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Original article

Spermatogenesis after gonadotoxic childhood treatment: follow-up of 12 patients

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ABSTRACT

STUDY QUESTION: What is the long-term impact of presumed gonadotoxic treatment during childhood on the patient's testicular function at adulthood?

SUMMARY ANSWER: Although most patients showed low testicular volumes and some degree of reproductive hormone disruption 12.3 (2.3–21.0) years after gonadotoxic childhood therapy, active spermatogenesis was demonstrated in the semen sample of 8 out of the 12 patients.

WHAT IS KNOWN ALREADY: In recent decades, experimental testicular tissue banking programmes have been set up to safeguard the future fertility of young boys requiring chemo- and/or radiotherapy with significant gonadotoxicity. Although the risk of azoospermia following such therapies is estimated to be high, only limited long-term data are available on the reproductive potential at adulthood.

STUDY DESIGN, SIZE, DURATION: This single-centre prospective cohort study was conducted between September 2020 and February 2023 and involved 12 adult patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: This study was carried out in a tertiary care centre and included 12 young adults (18.1–28.3 years old) who had been offered testicular tissue banking prior to gonadotoxic treatment during childhood. All patients had a consultation and physical examination with a fertility specialist, a scrotal ultrasound to measure the testicular volumes and evaluate the testicular parenchyma, a blood test for assessment of reproductive hormones, and a semen analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Testicular tissue was banked prior to the gonadotoxic treatment for 10 out of the 12 included patients. Testicular volumes were low for 9 patients, and 10 patients showed some degree of reproductive hormone disruption. Remarkably, ongoing spermatogenesis was demonstrated in 8 patients at a median 12.3 (range 2.3–21.0) years post-treatment.

LIMITATIONS, REASONS FOR CAUTION: This study had a limited sample size, making additional research with a larger study population necessary to verify these preliminary findings.

WIDER IMPLICATIONS OF THE FINDINGS: These findings highlight the need for multicentric research with a larger study population to establish universal inclusion criteria for immature testicular tissue banking.

STUDY FUNDING/COMPETING INTEREST(S): This study was conducted with financial support from the Research Programme of the Research Foundation-Flanders (G010918N), Kom Op Tegen Kanker, and Scientific Fund Willy Gepts (WFWG19-03). The authors declare no competing interests.

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This study was registered on 6 December 2019, and the first patient was enrolled on 8 September 2020.

Keywords: testicular tissue banking / fertility preservation / male infertility / gonadotoxic treatment / cancer

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WHAT DOES THIS MEAN FOR PATIENTS?

Since chemo- and radiotherapy can cause infertility, men are recommended to freeze sperm before the start of their treatment. For young boys who cannot yet produce mature spermatozoa, freezing a piece of testicular tissue (containing spermatogonial stem cells) is the only option to preserve their fertility. However, this technique is still experimental and only recommended for patients at significant risk of infertility. As the available data on the later fertility of these patients are still limited, it is difficult to establish inclusion criteria.

Therefore, this study evaluated the testicular function of 12 men who underwent chemo- and/or radiotherapy with gonadotoxic risk during childhood. Although most patients had small testicles and disturbed hormone levels at adulthood, 8 out of the 12 patients were able to produce mature spermatozoa. This suggests that a large proportion of patients might be able to have children after presumed gonadotoxic chemo- and/or radiotherapy during childhood. However, as it is currently not yet possible to predict which patients will remain fertile, more research is needed before universal inclusion criteria can be established.

Introduction

Chemotherapy, whether or not combined with radiotherapy, is commonly used to treat malignant diseases or as conditioning therapy for certain non-malignant disorders, e.g. sickle cell disease or thalassaemia. Continuous progress of these treatment protocols has increased the long-term survival rate of childhood cancer patients, up to 80% (Hudson, 2010). This long-term survival enables further investigation of the long-term effects of gonadotoxic treatments on the patient's quality of life. Depending on the dosage and duration, the gonadotoxic features of chemo- and radiotherapy can cause transient to permanent infertility (Kenney *et al.*, 2012; Vakalopoulos *et al.*, 2015; Stukenborg *et al.*, 2018a). Therefore, adult men are recommended to cryopreserve mature spermatozoa before starting any gonadotoxic treatment. Peripubertal patients are asked to provide a semen sample for banking, if possible. For prepubertal and early pubertal boys without active spermatogenesis, banking of immature testicular tissue containing spermatogonial stem cells (SSCs) is the only option to preserve their fertility.

