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Sperm Telomere Interactions Are Impaired in Testicular Cancer Before and After Adjuvant Therapy

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ABSTRACT

Background: No study has yet explored telomere length or interaction in sperm nuclei of testicular cancer (TC) patients exposed to chemotherapy or radiotherapy. However, sperm telomere dynamics have emerged as a potential marker in male infertility.

Objectives: We aimed to investigate in a pilot and exploratory study whether TC and its adjuvant treatments alter sperm telomeres and DNA integrity during a 2-year follow-up after treatment.

Materials and methods: This ancillary study is part of the multicentric prospective and longitudinal French GAMATOX study. Twenty-nine TC patients treated with orchiectomy and either adjuvant chemotherapy ($n = 14$) or radiotherapy ($n = 15$) were included, alongside ten fertile donors. Semen samples were collected before orchiectomy and adjuvant therapy, and at 3, 6, 12, and 24 months after treatment. Sperm telomere length (STL) and telomere interactions were assessed by quantitative fluorescent in situ hybridisation (FISH). Sperm DNA fragmentation (SDF) was measured using the TUNEL assay, aneuploidy by FISH, and chromatin condensation defects by aniline blue staining.

Results: Before adjuvant therapy, patients presented a higher number of sperm telomere signals compared with controls ($p = 0.004$), persisting 2 years after treatment ($p = 0.001$). STL was not affected by tumour histology. Extremely short telomeres and the highest number of telomere signals per nucleus were more frequently observed after chemotherapy. SDF and chromatin condensation defects were transient and paralleled the recovery of conventional semen parameters, regardless of treatment type. A moderate increase in sperm aneuploidy was observed 6 months after chemotherapy ($p = 0.03$).

Conclusion: TC patients presented impaired sperm telomere interactions before and after adjuvant therapy, as reflected by a persistently elevated number of telomere signals per nucleus, and a higher prevalence of extremely short sperm telomeres, especially after chemotherapy. While relative STL remained comparable to controls, these findings raise questions about the impact of telomere architecture on germ cell tumour biology and post-treatment fertility.

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1 | Introduction

Testicular cancer (TC) is the most frequent cancer in young males, representing 12% of cancers between 20 and 35 years. A continuous rise in the incidence is reported among industrialised countries [1–3], correlated with an increased demand for sperm cryopreservation for this specific indication [4–6]. TC is more likely to occur in men presenting testicular anomalies such as infertility with defective spermatogenesis, cryptorchidism, and gonadal dysgenesis [7]. A cohort effect seems to explain the increase in TC incidence, suggesting environmental toxic exposure [1, 8, 9]. TC most commonly presents as a firm, painless testicular enlargement or intratesticular nodule, often with few or no associated symptoms. In a proportion of cases, the lesion is identified incidentally during a scrotal ultrasound performed for another indication [3, 10, 11]. In 95% of cases, TC are testicular germ cell tumours (TGCT), divided into seminoma and non-seminoma tumours [12]. Orchiectomy is the local gold-standard treatment, which allows histopathological staging [13]. Adjuvant chemotherapy consists of BEP (bleomycin, etoposide, cisplatin) in all stages and types. However, in the case of stage I and some high-volume seminoma, carboplatin alone is used [13, 14]. The number of BEP cycles varies from 1 to 4, depending on the TC stage. Para-aortic radiotherapy between 20 (stage I) and 30 (stage II) Grays (Gy) is recommended in the case of lymphatic vessels or retroperitoneal lymph node involvement. Survival rate has drastically increased in the last 40 years, from 10% in the seventies to 90% at the end of the nineties, and is even superior to 95% nowadays, according to the histological type and stage [14]. Abdominopelvic imaging using CT and MRI has improved the follow-up of adult patients treated for TC for the precocious detection of recurrence that mainly occurs in retroperitoneal lymph nodes [15].

However, cancer treatment (e.g., chemotherapy, radiotherapy) has a recognised gonadal toxicity on both the endocrine and exocrine functions of the testis. Consequently, a major improvement in TC survival rate emphasises the importance of fertility preservation in the patient's care. Indeed, sperm cryopreservation, the gold standard procedure for pubertal males, is recommended from puberty and should be performed before orchiectomy, chemotherapy, radiotherapy and retroperitoneal lymph node dissection [6, 16].

In sperm cryopreservation, TC patients present impairment of semen parameters with diminished sperm concentration or total sperm count, reduced motility and normal sperm forms in comparison to patients with other types of cancer [6, 17–23] or healthy controls [6, 17, 21, 22, 24–28]. Time to recovery to pre-treatment semen values varies among longitudinal studies and can even take up to two years depending on treatment modalities and pre-treatment semen parameters [6, 24, 26, 29, 30]. In addition to the quantitative alterations of spermatogenesis, chemotherapy and radiotherapy can cause qualitative alterations in spermatozoa, specifically by affecting the sperm nucleus. Before chemotherapy, TC patients generally present a similar sperm DNA fragmentation (SDF) rate to controls [19–20, 24, 26, 28, 29, 31], but some reports show an increased level of SDF [17, 21, 26, 32]. After treatment, SDF tends to increase during the first year following chemotherapy [20, 21, 24, 29,

30, 33]. Sperm chromosome aneuploidy is mostly comparable to controls before treatment, rises during the first 6 months after the end of chemotherapy or radiotherapy, before reaching pre-treatment values at 24 months for chemotherapy and 12 months for radiotherapy [25, 27, 29].

Chemotherapy and radiotherapy are also known to alter telomere function in tumour and somatic cells [34, 35]. To the best of our knowledge, no study has yet explored telomere length or interaction in sperm nuclei of TC patients exposed to chemotherapy or radiotherapy. However, sperm telomere is a marker of emerging interest in infertile males. Indeed, a diminished sperm telomere length (STL) has been linked to oligozoospermia [36], younger age [37–40] and male infertility [36, 37, 41–44]. In addition, sperm telomere architecture and interaction have been investigated in a few studies [38, 39, 45–48].

