

Sperm DNA Integrity in Men Treated for Childhood Cancer

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Abstract

Purpose: It is not known whether childhood cancer and its treatment are associated with sperm DNA damage, which subsequently affects fertility and might be transmitted to the offspring. The aim of this study is to assess DNA fragmentation index (DFI) as an indicator of sperm DNA integrity in childhood cancer survivors (CCS), with treatment regimen taken into account.

Experimental Design: In 99 CCS and 193 age-matched healthy controls, DFI was assessed by using sperm chromatin structure assay.

Results: In the whole group of CCS, DFI was increased compared with the controls, with borderline statistical significance [mean difference, 1.8%; 95% confidence interval (95% CI), -0.0088%-3.7%]. Those treated with radiotherapy only (mean difference, 6.0%; 95% CI, 1.6–10%) or surgery only (mean difference, 2.9%; 95% CI, 0.083–5.8%) had statistically significantly higher DFI than the controls. The odds ratio (OR) for having DFI >20%, which is associated with reduced fertility, was significantly increased in CCS compared with the control group (OR, 2.2; 95% CI, 1.1–4.4). For the radiotherapy-only group, the OR was even higher (OR, 4.9; 95% CI, 1.3–18). DFI was not associated with dose of scattered testicular irradiation or type of chemotherapy given.

Conclusions: DFI was increased in CCS, with those treated with chemotherapy being the only exception. This sperm DNA impairment may be associated with the disease per se rather than due to the treatment, and may have negative consequences in terms of fertility and risk of transmission to the offspring.

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The survival rate of patients treated for childhood cancers has improved over the last decades due to the use of potent chemotherapy, improved surgical and radiation techniques, as well as diagnostic improvements. Close to 80% of patients survive childhood cancer (1, 2). Future treatment regimens must aim for survival of the highest possible number of patients, combined with the least impairment of their quality of life, including well-preserved reproductive function (3).

Irradiation and chemotherapy can have a persistent deleterious effect on reproductive function in both humans (4, 5) and animal models (6). The negative effects include impaired spermatogenesis, resulting in azoospermia or oligozoospermia (7–9). Increased number of

both autosomal and sex chromosome aberrations in sperm samples after irradiation and chemotherapy have been reported in humans; however, data suggest that these changes are transient (10–14). Less is known about more subtle sperm DNA damage such as strand breaks. To our knowledge, the largest study focusing on sperm DNA integrity in CCS was based on 23 subjects only (9). In this study, using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling, no increase in the proportion of sperms with DNA damage was seen compared with healthy controls. However, due to the limited number of subjects included, no definitive conclusions could be drawn.

Sperm DNA impairment may have implications on the fertility of (15), and/or may be transmitted to, the offspring. The probability of achieving pregnancy *in vivo* is decreased when the DNA fragmentation index (DFI), as assessed by using sperm chromatin structure assay (SCSA), is >20% and is close to zero if DFI is >30% (16–18). Use of assisted reproduction, such as intracytoplasmic sperm injection, which seems to be the most efficient fertilization method if DFI is high (16), can bypass the natural selection mechanisms and potentially increase the risk of transmitting cancer or therapy-related sperm DNA damage to the offspring. The mutagenic effects of both radiotherapy and chemotherapy are well known from animal studies

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Translational Relevance

Childhood cancer per se and/or its treatment can influence reproductive ability by being associated with impairment of sperm production as well as sperm DNA damage. The finding of increased proportion of sperm with DNA strand breaks in childhood cancer survivors not treated with chemotherapy or radiotherapy points to genomic instability as being possibly associated with malignancy in early life. The risk of DNA damage seems to increase in those treated by irradiation but not chemotherapy. This is a matter of concern because sperm DNA breaks may have a negative impact on fertility and through use of powerful assisted reproduction tools can be transmitted to the offspring, possibly resulting in malformations or diseases later in life.

(19–22). Previous human studies indicated no increased risk of adverse pregnancy outcomes for the partners of male childhood cancer survivors (CCS; refs. 23–25). However, in two recent studies on children of men treated for cancer, an increase in malformation rate was seen (26, 27).

Apart from the negative impact of the treatment on sperm DNA, the disease per se might be associated with genomic instability (28–32) and, therefore, even with sperm DNA defects—a hypothesis supported by reports connecting congenital anomalies to childhood malignancies.

In view of the above-mentioned findings on a possible link between childhood cancer and its treatment and sperm DNA defects, the aim of our study was to elucidate whether the disease per se and/or its different modalities

had an impact on sperm DFI in a group of men treated for childhood cancer.

