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

Running title: Sperm characteristics of patients with testicular cancer

Z Molnar MD[1,2*], A Mokanszki MSc, PhD[2], Z Kassai Bazsane MSc, PhD[1], H P Bhattoa MD, PhD[2], M Benyo MD, PhD[3], E Olah MD, PhD, DSc[4], A Jakab MD, PhD[1]

[1]Department of Obstetrics and Gynecology, Medical and Health Science Center, University of Debrecen, Debrecen, 4032, Hungary

[2]Department of Laboratory Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, 4032, Hungary

[3]Department of Urology, Medical and Health Science Center, University of Debrecen, Debrecen, 4032, Hungary

[4]Clinical Genetic Center, Department of Pediatrics, Medical and Health Science Center, University of Debrecen, Debrecen, 4032, Hungary

*Correspondence address: Department of Laboratory Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, 4032, Hungary.

Tel: +36-52-340-006, Fax: +36-52-417-631, E-mail: molnarzs@med.unideb.hu

Abstract

Study question: Are the quantitative and qualitative characteristics of semen samples of patients with testicular cancer (TC), prior to anticancer therapy, different from infertile oligozoospermic and normozoospermic age-matched men?

Summary answer: Sperm concentration in TC patients was significantly decreased with no difference in estimated numerical chromosome aberrations and nuclear decondensation compared to normozoospermic men, while the infertile, oligozoospermic men had significantly poorer sperm qualitative characteristics versus the TC group overall and oligozoospermic patients with testicular cancer.

What is known already: Spermatogenesis is altered in TC patients at the time of the diagnosis. However, the mechanism responsible for the decreased semen quantity in patients with testicular cancer is not well understood. Anticancer treatment may have gonadotoxic side effects and post treatment fertility cannot be predicted. 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.

Study design, size, duration: This is a cross-sectional study examining semen from 28 patients with testicular cancer, 20 oligozoospermic and 20 normozoospermic age-matched men attending the Andrology Center and the Sperm Cryopreservation Laboratory of the Medical and Health Science Center, University of Debrecen. Semen samples from patients with testicular cancer were collected after orchidectomy, but prior to anticancer treatment. Semen samples from TC patients recruited over a period of 4 years were studied. Based on their sperm concentration, TC patients were subgrouped into an oligozoospermic TC (TCO) and a normozoospermic TC (TCN) group.

For statistical analysis, the normal group (NZ+IO) comprised non-tumorous normozoospermic (NZ) and infertile oligozoospermic (IO) men.

Participants/materials/setting, method:

The ejaculates were assessed as per World Health Organization guidelines. Hyaluronic acid (HA) binding capacity was the functional test. To determine the numerical chromosome aberrations we used multicolour fluorescence in-situ hybridization. Aniline blue (AB) staining was performed as a nuclear decondensation marker test.

Main results and the role of chance:

The results did not reveal any significant difference in disomy of sex chromosomes and chromosome 17, diploidy and estimated numerical chromosome aberrations and AB staining results upon comparing the normozoospermic and testicular cancer groups, although the sperm concentration ($p<0.001$) and HA binding capacity ($p<0.001$) were lower in the TC group. Estimated numerical chromosome aberrations ($p<0.001$), AB staining ($p<0.001$) and HA-binding capacity ($p=0.019$) was lower in the infertile, oligozoospermic group as compared to the patients with testicular cancer. The TCO group had significantly better results in every examined parameter than the infertile, oligozoospermic group.

In the non-tumorous control group (NZ+IO), a significant ($p<0.001$) correlation (Spearman's rho=r) was found between sperm concentration and aneuploidy rate ($r=-0.642$), AB staining ($r=-0.876$) and HA binding ($r=0.842$); the HA-binding capacity was related to the aneuploidy rate ($r=-0.678$) and the AB staining ($r=-0.811$,); and there was significant correlation between aneuploidy and AB staining ($r=0.559$). In the TC group, apart from the negative correlation between sperm concentration and estimated chromosomal aberrations ($r=-0.642$), no other correlations were observed.

Limitations, reasons for caution: Data on confounders influencing sperm characteristics, such as smoking, occupational or environmental hazards, alcoholism, co-morbidities and other andrological conditions, were not collected.

