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ORIGINAL ARTICLE

Long-term effect of cytotoxic treatments on sperm DNA fragmentation in patients affected by testicular germ cell tumor

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Abstract

Introduction: Testicular germ cell tumor is the most frequent neoplasia in men of reproductive age, with a 5-year survival rate of 95%. Antineoplastic treatments induce sperm DNA fragmentation, especially within the first year post-therapy. Data in the literature are heterogeneous concerning longer follow-up periods, and the large majority is limited to 2 years.

Objective: To define the timing for the recovery of sperm DNA damage and the proportion of patients with severe DNA damage at 2 and 3 years from the end of therapy.

Materials and methods: Sperm DNA fragmentation was evaluated in 115 testicular germ cell tumor patients using terminal deoxynucleotidyl transferase dUTP nick end labeling assay coupled with flow cytometry before (T_0) and 2 (T_2) and 3 (T_3) years post-treatment. Patients were divided based on the type of treatment: carboplatin, bleomycin–etoposide–cisplatin, and radiotherapy. For 24 patients, paired sperm DNA fragmentation data were available at all time-points (T_0–T_2–T_3). Seventy-nine cancer-free, fertile normozoospermic men served as controls. Severe DNA damage was defined as the 95th percentile in controls (sperm DNA fragmentation = 50%).

Results: Comparing patients versus controls, we observed: (i) no differences at T_0 and T_3 and (ii) significantly higher sperm DNA fragmentation levels ($p < 0.05$) at T_2 in all treatment groups. Comparing pre- and post-therapy in the 115 patients, the median sperm DNA fragmentation values were higher in all groups at T_2, reaching significance ($p < 0.05$) only in the carboplatin group. While the median sperm DNA fragmentation values were also higher in the strictly paired cohort at T_2, about 50% of patients returned to baseline. The proportion of severe DNA damage in the entire cohort was 23.4% and 4.8% of patients at T_2 and T_3, respectively.

Discussion: Currently, testicular germ cell tumor patients are advised to wait 2 years post-therapy before seeking natural pregnancy. Our results suggest that this period may not be sufficient for all patients.

Conclusion: The analysis of sperm DNA fragmentation may represent a useful biomarker for pre-conception counseling following cancer treatment.

KEYWORDS
chemotherapy, cytotoxic therapy, sperm DNA fragmentation, spermatogenesis, testicular cancer

1 | INTRODUCTION

Testicular germ cell tumor (TGCT) is the most frequent malignancy in men of reproductive age.[1] Its incidence has risen by 1.5% worldwide in the past two decades, presenting sharp geographic differences with the highest incidence in Caucasian populations.[1–3] Thanks to the progress in diagnosis and treatment, TGCT is now highly curable with an overall 5-year survival rate of 95%.[4] The primary treatment is radical inguinal orchiectomy, which can be followed by chemotherapy (CT) and/or radiotherapy (RT), depending on the histology and staging.[5] CT usually includes platinum-based agents, such as carboplatin (CP) or a combination of bleomycin, etoposide, and cisplatin (BEP), while RT is recommended only in seminoma (stage IIA and IIB).[5]

Antineoplastic treatments exert their effect by inducing DNA fragmentation in proliferating cells. Hence, they often lead to spermatogenic impairment (severe oligozoospermia or azoospermia) as a short-term effect. In the large majority of cases, a full or partial recovery of the spermatogenesis occurs within 2 years from the end of therapy. Although routine semen parameters return or even ameliorate with respect to the pre-treatment values, concerns have been raised about the potential long-term effect of oncological treatments on sperm DNA integrity.[6]

The importance of better characterizing the nature and persistence of sperm DNA damage after oncological treatment is related to its potential consequences on the offspring's health. It is plausible that severe DNA damage (SDD) could not only negatively influence sperm fertilizing capacity but also be responsible for malformations in children born to cancer survivors. Data in the literature are extremely poor concerning the malformation rate in children from TGCT survivors. To date, the largest epidemiological study collecting information in children with paternal history of cancer reported an overall significant increase in the risk for congenital malformations.[7] However, the adjusted relative ratio did not show a significant increase in case of TGCT.[7] A more recent study based on a smaller population focusing only on children fathered by men treated for TGCT observed a 30% increased risk for malformations with respect to those born from fathers without this neoplasia.[8] However, in the same study, the authors did not observe an increased risk when comparing children conceived before and after CT and/or RT.[8]

The standard indication for attempting natural pregnancy after the end of cancer treatment is 24 months. However, some authors reported an increased sperm aneuploidy rate up to 18—24 months after therapy.[9–12] Another consequence of anticancer treatment is the induction of sperm DNA fragmentation (SDF). The most commonly used technique to evaluate SDF is the sperm chromatin structure assay (SCSA), which detects the susceptibility of spermatozoa to DNA denaturation and provides an indirect measure of SDF. Others used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and COMET assays, both directly detecting SDF. Literature data on SDF in TGCT patients prior to and after cytotoxic therapy are controversial. Several studies based on different assays (SCSA,[13–17] TUNEL,[15,18,19] or COMET[15,20,21]) observed increased SDF in TGCT patients prior to therapy (T_0) with respect to controls, suggesting two possible scenarios: (i) a direct effect of cancer itself on sperm DNA quality and (ii) a constitutional genomic instability of cancer patients.[7,8] However, some other studies based on SCSA[22–26] or TUNEL assay[13,23,27,28] did not find significant differences between SDF values at T_0 versus controls. The literature exploring the effects of CT and RT on SDF also produced conflicting results, especially concerning the time needed to repair such damages.[6] Most of the studies are limited to a 2-year follow-up[13,14,20,27,29] and among them, some authors who used SCSA[13,29] or TUNEL assay[13,18] observed a return of SDF levels to baseline after 2 years (T_2), while others using the same methods found that patients at T_2 still had significantly higher SDF than T_0 or controls.[22–24] Only five studies extended the survey over 3 years from the end of therapies, reporting a return of SDF values to baseline in the majority of cases.[18,22–25] However, if we consider paired samples, only three patients have been evaluated in a 3-year follow-up.[23] Regarding the type of cancer treatment, there is a general consensus among studies on the fact that RT is the most harmful treatment,[22,25,29] followed by BEP and CP.[27]