Subjects and Methods

Subjects

This study was based on a cohort of consecutive CCS in the region of southern Sweden reported to the Swedish Tumor Registry in the period 1970 to 2002. The inclusion criteria were as follows: (a) male gender, (b) diagnosed before 18 years of age, (c) still alive in September 2004, (d) no active oncological treatment for the last 4 years, and (e) between 18 and 45 years of age at the start of the study in 2004.

Written invitation was sent to 407 CCS. Among them, 140 declined to take part in the study; 106 did not reply, despite being sent a reminder; and 161 gave their consent. Ten men were excluded, one as he previously had undergone vasectomy and nine because their disease was considered nonmalignant (e.g., carcinoid in the appendix or spinal hemangioma). Thus, a total of 151 men (38% of eligible subjects) were included.

Median age (range) at diagnosis was 10 years (0.1–17 years) and 30 years (20–46 years) at the time of examination. Background characteristics for the participants were collected from their medical records. The distribution of diagnoses for the 151 participants is presented in Fig. 1, along with corresponding numbers for the whole of Sweden (2003-NORDCAN; <http://www.dep.iarc.fr/nordcan.htm>).

To determine whether the cohort of participants was representative for the whole group of CCS, data on fertility of the participants and nonparticipants were extracted from the Swedish Multi-Generation Register. This register has information on the number of children for each index person. For those alive after 1990, the register is virtually

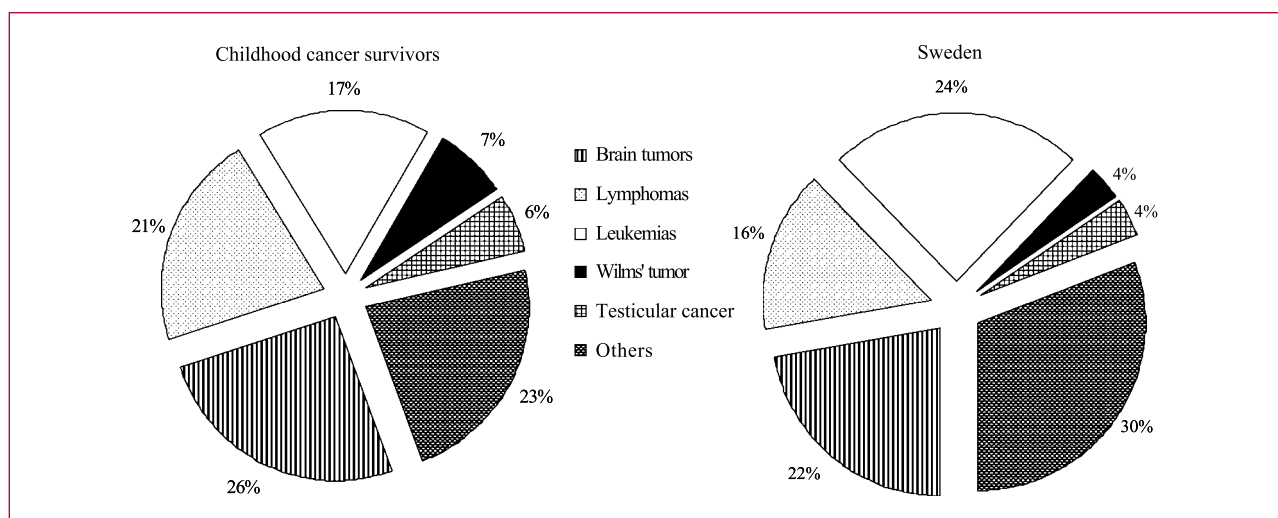


Fig. 1. Distribution of diagnoses for CCS included in the current study ($n = 151$) and the incidence frequency for males less than 19 years of age in Sweden in 2003.