Wider implications of the findings: This is the first study to demonstrate that sperm qualitative characteristics in anticancer therapy naïve oligozoospermic TC patients differ significantly from those in infertile oligozoospermic men and do not differ from those in normozoospermic men. Our results need to be validated in similar groups of men and in other patient groups with cancer where cryopreservation is advisable.

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Key words: testicular cancer, infertility, hyaluronic acid binding assay, aniline-blue staining, human sperm fluorescence in-situ hybridization

Introduction

Testicular germ cell carcinoma (TC) represents about 1% of malignant tumors in men, but is the most frequent solid tumor in men aged 15-35 years. The majority of patients with testicular cancer are diagnosed prior to conception of the first offspring (de Kretser, 2000). The use of chemo- and radiotherapy combined with surgery has resulted in increased survival rates, i.e. over 95% 5-year survival rate (Kliesch *et al.*, 1997).

Chemotherapeutic agents and radiotherapy are both known to be gonadotoxic (Howell and Shalet, 2001; Lass *et al.*, 2001) and may lead to defective sperm production resulting in oligozoospermia, severe oligozoospermia and even azoospermia, and as a consequence infertility (Hallak *et al.*, 1999). Since the majority of men treated for testicular carcinoma are in their reproductive years, the long-term therapy associated side effects are of increasing importance. Although, the recovery of spermatogenesis depends on the treatment modality, dose and individual susceptibility, the extent of recovery after treatment is difficult to predict (Gandini *et al.*, 2003). Therefore, sperm cryopreservation is recommended for patients with testicular cancer preceding treatment.

An increasing number of reports suggest that spermatogenesis may already be defective at the time of diagnosis of TC (Martin *et al.*, 1997; Hallak *et al*., 1999; Gandini *et al*., 2003; Agarwal and Allamaneni, 2005; van Casteren e*t al.*, 2007; Dohle, 2010; Rives *et al*., 2012). It remains unclear whether the etiology of oligozoospermia in most TC patients arises from testicular alteration due to the presence of malignancy or surgical removal, or as a result of the altered neuroendocrine environment (Spermon *et al.*, 2006). Although sperm cryopreservation is widely advocated, the seminal genetic alterations, functional characteristics and fertilization potential of TC patients has not been intensively investigated (Alvarez *et al.*, 1999; Spermon *et al.*, 2006).

The developing spermatids undergo complex changes during spermiogenesis in both the nuclear and cytoplasmic compartments; this includes meiosis, histone to protamine replacement, acrosome development, sprouting of the sperm tail and cytoplasmic extrusion (Kovanci *et al.*, 2001). In addition, there is a remodelling of the sperm plasma membrane during late spermiogenesis, a step that facilitates formation of the zona pellucida and hyaluronic acid (HA) binding sites (Huszar *et al.*, 2003; Jakab *et al*., 2005; Huszar *et al*., 2007). It has been previously proven by assays of cytoplasmic and nuclear biochemical markers that mature sperm selectively bind to solid state HA (Jakab *et al.*, 2005). We used this functional feature of sperm to examine fertilization potential (HA binding assay).

With maturation of the sperm there is increase in protamine at the expense of histones. Aniline blue (AB) staining is an efficient dye to detect histones, and its negative staining certifies sperm maturity. Previous studies have reported an association between diminished histone to protamine exchange, as detected by AB staining, of excess persistent histones in the nuclear compartment of sperm with arrested cellular maturity (Foresta *et al.*, 1992; Agarwal and Said, 2003; Sati *et al.*, 2008). Additionally, it has been demonstrated that chromatin condensation is impaired not only in malformed but also in morphologically normal spermatozoa (Hofmann and Hilscher, 1991). We used AB staining to detect sperm nuclear condensation.

Knowing that aberrant sperm have an increased frequency of chromosomal aneuploidies (McAuliffe *et al*., 2012), we investigated sperm aneuploidy frequencies of chromosome 17, X and Y using multi-colour fluorescence in-situ hybridization (FISH).

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 (Cooper *et al.* 2010; van der Steeg *et al.* 2011;

Mallidis *et al.* 2012). 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.

The aim of the present study is to evaluate the quality of semen in patients with testicular cancer before treatment for cancer. We used conventional and specific tests to determine the quantitative and qualitative changes in the ejaculated spermatozoa at diagnosis of testicular cancer and compared these findings to age-matched groups of infertile oligozoospermic and normozoospermic men.