The different conclusions in the literature may derive from the recruitment of relatively small cohorts and the composition of control populations. Given that the same method used by different authors gave contradictory results, it is possible that the observed differences derive from the set-up of a given method to detect SDF. In fact, SDF data reported in independent cohorts from the same laboratory show similar results.

Given the paucity of data on long-term effect of antineoplastic treatment in the literature, the objective of our study was to evaluate SDF

with TUNEL assay coupled with flow cytometry in TGCT patients in an up to 3-year longitudinal survey. We aimed to define the timing for the recovery of sperm DNA damage and the proportion of patients with SDD at 2 (T_2) and 3 (T_3) years from the end of therapy. In addition, we analyzed SDF in the largest cohort of patients having matched samples at the three different time-points.

2 | MATERIALS AND METHODS

2.1 | Subjects

We enrolled a total of 115 men affected by TGCT who underwent orchifunicolectomy at the University Hospital of Careggi (Florence, Italy) in the context of an onco-andrological follow-up every 12 months for up to 3 years. Only patients for whom we were able to analyze SDF were included in the study; hence, severe oligozoospermic and azoospermic patients were excluded. According to the testis histology, 66 patients were affected by pure seminoma, while 49 were affected by non-seminomatous germ cell tumor. Semen samples for SDF were analyzed before therapy (T_0) and 2 and 3 years after the end of therapy (T_2 and T_3, respectively). A total of 104 patients were treated with CT/RT, whereas 11 underwent active surveillance. For 56/104 patients, semen samples were available before therapy, and for 44/56, semen samples were available at different post-therapy time-points (Figure 1). Due to the sperm number request of TUNEL assay (at least 5 million spermatozoa) and the drop out of some patients, only for 24/44 patients we could perform a longitudinal follow-up including all time-points (T_0–T_2–T_3). Forty-eight out of 104 patients entered the study only 2 or 3 years (T_2 or T_3) after therapy (Figure 1).

The control group was composed of 79 men recruited in the frame of a previous European Academy of Andrology ultrasound study.[30] All of them achieved pregnancy within 12 months and were normozoospermic according to the 5th percentile of the World Health Organization (WHO) reference values.[31]

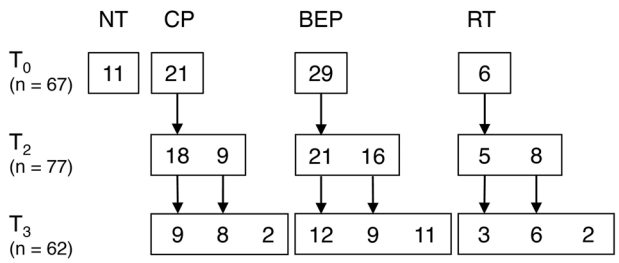

FIGURE 1 Flowchart illustrating the distribution of the 115 testicular germ cell tumor patients according to the type of cancer treatment and the time-points when the sperm analysis took place. The arrows connect the samples delivered by the same patient (paired samples at different time-points). T_0, before therapy; T_2, 2 years after the end of therapy; T_3, 3 years after the end of therapy. The total number of individuals participating in the four different treatment groups are as follows: 11 no cytotoxic therapy (NT), 32 carboplatin (CP), 56 bleomycin–etoposide–cisplatin (BEP), and 16 radiotherapy (RT).

TABLE 1 Patients' distribution according to the treatment regimen used in the entire cohort and in the paired longitudinal cohort

Treatment	Regimen	Number of patients: Entire cohort ($n = 115$)	Number of patients: Paired longitudinal cohort ($n = 24$)
CP	1 cycle	22	6
	2 cycles	10	3
BEP	1 cycle	9	5
	2 cycles	20	3
	3 cycles	22	4
	4 cycles	4	–
	6 cycles	1	–
RT	20 Gy	12	3
	36 Gy	4	–
NT	–	11	–

Abbreviations: BEP, bleomycin–etoposide–cisplatin; CP, carboplatin; RT, radiotherapy; NT, no cytotoxic therapy; Gy, Gray.

The project was approved by the regional ethics committee for clinical experimentations in Tuscany (ref. 27–11 and 2019-481), and written informed consent for participation was obtained from each subject.

2.2 | Cancer treatment

Patients were treated according to the current international[5] and national guidelines (http://media.aiom.it/userfiles/files/doc/LG/2017_LGAIOM_Testicolo.pdf) for testis cancer and were divided into four groups according to the treatment received, that is, CP, BEP, RT, and surgery only (no therapy, NT) (Table 1). RT was administered to the lumbar-aortic lymph nodes, with shielding of the remaining testicle, at a mean absorbed dose of 20 Gy ($n = 12$) or 36 Gy ($n = 4$).

2.3 | Semen analysis

All semen samples were obtained through masturbation after 2–7 days of sexual abstinence. After collection, samples were incubated at 37°C for 30–60 min for complete liquefaction and evaluated according to the WHO guidelines.[32] Apart from taking into consideration the total sperm count (TSC), we also combined quantitative and qualitative features and defined the following two sperm parameters: total motile sperm count (TMSC) and total number of spermatozoa with typical morphology (TTSC).