The present study is ancillary to the collaborative, multicentric and prospective research project “GAMATOX” (Gamete Male Toxicity) that initially enrolled 129 TC patients who were referred for sperm banking before treatment. A longitudinal follow-up was performed, consisting of semen examinations before TC treatment (T0), at 3 (T3), 6 (T6), 12 (T12), and 24 (T24) months after treatment [25, 26]. The primary objective of the GAMATOX research project was to evaluate the consequences of exposure to chemotherapy and radiotherapy on conventional semen parameters, sperm DNA fragmentation, chromatin integrity, and aneuploidy [25, 26]. We conducted a pilot and exploratory study to investigate telomere length, the number of telomere signals, and their interactions, along with other nuclear damages (i.e., DNA fragmentation, chromatin condensation defects, and aneuploidy), in spermatozoa from 29 TC patients enrolled in the GAMATOX study during spermatogenesis recovery after treatment. The objective was to assess whether TC itself, adjuvant chemotherapy, or radiotherapy specifically affects telomere characteristics within sperm nuclei.

2 | Materials and Methods

2.1 | Population and Study Design

This pilot and exploratory study explored 29 patients from the GAMATOX study with testicular cancer who underwent orchiectomy followed by adjuvant treatment. Of these patients, 14 received chemotherapy and 15 received radiotherapy. The chemotherapy regimen consisted of etoposide 120 mg/m² and cisplatin 20 mg/m² administered from days 1 to 5, with bleomycin 30 mg/m² given on days 2 and 9. Patients received between 1 and 4 cycles of chemotherapy. Radiotherapy involved para-aortic irradiation of 20 to 30 Gy, depending on histological type and staging. The GAMATOX study received support from a national research project (PHRC n° 200230222) and was approved by the institutional ethics review board. Inclusions occurred between 2010 and 2012 [25, 26]. Patient selection for the current study was based on their complete participation in the initial protocol. To strengthen the study, we included only patients for whom sperm samples were available at all or most follow-up time points (T0, T3, T6, T12, and T24), to ensure reliable longitudinal assessment.

Study participants were recruited from eight centres within the French network of Centres d'Etude et de Conservation des Oeufs et du Sperme (CECOS), namely Caen, Clermont-Ferrand, Grenoble, Marseille, Paris Cochin, Paris Tenon, Rouen, and Toulouse. Informed consent was obtained from all TC patients for the storage of any remaining cryopreserved spermatozoa not used in the initial GAMATOX study in the certified GERMETHEQUE biobank (BB-0033-00081) for future research purposes.

The control group for this study consisted of ten anonymous, healthy, and fertile sperm donors recruited from the Reproductive Biology Laboratory-CECOS of Rouen University Hospital. These individuals had no history of chronic illness or toxic exposure, normal semen parameters according to WHO guidelines [49], a normal blood karyotype (46, XY), and no history of infertility. Control donors were selected among individuals recruited during the study period and were aged between 24 and 43 years, with an abstinence period of 3–4 days. This age range is consistent with the typical age at which TC occurs in young adult males [1–3]. All participants provided written informed consent for the research use of surplus sperm straws that were not employed in ART procedures, in accordance with the maximum number of births permitted per donor under current French regulations. Sperm straws from control donors were also stored in the certified GERMETHEQUE biobank.

2.2 | Semen Analysis and Sperm Cryopreservation

As previously described, patients taking part in the study provided an extra semen sample in addition to the two to three samples required for the fertility preservation protocol [21, 22]. Semen analysis followed the World Health Organization (WHO) manual for the examination and processing of human semen [44] and was in accordance with the checklist published by Björndahl et al. [50]. Semen sampling occurred after 2–7 days of sexual abstinence. After performing conventional semen analysis [i.e., ejaculate volume (mL), pH, sperm concentration (SC, 10^6 /mL), total sperm number (TSN = $SC \times V$, 10^6 /ejaculate), sperm progressive motility (M, WHO grades a+b combined: %), sperm vitality (V, %)], the remaining semen sample from patients and controls was cryopreserved in straws with a cryoprotectant and stored in liquid nitrogen until use.

2.3 | Quantitative Fluorescent In Situ Hybridisation for Telomere Assessment

STL and the number of telomere signals (spots) were determined by Q-FISH using peptide nucleic acid (PNA) probes as previously described [43]. After fixation in methanol, decondensation of sperm nuclei was performed with 1 M NaOH for 3 min. The Telomere PNA FISH kit (DakoCytomation, K5326, Les Ulis, France) was used following the manufacturer's instructions (DakoCytomation). Cy3-labelled (532 nm, red fluorescent signal) PNA telomere probes were mixed before hybridisation with FITC-labelled (488 nm, green fluorescent signal) PNA centromere of chromosome 2 probes, kindly provided by DakoCytomation. Ten consecutive images captured at $\times 900$ magnification on a BX61 epifluorescence microscope (Olympus, Tokyo, Japan) at 9

different focal depths were stacked into a final composite image. Software Bioview Duet v2.3 image analyser (Nes Ziona, Israël) allowed image acquisitions and analyses. A manual analysis of at least 200 spermatozoa heads was performed to assess the number of telomere signals present in each nucleus as previously described [43]. Spermatozoa with only one centromere of chromosome 2, presenting exactly one green spot, are accounted for. The intensity of fluorescent signals was automatically analysed after correction for background autofluorescence, exposure and acquisition times with a specifically designed program for STL developed by the manufacturer (Bioview). Normalisation of the signal to chromosome 2 centromere probe intensity was performed using the following formula: (telomere probe intensity)/(centromere 2 probe intensity) $\times 100$, expressing a relative telomere length in fluorescent relative unit (FRU). We considered patients with severe impairment of STL or sperm telomere interactions (telomere signals) when they presented respectively (1) extremely short relative STL (esSTL) with a mean relative STL below the fifth percentile of controls or (2) the highest number of telomere signals per sperm nucleus (HnTS) with the mean number of telomere signals per nucleus above the 95th percentile of controls.

