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ESHRE Pages

ESHRE good practice recommendations on fertility preservation involving testicular tissue cryopreservation in children receiving gonadotoxic therapies†

ESHRE FP for Boys Working Group: Rod T. Mitchell [1,2,*], Cristina Eguizabal [3], Ellen Goossens [4], Michael Grynberg [5], Kirsi Jahnukainen [6,7], Nathalie Le Clef [8], Callista L. Mulder [9], Nina Neuhaus [10], Michael P. Rimmer [2,11], Jan-Bernd Stukenborg [7,12], Marianne D. van de Wetering[13], Ans M. M. van Pelt [9], and Christine Wyns [14]

[1]Royal Hospital for Children and Young People, Edinburgh, UK
[2]Centre for Reproductive Health, Institute of Regeneration and Repair, University of Edinburgh, Edinburgh, UK
[3]Advanced Therapies Unit, Basque Center for Blood Transfusion and Human Tissues, Galdakao, Spain; Cell Therapy, Stem Cells and Tissues, Biobizkaia Health Research Center, Barakaldo, Spain
[4]Research Group Genetics, Reproduction and Development (GRAD), Biology of the testis (BITE), Vrije Universiteit Brussel (VUB), Brussels, Belgium
[5]Service de Médecine de la Reproduction et Préservation de la Fertilité, Hôpital Antoine Béclère, Clamart, France
[6]New Children's Hospital, Paediatric Research Centre, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[7]NORDFERTIL Research Lab Stockholm, Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet, and Karolinska University Hospital, Solna, Sweden
[8]European Society of Human Reproduction and Embryology (ESHRE), Strombeek-Bever, Belgium
[9]Reproductive Biology Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands; Amsterdam Reproduction & Development Research Institute, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
[10]Centre of Reproductive Medicine and Andrology, University Hospital Münster, Münster, Germany
[11]Edinburgh Fertility Centre, Simpsons Centre for Reproductive Health, Royal Infirmary of Edinburgh, Edinburgh, UK
[12]NORDFERTIL Research Lab Uppsala, Department of Organismal Biology, Uppsala University, Uppsala, Sweden
[13]Prinses Máxima Centrum voor kinderoncologie BV, Utrecht, The Netherlands
[14]Cliniques Universitaires Saint-Luc, Université catholique de Louvain, Brussels, Belgium

*Correspondence address. ESHRE Central office, BXL7—Building 1, Nijverheidslaan 3, B-1853 Strombeek-Bever, Belgium. E-mail: guidelines@eshre.eu; Rod.Mitchell@ed.ac.uk https://orcid.org/0000-0003-4650-3765 (RTM)

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ABSTRACT

STUDY QUESTION: How should fertility preservation in child and adolescent males receiving gonadotoxic therapies be managed?

SUMMARY ANSWER: There were 44 recommendations formulated to provide guidance on all aspects of fertility preservation in prepubertal boys and adolescent males in whom it is not possible to obtain sperm by established methods including semen cryopreservation and surgical sperm extraction; the recommendations covered topics from setting up a fertility preservation program, determining who is eligible, and counselling, to the practical aspects of the testicular tissue biopsy and cryopreservation.

WHAT IS KNOWN ALREADY: For young males facing gonadotoxic treatment, there are limited options for fertility preservation. For those who are unable to produce sperm (children and adolescents) testicular tissue cryopreservation is being increasingly offered prior to gonadotoxic treatment for potential future clinical use to restore fertility.

STUDY DESIGN, SIZE, DURATION: This Good Practice Recommendations (GPR) article was developed according to a predefined methodology for ESHRE good practice recommendations. Recommendations are supported by data from the literature, if available.

PARTICIPANTS/MATERIALS, SETTING, METHODS: ESHRE appointed a European multidisciplinary working group with expertise in paediatric oncology, paediatric endocrinology, human tissue banking, and surgery, as well as reproductive specialists and researchers, who have demonstrated leadership and expertise in fertility preservation in prepubertal boys and adolescent males. The recommendations were formulated based on the expert opinion of the working group, while taking into consideration the published data. The draft document was then open to ESHRE members for online peer review and was revised in light of the comments received.