2.4 | TUNEL/propidium iodide assay

SDF was determined on fresh semen samples of controls and patients through TUNEL assay coupled with flow cytometry and combined with

nuclear staining using propidium iodide, which allows the exclusion of semen apoptotic bodies. SDF has been determined as previously described by Muratori et al.[32] Sample measurements were acquired using a FACScan flow cytometer (Becton Dickinson) equipped with a 15-m Wargon-ion laser for excitation. The inter- and intra-individual variability of the method has been previously reported.[32] In particular, the method has an intra-individual coefficient of variation of 12.9% (when the time interval between measurements is 12 months) and 14% (with a time interval of 2 years), as previously described by the authors.[32]

2.5 | Statistical analysis

Statistical analysis was performed using the SPSS 27.0.1 software (SPSS, Chicago, IL, USA). Semen parameters and SDF values are not normally distributed (Kolmogorov–Smirnov test); therefore, descriptive values are expressed as medians and interquartile ranges (IQR). The Mann–Whitney *U*-test was used to make comparisons between different groups: (i) patients at T_0 versus controls and (ii) patients at T_0 versus T_2 and T_3 in the three treatment groups. The same comparisons were made in the paired longitudinal cohort using the Wilcoxon signed-rank test for correlated samples. Fisher's exact test was used to make intergroup comparisons of the proportion of patients with SDF higher than the threshold for SDD. A *p*-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Baseline comparison between patients affected by TGCT and controls

The results concerning the baseline values are reported in Table 2. Prior to oncological treatment, TGCT patients exhibited significantly lower values for sperm concentration, TSC, TMSC, and TTSC than controls ($p < 0.001$). On the contrary, similar values of SDF were observed in the two groups, even after adjusting for age. Routine sperm parameters and SDF values showed no difference between the two TGCT histotypes.

3.2 | Effect of treatments on sperm DNA fragmentation

3.2.1 | Analysis of the entire study population

We analyzed the effect of three different treatment types in the entire cohort of patients ($n = 115$): CP, BEP, and RT after 2 (T_2) and 3 (T_3) years from the end of the therapy. We observed a more evident increase in SDF at T_2 in those patients who received higher doses of CT (CP 2 cycles or BEP 3 or 4 cycles); however, the difference was not statistically significant (Table S1). Therefore, for further statistical analysis, patients receiving CT were divided into two groups, CP and BEP,

TABLE 2 Comparison of routine sperm parameters and sperm DNA fragmentation (SDF) values in testicular germ cell tumor patients (seminoma and non-seminoma) at baseline (T_0) versus controls

	Age (years)	Volume (mL)	Sperm concentration (spermatozoa/mL)	TSC	TMSC	TTSC	SDF (%)
Ctrl ($n = 79$)	36[a,b,c] (33–38)	3 (2.2–4.3)	63.9[a,b,c] (45.5–120)	192.6[a,b,c] (135.5–324.8)	113.3[a,b,c] (72.7–165.8)	13.6[a,b,c] (8.1–22.8)	28 (22–38)
Patients ($n = 67$)	31[a] (25–35)	3.1 (2.4–4.8)	25.4[a] (11.2–58)	90[a] (33.8–171)	50.4[a] (13.2–99.9)	2.9[a] (0.7–9.7)	27.7 (21.8–39.2)
Seminoma ($n = 39$)	33[b,d] (29–35)	3 (2.3–4.6)	32[b] (15.9–68)	93.4[b] (34.6–225.3)	57.3[b] (13.9–119.4)	2.8[b] (0.6–9.7)	27.5 (21–40.7)
Non-seminoma ($n = 28$)	26[c,d] (22–33)	3.3 (2.6–5.3)	24.6[c] (10–33)	75[c] (32–163.9)	40.3[c] (10.5–81.9)	3.3[c] (0.7–9.6)	27.8 (22.2–38.4)

Note: Data are expressed as median (IQR).
Abbreviations: Ctrl, controls; IQR, interquartile range; TMSC, total motile sperm count; TSC, total sperm count; TTSC, total number of spermatozoa with typical morphology.
[a]Patients versus controls ($p < 0.001$).
[b]Seminoma versus Ctrl ($p < 0.001$).
[c]Non-seminoma versus Ctrl ($p < 0.001$).
[d]Seminoma versus non-seminoma ($p < 0.001$).

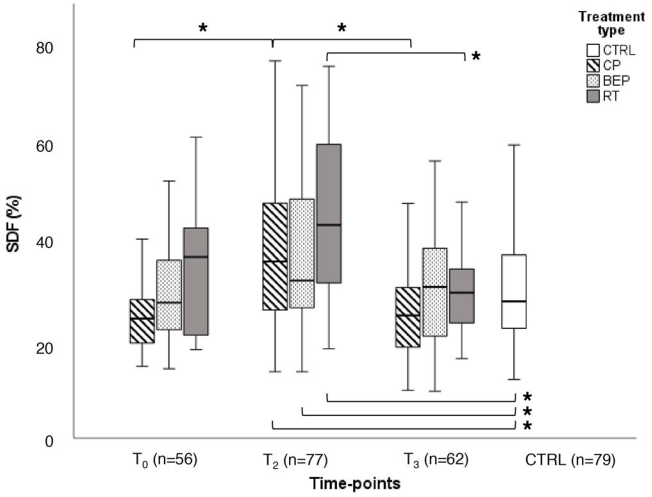

FIGURE 2 Sperm DNA fragmentation (SDF) trend in the entire cohort at the three time-points in the three cytotoxic treatment groups. Bars indicate median values, boxes represent interquartile intervals, and whiskers represent maximum and minimum SDF values observed in the cohorts. T_0, before therapy (excluding 11 patients who did not underwent cytotoxic treatments); T_2 and T_3, 2 and 3 years after the end of therapy, respectively. Different numbers of patients were evaluated at each time-point—T_0: 21 carboplatin (CP), 29 bleomycin–etoposide–cisplatin (BEP), and 6 radiotherapy (RT); T_2: 27 CP, 37 BEP, and 13 RT; T_3: 19 CP, 32 BEP, and 11 RT. *$p < 0.05$.