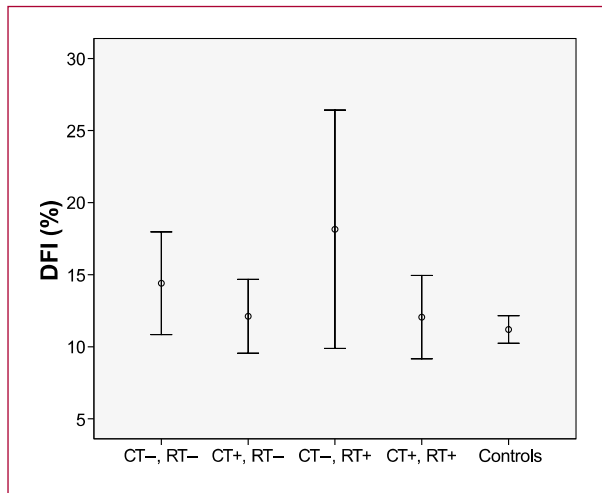


Fig. 2. Sperm DFI in CCS depending on the treatment given. The circles show the mean and the bars show 95% CI of the mean. CT, chemotherapy; RT, radiotherapy.

complete with respect to parents as well as offspring. Non-biological relations are flagged in the register. The distribution of men having 0, 1, 2, or 2+ children was 71%, 12%, 15%, and 3.1% among the participants and 73%, 9.5%, 13%, and 5.1% among the nonparticipants, respectively.

Through a questionnaire, we collected information regarding smoking habits and alcohol consumption.

The study was approved by the Ethics Committee of Lund University, and all subjects provided written informed consent.

Cancer therapy groups

The subjects were allocated to four groups according to the type of treatment received: (a) surgery only, (b) radiotherapy only, (c) chemotherapy only, and (d) radiotherapy and chemotherapy combined (Fig. 2).

Controls

The control group comprised 193 Norwegian men from the general population, recruited for a study of seasonal variation in semen quality, which was not found among these men living north and south of the Arctic Circle (33). These men were not selected due to proven fertility or infertility, therefore representing semen quality in men in general. This group was similar in age and ethnicity compared with the study population. Median age (range) for the controls was 28 years (19–40 years).

Sample collection

The men were asked to deliver a semen sample by masturbation after an abstinence period of 2 to 3 days. The sample was analyzed within 1 hour after ejaculation, and semen quality was assessed as recommended by WHO (34).

Sixteen CCS were unable to provide an ejaculate. Of the remaining samples, 31 samples showed azoospermia, 3 samples contained too few sperms, and 2 samples had

insufficient amount of semen for analysis. An aliquot of 200 μ L was frozen and kept at -80°C for subsequent analysis of sperm DNA integrity by SCSA for the 99 samples from the CCS and all the controls.

Sperm chromatin structure assay

The details of this method have previously been described (35). Briefly, the SCSA is based on the phenomenon that DNA with strand breaks has a tendency to be denatured when exposed to an acid detergent, whereas normal DNA remains stable. The SCSA measures the *in situ* ability of sperm DNA to be denatured using a metachromatic dye, acridine orange, which differentially stains double- and single-stranded nucleic acids. Upon blue-light excitation, acridine orange intercalated into the intact (double-stranded) DNA emits green fluorescence, whereas acridine orange bound to denatured (single stranded) DNA emits red fluorescence. The extent of DNA-denaturing ability is expressed as the DFI, which is the ratio of red to total (red plus green) fluorescence intensity. DFI hereby expresses the proportion of cells containing denatured DNA (17).

Five thousand cells were analyzed by FACSsort (Becton Dickinson).

DFI was calculated using the List View software (Phoenix Flow Systems). An intralaboratory coefficient of variation of 4.5% was found after repeated measurements of the same reference sample.

Gonadal radiation dose

The dose outside the radiotherapy target area, called peripheral dose, was calculated. The peripheral dose was used to estimate the scattered dose to the testes. The program Peridose, which distinguishes between orthogonal and tangential beams and accounts for the use of wedges and shielding blocks, was used (36). The separate contribution of leakage radiation and collimator scatter to the total peripheral dose was also calculated (37).

The model was verified using diode measurements of the dose outside the primary beam and compared with a simple model in a solid water phantom. The correspondence between the calculation and the measurement was within $\pm 10\%$.

The treatment techniques for the CCS in our study have mainly been rectangular, perpendicularly incident beams with none or small shielding blocks. An exception is the large, irregular mantle field.

Data used for the calculations for each patient were collected from their medical charts, regarding photon energy, beam sizes, beam arrangement, dose to d_{max} , and target dose. For the estimation of the peripheral dose to the gonads, the distance between the lower level of the beam edge and the gonads was estimated for each treatment beam. This information was collected from the localization and verification films of each patient.