In 2002, the Universitair Ziekenhuis (UZ) Brussel was one of the first worldwide to launch a clinical programme for immature testicular tissue banking (TTB). Over the years, immature TTB has been implemented in many fertility centres around the world (Picton *et al.*, 2015; Goossens *et al.*, 2020). Nevertheless, until fertility restoration methods following TTB become clinically available, this technique is still considered experimental. Therefore, our centre only recommends immature TTB to patients with a significant risk of treatment-related infertility [e.g. chemotherapy with a cyclophosphamide equivalent dose (CED) $\geq$4000 mg/m^2, total body irradiation (TBI) (Green *et al.*, 2014a; Poganitsch-Korhonen *et al.*, 2017; Mulder *et al.*, 2021)]. However, there is currently still much uncertainty as to which gonadotoxic treatments truly give a significant risk of infertility at adulthood (Delgouffe *et al.*, 2022). Owing to this lack of consensus, fertility centres continue to use their own inclusion criteria for TTB (Wyns *et al.*, 2011; Picton *et al.*, 2015; Stukenborg *et al.*, 2018b; Braye *et al.*, 2019; Valli-Pulaski *et al.*, 2019; Goossens *et al.*, 2020; Rives *et al.*, 2022).

Ample research has already been performed on the fertility status of adult men following chemotherapy treatment. Long-term data on the fertility outcomes after gonadotoxic treatment during childhood, however, are still scarce (Uijldert *et al.*, 2017; Borgström *et al.*, 2020; Kanbar *et al.*, 2020; Mathiesen *et al.*, 2021). Therefore, this study aimed at investigating the testicular function of young adults who received chemo- and/or radiotherapy with gonadotoxic risk to treat a (non-)malignant disease during childhood and were therefore proposed TTB.

Materials and methods

This single-centre prospective cohort study was conducted between September 2020 and February 2023 at the UZ Brussel.

Ethical approval

Ethical approval for this study has been obtained from the Institutional Ethical Review Board of the UZ Brussel (B.U.N. 143201941462). Patient data were anonymized after collection for further data processing. Written informed consent from the patients or their parents was required for participation in this study.

Patients

This study included young adults who underwent gonadotoxic treatment during childhood to treat a malignant or non-malignant disease. Upon diagnosis, these young adults were referred to the fertility preservation programme of the UZ Brussel for immature TTB because of the significant infertility risk related to their treatment protocol (Delgouffe *et al.*, 2022). Our fertility preservation programme and immature TTB procedure were previously described (Braye *et al.*, 2019). The biopsy procedure was combined with the placement of a central venous catheter or other procedures to minimize the need for general anaesthesia. After the biopsy procedure, the testicular tissue was cut into small fragments (±6 mm^3). One fragment was used to perform immunohistochemical staining for melanoma-associated antigen 4 (MAGE-A4) to confirm the presence of spermatogonia in the testicular biopsies. The remaining fragments were cryopreserved using a slow freezing protocol (Braye *et al.*, 2019). For 4 patients, testicular biopsies were cryopreserved as testicular cell suspensions by using ethylene glycol, as previously published (Frederickx *et al.*, 2004).

Both the patients who accepted and those who refused immature TTB were invited to participate in the study once they turned 18 years old. Patients undergoing treatment during the study period because of relapse were excluded. An invitation letter was sent to the parents of the patients by the study nurses of the oncofertility department. When there was no response to the invitation letter after 3 weeks, the parents were contacted by telephone or e-mail.

Data collection

All patients were invited for a consultation and physical examination with a fertility specialist, a scrotal ultrasound to measure testicular volume, a blood test for assessment of reproductive hormones, and a semen analysis (Fig. 1). Additionally, relevant background data, including data on the MAGE-A4 staining, were collected from the patient's medical records.