MAIN RESULTS AND THE ROLE OF CHANCE: The multidisciplinary working group formulated 44 recommendations to provide guidance on all aspects of fertility preservation by testicular tissue cryopreservation, from setting up a fertility preservation program, determining who is eligible and counselling, to the practical aspects of the testicular tissue biopsy and cryopreservation.

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LIMITATIONS, REASON FOR CAUTION: These guidelines offer valuable direction for healthcare professionals taking care of children and adolescents facing gonadotoxic treatment. Their purpose is to promote knowledge among clinicians and to enable patients to make informed decisions based on realistic expectations.

WIDER IMPLICATIONS OF THE FINDINGS: This article provides not only good practice advice but also highlights the areas that need further research. This research, when well-conducted, will be key to making progress in the area of fertility preservation in children and adolescents.

STUDY FUNDING/COMPETING INTERESTS: All costs relating to the development process, including meetings and technical support for this project, were funded by the European Society of Human Reproduction and Embryology (ESHRE). There was no external funding of the development process or manuscript production. RTM was supported by a UKRI Future Leader Fellowship. The other authors disclosed no conflicts of interest.

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Introduction

For young males facing gonadotoxic treatment, there are limited options for fertility preservation. For those who are unable to produce sperm (children and adolescents), testicular tissue cryopreservation is being increasingly offered to patients prior to gonadotoxic treatment for potential future clinical use to restore fertility. The number of young males undergoing testicular tissue cryopreservation is increasing year on year, and a recent survey of centres offering this procedure has indicated that >3000 patients have had this procedure performed (Duffin *et al.*, 2024). The number has doubled in the last 5 years. It is expected that it will continue to rise as the number of centres offering the procedure increases and the availability becomes more widespread, although clinical applications using cryopreserved testicular tissue remain to be proven. However, there is no clear consensus on the best clinical practice for testicular tissue cryopreservation from the perspective of the cryopreservation approach, tissue quality control, clinical assessment, and follow-up for patients who are having tissue stored.

This Good Practice Recommendations article therefore aims to provide guidance on all aspects of fertility preservation in prepubertal boys and adolescent males in whom it is not possible to obtain sperm by established methods including semen cryopreservation and surgical testicular sperm extraction (TESE). The recommendations cover topics from setting up a fertility preservation program, determining who is eligible, and counselling, to the practical aspects of the testicular tissue biopsy and cryopreservation.

Materials and methods

The current document was developed according to the manual for development of ESHRE Good Practice Recommendations (Vermeulen *et al.*, 2019).

A working group was composed of members of the ESHRE Special Interest Group (SIG) Fertility Preservation, Andrology and Stem cells, and invited experts in the field, ensuring representation of clinical and laboratory expertise, and geographical balance, supported by a methodological expert (NLC). In the first meetings, the working group reached agreement on a list of questions to be addressed in this recommendations article. A literature search of PUBMED/MEDLINE and Cochrane library was performed. Papers published up to 22 September 2024 were included. All titles and abstracts were screened to identify relevant papers, for which full text papers were collected and summarized. For each question, the current paper includes a short narrative summary of published data incorporated. Testicular tissue integrity and survival of spermatogonial stem cells were the critical outcomes; important outcomes were the survival and function of somatic cells. Further, information on other technical and practical aspects of possible relevance for the clinic and the patient was included. At working group meetings, the evidence and draft recommendations were presented by the assigned working group member and discussed until consensus was reached within the group.

In this good practice recommendations article, in line with the research, terminology and discussion is focused on boys and men. The working group recognizes that there are individuals who do not identify with the terms used in the literature. For the purposes of this recommendations article, we use the terms 'boys', 'males', and 'parents/caregivers', however, it is not intended to isolate, exclude, or diminish any individual's experience nor to discriminate against any group.

Abbreviations used throughout this article are listed in Supplementary Table S1. An overview table with all recommendations formulated by the ESHRE working group on Fertility Preservation in boys, and discussed in this Recommendations for Good Practice paper, can be found in Supplementary Table S2.

The final draft was published on the ESHRE website between 9 December 2024 and 13 January 2025 for stakeholder review. There were 172 comments received from 22 reviewers and these were incorporated where relevant. The review report is available on www.eshre.eu/guidelines. The experts who participated in the stakeholder review are listed in Supplementary Table S3.