The scattered dose to the testes was only calculated for those patients who received irradiation only ($n = 12$)

Table 1. Background characteristics for the nonazoospermic CCS and controls

	CCS (n = 99)	Controls (n = 193)
Age at diagnosis (y)		
Mean (SD)	9.4 (5.7)	NA
Median (range)	9.0 (0.13–17)	NA
Age at examination (y)		
Mean (SD)	30 (6.9)	29 (5.2)
Median (range)	30 (20–46)	28 (19–40)
Distribution		
Smokers (%)	11	20
Weekly alcohol consumption, g/wk (%)		
0–99	61	61
100–199	33	18
≥200	6.1	21

Abbreviation: NA, not applicable.

because it was not possible to discriminate between the effects of radiotherapy and chemotherapy on sperm DFI in cases where both was given.

Statistical analysis

The group-descriptive values were expressed as medians (range) and means (SD).

In relation to their smoking habits, the participants were allocated to three groups: (a) nonsmokers, (b) 1 to 10 cigarettes per day, and (c) ≥11 cigarettes per day. For alcohol consumption, three groups were defined: (a) 0 to 99 g of alcohol per week, (b) 100 to 199 g of alcohol per week, and (c) ≥200 g of alcohol per week (Table 1).

Primarily, we compared all the CCS with the controls. A univariate linear model was used for comparison of sperm concentration, as well as for DFI, between CCS and controls, using age, smoking, and alcohol consumption as confounders.

The results are given as mean difference, with 95% confidence interval (CI) of the difference. $P < 0.05$ was considered statistically significant.

Subsequently, DFI in each of the four groups defined according to the type of treatment was compared with that in the controls. Because the group that had received radiotherapy only was relatively small and contained outliers regarding DFI values, both a parametric test (univariate linear model with age, smoking, and alcohol consumption as confounders) and a nonparametric test (Mann-Whitney U test) were used for statistical analysis.

Furthermore, using binary logistic regression, the controls were compared with the whole CCS group, as well as with the four therapy groups regarding the odds ratio (OR) for having a DFI >20%, a level recently shown to

be associated with impairment of fertility both in assisted reproduction (16) and in natural conception (38).

Subsequently, we divided the CCS who had received chemotherapy only into three groups according to the known gonadotoxic effect of the drugs (39): (a) treatment with any kind of alkylating agent/cisplatin, regardless of additional therapy; (b) no treatment with alkylating agents, but with *Vinca* alkaloids, regardless of additional therapy; and (c) cytotoxic drugs other than alkylating agents or *Vinca* alkaloids. The OR for having DFI >20% was also calculated for these subgroups.

Finally, Spearman's ρ was used to correlate DFI to the calculated gonadal radiation dose for the CCS who received irradiation only.

Statistical analysis was done using SPSS 17.0 software (SPSS).

Results

CCS versus controls

The samples from the CCS showed significantly lower sperm concentrations than the controls (mean difference, $14 \times 10^6/\text{mL}$; 95% CI, 0.61×10^6 – $27 \times 10^6/\text{mL}$, $P = 0.04$; Table 2). The DFI for the CCS was higher than in the control group; this difference was close to being statistically significant (mean difference, 1.8%; 95% CI, –0.0088% to 3.7%, $P = 0.051$; Table 3). The risk of having DFI >20% was significantly increased in CCS compared with the control group (OR, 2.2; 95% CI, 1.1–4.4, $P = 0.032$).

Impact of different treatment modalities

Surgery only. The group of CCS that was treated with surgery only had no significant difference in sperm concentration compared with the control group. However, this group had significantly higher DFI than the controls (mean difference, 2.9%; 95% CI, 0.083–5.8%, $P = 0.044$; Table 3). The OR for having DFI >20% was 2.7 (95% CI, 1.1–6.4, $P = 0.03$).

Radiotherapy only. CCS who had received radiotherapy only did not differ significantly from the controls in terms of sperm concentration. The DFI for these men was significantly higher than for the controls (mean difference, 6.0%; 95% CI, 1.6–10%, $P = 0.008$; Table 3). The difference was also significant when analyzed by the nonparametric test, which is not sensitive to inclusion of outliers. The OR for having DFI >20% was 4.9 (95% CI, 1.3–18, $P = 0.017$). There was no statistically significant difference in DFI between the radiotherapy-only and the surgery-only groups. Furthermore, there was no statistically significant correlation between the calculated testicular radiation dose and DFI ($\rho = 0.55$; $P = 0.064$). The patient characteristics for this group regarding the diagnoses, radiation doses and fields, as well as sperm parameters are given in Table 4.