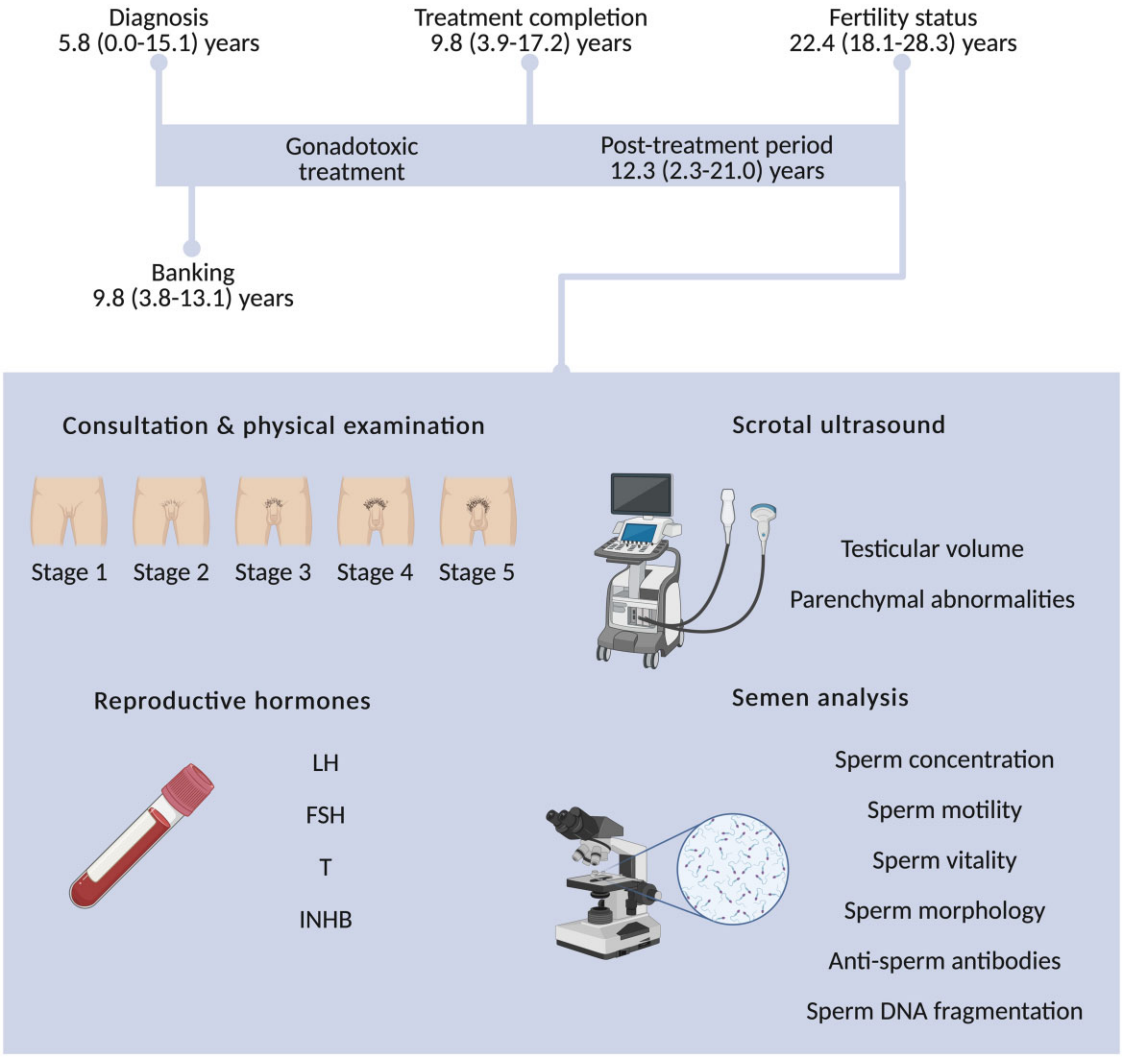

Figure 1. Schematic overview of the study design to investigate the testicular function after gonadotoxic childhood treatment. T: testosterone; INHB: inhibin B.

During the consultation, medical data related to diagnosis, treatment received during childhood, and TTB were verified with the patient. Relevant information about the patient's lifestyle and general health status (smoking, alcohol and drug use, obesity, etc.) was also recorded. The physical examination consisted of an assessment of the patient's pubertal maturation according to Tanner stages (Marshall and Tanner, 1970).

Testicular growth and parenchymal abnormalities—such as fibrotic lesions and calcifications—were assessed by a radiologist on scrotal ultrasound using a 14L5 linear transducer (Canon Medical Systems N.V. Belgium, Zaventem, Belgium). Testicular volumes were calculated using the formula of Lambert (length × width × height × 0.71) (Hsieh *et al.*, 2009) and scored according to reference values (Sotos and Tokar, 2017).

A morning blood sample was collected to evaluate the patient's reproductive hormone levels [LH, FSH, testosterone, and inhibin B (INHB)]. Blood samples were analysed at the Clinical Chemistry & Radioimmunology laboratory of the UZ Brussel using the Elecsys LH, FSH, and testosterone assay on the Cobas e immunoassay analyser (Roche diagnostics, Machelen, Belgium) or the Inhibin B Gen II ELISA kit (Diagnostics Systems Laboratories, Webster, TX, USA).

After an abstinence period of 2–7 days, a semen analysis was performed according to the 2021 World Health Organization (WHO) criteria (World Health Organization, 2021) to assess the sperm concentration, motility, vitality, and morphology, the presence of anti-sperm antibodies, and the sperm DNA fragmentation index (DFI). This sperm analysis complied with the published checklist unless stated otherwise (Björndahl *et al.*, 2015). A macroscopic examination consisted of measures of semen volume, pH, and viscosity as well as the appearance and liquefaction of the ejaculate. Sperm concentration and motility were examined by evaluating at least 200 spermatozoa (if possible) in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). At low sperm concentration ($<2 \times 10^6$/ml), the semen sample was concentrated by centrifugation at 600 *g* for 10 min at room temperature. Sperm vitality was assessed using an Eosin Y test (VWR International BV, Leuven, Belgium) to distinguish live (Eosin Y negative) from dead (Eosin Y positive) spermatozoa. Sperm morphology (head, neck, midpiece, and tail) was determined using the Kruger strict criteria (Kruger *et al.*, 1986) after a modified Shorr-Papanicolaou staining (VWR International BV, Leuven, Belgium). The presence of small/large vacuoles and small acrosomes was visually assessed. Acrosomes were considered small if they comprised <40% of the head area. Sperm heads containing large vacuoles, or more than 2 small vacuoles were scored as vacuolated. The mixed antiglobulin reaction test (SpermMar Test