Results

Fertility preservation programme for testicular tissue cryopreservation

What clinical expertise is required to start a program?

Evidence

Several of the identified studies point to the importance of a multidisciplinary team for providing a fertility preservation (FP) program (Loren *et al.*, 2013b; Kim *et al.*, 2014; Picton *et al.*, 2015; Sadri-Ardekani *et al.*, 2016; Carlson *et al.*, 2017; Oktay *et al.*, 2018; Keim-

Malpass *et al.*, 2018; Anazodo *et al.*, 2019; Moravek *et al.*, 2019; Stern and Agresta, 2019; Goossens *et al.*, 2020; Norton and Wright, 2020; Crespi *et al.*, 2021; Joshi *et al.*, 2021; Sehring *et al.*, 2021; Ludemann *et al.*, 2023). This most frequently includes clinical expertise in oncology, reproductive endocrinology, paediatric surgery, urology, nursing, genetic counselling for heritable diseases, laboratory medicine and pathology, mental health professionals, ethicists, administrators and social workers. A member of clinical staff (nurse, nurse practitioner, physician associate or physician) acting as a 'Divisional Champion' (Carlson *et al.*, 2017; Moravek *et al.*, 2019) and the use of Multi-collaborative Care Pathways (Wyns *et al.*, 2015; Goossens *et al.*, 2020) are also considered of importance for permitting early identification and referral of patients. Access to a fertility specialist is also reported to be important for the success of a FP programme (Loren *et al.*, 2013a; Stern and Agresta, 2019).

In a thematic analysis of 11 studies investigating the role of nurses, the authors show that registered nurses represent a strong solution to providing guideline-concordant FP care (Crespi *et al.*, 2021); this includes an important role for oncology nurses (King *et al.*, 2008; Krouwel *et al.*, 2017; Norton and Wright, 2020) and those employed as a patient navigator (Kim *et al.*, 2014; Keim-Malpass *et al.*, 2018; Anazodo *et al.*, 2019; Moravek *et al.*, 2019; Joshi *et al.*, 2021; Sehring *et al.*, 2021; Ludemann *et al.*, 2023).

Other contributors to an FP programme may include patient advocates (Loren *et al.*, 2013b, Quinn *et al.*, 2008) and a scientific team, with both clinical and research engagement (Moravek *et al.*, 2019; Stern and Agresta, 2019; Joshi *et al.*, 2021).

Recommendations

Testicular tissue cryopreservation in this patient population requires multi-disciplinary expertise. The team should include clinical expertise in gonadotoxic therapies, gonad surgery, and laboratory expertise including pathology, testis tissue cryopreservation and reproduction/fertility, in support of the treating physician.

To optimize this multi-disciplinary care pathway, the addition of a geneticist and psychologist is advised

What facilities are required to start a program?

Evidence

No studies could be retrieved from literature to answer this question.

Recommendation

Testicular tissue cryopreservation in this patient population requires access to a sterile environment (laboratory or clean room, see quality control section) to process the tissue, a place to store cryopreserved material with an internal liquid nitrogen level alarm system and room oxygen monitoring system (e.g. clinical tissue bank), operating theatre, and clinical facilities providing care to patients receiving therapies. This should be provided in accordance with local and national regulations.

Eligibility

Who is eligible?

Evidence

A total of 24 studies have reported results of semen analysis in adult survivors of childhood cancer after at least a median follow-up of 10 years (Table 1). The results of another 11 studies with a median follow-up of less than 10 years are summarized in Supplementary Table S4.

The majority of studies with at least a median follow-up of ten years report an association between increasing alkylating agent exposure and/or cyclophosphamide equivalent dosing (CED) (Green *et al.*, 2014) and the risk of azoospermia. Whilst there is some variability in findings between studies, CED <4 g/m^2 is usually associated with normozoospermia in adulthood, while exposure to >8 g/m^2 is associated with a significantly increased risk of oligozoospermia and >10 g/m^2 with azoospermia. A study with extended follow-up and serial sperm analyses reported evidence of spermatogenic recovery after long-term follow-up, even following exposure to high doses of alkylating agents (Korhonen *et al.*, 2025). Radiotherapy to the pelvis or direct radiotherapy to the testis results in a significant risk of subsequent azoospermia with limited options for recovery. However, the current evidence is insufficient to accurately define dose thresholds for testicular radiotherapy or scattered radiation exposure associated with the risk of azoospermia. Limited data shows that scattered radiation to the testis of less than 1 Gy does not lead to azoospermia or reduced paternity (Korhonen *et al.*, 2025).