Materials and Methods

Patients

Patients with testicular cancer 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 men (IO)

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

Normozoospermic men (NZ)

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

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).

Semen analysis

Semen specimen was collected after a requested abstinence of two to four days. Semen analysis was performed manually according to the World Health Organization guidelines (WHO, 2010) (Table I.).

FISH analysis

Smears of semen samples (10 μL) were fixed with methanol: acetic acid (3:1) for 10 min, air dried, dehydrated in a series of 70, 85 and 98% ethanol for 2 min each, and stored at – 20 °C until FISH was performed.

For decondensation, the sperm slides were warmed to room temperature, and in order to render the sperm chromatin accessible to DNA probes, they were treated first with 10 mmol/L dithiothreitol (Sigma, St. Louis, MO, USA) in 0.1 mol/L Tris-HCl, pH 8.0, for 30 min, then with 10 mmol/L lithium diidosalicylate (Sigma, St. Louis, MO, USA) in Tris-HCl for 1-3 h. After decondensation, the sperm slides were dehydrated in a series of 70, 85 and 98 % ethanol. 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 sample and the probe denaturation were carried out at 76°C for 3 min. The hybridization was carried out at 37°C in a moist chamber for 16-18 h (Hybrite, Abbott/Vysis, Des Plaines, IL, USA). Post hybridization washes were performed with 50% formamide/ 2X saline sodium citrate (SSC) at 42 °C for 15 min. The slides were then washed with 2X SSC at room temperature for 10 min and 2X SSC/ 0.1 % NP-40 for 5 min. After washing, the nuclei were counterstained with 4'-6' diamidino-2 - phenylindole (DAPI) (Abbott/Vysis, Des Plaines, IL, USA).

The sperm FISH analysis was carried out by scoring a minimum of 5 000 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

187 performed using a Zeiss Axioplan2 (Carl Zeiss, Jena, Germany) fluorescence microscope, and the
188 images were captured and analyzed by ISIS software (Metasystems, Althussheim, Germany).
189 We chose chromosome 17 as its aneuploidy frequency matches the average aneuploidy of all
190 autosomal chromosomes (i.e., 0.12%) (Downie *et al*., 1997; Egozcue *et al.*, 1997, Jakab *et al.*,
191 2003, Jakab *et al.*, 2005, Mokanszki *et al*, 2012).
192 The sperm disomy frequencies and estimated numerical chromosome aberrations were calculated
193 as described previously (Egozcue *et al*., 1997).
194 Sperm HA-binding assay
195 The semen sample was maintained at room temperature (18-28˚C) for 30 to 60 minutes to allow
196 it to liquefy. The HA-binding test (MidAtlantic Diagnostics, Martlon, NJ, USA) was carried out
197 at room temperature: the sample was mixed and 10 μL was pipetted onto the center of the
198 chamber. The CELL-VU gridded cover slip was placed over the chamber, taking care to avoid air
199 bubble formation. As per the protocol, the chamber was incubated at room temperature for at
200 least 10 minutes, but not more than 20 minutes; this period proved to be necessary for sperm to
201 bind to HA. The number of bound, motile sperm and total, motile sperm was scored. The ratio of
202 HA binding motile sperm was calculated as follows: %Bound = 100 X Bound Motile / Total
203 Motile.
204 AB staining of the sperm head
205 The slides were stained by 5% AB solution (Sigma Co., St. Louis, MO, USA) acidified to
206 approximately pH 3.5 with acetic acid for 5 minutes, and then washed with tap water and allowed
207 to completely air dry at room temperature (Huszar *et al*., 2003). Mature spermatozoa, having
208 completed histone-protamine replacement, stained very lightly with AB (light=mature
209 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 x 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 (Sellami *et al*., 2013).

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. The 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).

Results

Patients with testicular cancer

Mean age of the TC patients was 28.9 years (range: 18-47 years), and mean sperm concentration was 27.7 $x10^6$/mL (range: 6-65 $x10^6$/mL) (Table I., Table II.). Patients were grouped as per sperm concentration as being oligozoospermic (TCO) (sperm concentration <15 $x10^6$/mL) or normozoospermic (TCN) (sperm concentration ≥15 $x10^6$/mL). The histological subtype of tumor was also used to further subgroup the patients (i.e., seminoma (n=16) and non-seminoma (n=12)).