2.4 | TUNEL Assay

After fixation in methanol, spermatozoa were spread onto glass slides before TUNEL assay to analyse SDF using the in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and as previously described [43]. SDF was assessed in 500 spermatozoa at $\times 1000$ magnification using an epifluorescence microscope (DMRB, Leica, Solms, Germany). The test was considered positive (i.e., sperm DNA considered as fragmented) when the green fluorescence signal occupied more than 50% of the sperm head area. The ratio between the number of sperm nuclei positive for the TUNEL assay and the total number of explored spermatozoa was used to calculate the percentage of SDF.

2.5 | Aniline Blue Staining

Sperm chromatin condensation was evaluated after staining with Aniline blue (AB) staining at 5% pH 3.5 for 5 min (Gurr by VWR Chemicals BDH, Fontenay-sous-Bois, France), as previously described [48]. Five hundred spermatozoa were analysed at $\times 1000$ magnification under a light microscope (Leitz DMRD, Leica). For each spermatozoon, the test was considered positive (i.e., sperm nuclei considered to contain abnormal chromatin condensation) when the dark blue staining occupied more than 50% of the head area. For each patient, the ratio between the number of sperm nuclei positive for AB staining and the total number of explored spermatozoa was used to determine the percentage of spermatozoa with abnormal chromatin condensation.

2.6 | FISH Assay

A three-colour fluorescence in situ hybridisation (FISH) was performed to assess the frequency of chromosomal abnormalities, as previously reported [25], using α -satellite centromeric probes

specific for chromosomes 18 (CEP 18 Spectrum Aqua Blue, Abbott, Rungis, France), X (CEP X Spectrum Green, Abbott) and Y (CEP Y Sat III Spectrum Orange, Abbott). A minimum of 5000 sperm nuclei was examined at $\times 1000$ magnification using a BX61 epifluorescence microscope (Olympus), according to criteria previously described [22]. The sum of the rates of XX, YY and XY diploid spermatozoa corresponds to the diploidy rate. The sum of the rates of presumed disomic XX, YY, 1818 and hyperhaploid XY spermatozoa corresponds to the aneuploidy rate. The addition of aneuploidy and diploidy rates is equal to the total chromosome abnormalities rate.

2.7 | Statistical Analysis

Statistical analysis was performed using R 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Values for quantitative variables are shown as mean \pm standard deviation. Values for qualitative variables are shown in the number of occurrences and the percentage. First, data were compared between the TC patients and controls using the nonparametric Mann–Whitney test adjunct with Holm correction to consider multiple comparisons. Second, sperm characteristics (conventional semen parameters and sperm nuclear alterations) of TC patients were compared between pre- and post-treatment analyses performed at T3, T6, T12 and T24 using the Wilcoxon signed-rank sum test. Finally, treatment regimens were compared using the Mann–Whitney test. Longitudinal analyses assessing sperm characteristics in TC patients during the follow-up were performed using the Friedman test.

3 | Results

3.1 | Population Description

Considering the selection criteria mentioned above, the current study included 29 TC patients from the initial cohort of 129 TC patients originating from the GAMATOX research project. All the semen samples at T0 were collected before orchiectomy and adjuvant chemotherapy or radiotherapy. Among the TC patients included in the current study, 14 of them (14/29, 48%) received chemotherapy in addition to orchiectomy, 3 of them (3/29, 10%) had 1 or 2 BEP cycles and 11 of them (11/29, 38%) had more than 2 BEP cycles; 15 TC patients (15/29, 52%) received radiotherapy in addition to orchiectomy. Fourteen patients (14/29, 48%) had a pure seminoma tumour; 13 of them (13/29, 44.8%) were in the radiotherapy group. Fifteen patients (15/29, 52%) had a non-seminoma tumour and received either chemotherapy (14/15, 93%) or radiotherapy (1/15, 7%). Patients receiving radiotherapy did not miss any semen examinations during follow-up, whereas two patients in the chemotherapy group were lost to follow-up. The first patient missed both the T12 and T24 sample collections, and the second patient missed the T24 sample collection. Three other patients in the chemotherapy group did not complete the T3 sample collection but were subsequently successful.

3.2 | Conventional Semen Parameters before and After TC Treatment

The data of the conventional semen parameters are presented in Tables 1 and 2, as well as in Table S1.

At T0, SC varied from 3 to 180×10^6 /mL among TC patients and was significantly lower in TC patients than in controls ($p = 0.01$) (Table 1). SC did not differ significantly between the chemotherapy and radiotherapy groups ($p = 0.84$) (Table 2). Eight patients (8/29, 28%) presented oligozoospermia, defined as $SC < 15 \times 10^6$ /mL (WHO, 2010), with two patients (2/14, 14%) in the chemotherapy group and six patients (6/15, 40%) in the radiotherapy group (Table 2). Sperm motility did not differ significantly between TC patients and controls ($p = 0.85$), nor between treatment groups ($p = 0.28$) (Tables 1 and 2). Six patients (6/29, 20%) presented asthenozoospermia, defined as a percentage of a+b motile sperm less than 32% (WHO, 2010), one patient (1/15, 7%) from the radiotherapy group and five patients (5/14, 36%) from the chemotherapy group. TSN and sperm vitality did not differ significantly between the treatment groups (Table 2). Conventional semen parameters were not different between pure seminoma and non-seminoma patients before TC treatment (Table S1).

At T3, a drastic decline in both SC and TSN was observed in both treatment groups; however, a more pronounced reduction was evident in the chemotherapy group compared with the radiotherapy group ($p = 6.10^{-6}$ and $p = 5.10^{-6}$, respectively) (Table 2). All patients in the chemotherapy group exhibited oligozoospermia, with 12 of them (12/14, 86%) demonstrating severe oligozoospermia, characterised by $SC < 1 \times 10^6$ /mL. SC remained significantly lower in the chemotherapy group than in the radiotherapy group until T12 ($p = 0.03$). During the entire follow-up period, SC remained lower in patients than in the control group ($p = 0.025$) (Table 1). Furthermore, both SC and TSN were found to be significantly higher in pure seminoma than in non-seminoma tumours ($p = 3.10^{-4}$ and $p = 2.10^{-4}$, respectively) (Table S1). Finally, close to 93% of patients (14/15) with non-seminoma tumours presented with oligozoospermia compared with 57% (8/14) in the pure seminoma group ($p = 0.0352$) (Table S1).

