testicular cancer patients.

6. Our correlation findings supports earlier studies and perhaps confirm that in men without cancer sperm concentration aneuploidy frequency, nuclear condensation and fertilization potential detected by HA-binding capacity are related characteristics of the semen In contrast, in the testicular cancer group,

with an exception of a significant correlation between sperm concentration and estimated numerical chromosome aberration, no correlations were found between the other studied parameters. This finding may suggest that sperm concentration per se cannot predict the quality of the sperm of the patients with testicular cancer.

7. Oligozoospermia in patients with testicular cancer differ from oligozzospermia in infertile men in qualitative parameters. Testicular cancer patients semen did not carry elevated numrical chromosome aberrations or chromatin condensation failure. The fertilisation potential examined with hialuronian acid binding capacity is was worse in the testicular acncer group compared to normozoospemic samples, but it was statisticly significantly better than in the infertile group.

Presentations:

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Register number: DEENKÉTK/177/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Zsuzsanna Molnár
Neptun ID: F68SOC
Doctoral School: Doctoral School of Clinical Medicine
MTMT ID: 10044155

List of publications related to the dissertation

1. **Molnár, Z.**, Berta, E., Benyó, M., Póka, R., Kassai, Z., Flaskó, T., Jakab, A., Bodor, M.: Fertility of testicular cancer patients after anticancer treatment: Experience of 11 years.
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Total IF of journals (all publications): 14.949
Total IF of journals (publications related to the dissertation): 5.632

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

22 July, 2014