Investigation of chromosome numerical aberrations by FISH

The results of the FISH analysis are presented in Table II. In the 68 individuals, we evaluated a total of 342 000 spermatozoa, i.e., on average 5 030 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 as compared to the normozoospermic 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 normozoospermic and TC, TCO and TCN, NZ and TCN, and NZ and TCO groups (Table II.)

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.

Sperm HA binding assay results

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

In the normozoospermic group, only 2 (10%) semen samples had HA-binding capacity below the clinical cut-off of <60%, i.e., 55% and 58%, with a sperm concentration of $26x10^6$/mL and $30x10^6$/mL, respectively. In the oligozoospermic group, 16 samples (80%) had low HA-binding capacity, and 10 (35.7%) of the TC samples had HA-binding capacity under 60%.

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 normozoospermic group, 45.7% in the oligozoospermic group, 23.0% in the TC group, 19.9% in the TCN group and 30.7% in the TCO group (Table II).

The difference in AB staining was found to be significant between the 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 TCN ($p=0.005$), and IO and TCN ($p=0.011$) groups. There was no difference between NZ and TC, and NZ and TCO groups (Table II).

Among the 20 normozoospermic men, 2 (10%) had more than 20% AB-stained cells i.e., 48.6% and 24.6% with sperm concentration of $18x10^6$/mL and 26 $x10^6$/mL, respectively. All the oligozoospermic patients had higher than normal AB-stained sperm proportion (28-68%). In the testicular cancer group, the percentage of AB stained sperm was 5-53%. Normal results were found in 17 (60.7%) of the TC patients.

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 $22.7x10^6$/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 diplody (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 analysis was 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=-0.876); estimated numerical chromosome aberrations and HA-binding

290 capacity (Spearman's rho=-0.678) and AB stained cells (Spearman's rho=0.559); HA-binding
291 capacity and AB stained proportion of cells (Spearman's rho=-0.811) (Figure I.).

292 In the TC group, we found a significant correlation only between the sperm concentration and
293 estimated chromosomal aberrations (Spearman's rho=-0.642). No other significant correlations
294 were observed between the other parameters examined.

295

Discussion

To the best of our knowledge, this is the largest study to date which has investigated the pretreatment semen parameters of patients with testicular cancer, using not only conventional test (sperm concentration), but also evaluating the sperm integrity at multiple levels, i.e., membrane remodeling (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 testicular cancer to infertile oligozoospermic men and normozoospermic men. In addition, we examined the correlation of numerical chromosomal aberrations, and functional and structural characteristics of semen in the non-cancer (normozoospermic and oligozoospermic men) and testicular cancer group. We also analyzed whether the histological subtype of testicular cancer 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 normozoospermic and testicular cancer groups, although sperm concentration, HB capacity and AB staining results were significantly lower in TC group. Further, aneuploidy rate and AB staining results for oligozoospermic TC patients did not differ from those of normozoospermic men. Significant difference between the TCO and TCN group was found only in sperm concentration and AB staining. In every examined parameter there was a significantly lower value in the infertile, oligozoospermic group as compared to patients with testicular cancer. Even the TCO group had significantly better results than the infertile, oligozoospermic 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 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 the hybridization at FISH, as reported in a double probing study (Ovari *et al*., 2010). Our correlation 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 (Mokanszki *et al*, 2012; Pylyp *et al*. 2013). 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. This assumption definitely deserves further investigation.

Recently, it has been hypothesized that a common defect is implicated in the pathogenesis of testicular cancer and decreased spermatogenesis (e.g. testicular dysgenesis syndrome) (Jacobsen et al., 2000). 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 don't 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 as compared to more distant parts of the testis (Berthelsen JG and Skakkebaek, 1983). Carcinoma in situ (CIS) in seminiferous tubules at the periphery of germ cell tumors may be an underlying cause of deteriorating semen quality. Contralateral CIS in testicular germ cell tumors was found in 5% of

patients, and CIS was significantly associated with poor spematogenesis and testicular atrophy (Daugaard G *et al*., 1992; Dieckmann *et al*., 2007).