IgG and IgA, FertiPro N.V., Beernem, Belgium) was performed on the fresh semen sample to search for motile spermatozoa with surface-bound antibodies. Microscopic examinations were performed on a phase-contrast Olympus BX 43 microscope (Olympus Belgium S.A./N.V., Antwerp, Belgium) at ×200 magnification. Sperm DNA fragmentation was assessed using the GoldCyto Sperm DNA kit (Microptic S.L., Barcelona, Spain). The test is based on the principle that, following acid denaturation and removal of nuclear proteins, sperm will produce the characteristic halo of dispersed DNA loops. The presence of the halo was assessed in about 500 cells using the Sperm Class Analyzer CASA system (Microptic S.L., Barcelona, Spain). The spermatozoa with fragmented DNA either show a minimal or no dispersion halo. The DFI represents the percentage of spermatozoa showing DNA fragmentation per number of cells analysed. DNA fragmentation testing was only feasible for patients with a sperm concentration of at least 5×10^6/ml of which sufficient semen remained after performing the previously mentioned tests. In case of azoospermia, a second semen analysis was performed shortly after the first one to confirm the azoospermia. In addition, in case of a low motile sperm concentration ($<0.5 \times 10^6$/ml motile sperm) (Hargreave and Elton, 1983), the complete semen analysis was repeated 1 year later.

Statistical analysis

Descriptive statistics were performed with MS Excel version 16.74 (Microsoft, Redmond, WA, USA). Patient's age (years) at diagnosis, at testicular biopsy and at treatment completion, and the follow-up period post-treatment (years) are presented as median (range). A Spearman correlation test was performed in GraphPad Prism version 9.3.1 (GraphPad software, La Jolla, CA, USA) to analyse the degree of association between the physical examination data and semen analysis results. A value of $P < 0.05$ was considered statistically significant.

Results

Patient background

A total of 40 young adults who underwent gonadotoxic treatment during childhood and were proposed TTB at the UZ Brussel met the previously mentioned selection criteria and were invited to participate in this study. Twelve patients (30%) decided not to participate in the study and 16 patients (40%) did not reply. The remaining 12 young adults (30%) accepted to participate in this study.

Table 1 displays the patients' clinical background information. During childhood, 7 patients were treated with myeloablative conditioning therapy prior to haematopoietic stem cell transplantation (HSCT) for sickle cell disease (4/7), chronic granulomatous disease (1/7), acute lymphoblastic leukaemia (1/7), and thalassaemia major (1/7). The patients with sickle cell disease were also treated with hydroxyurea for an average of 3 (2–6) years. For the remaining 5 patients, childhood treatment consisted of non-conditioning chemo- and/or radiotherapy for acute lymphoblastic leukaemia (2/5), nasopharyngeal tumour (1/5), Hodgkin's lymphoma (1/5), and non-Hodgkin's lymphoma (1/5). The median age at diagnosis was 5.8 (neonatal–15.1) years and 9.8 (3.9–17.2) years at treatment completion (Fig. 1). When applicable, the CED was calculated to estimate the gonadotoxic risk of the patient's treatment (Table 1). The CED is a method that quantifies cumulative alkylating chemotherapy and was first described by Green et al. (2014b). A CED of $\geq$4000 mg/m^2, a cisplatin dose $\geq$500 mg/m^2, or TBI are considered as treatments with a significant risk of infertility (Green et al., 2014a; Lambertini et al., 2016; Poganitsch-Korhonen et al., 2017).

An immature TTB for fertility preservation was performed in 10 out of the 12 patients at a median age of 9.8 (3.8–13.1) years (Fig. 1). Most patients underwent testicular biopsy before puberty and within 2 months after their diagnosis, except for the patients with a non-malignant haematological disorder. Of these 10 patients, 5 underwent a hemi-orchiectomy and 4 a unilateral orchiectomy (Table 1). For 1 patient, the size of the biopsy was not recorded. There were no complications recorded related to the biopsy procedure. The MAGE-A4 immunohistochemical results at the time of TTB identified 3 patients with a normal to high number of spermatogonia (Patients 4, 5, and 12) and 2 patients with a low number of spermatogonia (Patients 8 and 10) according to the reference values of Masliukaite et al. (2016) (Supplementary Table S1). In 1 patient (Patient 9), no spermatogonia could be found. For the remaining 6 patients, MAGE-A4 staining was not performed because the biopsied tissue was banked as a cell suspension (n = 4), or the patient did not bank testicular tissue (n = 2). At the time of fertility assessment, 12.3 (2.3–21.0) years post-treatment, the patients had a median age of 22.4 (18.1–28.3) years (Fig. 1).