independent of the number of cycles received. In all three treatment groups, we observed an improvement in routine semen parameters such as TSC, TMSC, and TTSC at T_2 and T_3 with respect to pre-therapy. Statistical significance was obtained for TSC and TMSC only in the BEP group (T_0 versus T_2, $p < 0.05$, Table S2A). Concerning SDF, by comparing pre- and post-therapy data, we observed higher median SDF levels in patients at T_2 than at T_0, with statistical difference only in the CP group. SDF at T_3 decreased in all the three treatment groups, with median values lower than T_2. However, only CP and RT groups exhibited a significant reduction in median SDF values at T_3 with respect to T_2 (Figure 2 and Table S2A).

The comparison of patients after therapy versus cancer-free, fertile normozoospermic controls showed that in all the three treatment groups, patients had significantly higher SDF at T_2 (Figure 2 and Table S2A). At T_3, no differences in SDF levels have been observed between controls and patients in all the three treatment categories (Figure 2 and Table S2A).

In order to define putative SDD, we used an SDF threshold of 50%, corresponding to the 95th percentile in controls. At T_0, 8.9%; at T_2, 23.4%; and at T_3, 4.8% of patients exhibited SDD. With respect to T_0, we observed in all three treatment groups an increase in the proportion of patients having SDD at T_2, reaching statistical significance only in the BEP group (T_0 6.9% vs. T_2 24.3%, $p < 0.05$). After 3 years from the end of therapy, a reduction in patients with SDD was observed in all groups being significant in the BEP group (T_3 vs. T_2, $p < 0.05$). However, three patients treated with three cycles of BEP showed SDD after 3 years of therapy. No patient with SDD was observed in the CP and RT cohorts (Table 3).

TABLE 3 Proportion of patients belonging to the entire cohort, showing severe DNA damage (SDD) at various time-points (pre- and post-therapy)

	n with SDD/total *n* (% patients with SDD)		
Treatment groups	T_0	T_2	T_3
CP	2/21 (9.5)	5/27 (18.5)	0/19 (0)
BEP	2/29 (6.9)	9/37 (24.3)	3/32 (9.4)
RT	1/6 (16.7)	4/13 (30.8)	0/11 (0)

Note: SDD was defined as sperm DNA fragmentation >95th percentile in the control group.
Abbreviations: BEP, bleomycin–etoposide–cisplatin; CP, carboplatin; RT, radiotherapy; T_0, before therapy; T_2 and T_3, 2 and 3 years after the end of therapy, respectively.

3.2.2 | Analysis of the longitudinal cohort with paired semen samples

We performed a subgroup analysis in 24 patients for whom SDF data were available at all three time-points. In analogy to the entire cohort, we observed an improvement in semen parameters at T_2 and T_3 in all treatment groups without reaching statistical significance (Table S2B).

Regarding median SDF values in the two groups who received CT (CP or BEP), we observed the same trend in the entire cohort: median SDF values at T_2 were higher than those at T_0, while at T_3, they were similar to those at T_0. However, statistical significance ($p < 0.05$) was reached only in the CP and BEP groups when comparing T_2 versus T_3 (Figure S1 and Table S2B). We could not perform any statistics on the RT group because of the small number of patients ($n = 3$).

At the individual level, when we compared T_0 versus T_2, only 4/9 of the CP group, 6/12 of the BEP group, and 1/3 of the RT group showed an increase in SDF. The remaining individuals had either lower or similar values with respect to T_0 (Figures S2–S4). Given that data on SDF were available for each patient at all the three time-points, we were interested in defining the proportion of patients who returned to baseline SDF values after 2 or 3 years from the end of therapy. Overall, 45.8% of patients did not return to baseline at T_2 and about 12.5% of these patients still exhibited SDF values at T_3 higher than T_0. In Figure 3, the proportion of patients returning to pre-treatment values after CT is reported. In both CP and BEP groups, a similar percentage of patients did not normalize at T_2, whereas at T_3 only in the BEP group were present patients with higher than T_0 SDF values. In order to evaluate whether clinical parameters may predict a higher risk for "non-normalization" at T_2, we analyzed the data as a function of body mass index (BMI), age, tumor histotype, semen phenotype at baseline, and type and number of cycles of CT (Table S3). No significant