At T6, both SC and TSN exhibited an upward trend, yet remained significantly lower than their respective pre-treatment values (Table 1). The number of patients with severe oligozoospermia was substantially reduced at T6 (4/29, 14%) in comparison to T3 (15/29, 52%), particularly within the chemotherapy group, where only 21% of patients (3/14) exhibited severe oligozoospermia (Table 2).

At T12, both SC and TSN continued to rise, reaching pre-treatment values in both chemotherapy and radiotherapy groups. Concurrently, the proportion of patients with oligozoospermia continued to decrease (13/28, 46%), and none of these patients presented severe oligozoospermia (Table 1). The radiotherapy group demonstrated a more rapid recovery from the SC and TSN values observed before treatment. The SC and TSN of the radiotherapy group were significantly higher compared with the chemotherapy group ($p = 0.03$ for SC and $p = 0.01$ for TSN, respectively) (Table 2).

At T24, 19 patients (19/27, 66%) recovered pre-treatment SC (Table 1), 11 patients (11/15, 73%) after radiotherapy and 8 patients (8/12, 67%) after chemotherapy. Twenty-five patients (25/27, 93%) achieved normozoospermia, defined as $SC \geq 15 \times 10^6$ /mL (WHO, 2010) (Table 2). At T24, no difference in semen parameters

TABLE 1 | Conventional semen parameters in controls and patients before and after TC treatment.

Semen parameters	Controls <i>n</i> = 10	Patients				
		Before treatment (T0) <i>n</i> = 29	After treatment			
			3 months (T3) <i>n</i> = 29	6 months (T6) <i>n</i> = 29	12 months (T12) <i>n</i> = 28	24 months (T24) <i>n</i> = 27
Sperm count (10^6 /mL)	65.9 ± 16.3 ^a	41.9 ± 40.3 ^{a,b,c}	12.9 ± 16.8 ^b	20.6 ± 38.1 ^c	25.0 ± 21.8 ^c	45.5 ± 31.5 ^a
Total sperm number (10^6 /ejaculate)	ND	185.8 ± 222.2 ^{b,c}	49.4 ± 76.3 ^b	89.7 ± 201 ^c	102.3 ± 105.5	167.0 ± 116.7
Sperm progressive motility (WHO a + b. %)	44.0 ± 4.6	43.2 ± 5.5	34.0 ± 21.4	37.4 ± 17.3	39.9 ± 15.9	40.7 ± 13.2
Vitality (living spermatozoa %)	ND	67.0 ± 11.6	67.2 ± 15.4	66.6 ± 19.8	67.6 ± 13.3	69.7 ± 12.3
Oligozoospermia						
Severe <math><1 \times 10^6</math>/mL, <i>n</i> (%)	0 (0)	0 (0) ^{d,e}	15 (52) ^{d,e,f,g,h}	4 (14) ^{d,f}	0 (0) ^{d,g}	0 (0) ^{d,h}
<math><15 \times 10^6</math>/mL, <i>n</i> (%)	0 (0)	8 (28) ^{i,j,k,l}	25 (86) ^{i,j,m,n}	23 (79) ^{i,k,o,p}	13 (46) ^{i,m,o,p,q}	1 (4) ^{j,l,n,p,q}

Note: Data are expressed as mean ± standard deviation.

Abbreviations: *n*: number; ND: not determined.

^aTC patients before treatment versus controls, *p* < 0.05.

^{b,c}TC patients before and after treatment, (b) *p* < 0.00005, (c) *p* < 0.0005.

^{d,e,f,g,h}Severe oligozoospermia before and after treatment, (d, e, g, h) *p* < 0.0001, (f) *p* = 0.0045.

^{i,j,k,l,m,n,o,p,q}Oligozoospermia before and after treatment, (i, j, n, p) *p* < 0.0001, (k) *p* = 0.0002, (l) *p* = 0.0257, (m) *p* = 0.002, (o) *p* = 0.014, (q) *p* = 0.004.

was observed between patients treated with chemotherapy or radiotherapy and compared with pre-treatment values (Tables 1 and 2).

Longitudinal analysis confirmed a significant effect of time after the end of TC treatment on SC and TSN recovery (*p* = 4.10⁻⁸ and *p* = 1.10⁻⁸, respectively) and a significantly lower motility at T3 (*p* = 0.006) (Tables 1 and 2). Two years after TC treatment, sperm motility and vitality were significantly higher in pure seminoma than in non-seminoma tumours (*p* = 0.04 and *p* = 0.04, respectively) (Table S1).

3.3 | Sperm Nuclear Alterations before and After TC Treatment

The data on sperm nuclear alterations are presented in Table 3 for control subjects and for patients before treatment and during the follow-up, and in Table 4 when comparing TC patients according to the adjuvant treatment received (chemotherapy or radiotherapy). Table S2 reports sperm nuclear alterations considering pure seminoma and non-seminoma tumours.

At T0, the number of telomere signals per sperm nucleus was significantly higher in TC patients than in controls (*p* = 0.004) (Table 3). This parameter did not differ significantly between treatment groups (*p* = 0.50) (Table 4) or between pure seminoma and non-seminoma patients (*p* = 0.33) (Table S2). No difference in the relative STL was observed between TC patients and controls (*p* = 0.31) (Table 3), between treatment groups (*p* = 0.16) (Table 4), or between pure seminoma and non-seminoma tumours (*p* = 0.29) (Table S2). The rates of SDF,

chromatin condensation defects, chromosome aneuploidy or total numerical chromosome abnormalities did not differ significantly between TC patients and controls (Table 3), treatment groups (Table 4), or pure seminoma and non-seminoma tumours (Table S2).

At T3, the number of telomere signals per sperm nucleus and the relative STL did not differ significantly from the pre-treatment values. The same results were observed for SDF and chromatin condensation defects (Table 3). Aneuploidy and total chromosomal abnormality rates increased at T3 compared with pre-treatment values (*p* = 0.01 and *p* = 0.001, respectively). The rate of total chromosomal abnormalities was significantly higher in patients compared with controls at T3 (*p* = 0.016) (Table 3). No differences were observed between treatment groups (Table 4) or histological types for the different sperm nuclear alterations examined (Table S2).