Testicular function at adulthood

Most patients lived a healthy lifestyle with a normal BMI ranging from 19 to 25 kg/m^2. However, 2 patients were daily smokers (Patients 3 and 4), and 2 other patients were obese (Patients 6 and 9 with a BMI of 37 and 34 kg/m^2, respectively, and development of type 2 diabetes for Patient 9). None of the patients needed testosterone substitution treatment during pubertal development. All patients were postpubertal at consultation based on the secondary sexual characteristics described by Marshall and Tanner (1970). None of the included patients had already attempted to conceive or successfully fathered children.

Data on testicular volume and reproductive hormone levels of the young adults are specified in Table 2. Small testicular volumes below the reference limit of 15.2 ml were detected for 9 patients. In the 5 patients who underwent a hemi-orchiectomy, the volume of the biopsied testis was 1–5 ml smaller than the contralateral testis. In Patient 4, this difference was 19 ml. Testicular abnormalities were observed in only 2 cases: Patient 8 presented a discrete hydrocele in both testes and Patient 10 had a lightly lobed biopsied testis (by scar formation or chance).

The morning hormonal determinations revealed high LH serum levels for 6 patients, high FSH serum levels for 4 patients, low INHB serum levels for 9 patients, and normal serum testosterone levels for all patients. The 4 patients with elevated FSH levels all showed decreased INHB serum levels.

Ongoing spermatogenesis with production of spermatozoa was observed in 8 young adults (Table 3). Sperm concentration analysis revealed 3 patients with normozoospermia (Patients 2, 3, and 5), 5 with oligozoospermia (severe: Patients 1, 4, and 7; moderate: Patients 10 and 11), and 4 with confirmed azoospermia (Patients 6, 8, 9, and 12) of which 2 (Patients 6 and 9) were overweight and 1 (Patient 9) presented with Sertoli cell-only syndrome at the time of banking. The 2 patients who did not undergo banking had severe oligozoospermia (Patient 1) or normozoospermia (Patient 3). When only considering the 7 patients who underwent conditioning therapy, 3 were azoospermic (Patients 6, 8, and 9), 2 were oligozoospermic (Patients 7 and 11), and 2 were normozoospermic (Patients 2 and 5).

A total of 7 patients produced a viscous semen sample. Normal forward sperm motility was observed in the 3

Table 1 Clinical background of 12 patients who underwent gonadotoxic childhood treatment.

Patient	Diagnosis	HSCT	Age at diagnosis (years)	Conditioning regimen	Chemotherapy and other treatments	Immature testicular tissue banking	Age at banking (years)	MAGE-A4 staining at banking
1	Acute lymphoblastic leukaemia	No	15.1	/	EORTC 58081 VHR: Chemotherapy CED 4000 mg/m^2	No	/	/
2	Sickle cell disease	Yes	0.0	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	• Hydroxyurea (2–3 years) before TTB • Conditioning CED 7992 mg/m^2	Orchiectomy left	4.2	N/A
3	Non-Hodgkin's lymphoma	No	6.7	/	FAB LMB96—group c: Chemotherapy CED 5300 mg/m^2	No	/	/
4	Nasopharyngeal tumour	No	10.5	/	NPC-2003-GPOH: Cisplatin 420 mg/m^2 and local radiotherapy (59 Gy)	Hemi-orchiectomy left	10.5	High number of spermatogonia
5	Chronic granulomatous disease	Yes	4.8	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	Conditioning CED 7992 mg/m^2	Hemi-orchiectomy left	12.0	Spermatogonia, spermatocytes
6	Sickle cell disease	Yes	2.0	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	• Hydroxyurea (6 years) before TTB • Conditioning CED 7992 mg/m^2	Yes, size and side N/R	12.4	N/A
7	Sickle cell disease	Yes	0.0	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	• Hydroxyurea (2 years) before TTB • Conditioning CED 7992 mg/m^2	Orchiectomy left	3.8	N/A
8	Acute lymphoblastic leukaemia	Yes	7.7	TBI 12 Gy	• EORTC 58081 VHR before TTB: • Chemotherapy CED 4000 mg/m^2 • Conditioning: TBI	Hemi-orchiectomy right	8.2	Low number of spermatogonia
9	Sickle cell disease	Yes	3.4	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	• Hydroxyurea (3 years) before TTB • Conditioning CED 7992 mg/m^2	Orchiectomy left	9.6	Sertoli cell only
10	Hodgkin's lymphoma	No	9.9	/	Euronet PHL c1- TG2: Chemotherapy CED 1000 mg/m^2	Hemi-orchiectomy left	10.0	Low number of spermatogonia
11	Thalassaemia major	Yes	0.0	Busulphan 16 mg/kg and cyclophosphamide 200 mg/kg	Conditioning CED 7992 mg/m^2	Orchiectomy right	4.1	N/A
12	Acute lymphoblastic leukaemia (Philadelphia positive)	No	13.1	/	EsPhALL VHR: Chemotherapy CED 5117 mg/m^2	Hemi-orchiectomy right	13.1	High number of spermatogonia, differentiation

HSCT: haematopoietic stem cell transplantation; MAGE-A4: melanoma-associated antigen 4; CED: cyclophosphamide equivalent dose; TBI: total body irradiation; N/R: not recorded; N/A: not available; TTB: testicular tissue banking. The age at conditioning regimen or mainline treatment corresponds to the age at banking, as the testicular tissue was collected just before the start of the gonadotoxic treatment. Earlier treatment is listed as 'before TTB' in the column 'Chemotherapy and Other Treatments'.