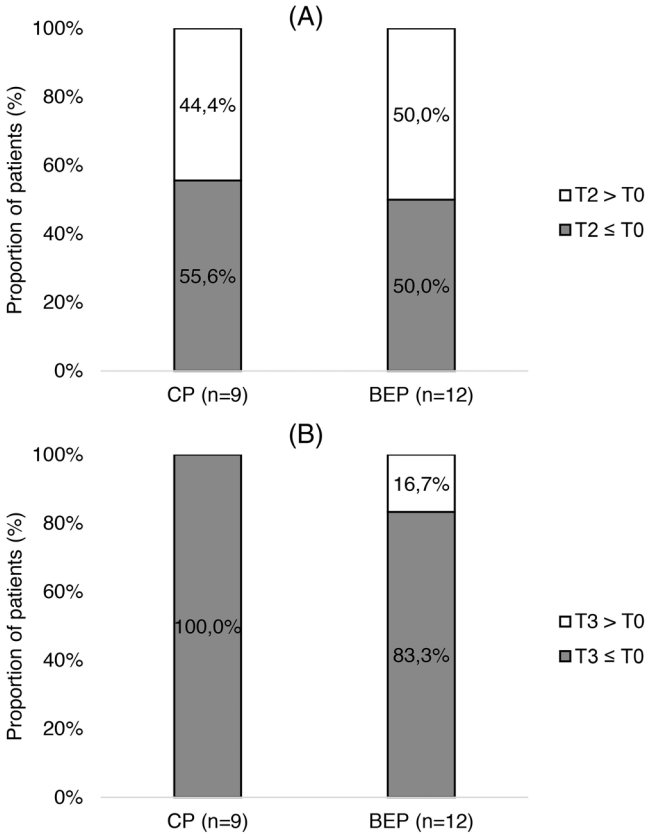

FIGURE 3 Evaluation of the proportion of patients with sperm DNA fragmentation returning to pre-treatment values ($\leq T_0$) in the paired cohort. Patients were divided according to the different treatment categories: (A) after 2 years and (B) after 3 years from the end of the therapies. BEP, bleomycin–etoposide–cisplatin; CP, carboplatin; T_0, before therapy; T_2 and T_3, 2 and 3 years from the end of therapy, respectively. Radiotherapy was not included in the analysis because of the limited number of individuals ($n = 3$).

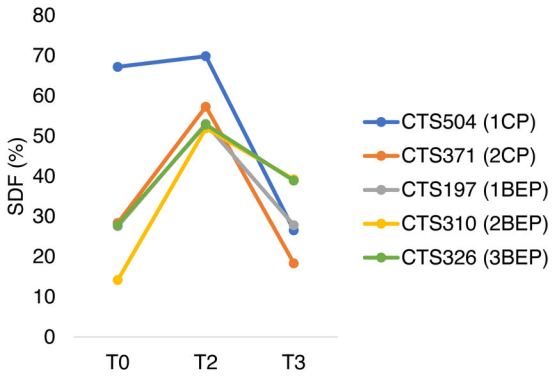

FIGURE 4 Sperm DNA fragmentation (SDF) trend over the observation period in patients with severe DNA damage (SDF ≥50%) at T_2. BEP, bleomycin–etoposide–cisplatin; CP, carboplatin; T_0, before therapy; T_2 and T_3, 2 and 3 years after the end therapy, respectively.

associations were found with any of the above parameters, including the type and number of cycles received.

Similar to the entire cohort, we defined the proportion of subjects with SDD in this paired longitudinal group. The percentage of patients with SDD was 20.8% at T_2, and the majority of them were treated with BEP. Interestingly, 1/5 patients with SDD at T_2 showed SDD at T_0, while at T_3, all the five patients presented an SDF value below 50% (Figure 4).

4 | DISCUSSION

The treatment of TGCT is based mainly on three types of anti-cancer regimens, such as CP, BEP, and/or RT. Their anti-neoplastic effect is exerted through the induction of DNA damage on actively proliferating cells followed by their apoptosis. As a result, TGCT survivors experience a temporary disruption of spermatogenesis, especially in the first year after the completion of therapy. However, after 2 years, there is a substantial improvement of the quantitative and qualitative sperm parameters.[13,18,22,23,33] In our study, we also observed a progressive improvement of the TSC, TMSC, and TTSC during the observation period up to 36 months.

The recovery of spermatogenesis in the large majority of cases allows couples to plan natural pregnancies instead of undergoing assisted reproductive techniques with the cryopreserved samples. However, concerns were raised about the genetic integrity of spermatozoa after genotoxic treatments, especially based on the observations about the persistence of increased aneuploidy rate and DNA fragmentation in some patients up to 18/24 months.[9–13] It is well known that etoposide, a component of the BEP regimen, inhibits both topoisomerase II activity and the disjunction of recombinant chromosomes and sister chromatids, leading to aneuploidy.[35,36] On the other hand, platinum-based drugs, that is, cisplatin and CP, and RT are known to cause DNA strand breaks. Several authors proposed the evaluation of SDF as a biomarker to monitor such effects with a follow-up usually limited to 24 months. Among the five studies that extended the survey to 3 years after therapy, three of them analyzed overlapping cohorts,[22–24] and in the other two papers, it is not possible to extrapolate the results at T_3 because the authors did not standardize the time-points after therapy.[18,25] Given the paucity of data on longer follow-up periods, our main objective was to obtain novel insights into the persistency of DNA damage over 3 years. To this purpose we evaluated SDF by applying TUNEL assay coupled with flow cytometry prior to and after 2 and 3 years from the end of cytotoxic therapy in a total of 115 patients.

Pre-therapy SDF values were similar between seminoma versus non-seminoma patients, in accordance with other authors.[25,28,29] Our results also support the majority of the studies based on TUNEL[13,23,27,28] and 5/11 studies based on SCSA,[22-26] which reported similar SDF levels in patients at T_0 versus healthy controls. On the other hand, other SCSA- and all COMET-based studies[13-19,21] reported higher SDF values in patients with respect to controls. These discrepancies may derive from differences in the methodologies and their respective targets (double or single strand breakage, chromatin integrity or susceptibility to denaturing agents) and clinical characteristics of the controls (fertile and/or normozoospermic or volunteers from the general population). Moreover, the analysis of fresh versus

frozen semen samples may also influence the results. Indeed, in a previous study from our laboratory, we demonstrated that spermatozoa from oncological patients have higher DNA damage after thawing in respect to non-cancer patients, implying a higher susceptibility to thermic shock in oncological patients.[36] In our current study, in order to obtain information on the genomic integrity of spermatozoa in the context of natural conception, we analyzed fresh semen samples at all time-points.