At T6, the number of telomere signals per sperm nucleus and the relative STL did not differ significantly from pre-treatment values and when considering treatment groups and histological types. Aneuploidy and total chromosomal abnormality rates remained elevated compared with pre-treatment values (*p* = 0.01 and *p* = 0.001, respectively) (Table 3). The diploidy rate was higher in the radiotherapy group than in the chemotherapy group (*p* = 0.03) (Table 4), while pure seminoma or non-seminoma tumours did not induce any change (Table S2). Total sperm chromosome abnormality and diploidy rates were significantly higher in patients compared with controls (*p* = 0.019 and *p* = 0.02, respectively) (Table 3). There were no differences in SDF and chromatin condensation defects between patients and controls, treatment groups or histological types (Tables 3 and 4; Table S2).

TABLE 2 | Evolution of conventional semen parameters in chemotherapy and radiotherapy groups during the entire follow-up.

Semen parameters	Before treatment				After treatment					
	(T0)		3 months (T3)		6 months (T6)		12 months (T12)		24 months (T24)	
	CT n = 14	RT n = 15	CT n = 14	RT n = 15	CT n = 15	RT n = 15	CT n = 13	RT n = 15	CT n = 12	RT n = 15
Adjuvant treatment										
Sperm count (10^6 /mL)	33.5 ± 22.6	49.7 ± 51.4	0.34 ± 0.5 ^a	22.0 ± 17.1 ^a	11.0 ± 23.9 ^b	29.5 ± 46.9 ^b	15.1 ± 13.0 ^c	33.6 ± 24.4 ^c	49.9 ± 33.4	42.0 ± 30.6
Total sperm number (10^6 /ejaculate)	123.6 ± 105	243.8 ± 284	1.14 ± 1.6 ^a	84.6 ± 85 ^a	40.2 ± 75 ^b	135.9 ± 266 ^b	53.4 ± 51 ^c	144.6 ± 122 ^c	191.0 ± 149	147.9 ± 83
Sperm progressive motility (WHO a + b, %)	40.8 ± 12.3	45.5 ± 10.4	25.9 ± 26.4	39.9 ± 15.2	32.4 ± 17.3	42.1 ± 16.4	36.6 ± 14.6	42.7 ± 16.8	36.2 ± 9.3	44.3 ± 14.9
Vitality (living spermatozoa, %)	64.5 ± 12.9	69.4 ± 10.2	62.0 ± 23.3	69.3 ± 11.3	60.8 ± 25.6	70.8 ± 13.7	67.2 ± 10.8	67.9 ± 15.3	68.2 ± 15.5	70.9 ± 9.4
Oligozoospermia										
Severe $<1 \times 10^6$ /mL (n, %)	0 (0) ^{d,e}	0 (0)	12 (86) ^{d,e,f,g,h}	0 (0)	3 (21) ^{d,f}	1 (7)	0 (0) ^{d,g}	0 (0)	0 (0) ^{d,h}	0 (0)
$<15 \times 10^6$ /mL (n, %)	2 (14) ^{i,j,k,l}	6 (40)	14 (100) ^{j,m}	8 (53)	13 (93) ^{k,n}	10 (67)	10 (69) ^{l,o}	4 (27)	1 (8)	1/15 (7) ^{m,n,o}

Note: Data are expressed as mean ± standard deviation.

Abbreviations: CT: chemotherapy; n: number; RT: radiotherapy.

^{a,b,c}Chemotherapy group versus radiotherapy at each time point of follow-up, (a) $p < 0.0005$, (b, c) $p < 0.05$.

^{d,e,f,g,h}Severe oligozoospermia before and after treatment in patients treated by chemotherapy, (d, e, g, h) $p < 0.00001$, (f) $p = 0.018$.

^{i,j,k,l,m,n,o}Oligozoospermia before and after treatment, (i, j, k, m, n) $p < 0.00001$, (l) $p = 0.0018$, (o) $p = 0.001$.

At T12 and T24, sperm nuclear alterations (i.e., relative STL, number of telomere signals, aneuploidy, diploidy, total chromosome abnormality, SDF, chromatin condensation defect) were overall comparable to pre-treatment values and did not significantly differ across treatment groups (Tables 3 and 4) or histological types (Table S2), except for the number of telomere signals at T24, which was significantly higher in patients with seminoma compared with those with non-seminoma tumours. At the end of the follow-up, 8/12 (67%) patients in the chemotherapy group and 8/15 (53%) in the radiotherapy group achieved pre-treatment STL values.

Throughout the follow-up, except at T6, the number of telomere signals per sperm nucleus remained significantly higher in TC patients compared with controls ($p = 4.10^{-3}$ at T0, $p = 7.10^{-4}$ at T3, $p = 0.05$ at T6, $p = 3.10^{-4}$ at T12, $p = 1.10^{-3}$ at T24, respectively) (Table 3). The treatment received did not alter the number of telomere signals per sperm nucleus at any time point (Table 4). At T24, pure seminoma patients had a higher mean number of telomere signals per sperm nucleus than non-seminoma patients, as mentioned above ($p = 0.04$) (Table S2).