Chemotherapy only. Compared with controls, CCS who had received chemotherapy only had significantly lower sperm concentrations (mean difference, $31 \times 10^6/\text{mL}$; 95% CI, 12×10^6 – $50 \times 10^6/\text{mL}$, $P = 0.002$; Table 2). No significant difference in DFI was seen between this group

Table 2. Sperm concentration for controls and for CCS in relation to treatment modalities

	<i>n</i>	Median sperm concentration × 10 ⁶ /mL (range)	Mean sperm concentration × 10 ⁶ /mL (SD)	Mean difference* × 10 ⁶ /mL	95% CI* × 10 ⁶ /mL	<i>P</i> *
Controls	193	59 (2.0–270)	67 (48)	Reference group		
CCS (all)	99	34 (0.5–296)	58 (65)	–14	–27 to –0.61	0.04
CT–, RT–	30	36 (4.0–296)	76 (81)	–0.057	–21 to 21	1.0
CT–, RT+	12	64 (7.0–283)	91 (86)	9.8	–21 to 40	0.53
CT+, RT–	26	27 (3.0–183)	35 (36)	–31	–50 to –12	0.002
CT+, RT+	31	34 (0.5–223)	46 (50)	–23	–41 to –4.4	0.015

Abbreviations: CT, chemotherapy; RT, radiotherapy.

*Adjusted for age, smoking, and alcohol consumption.

and the controls (mean difference, 0.97%; 95% CI, –1.8% to 3.7%; *P* = 0.48).

None of the three groups, defined according to the gonadotoxicity of the chemotherapy given, showed significantly increased risk for having DFI >20%, compared with the controls, regardless if they had received chemotherapy only or if additional radiotherapy had been given.

Radiotherapy and chemotherapy combined. Sperm concentration was significantly decreased (mean difference, 23×10^6 /mL; 95% CI, 4.4×10^6 – 41×10^6 /mL, *P* = 0.015; Table 2), whereas there was no significant difference in DFI between the group that had received chemotherapy in combination with radiotherapy and the control group (mean difference, 0.79%; 95% CI, –1.8% to 3.4%; *P* = 0.55). Furthermore, the OR for having DFI >20% was not significantly increased in this group.

Discussion

In this study on sperm DNA integrity in 99 CCS, we found increased DFI in all patient categories, except in those treated with cytotoxic drugs. Looking at the two groups with impairment of sperm DNA integrity, we found that the increase in DFI was somewhat more pronounced in CCS who received irradiation only compared with those who were treated with surgery only; however, this difference did not reach the level of statistical

significance. The same was true for the association between the scattered irradiation dose and the DFI, which was only borderline significant, possibly due to the limited number of subjects included. These results show that childhood cancer per se is associated with increased level of sperm DNA damage. Furthermore, our results might indicate that the level of DFI might be additionally increased by irradiation and reduced by cytotoxic treatment.

In the only previous study on sperm DNA integrity in CCS, no increased sperm DNA damage was observed (9). The discrepancy between the two studies might be explained by differences in the methods to detect sperm DNA damage. Another reason could be the significantly higher power of the present investigation, which comprises four times as many subjects as the first study.

The increased DFI in the group that received neither radiotherapy nor chemotherapy might imply that childhood cancer patients have some kind of genomic instability. Defects in the DNA repair mechanisms have been shown to be associated with the risk of childhood cancer (40), and these mechanisms could also be responsible for the increased proportion of sperm DNA breaks in CCS. Results from some epidemiologic studies also seem to support this hypothesis. For example, in a recent register study, an increased risk of congenital malformations was found in the offspring of cancer survivors, even in those treated with surgery only (27). Another study pointing in this

Table 3. DFI for controls and for CCS in relation to treatment modalities

	<i>n</i>	Median DFI, % (range)	Mean DFI,* % (SD)	Mean difference*, %	95% CI,* %	<i>P</i> *
Controls	193	9.2 (2.0–49)	11 (7.5)	Reference group		
CCS (all)	99	10 (3.0–50)	13 (7.5)	1.8	–0.0088 to 3.7	0.051
CT–, RT–	30	13 (3.0–45)	14 (7.4)	2.9	0.083 to 5.8	0.044
CT–, RT+	12	15 (5.0–50)	17 (7.5)	6.0	1.6 to 10	0.008
CT+, RT–	26	10 (3.0–27)	12 (6.6)	0.97	–1.8 to 3.7	0.48
CT+, RT+	31	10 (3.0–34)	12 (6.9)	0.79	–1.8 to 3.4	0.55

*Adjusted for age, smoking, and alcohol consumption.