Several surrogate markers of spermatogenesis, such as serum FSH, inhibin B, and adult testicular volume provide indirect measures of spermatogenic capacity.

There were 13 studies identified that reported on the value of follicle stimulating hormone (FSH) or Inhibin B in predicting azoospermia (Supplementary Table S5a) and an additional 28 studies that described FSH levels in relation to gonadotoxic treatment received during childhood (Supplementary Table S5b). The majority of studies that included semen analysis showed an association between raised FSH and impaired semen parameters (Supplementary Table S5a). A meta-analysis of all available data reported a threshold of 10.4 IU/l had a sensitivity of 82% and specificity of 84% in predicting azoospermia (Kelsey *et al.*, 2017).

Another 45 studies reporting on effects of gonadotoxic therapies on Leydig cell function are summarized in Supplementary Table S6.

There were four studies identified that reported on the value of testicular volume in predicting azoospermia (Supplementary Table S7a) and an additional 26 studies that described testicular volume in relation to gonadotoxic treatment received during childhood (Supplementary Table S7b). Overall, there was a tendency towards lower adult testicular volume in childhood cancer survivors compared to healthy controls and a reduced adult testicular volume with regimens involving increasing doses of alkylating agent (Supplementary Table S7b). The only study with repeated testicular volume measurements (Korhonen *et al.*, 2025) found normalization of adult testicular volume in survivors treated exclusively with chemotherapy. No significant differences in adult testicular volume were observed based on the type of chemotherapy, whether alkylating or non-alkylating, or the dose of alkylating agents, despite low volumes during puberty. This highlights the importance of extended follow-up to capture the recovery of testicular volume and late recovery of spermatogenesis. The four studies that included semen analysis showed an association between testicular volume and semen parameters (Supplementary Table S7a). An adult testicular volume (unilateral) of ≥17 ml (Jahnukainen *et al.*, 2011) or ≥15 ml was predictive of non-azoospermia (Romerius *et al.*, 2011; Korhonen *et al.*, 2025), whilst a testicular volume of <12 ml was predictive of azoospermia (Mathiesen *et al.*, 2020).

A total of 17 studies that reported on the association between gonadotoxic therapy and paternity are summarized in Table 2. Several studies report a reduced paternity in childhood cancer survivors compared to healthy controls. Overall, there was a

Table 1. Studies reporting on semen analysis results after long-term follow-up (median ≥10 years) of childhood cancer survivors, arranged in descending order of median follow-up duration.