Table 2 Adult testicular volumes and reproductive hormone levels after gonadotoxic childhood treatment.

Patient	Diagnosis	HSCT	Post-treatment period (years)	Immature testicular tissue banking	Age at follow-up (years)	Testicular volume: left/right (mL)	LH (IU/l)*	FSH (IU/l)*	T (μg/l)	INHB (ng/l)*
1	Acute lymphoblastic leukaemia	No	2.3	No	19.6	**14/13**	**16.6**	**26.2**	6.8	**<10.0**
2	Sickle cell disease	Yes	18.6	Orchiectomy left	22.9	0#/19	5.0	4.1	3.9	**84.8**
3	Non-Hodgkin's lymphoma	No	21.0	No	28.3	25/29	3.9	1.8	6.2	316.0
4	Nasopharyngeal tumour	No	8.8	Hemi-orchiectomy left	19.7	**8#**/27	**10.4**	7.3	6.0	178.4
5	Chronic granulomatous disease	Yes	12.7	Hemi-orchiectomy left	25.6	**6#/10**	6.3	5.9	6.25	**63.9**
6	Sickle cell disease	Yes	11.9	Yes, size and side N/R	24.5	**6/7**	**11.8**	**15.3**	5.4	**12.6**
7	Sickle cell disease	Yes	19.3	Orchiectomy left	23.2	0#/**8**	**9.1**	6.8	5.7	**26.7**
8	Acute lymphoblastic leukaemia	Yes	10.0	Hemi-orchiectomy right	18.3	**9/7#**	8.0	**17.3**	4.1	**26.0**
9	Sickle cell disease	Yes	13.9	Orchiectomy left	23.6	0#/**9**	**15.0**	**14.0**	3.5	**<10.0**
10	Hodgkin's lymphoma	No	7.8	Hemi-orchiectomy left	18.1	**7#/12**	4.2	6.5	5.5	103.7
11	Thalassaemia major	Yes	17.8	Orchiectomy right	21.9	24/0#	**9.4**	6.6	4.1	**93.4**
12	Acute lymphoblastic leukaemia (Philadelphia positive)	No	4.5	Hemi-orchiectomy right	19.8	**11/7#**	6.5	9.7	2.3	**58.2**

HSCT: haematopoietic stem cell transplantation; T: testosterone; INHB: inhibin B; N/R: not recorded. Abnormal values are marked in bold. The volume of the testis that was biopsied for fertility preservation is indicated with #. Hormone values marked with * are significantly correlated with the semen concentrations values from Table 3: LH ($r = -0.6336$; $P = 0.0310$), FSH ($r = -0.8969$; $P = 0.0002$), and INHB ($r = 0.7612$; $P = 0.0058$). Reference values: ≥15.2 ml (postpubertal testicular volume), 1.7–8.6 IU/l (LH), 1.5–12.4 IU/l (FSH), 1.88–8.8 μg/l (testosterone), 95.0–323.0 ng/l (INHB). Detection limits: 0.05 IU/l for LH, 0.3 mIU/ml for FSH, 25 pg/ml for T, and 2.91 pg/ml for INHB.

normozoospermic patients and 1 patient with severe oligozoospermia. The other 4 patients with spermatozoa in the ejaculate had a lower forward sperm motility. All patients with ejaculated spermatozoa, except Patient 2, had morphologically abnormal spermatozoa with small acrosomes and nuclear vacuoles as the most dominant defects. A normal DFI was reported for the 5 patients in which DNA fragmentation testing was feasible. None of the patients with motile spermatozoa tested positive for antisperm antibodies.

With the current number of patients in this study, no correlation could be found between the sperm concentration and the age at biopsy ($r = -0.3189$; $P = 0.3656$), biopsy size ($r = -0.0272$; $P = 0.9374$), post-treatment period ($r = 0.4164$; $P = 0.1776$), treatment intensity ($r = -0.1561$; $P = 0.6254$), or serum testosterone levels ($r = 0.4307$; $P = 0.1622$). The Spearman correlation test revealed a significant negative correlation between sperm concentration and LH ($r = -0.6336$; $P = 0.0310$) and FSH ($r = -0.8969$; $P = 0.0002$) serum levels, as well as a significant positive correlation with INHB ($r = 0.7612$; $P = 0.0058$) serum levels.