Concerning the effects of CT and RT on SDF, data in the literature clearly show that 1 year after the completion of treatment, SDF values are significantly higher than T_0, while data on T_2 are more heterogeneous.[6] We therefore focused our attention on the long-term effect of cancer therapies. Since sperm parameters and SDF values were not significantly different between subgroups defined according to the number of cycles of a given treatment (low vs. high dosage), patients were divided into three treatment groups: CP, BEP, or RT. For all the three treatment categories, we observed an increase in SDF at T_2 with respect to baseline, reaching statistical significance only in the CP group. Data on the effect of CP are extremely scarce in the literature because there is only one Italian study reporting the effect of this treatment without finding a significant difference between T_0 versus T_2 and between CP and BEP at T_2.[27] We also found similar median SDF values between CP and BEP, 36.2% (IQR 25.7–49.1) and 32.3% (IQR 26.3–50.5), respectively. Although there was no significant difference between the three types of treatments at T_2, the highest median SDF values (43.6% [IQR 28.1–61.5%]) were observed in the 13 patients who underwent RT. This observation is in line with other studies reporting a higher impact of RT on DNA fragmentation with respect to CT at different time-points.[23,25,29] As stated above, we did not observe significant differences in SDF levels between patients receiving low doses versus high doses of CT at T_2. Ståhl et al.[23] reported a similar finding after the same 24-month interval. The effect of treatment intensity (1–2 cycles vs. 3—4 cycles BEP) seems to be relevant only after a few months from the end of therapies, that is, significant increase in SDF was observed when evaluated at T_0 versus 3—6 months in patients receiving high doses of BEP.[29]

When we compared the T_2 values with a group of tumor-free, normozoospermic, fertile men, we found a significantly higher median SDF level in all three treatment groups. O'Flaherty et al.[14] has also reported higher SDF values in patients treated with BEP than controls at T_2, indicating that the genotoxic effect of cancer therapies may extend over 24 months. We were therefore interested in defining sperm DNA damage after an additional year by extending the follow-up to T_3. We observed a reduction in median SDF levels at T_3 compared to T_2 in all the three treatment categories, reaching statistical significance in the CP and RT groups. Moreover, median SDF levels at T_3 were not significantly different from both baseline and controls.

One of the biggest challenges we are facing in this field is the difficulty to perform longitudinal studies with paired semen samples. In fact, except for very few papers reaching up to 24 months of observation, the large majority of the literature is based on the analysis of unmatched samples at different time-points with a risk of intrinsic biases.[13,14,20,22–24,27,29] The analysis of median values derived from a paired sample set (from T_0 to T_3) allows a more precise evaluation of the treatment effect, and most importantly, allows us to define for each individual the timing of the "normalization" of SDF values, that is, the return to the baseline values. For this purpose, we selected from the entire cohort those 24 patients for whom SDF values of pre- and post-therapy (T_2 and T_3) were available. Although it is a relatively small cohort, this is the largest one that has been evaluated in a 3-year post-therapy survey. Indeed, in the literature, only three patients with paired samples were analyzed at T_0, T_2, and T_3.[23] In our selected cohort, almost half of the patients (11/24) exhibited higher SDF at T_2 than T_0 and among them, except for three patients, all the remaining patients returned to or below the pre-therapy levels at T_3. Hence, at T_3, only 12.5% (3/24) of patients displayed higher SDF values than T_0, although they showed an average 10%—14% decrease with respect to T_2. Given the progressive amelioration of DNA damage also in these patients, we can speculate that their SDF value will return to baseline within the subsequent 12 months.

In order to evaluate whether we can predict who is more susceptible to a longer persistence of DNA damage, we compared patients returning to baseline versus those who remained higher after 2 years as a function of selected clinical parameters at diagnosis (age, BMI, TGCT histotype, clinical and pathological stage, and semen phenotype) and treatment regimens. None of the clinical parameters, including treatment intensity, seems to be associated with an increased risk for non-normalization of SDF values.

The evaluation of the median SDF values is useful for providing a general notion about the persistence of post-therapy DNA damage. If we consider the observed significantly higher median values at T_2 with respect to the control group, we should conclude that the currently advised waiting time for natural pregnancy should be further expanded. However, we should take into consideration the high inter-individual variability, that is, about 50% of patients do not show increased SDF at T_2. In addition, for personalized counseling, it should be important to define the severity of the post-therapy DNA damage. For this purpose, we established an arbitrary threshold for putative SDD corresponding to SDF values above the 95th percentile in controls. A similar approach was taken by Bujan et al.[13] who defined SDD as the SDF value above the 90th percentile in controls. We observed that 8.9% of patients had SDD before therapy, with a significant increase to 23.4% ($p = 0.033$) in the second year after treatment. Although numbers are low, the highest incidence of SDD was observed in the RT group (30.8%). Bujan et al.[13] also found an increase from 11% at T_0 to 15% at T_2. After 3 years, SDD was observed in only 3/62 patients, and all of them were treated with three cycles of BEP. Unfortunately, we do not have their SDF values prior to therapy; therefore, it remains an open question, whether these patients have an intrinsically high DNA fragmentation or there is a longer persistency of the genotoxic effect because of the higher doses of BEP. Concerning the second option, it is worth noticing that the four patients belonging to the paired cohort, who received three cycles of BEP, did not show persistent DNA damage. In addition, interindividual differences may also depend on the individual efficiency of the DNA repair machinery. Among the three patients with persistent SDD, we did not identify

shared clinical features that would distinguish them from the remaining individuals. Patient with code CRR-116 was 35 years old and affected by seminoma, while the other two were younger (25 and 27 years old) and had non-seminoma. CRR-116 was the only one with a history of bilateral cryptorchidism and he was severely oligozoospermic at T_2 (TSC = 1.08×10^6 spermatozoa); hence, we were unable to perform the TUNEL assay. For the other two patients, SDF was already above 50% at T_2. Our results on the persistence of DNA damage even after 3 years call for further investigation on larger study populations with paired samples.