Table 4. Details of diagnosis, radiation treatment, and sperm parameters for the 12 CCS treated with radiotherapy but not chemotherapy

Diagnosis	Age at diagnosis (y)	Age at examination (y)	PTV-1	Radiation dose to PTV-1 (Gy)	PTV-2	Radiation dose to PTV-2 (Gy)	Sperm concentration ($\times 10^6/\text{mL}$)	Sperm DFI (%)	Dose to testicles (Gy)
Teratoma	15	38	Pineal gland	48	Craniospinal	32	37	50	0.1
Ependymoma	9.2	35	Spine (L5-S1)	55	Craniospinal	30	19	28	1.2
Neuroblastoma	0.13	31	Right side of neck	20	—	—	7.2	27	0
Pinealocytoma	14	38	Pineal gland	50	Craniospinal	35	63	26	0.2
Glioma	2.7	34	Brain stem	40	—	—	86	17	0.02
(relapse)	16			30	—	—			0.015
Germinoma	15	33	Pineal gland	55	Craniospinal	35	9.9	16	0.2
Germinoma	18	30	Pineal gland	45	Craniospinal	30	190	15	0.2
Hodgkin's lymphoma	6.8	40	Left side of neck	40	Upper mantle	40	65	10	0.1
Astrocytoma	2.1	36	Brain stem	35	—	—	63	8.1	0.03
Astrocytoma	13	46	Left frontal lobe	45	—	—	200	8.0	0
Malignant melanoma	17	21	Left eye	100 (locally)	—	—	78	7.0	0
Astrocytoma	13	31	Right temporal lobe	58	Cerebrum	40	280	5.0	0

Abbreviations: PTV, planning target volume (44); PTV-1, (boost volume) contains the demonstrated tumor and its local estimated subclinical extension; PTV-2, PTV-1 and estimated subclinical extensions at a distance.

direction is the report on increased risk of cancer in fathers to children born with cleft lip/palate. The study did not include parental cancer diagnosed until after their index children were born and was, therefore, not confounded by treatment for cancer rather suggesting a common genetic association (41).

In contrast to the finding of increased DFI in CCS treated with irradiation or surgery only, patients treated with chemotherapy only or with radiotherapy and chemotherapy did not differ from controls in terms of sperm DNA integrity. The observation of sperm DFI-lowering effect of chemotherapy is in line with previous observations of lower DFI and improved chromatin packaging in men given cytotoxic drugs for testicular germ cell cancer (42, 43). The biological mechanism behind this association is not known. However, one could speculate whether cytotoxic compounds might increase apoptosis and, thereby, elimination of germ cells with increased levels of DNA strand breaks.

Apart from the biological aspects of our findings, discussed above, the results of this study also have some clinical implications. Although it seems reassuring that the mean DFI, as seen in the whole group of CCS, was only slightly elevated compared with the controls (14% and 11%, respectively), the OR for having DFI >20%, previously shown to be associated with decreased fertility *in vivo* (16), was 2.2 in cancer survivors compared with controls. Because the increase in DFI was most pro-

nounced in those groups not having decreased sperm counts due to the cancer treatment given, an impairment of sperm DNA integrity may contribute to fertility problems in those CCS in whom the standard sperm parameters are not significantly affected.

Although the study includes almost 100 CCS, the heterogeneity of diagnoses and the treatments given, along with the considerable variation in age at diagnosis, does not allow a more refined analysis on the impact of specific treatment modalities and/or age at puberty, on sperm DNA integrity. Even when dividing the material into the main therapeutic groups—surgery, irradiation, and chemotherapy—the cohorts were still relatively small, implying the risk of type II error for some of the comparisons done in this study. This might also be the reason why we did not find a statistically significantly higher DFI in those men treated with irradiation only compared with CCS who received neither chemotherapy nor radiotherapy.

Another weak point of our current study was that only 38% of the eligible patients accepted to take part. Nevertheless, from a fertility point of view, the participants were representative of the whole group of CCS survivors, and the distribution of diagnoses among the participants was similar to the cancer incidence in Sweden. Therefore, we do not believe that our results are due to selection bias. In conclusion, we found that the proportion of DNA

strand breaks in spermatozoa from men treated for childhood cancer was significantly increased in men who had not received chemotherapy. From the clinical point of view, the study shows that impairment of DNA integrity may contribute to infertility problems in CCS. Furthermore, our data might indicate that childhood cancer is associated with genomic instability, and the consequences of possible transmission of sperm DNA damage to the offspring need to be considered.

Disclosure of Potential Conflicts of Interest

None of the authors have any financial or personal relationships with people or organizations that could inappropriately influence the work.

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