Discussion

This study aimed to evaluate the testicular function of young adults who were eligible for immature TTB prior to chemo- and/or radiotherapy with gonadotoxic risk. Similar to previous reports (Duca et al., 2019; Borgström et al., 2020), our study recorded smaller adult testicular volumes after childhood gonadotoxic treatment in 9 out of the 12 included patients. Besides, our data show that the biopsied testes have grown, as was also seen in earlier studies (Uijldert et al., 2017; Borgström et al., 2020; Kanbar et al., 2020; Braye et al., 2022). In agreement with the medium-term results of Uijldert et al. (2017), our study suggests that a testicular biopsy does not hamper further development of the testicular parenchyma. Furthermore, the biopsy size did not have a significant effect on the sperm parameters in our patients. These first results suggest that it is safe to perform a (hemi-)orchidectomy to collect sufficient tissue for fertility restoration. However, as the absence of significant differences may be the effect of the small sample size, additional studies are needed to confirm our results.

At adulthood, 10 of the 12 patients included in this study had some degree of reproductive hormone impairment. The high LH, high FSH, and low INHB serum levels significantly correlated with a lower sperm concentration. However, as previously described, low INHB levels alone were not sufficient to predict the patient's fertility status (Meachem et al., 2001; Bordallo et al., 2004; Rendtorff et al., 2012; Anderson et al., 2015).

Older studies report a rather high rate (66–83%) of infertility in survivors of childhood cancer (Ahmed et al., 1983; Byrne et al., 1987; Siimes et al., 1995; Green et al., 2010; Romerius et al., 2011). Notably, since the introduction of the WHO laboratory guidelines in 1980, it became standard to include a centrifugation step whenever azoospermia was observed (Ron-El et al., 1997). Therefore, in the past, cryptozoospermia (a condition where azoospermia is diagnosed after microscopic evaluation of the semen, but spermatozoa can be observed in the pellet obtained after centrifugation) potentially remained underdiagnosed in childhood cancer survivors. More recent, yet small, studies found that 8/27 patients (Kanbar et al., 2020), 4/6 patients (Borgström et al., 2020), and 15/20 (Mathiesen et al., 2021) were azoospermic after gonadotoxic treatment during childhood. Remarkably, in the present study, only 4/12 young adults were azoospermic at 12.3 (2.3–21.0) years post-gonadotoxic treatment during childhood. Luckily, testicular tissue was banked for all these patients. The remaining 8 patients had ongoing spermatogenesis, of which 3 were normozoospermic and 5 presented with moderate to severe oligozoospermia. The considerable difference between our results and the studies from Borgström et al. (2020) and Mathiesen et al. (2021) could be explained by the gonadotoxicity of the treatments as they only included patients who underwent high-dose chemotherapy or TBI as conditioning therapy prior to HSCT. The study population of Kanbar et al. (2020), however, was similar to ours with CEDs ranging from 0 to 26 644 mg/m^2 (median: 4252 mg/m^2), resulting in similar outcomes.

Although spermatogenesis can take up to several years to recover after gonadotoxic treatment (Vakalopoulos et al., 2015), the time after treatment did not correlate with the sperm concentration at adulthood in the present study. Besides this, no link was found between the age at testicular biopsy and the sperm concentration. Interestingly, in the study of Kanbar et al. (2020), there was a higher incidence of primary testicular failure (defined by serum FSH levels ≥ 10 IU/l) after gonadotoxic treatment in patients who were peripubertal compared to those who were prepubertal at the time of TTB. However, another publication found

Table 3 Semen parameters following gonadotoxic treatment during childhood.

Patient	Diagnosis	HSCT	Immature testicular tissue banking	Age at follow-up (years)	Abstinence time (days)	Semen volume (ml)	Concentration (10^6/ml)	Progressive motility (%)	Normal morphology (%)	DFI (%)
1	Acute lymphoblastic leukaemia	No	No	19.6	6	2.0	**0.00005***	**0**	/	/
2	Sickle cell disease	Yes	Orchiectomy left	22.9	7	2.0	36.2	40	6; small acrosome and vacuoles	20
3	Non-Hodgkin's lymphoma	No	No	28.3	**0**	3.3	36.5	52	**3**; small acrosome and vacuoles	10
4	Nasopharyngeal tumour	No	Hemi-orchiectomy left	19.7	N/R	1.5	**3.4**	75	**1**; small acrosome and vacuoles	24
5	Chronic granulomatous disease	Yes	Hemi-orchiectomy left	25.6	4	2.9	28.4	62	**3**; small acrosome and vacuoles	12
6	Sickle cell disease	Yes	Yes, size and side N/R	24.5	2	2.9	**0.0***	/	/	/
7	Sickle cell disease	Yes	Orchiectomy left	23.2	2	3.0	**1.5**	**13**	**0**; small acrosome and vacuoles	/
8	Acute lymphoblastic leukaemia	Yes	Hemi-orchiectomy right	18.3	5	4.5	**0.0***	/	/	/
9	Sickle cell disease	Yes	Orchiectomy left	23.6	2	1.8	**0.0***	/	/	/
10	Hodgkin's lymphoma	No	Hemi-orchiectomy left	18.1	2	1.4	**5.1**	**18**	**3**; small acrosome and vacuoles	/
11	Thalassaemia major	Yes	Orchiectomy right	21.9	2	**1.0**	**7.3**	**11**	**1**; vacuoles	13
12	Acute lymphoblastic leukaemia	No	Hemi-orchiectomy right	19.8	5	**0.8**	**0.0***	/	/	/