In conclusion, our results provide further evidence for the long-lasting deleterious effect of cytotoxic treatment on the integrity of sperm DNA. Currently, couples are advised to wait for 2 years from the end of antineoplastic treatment before seeking natural pregnancy. However, such a time interval might not be sufficient for all patients because according to our data, SDD might persist for a longer period. Hence, the analysis of SDF may represent a useful biomarker for the detection of persistent genotoxic effects. Measuring this parameter at T_2 may help in personalizing pre-conceptional counseling on the use of cryopreserved versus fresh spermatozoa, especially when SDD is observed.

4.1 | Limitations of the study

The main limitation of the study is related to the method. The TUNEL assay coupled with flow cytometry requires a minimum of 5 million spermatozoa to be performed, implying that severe oligozoospermic or cryptozoospermic patients could not be included in the study. Therefore, although the sperm concentration and TSC are significantly lower in TGCT patients than in controls, we cannot provide information on subjects with severely impaired spermatogenesis before and/or after cytotoxic treatment.

AUTHOR CONTRIBUTIONS

Csilla Krausz designed and supervised the study. Ginevra Farnetani performed statistical analysis and wrote the manuscript together with Csilla Krausz. Maria Grazia Fino and Selene Degl'Innocenti performed semen analysis. Antoni Riera-Escamilla, Francesca Cioppi, Elena Casamonti, Selene Degl'Innocenti, and Sara Marchiani performed the TUNEL assay. Matteo Vannucci, Viktoria Rosta, Maria Grazia Fino, and Csilla Krausz recruited the patients and provided their clinical data. Serena Vinci, Matilde Spinelli, Maria Grazia Fino, and Leila Turki contributed to the management of the database. Francesco Lotti and Sara Marchiani are responsible for the recruitment of controls and their data acquisition. Monica Muratori and Lara Tamburrino contributed to the interpretation of TUNEL data. All authors critically reviewed the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Znaor A, Skakkebaek NE, Rajpert-De Meyts E, et al. Global patterns in testicular cancer incidence and mortality in 2020. *Int J Cancer*. 2022;151(5):692-698. https://doi.org/10.1002/IJC.33999
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA Cancer J Clin*. 2021;71(1):7-33. https://doi.org/10.3322/CAAC.21654
3. Park JS, Kim J, Elghiaty A, Ham WS. Recent global trends in testicular cancer incidence and mortality. *Medicine*. 2018;97(37):e12390. https://doi.org/10.1097/MD.0000000000012390
4. Cheng L, Albers P, Berney DM, et al. Testicular cancer. *Nat Rev Dis Primers*. 2018;4(1):1-24. https://doi.org/10.1038/s41572-018-0029-0
5. Albers P, Albrecht W, Algaba F, et al. Guidelines on testicular cancer: 2015 update. *Eur Urol*. 2015;68(6):1054-1068. https://doi.org/10.1016/J.EURURO.2015.07.044
6. Paoli D, Pallotti F, Lenzi A, Lombardo F. Fatherhood and sperm DNA damage in testicular cancer patients. *Front Endocrinol*. 2018;9:506. https://doi.org/10.3389/FENDO.2018.00506
7. Ståhl O, Boyd HA, Giwercman A, et al. Risk of birth abnormalities in the offspring of men with a history of cancer: a cohort study using Danish and Swedish national registries. *J Natl Cancer Inst*. 2011;103(5):398. https://doi.org/10.1093/JNCI/DJQ550
8. Al-Jebari Y, Glimelius I, Nord CB, et al. Cancer therapy and risk of congenital malformations in children fathered by men treated for testicular germ-cell cancer: a nationwide register study. *PLoS Med*. 2019;16(6):e1002816. https://doi.org/10.1371/JOURNAL.PMED.1002816
9. Tempest HG, Ko E, Chan P, Robaire B, Rademaker A, Martin RH. Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. *Hum Reprod*. 2008;23(2):251-258. https://doi.org/10.1093/HUMREP/DEM389
10. Burrello N, Vicari E, la Vignera S, et al. Effects of anti-neoplastic treatment on sperm aneuploidy rate in patients with testicular tumor: a longitudinal study. *J Endocrinol Invest*. 2011;34(6):e121-e125. https://doi.org/10.1007/BF03346719
11. Martin RH, Ernst S, Rademaker A, Barclay L, Ko E, Summers N. Analysis of sperm chromosome complements before, during, and after chemotherapy. *Cancer Genet Cytogenet*. 1999;108(2):133-136. https://doi.org/10.1016/S0165-4608(98)00125-3
12. de Mas P, Daudin M, Vincent MC, et al. Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Hum Reprod*. 2001;16(6):1204-1208. https://doi.org/10.1093/HUMREP/16.6.1204
13. Bujan L, Walschaerts M, Moinard N, et al. Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. *Fertil Steril*. 2013;100(3):673-680.e2. https://doi.org/10.1016/J.FERTNSTERT.2013.05.018
14. O'Flaherty CM, Chan PT, Hales BF, Robaire B. Sperm chromatin structure components are differentially repaired in cancer survivors.