It has been reported that patients with malignancy have poor sperm quality prior to anticancer treatment (Meirow et Schenker, 1995; Hallak *et al*., 1999; Gandini *et al*., 2003; Trottmann *et al*., 2007, Molnar *et al*., 2014). In a Dutch study (van Casteren *et al*., 2010), 764 male cancer patients' semen samples were evaluated: 64% of the samples were abnormal, and men with testicular germ cell cancer (n=292) had the lowest sperm concentrations. In our study cohort, although significantly lower than the NZ group, the mean sperm concentration ($27.7x10^6$/mL) in the TC group was above the WHO (2010) reference value. The French National study published data on 1158 men with testicular cancer. 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 suggests 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 oligozoospermic and normozoospermic samples of patients with testicular cancer did not differ qualitatively. This may explain the 33-56% success rate of ART procedures using banked semen from cancer patients (van Casteren *et al.,* 2008; Dohle, 2010). Additionally, it has been reported that cryopreservation does not affect the frequency of chromosomal abnormalities or alter the sex ratio in human sperm (Martin *el al*., 1991).

One previous study (Tempest *et al*., 2007) compared sperm aneuploidy, using multicolour FISH, in 5 patients with testicular cancer before treatment with healthy normozoospermic 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 testicular cancer before treatment (n=28), but also compared them with a normozoospermic and an oligozoospermic 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 (McDowell *et al.,* 2013). Different tests are available to evaluate sperm DNA fragmentation, i.e., the acridine orange staining test, the sperm chromatin structural assay (SCSA), terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), and the comet assay. Ståhl et al (2006) investigated sperm DNA integrity in patients with testicular cancer using SCSA and TUNEL and their results suggest, in agreement to our observations, that the use of cryopreserved semen carries no increased risk of transmitting damaged DNA to offspring. Yagci et al (2010) found good agreement between the HA bound fraction of sperm and high DNA integrity as detected by acridine orange staining method. This study demonstrated that testing with the sperm HA-binding assay does reflect the proportion of sperm with high DNA integrity (Yagci *et al.,* 2010).

In our study, the HA binding capacity was decreased in the TC group compared to the normozoospermic 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 decreased nuclear condensation.

We found that AB stained cell proportion was not significantly different between the normozoospermic and TC groups, but was significantly higher in the IO group, as compared to the other two groups. It is known that a higher and lower proportion of AB stained sperms is associated with recurrent pregnancy loss (Ruixue *et al*., 2013) and fertility, respectively (Auger *et al*., 1990, Agarwal and Said, 2003). A recent study examined human sperm vacuoles and found that their presence is associated with failure of chromatin condensation as detected by AB staining (Boitrelle *et al.,* 2013). It has been demonstrated (Huszar *et al*., 2003; Jakab *et al*., 2005) 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.

Molecular components of sperm, as biomarkers of testicular damage, have the potential to be important indicators of reproductive toxicity. Measurement of messenger ribonucleic acid (mRNA) alterations may serve as one option in this regard (Dere et al. 2013). Furthermore, sperm proteomics-based studies have explored the potential diagnostic biomarkers of asthenozoospermia (Siva et al., 2010). Additionally, studies into the molecular basis of epigenetic regulation may help in understanding spermiogenesis further (Dere et al., 2013,). Another study presents maternally imprinted gene methylation as a new biomarker in andrology, where correlation with clinical parameters was also found (Kläver et al., 2013).

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-naïve oligozoospermic TC patients differ significantly from those in infertile oligozoospermic men and do not differ from those in normozoospermic men. Our results definitively need to be validated in

414 further studies of similar groups of patients and in patients with other types of tumor where
415 cryopreservation is advisable.
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419 **Authors' roles**

420 All authors (ZM, AM, ZKB, HPB, MB, EO, AJ) participated in the following:

421 1) substantial contributions to conception and design, or acquisition of data, or analysis and

422 interpretation of data

423 2) drafting the article or revising it critically for important intellectual content

424 3) final approval of the version to be published.

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428 Excellence Program'.

429 **Conflict of interest**

430 No conflict of interest are declared.

431 Legend of figure

432 Figure 1. Correlation analysis was performed between the sperm concentration, hyaluronic acid

433 (HA) binding capacity, estimated chromosome aberrations and aniline blue (AB) staining in the

434 control group (normozoospermic men (n=20) + infertile, oligozoospermic men (n=20)).

435 Spearmen's rho was calculated, $p<0.005$ was considered as statistically significant result.

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