At T24, although the relative STL reached pretreatment values in both treatment groups, 42% (4/12) of patients in the chemotherapy group and 20% (3/15) of patients in the radiotherapy group had esSTL ($p = 0.6618$) (Table S3). In comparison, 50% (7/14) of patients in the chemotherapy group and 40% (6/15) of patients in the radiotherapy group had esSTL at T0 ($p = 0.7152$). During the follow-up, 33% (9/27) of patients with STL starting below the fifth percentile of control males crossed the threshold. Conversely, 11% (3/27) of patients showed the opposite trend, while 19% (5/27) of patients remained thoroughly below the fifth percentile. In addition, at T24, nearly 67% (8/12) of patients in the chemotherapy group and 87% (13/15) of patients in the radiotherapy group had HnTS ($p = 0.3575$). In comparison, respectively 64% (9/14) of patients in the chemotherapy group and 93% (14/15) of patients in the radiotherapy group had HnTS at T0 ($p = 0.0801$) (Table S3). A total of 6/27 (22%) patients ended up with both esSTL and hnTS, with 4/12 (33%) after chemotherapy and 2/15 (13%) after radiotherapy ($p = 0.3575$). Of these, all had normal SC and no significant changes in conventional semen parameters and other sperm nuclear changes at the end of the follow-up. In addition, 33% (9/27) of patients in both groups had a total chromosome abnormality rate above the 95th percentile of controls. Severe SDF, namely SSDF, higher than the 95th percentile of controls, was observed in 5/12 (42%) and 4/15 (27%) patients following chemotherapy and radiotherapy, respectively ($p = 0.4479$). Severe sperm chromatin condensation defects, higher than the 95th percentile of controls, were observed in 6/12 (50%) and 6/15 (40%) patients after chemotherapy and radiotherapy, respectively ($p = 0.7068$). Comparison with pre-treatment values showed no significant difference (Table S3).

4 | Discussion

This pilot and exploratory study showed for the first time a higher number of telomere signals per sperm nucleus in TC patients compared with controls, both before and after adjuvant treatment, even in the case of recovery of normal conventional semen parameters. In addition, the relative STL of TC patients

TABLE 3 | Sperm nuclear alterations in controls and TC patients during the entire follow-up.

Sperm nuclear parameters	Patients					
	Controls <i>n</i> = 10	Before treatment	After treatment			
		(T0) <i>n</i> = 29	3 months (T3) <i>n</i> = 18	6 months (T6) <i>n</i> = 23	12 months (T12) <i>n</i> = 24	24 months (T24) <i>n</i> = 27
Number of telomeres (fluorescent signals per sperm nucleus)	15.2 ± 1.4 ^a	18.11 ± 2.7 ^a	18.97 ± 3.4 ^a	17.3 ± 3.1	18.38 ± 2.9 ^a	18.0 ± 3.2 ^a
Telomeres > 23/sperm nucleus (%)	3.78 ± 3.3 ^a	10.52 ± 10.9	10.54 ± 6.2 ^a	10.21 ± 14.2	11.98 ± 13.8 ^a	11.16 ± 15.0
STL (fluorescent relative units, FRU)	52.13 ± 15.1	47.58 ± 16.8	47.32 ± 16.5	46.35 ± 14.8	48.23 ± 14.3	48.73 ± 16.1
SDF (TUNEL positive spermatozoa, %)	7.42 ± 4.6	8.18 ± 4.2	15.63 ± 8.5	14.39 ± 7.4	13.16 ± 7.0	11.94 ± 7.3
Chromatin condensation defect (Aniline blue-stained spermatozoa, %)	9.6 ± 2.8	11.9 ± 6.7	8.54 ± 3.9	10.35 ± 6.4	9.23 ± 4.5	8.43 ± 4.0
	<i>n</i> = 10	<i>n</i> = 29	<i>n</i> = 17	<i>n</i> = 26	<i>n</i> = 27	<i>n</i> = 27
Aneuploidy (%)	0.47 ± 0.2	0.48 ± 0.2 ^{b,c}	0.84 ± 0.7 ^b	0.82 ± 0.7 ^c	0.56 ± 0.4	0.51 ± 0.3
Diploidy (%)	0.19 ± 0.1 ^d	0.21 ± 0.2 ^{b,c}	0.35 ± 0.3 ^b	0.37 ± 0.3 ^{c,d}	0.31 ± 0.3	0.25 ± 0.2
Total chromosomal abnormalities (%)	0.65 ± 0.3 ^d	0.68 ± 0.3 ^c	1.19 ± 0.7 ^{c,d}	1.19 ± 0.8 ^{c,d}	0.87 ± 0.5	0.75 ± 0.4

Note: Data are expressed as mean ± standard deviation except for the sperm relative telomere length, expressed as median ± standard deviation.

Abbreviations: *n*: number; nd: not determined; SDF: sperm DNA fragmentation; STL: sperm telomere length.

^{a,d}TC patients before or after treatment versus controls, (a) *p* < 0.005, (d) *p* < 0.05.

^{b,c}TC patients before and after treatment at each time point of follow-up, (b) *p* < 0.05, (c) *p* < 0.005.

remained stable throughout the 2-year follow-up period, considering the adjuvant treatment received or the histological type, and remained comparable to controls. All assessed sperm nuclear alterations returned to pre-treatment values after 2 years. Finally, as in the overall GAMATOX study, sperm count remained lower in patients than in controls throughout the follow-up period, with a recovery time for conventional semen parameters of 24 months [26].

Telomeres consist of a repeated DNA telomere sequence (TTAGGG) and its sheltering protein complex [51]. In somatic cells, telomere erosion functions as a biological clock that limits the number of cell divisions in normal cells [52]. Cancer cells, despite having initially shorter telomeres, require telomere maintenance via telomerase activation to support tumour progression [53]. In human spermatozoa, telomeres are organised in species-specific patterns, mainly as doublets or quadruplets [54, 55]. Thus, the expected number of telomere signals per sperm nucleus ranges from 12 to 23 [56]. Fewer telomere signals may reflect chromatin condensation defects, whereas a higher number suggests disrupted telomere–telomere interactions [45]. Elevated telomere signal numbers have been associated with increased SDF [39, 45]. We previously reported that the number of telomere signals per sperm nucleus is increased in infertile patients [48], findings which have been corroborated in other cohorts of infertile males [46]. Moreover, shorter STL has been reported in oligozoospermic and infertile men [36, 40, 42–45, 48, 56–58], establishing STL as an emerging biomarker of semen quality [36, 59–61], although its predictive value for ART outcomes remains debated [36, 44, 62–65]. To date, no study has evaluated sperm telomeres in TC patients. However, BEP chemotherapy has been shown to impair telomere length in the male germline

of rats, including spermatozoa [66]. Cisplatin shortens telomeres in spermatogonial cells both directly, through DNA damage at telomeric regions, and indirectly, via inhibition of telomerase activity and transcription [67]. Similar effects have been observed with bleomycin [67]. Furthermore, exposure of human testicular cancer cells to cisplatin reduces telomerase activity in vitro [68].