J Androl. 2012;33(4):629-636. https://doi.org/10.2164/JANDROL.111.015388

15. O'Flaherty C, Vaisheva F, Hales BF, Chan P, Robaire B. Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Hum Reprod*. 2008;23(5):1044-1052. https://doi.org/10.1093/HUMREP/DEN081
16. Fosså SD, de Angelis P, Kraggerud SM, Evenson D, Theodorsen L, Clausen OPF. Prediction of posttreatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. *Cytometry*. 1997;30:192-196. https://doi.org/10.1002/(SICI)1097-0320(19970815)30:4
17. Kobayashi H, Larson K, Sharma RK, et al. DNA damage in patients with untreated cancer as measured by the sperm chromatin structure assay. *Fertil Steril*. 2001;75(3):469-475. https://doi.org/10.1016/S0015-0282(00)01740-4
18. Spermon JR, Ramos L, Wetzels AMM, et al. Sperm integrity pre- and post-chemotherapy in men with testicular germ cell cancer. *Hum Reprod*. 2006;21(7):1781-1786. https://doi.org/10.1093/HUMREP/DEL084
19. Gandini L, Lombarde F, Paoli D, et al. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod*. 2000;15(4):830-839. https://doi.org/10.1093/HUMREP/15.4.830
20. O'Flaherty C, Hales BF, Chan P, Robaire B. Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. *Fertil Steril*. 2010;94(4):1374-1379. https://doi.org/10.1016/J.FERTNSTERT.2009.05.068
21. Kumar K, Lewis S, Vinci S, et al. Evaluation of sperm DNA quality in men presenting with testicular cancer and lymphoma using alkaline and neutral Comet assays. *Andrology*. 2018;6(1):230-235. https://doi.org/10.1111/ANDR.12429
22. Ståhl O, Eberhard J, Jepson K, et al. The impact of testicular carcinoma and its treatment on sperm DNA integrity. *Cancer*. 2004;100(6):1137-1144. https://doi.org/10.1002/CNCR.20068
23. Ståhl O, Eberhard J, Jepson K, et al. Sperm DNA integrity in testicular cancer patients. *Hum Reprod*. 2006;21(12):3199-3205. https://doi.org/10.1093/HUMREP/DEL292
24. Ståhl O, Eberhard J, Cavallin-Ståhl E, et al. Sperm DNA integrity in cancer patients: the effect of disease and treatment. *Int J Androl*. 2009;32(6):695-703. https://doi.org/10.1111/J.1365-2605.2008.00933.X
25. Smit M, van Casteren NJ, Wildhagen MF, Romijn JC, Dohle GR. Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod*. 2010;25(8):1877-1883. https://doi.org/10.1093/HUMREP/DEQ104
26. McDowell S, Harrison K, Kroon B, Ford E, Yazdani A. Sperm DNA fragmentation in men with malignancy. *Fertil Steril*. 2013;99(7):1862-1866. https://doi.org/10.1016/J.FERTNSTERT.2013.02.015
27. Ghezzi M, Berretta M, Bottacin A, et al. Impact of Bep or carboplatin chemotherapy on testicular function and sperm nucleus of subjects with testicular germ cell tumor. *Front Pharmacol*. 2016;7:122. https://doi.org/10.3389/FPHAR.2016.00122
28. Ribeiro TM, Bertolla RP, Spaine DM, Fraietta R, Ortiz V, Cedenho AP. Sperm nuclear apoptotic DNA fragmentation in men with testicular cancer. *Fertil Steril*. 2008;90(5):1782-1786. https://doi.org/10.1016/J.FERTNSTERT.2007.08.012
29. Paoli D, Gallo M, Rizzo F, et al. Testicular cancer and sperm DNA damage: short- and long-term effects of antineoplastic treatment. *Andrology*. 2015;3(1):122-128. https://doi.org/10.1111/J.2047-2927.2014.00250.X
30. Lotti F, Frizza F, Balercia G, et al. The European Academy of Andrology (EAA) ultrasound study on healthy, fertile men: scrotal ultrasound reference ranges and associations with clinical, seminal, and biochemical characteristics. *Andrology*. 2021;9(2):559-576. https://doi.org/10.1111/ANDR.12951
31. WHO. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. 5th ed. 2010.
32. Muratori M, Marchiani S, Tamburrino L, et al. DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters. *Fertil Steril*. 2015;104(3):582-590.e4. https://doi.org/10.1016/J.FERTNSTERT.2015.06.005
33. Gandini L, Sgrò P, Lombardo F, et al. Effect of chemo- or radiotherapy on sperm parameters of testicular cancer patients. *Hum Reprod*. 2006;21(11):2882-2889. https://doi.org/10.1093/HUMREP/DEL167
34. Rose D, Thomas W, Holm C. Segregation of recombined chromosomes in meiosis I requires DNA topoisomerase II. *Cell*. 1990;60(6):1009-1017. https://doi.org/10.1016/0092-8674(90)90349-J
35. Downes CS, Mullinger AM, Johnson RT. Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. *Proc Natl Acad Sci U S A*. 1991;88(20):8895. https://doi.org/10.1073/PNAS.88.20.8895
36. Tamburrino L, Cambi M, Marchiani S, et al. Sperm DNA fragmentation in cryopreserved samples from subjects with different cancers. *Reprod Fertil Dev*. 2017;29(4):637-645. https://doi.org/10.1071/RD15190

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