HSCT: haematopoietic stem cell transplantation; DFI: DNA fragmentation index; SCO: Sertoli-cell-only: N/A: not available; N/R: not recorded. Abnormal values are marked in bold. Reference values: 2–7 days (abstinence time), ≥1.4 ml (semen volume), ≥16 × 10^6/ml (concentration), ≥30% (progressive motility), ≥4% (normal morphology), <30% (DNA fragmentation). *After centrifugation (600 *g*).

no difference in the prevalence of primary hypogonadism at the time of cancer diagnosis in patients older or younger than 10 years (Brignardello *et al.*, 2016). Yet, the results of Kanbar *et al.* (2020) are consistent with previous research, which revealed that peripubertal testicles are more vulnerable to gonadotoxic treatments than prepubertal ones (Matus-Ridley *et al.*, 1985). This was also highlighted in the study by Mathiesen *et al.* (2021), which showed that patients who were peri- to postpubertal during gonadotoxic treatment (10/12) were more likely to become azoospermic than prepubertal patients (5/8). This might be the case as Sertoli cells are possibly more sensitive to gonadotoxic damage during their proliferation peak around the onset of puberty. Since most peripubertal patients can bank sperm, almost all patients included in the present study were prepubertal at the time of biopsy. For this reason, it remains difficult to draw any conclusions.

Besides sperm concentration, other semen parameters were examined. Among the included patients, 7 had a viscous sperm sample, which is remarkably higher than the normal prevalence (12–29%). Higher viscosity may negatively impact motile sperm count (Du Plessis *et al.*, 2013). In 4 of the 5 oligozoospermic patients, insufficient progressive sperm motility was observed. All but one patient showed morphologically abnormal spermatozoa with small acrosomes and large or numerous small vacuoles as dominant defects. Smaller acrosomes might reduce the sperm fertilization capacity and pregnancy rates, even when used in ICSI cycles (Zahiri and Ghasemian, 2019). The excessive presence of vacuoles could further affect this, as they are associated with lower implantation and pregnancy rates, among others (Berkovitz *et al.*, 2006; Setti *et al.*, 2014).

A feared consequence of gonadotoxic treatment is the possible DNA damage to sperm, of which DNA fragmentation is one of the most common disruptions. An increased DFI could affect the fertilization rates, embryo quality, and pregnancy rates (Cissen *et al.*, 2016). However, a normal DFI was present in all patients with a sufficiently high sperm concentration to perform the test. It is worth noting that the DFI could not be determined in the patients whose sperm production was most affected, which could create a possible bias. Another question that arose was whether patients would produce anti-sperm antibodies owing to the disruption of the blood–testis barrier by the biopsy procedure (Leathersich and Hart, 2022). Nevertheless, anti-sperm antibodies were not detected in any of the patients.

All taken together, our results suggest that a large proportion of young boys undergoing gonadotoxic treatment might be able to conceive during adulthood via ART or naturally. Therefore, it is suggested to delay the auto-transplantation of immature testicular tissue until adulthood to avoid unnecessary invasive surgery. Owing to the limited number of patients included in this study and the differences in terms of their medical backgrounds, a substantial number of important questions remain unanswered: What are the effects of disease and treatment protocol on the testicular function? Does the size of the testicular biopsy and/or the pubertal stage at banking affect the fertility? Does the testicular function improve with time post-treatment? These questions highlight the urgent need for more multicentric research with a larger study population to establish universal inclusion criteria for TTB as they currently vary amongst fertility centres (Green *et al.*, 2014a; Skinner *et al.*, 2017; Brougham *et al.*, 2019; Delgouffe *et al.*, 2022). However, as it is not possible yet to predict which patients will maintain their fertility and which will not, immature TTB is still recommended for patients at significant risk of infertility.

Supplementary data

Supplementary data are available at Human Reproduction Open online.

Data availability

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared upon reasonable request to the corresponding author.

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Authors' roles

E.D. performed the semen analyses, data collection and processing, and wrote the original draft of the manuscript. A.B. contributed to the supervision of the research, validation of the results, and review and editing of the article. V.V. was responsible for the patient consultations and contributed to validation of the results, review and editing of the article. C.E. oversaw the scrotal ultrasound analyses and aided with the validation of the results, review and editing of the article. I.M., A.F., C.D., H.T., and I.G. helped with the validation of the results and critical revision of the article. E.G. contributed to the study conceptualization and design, funding acquisition, supervision of the research and validation of the results, review and editing of the article.

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Conflict of interest

The authors declare no conflict of interest.

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