Our study clearly showed sperm telomere alterations at T0 before TC treatment (increased telomere signals and eSTL). Several mechanisms may explain how TC affects sperm telomere integrity. Histological subtype may influence telomere dynamics in TC. Seminoma cells have been shown to possess shorter telomeres than non-malignant cells, while non-seminoma tumours display longer telomeres [48]. Although bioinformatic analyses from The Cancer Genome Atlas support this hypothesis [69], we could not confirm it, likely because of our focus on ejaculated spermatozoa, whereas prior studies examined testicular tumour tissue [53]. In addition, germ cell tumours arise from cells with pre-existing genomic and epigenetic instability, which may extend to adjacent non-malignant germ cells, potentially impairing telomere maintenance [70, 71]. TC and its associated microenvironment generate increased oxidative stress, a major driver of telomere attrition because of the susceptibility of guanine-rich telomeric DNA to reactive oxygen species [72]. The tumour may compromise Sertoli and Leydig cell function (via paracrine cytokines or altered vascular supply) as well as Sertoli and germ cell interaction, thereby disrupting the trophic support and redox homeostasis of spermatogonial stem cells and early spermatocytes, which are particularly reliant on telomere maintenance mechanisms. Indeed, telomere length is influenced by the maturation stage of germ cells [73, 74]. As germ-cell telomerase is typically active during spermatogenesis,

TABLE 4 | Sperm nuclear alterations in TC patients during the entire follow-up period considering the type of treatment (chemotherapy or radiotherapy).

Sperm nuclear parameters	Before treatment						After treatment					
	(T0)		3 months (T3)		6 months (T6)		12 months (T12)		24 months (T24)			
	CT	RT	CT	RT	CT	RT	CT	RT	CT	RT		
Adjuvant treatment	n = 14	n = 15	n = 2	n = 14	n = 9	n = 14	n = 10	n = 14	n = 12	n = 15		
Number of telomeres (fluorescent signals per sperm nucleus)	17.8 ± 3.0	18.4 ± 2.6	18.0 ± 0.7	19.1 ± 3.7	17.8 ± 4.1	17.0 ± 2.4	18.5 ± 2.9	18.3 ± 2.9	17.1 ± 2.1	18.7 ± 3.8		
Telomeres > 23/sperm nucleus (%)	10.7 ± 12.5	10.3 ± 9.6	9.3 ± 0.2	10.7 ± 6.7	15.5 ± 21.7	7.18 ± 6.9	11.7 ± 16.8	12.2 ± 11.9	7.28 ± 9.9	14.3 ± 17.8		
STL (fluorescent relative units, FRU)	42.5 ± 11.0	52.3 ± 20.1	47.7 ± 11.6	47.3 ± 17.3	43.1 ± 15.4	48.2 ± 14.7	43.3 ± 15.9	51.8 ± 12.4	46.1 ± 13.3	50.9 ± 18.2		
	n = 14	n = 15	n = 3	n = 14	n = 8	n = 15	n = 11	n = 15	n = 12	n = 15		
SDF (TUNEL positive spermatozoa, %)	9.4 ± 5.3	7.0 ± 2.5	17.1 ± 9.2	15.3 ± 8.6	12.3 ± 7.4	15.5 ± 7.4	12.7 ± 8.3	13.5 ± 6.3	12.2 ± 8.9	11.7 ± 6.1		
Chromatin condensation defect (Aniline blue-stained spermatozoa, %)	12.6 ± 8.6	11.3 ± 4.5	10.3 ± 3.1	8.2 ± 4.0	11.5 ± 8.8	9.7 ± 4.9	10.3 ± 4.9	8.4 ± 4.1	9.2 ± 4.0	7.8 ± 4.0		
	n = 14	n = 15	n = 3	n = 15	n = 11	n = 15	n = 12	n = 15	n = 12	n = 15		
Aneuploidy (%)	0.44 ± 0.3	0.51 ± 0.2	2.81 ± nd	0.71 ± 0.4	0.66 ± 0.3	0.92 ± 0.9	0.52 ± 0.3	0.59 ± 0.4	0.53 ± 0.3	0.49 ± 0.2		
Diploidy (%)	0.18 ± 0.1	0.24 ± 0.2	0.35 ± nd	0.35 ± 0.3	0.28 ± 0.3 ^a	0.44 ± 0.3 ^a	0.29 ± 0.3	0.32 ± 0.2	0.22 ± 0.2	0.27 ± 0.2		
Total chromosomal abnormalities (%)	0.62 ± 0.3	0.75 ± 0.3	3.16 ± nd	1.06 ± 0.5	0.94 ± 0.5	1.36 ± 0.9	0.81 ± 0.6	0.91 ± 0.5	0.75 ± 0.5	0.76 ± 0.4		

Note: Data are expressed as mean ± standard deviation, except for STL expressed as median ± standard deviation.

Abbreviations: CT: chemotherapy; n: number; R: radiotherapy; SDF: Sperm DNA fragmentation; STL: Sperm telomere length.

^aChemotherapy group versus radiotherapy groups, (a) $p < 0.05$.

any tumour-induced dysregulation of telomerase may impair telomere elongation or protection during germ-cell divisions [75]. Endocrine alterations frequently observed in TC, such as disturbed testosterone or gonadotropin levels [76], may also impact spermatogenic dynamics and telomere regulation.

Patients treated with chemotherapy showed the most severe telomeric alterations: 42% presented esSTL, compared with 20% after radiotherapy. The combined prevalence of HnTS and esSTL reached 33% in the chemotherapy group versus 13% in the radiotherapy group. Sperm count reduction was also more pronounced after chemotherapy. Several hypotheses may explain the differential effects of chemotherapy and radiotherapy on sperm telomeres: (1) telomere elongation mechanisms could be activated in damaged but surviving spermatogonial stem cells (SSCs) after radiotherapy; (2) SSCs with longer telomeres may be more likely to survive, while those with shorter telomeres may undergo apoptosis; or (3) germline variants in telomere maintenance genes could confer resistance to chemotherapy-induced damage. Similarly, a differential susceptibility of SSCs to gonadotoxic treatment has also been proposed to explain varying levels of sperm DNA damage in survivors of Hodgkin versus non-Hodgkin lymphomas [77]. Moreover, oxidative stress, which is significantly elevated in spermatozoa from TC patients, is a well-established contributor to telomere shortening [78]. The presence of esSTL and potentially HnTS may compromise telomere function and cellular replicative potential. Extremely short telomeres are a hallmark of telomere biology disorders, which include a wide range of severe systemic conditions such as bone marrow failure, pulmonary and hepatic diseases, malignancies, and others [79]. Sperm telomere markers (telomere length and interaction, hnTS and esSTL) provide complementary information to other sperm nuclear alterations, as they reflect a distinct dimension of chromosomal integrity related to chromosome-end stability. DNA fragmentation and chromatin condensation defects are mainly associated with impaired fertilisation, abnormal embryo development, and an increased risk of early pregnancy loss [80, 81]. Aneuploidy in spermatozoa contributes to the risk of chromosomal abnormalities in the conceptus and may underlie spontaneous miscarriages or implantation failure [82]. In contrast, telomere alterations appear to be more specifically related to the health and long-term outcomes of the conceptus. Several studies have suggested that abnormal sperm telomere length or interaction may influence not only early embryonic development but also the genomic stability and health of the offspring, potentially contributing to disease susceptibility in adulthood [83, 84]. In TC survivors, sperm telomere abnormalities raise concerns regarding possible consequences for offspring, particularly when conception occurs post-treatment. Additionally, altered telomere architecture may contribute to the biology of germ cell tumours and warrants further investigation.

Regarding SDF, our findings were consistent with those from the initial GAMATOX study. No significant variation was observed before or after treatment during the follow-up, supporting the representativeness of our study population [26]. The slight post-treatment increase in SDF may not have reached significance because of the limited sample size. The increase in SDF following chemotherapy in TC patients has been documented in numerous studies, with results indicating that this phenomenon may be either transient [21, 24, 30, 33] or persistent for several years

after treatment [29]. BEP appears particularly detrimental, with a dose-dependent effect shown in animal models [85]. The combination of chemotherapy and radiotherapy resulted in a greater rate of SDF [30]. We observed a high variability of SDF measured between patients. This raises concerns about individual susceptibility to chemotherapy-induced sperm DNA damage. Regarding severe sperm DNA damage, namely SSDF, we did not observe any difference before and after TC treatment. We report a rate of SSDF 2 years after treatment of 33%, in line with the 23% previously reported [24]. Contrary to Farnetani et al. [24], we did not observe a post-treatment increase in SSDF rates. In addition, chromatin condensation defects remained comparable between TC patients and controls, and between treatment groups, consistent throughout follow-up, although these defects have been reported to be less frequent in TC patients compared with normozoospermic infertile men pre- and post-BEP [32]. The transient rise in sperm aneuploidy at 3- and 6-month post-treatment aligns with previous reports [26, 27, 29]. Chemotherapy also appears to induce both transient and persistent sperm DNA methylation [86]. Fortunately, the offspring of TC survivors do not show a higher rate of congenital abnormalities than the general population [87].

This study presents several notable strengths. The comprehensive and integrative design, combining multiple validated methodologies to assess a wide range of sperm nuclear quality parameters, constitutes a key methodological strength of this pilot longitudinal investigation and ensures the robustness of the generated dataset. Moreover, to our knowledge, this is the first study to specifically investigate sperm telomere characteristics in patients with TC. Reproducibility of previous GAMATOX findings regarding semen parameters, SDF, chromatin condensation defects, and aneuploidy strengthens the generalizability of our results. The multicentre design and inclusion of different histological types enhance robustness. Centralised analysis minimised inter-laboratory variation. The two-year follow-up period is a strength, with minimal loss to follow-up. Although telomere assessment was conducted on cryopreserved spermatozoa, previous studies have reported that the freezing procedure does not affect sperm telomere length [88]. While the longitudinal design is a major asset, the cohort of 29 TC patients and 10 fertile donors does not allow broad generalisation because of the relatively small sample size. The non-random selection of TC patients from the GAMATOX cohort, based on the availability of complete semen samples and sufficient material for nuclear analyses, may have introduced selection bias. Moreover, age was not considered in this study and could represent a potential confounding factor. Nevertheless, this pilot investigation provides valuable exploratory data to guide future confirmatory studies.

5 | Conclusion

Our pilot and exploratory study raises the question of which spermatozoa are most suitable for conception in TC survivors: those cryopreserved before treatment or fresh samples collected after spermatogenesis recovery. As conventional and nuclear sperm parameters generally returned to pre-treatment values after 2 years, cryopreserved sperm should be reserved for patients with persistent abnormalities. However, inter-individual variability and treatment-specific effects, particularly on DNA

integrity, call for personalised evaluation post-treatment. The persistent increase in telomere signals per sperm nucleus suggests altered telomere interactions in TC patients, potentially linked to both treatment effects and tumour biology, and warrants further investigation. In addition, the higher prevalence of esSTL after chemotherapy may reflect long-lasting telomeric damage and raises concerns about its potential impact on fertility and offspring health.

Author Contributions

Benoit Berby performed the investigation, formal analysis, and drafted the original manuscript. Louis Bujan was responsible for the study conceptualisation, data curation, and edited and modified the paper. Nathalie Rives was responsible for the study conceptualisation, methodology, investigation, data curation, supervision, and edited and modified the manuscript. Sylvianne Hennebicq, Florence Brugnon, Isabelle Berthaut, Myriam Daudin, Ludovic Dumont, Christine Rondanino, and Aurélie Feraille were responsible for data curation, edited and modified the paper. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1: Evolution of conventional semen parameters in seminoma and non-seminoma tumours during the entire follow-up. **Table S2:** Evolution of sperm nuclear alterations in seminoma and non-seminoma tumours during the entire follow-up. **Table S3:** Evolution of sperm nuclear alterations before and after TC treatment as a function of the 5th or 95th percentile of controls and considered as severe sperm nuclear alterations