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Green *et al.* (2017)	241	CRT: 6.6 ± 4.4 Non-CRT: 7.5 ± 5.0	32.9 ± 7.8	*After diagnosis* CRT: 26.3 ± 6.3 Non-CRT: 18.7 ± 6.0	**CED (g/m^2)** / **Non-CRT (n = 82)** / **CRT (n = 91)** >0–<4 / 10 (12%) / 7 (8%) ≥4–8 / 18 (22%) / 17 (19%) ≥8–12 / 50 (61%) / 50 (55%) ≥12 / 4 (5%) / 17 (18%) CT±CRT	173	Risk of azoospermia or oligozoospermia was not statistically associated with CRT exposure at a dose of >0–20 Gy (RR 0.99, 95% CI 0.70–1.28) or 20–26 Gy (RR 1.09, 95% CI 0.81–1.46). RR for oligozoospermia or azoospermia was increased for those 5–9 years of age at diagnosis compared to those 0–4 years of age at diagnosis, and for CED ≥8 –<12 g/m^2, and CED ≥12 g/m^2 compared to CED >0 –<4 g/m^2. RR for low sperm count was increased for those 5–9 years of age at diagnosis compared to those 0–4 years of age at diagnosis and for CED ≥8 g/m^2 compared to CED >0 –<4 g/m^2.
Hamre *et al.* (2012)	64	Median 13.3 (3.0–17.8)	Median 33.6 (19.0–54.5)	Median 22.0 (8.5–37.0)	**Low-gonadotoxicity** NHL/NHL: Radiotherapy only ABVD/EBVP and similar **Medium-gonadotoxicity** NHL: CHOP/COP ≤8 courses alone; CHOP ≤8 courses combined with Mtx BFM 90/93; Other regimen, total dose cyclophosphamide ≤6 g/m^2 HL: MVPP or ChlVPP ≤4 courses; MVPP or ChlVPP ≤4 combined with ABVD or EBVP; OEPA/OPPA + 0–4 COPP **High-gonadotoxicity** NHL: HDT with TBI and high-dose cyclophosphamide as conditioning regimen. HDT with BEAM as conditioning regimen. Other regimen, total dose cyclophosphamide >6 g/m^2 HL: HDT with TBI and high-dose cyclophosphamide as conditioning regimen. HDT with BEAM as conditioning regimen. MVPP or LVPP ≥4 courses	42	12/42 males presented with azoospermia 7/42 were oligozoospermic 23/42 were normospermic The proportion of azoospermia increased with treatment burden.
Green *et al.* (2014)	214	Median 7.7 (0.01–20.3)	Median 29.0 (18.4–56.1)	Median 21.0 (10.5–41.6)	CT with alkylating agent,	214	Azoospermia was identified in 53/214 (25%), oligozoospermia in 59/214 (28%), normospermia in 102/214 (48%). Mean CED was 10.83 g/m^2 (±7.27) for those with azoospermia, 8.48 g/m^2 (±4.26) for those with oligozoospermia, and 6.63 g/m^2 (±3.58) for those with normospermia. Of the 35 patients with a CED of <4 g/m^2, 31 (89%) were normospermic. CED and sperm concentration were negatively correlated ($r = -0.37$, $P < 0.0001$).

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Korhonen *et al.* (2025)	255	Median 6.1 (3.2–11.4)	Median 27 (25–30)	Median 21 (15–23)	**CED** (g/m^2) +/- CRT **(n = 253)** <4 116 (46%) ≥4 –<12 63 (25%) ≥12 74 (29%) **Testicular RT** (Gy) **(n = 253)** >0 –<1 71 (28%) ≥1 –<10 7 (3%) ≥10 71 (28%) **HSCT** **(n = 254)** No 202 (80%) Yes 52 (20%)	92	The highest sperm counts typically occurred in samples obtained 10–30 years post-therapy. Increasing trend in total sperm counts with time occurred in repeated semen samples of patients treated exclusively with CT. Testicular RT ≥1 Gy (median dose 12 Gy) and a CED of ≥12 g/m^2 were independent risk factors for having azoospermia at adulthood.
Jahnukainen *et al.* (2011)	75	Median 5 (1–15)	Median 29 (26–38)	Median 20 (11–30)	*Cumulative values* CRT: 24 (18–48) Gy Spinal RT: 6 Gy, n = 1 Testicular RT: 24 (10–24) Gy Cyclophosphamide: 6.9 (1.2–29.0) g/m^2	47	0–10 g/m^2 cumulative dose of cyclophosphamide (n = 47): no statistical difference in sperm count, mobility or morphology Prophylactic CRT did not reduce semen quality No spermatozoa in the semen samples after 24 Gy testicular irradiation (n = 15) or after >20 g/m^2 of cyclophosphamide (n = 2).
Romerius *et al.* (2011)	129	Median 10 (0.10–17)	Median 29 (20–46)	Median 19 (4–36)	**Treatment** **Number** Brain surgery 16 Other surgery 16 CT alone 35 RT to testes 1 RT alone (not testes) 13 CT+RT 48 CT previously shown to imply a 'high risk' of azoospermia **Agent** **Cumulative dose** Carmustine 1 g/m^2 Lomustine 500 mg/m^2 Chlorambucil 1.4 g/m^2 Cisplatin 500 mg/m^2 Cyclophosphamide 19 g/m^2 Melphalan 140 mg/m^2 Procarbazine 4 g/m^2	129	18% of childhood cancer survivors were azoospermic. Those treated with chemotherapy only, as well as those who had received a combination of CT and RT, had the highest proportion of azoospermia (14%, 95% CI 4.8–30 and 33%, 95% CI 20–48, respectively). Of the CCS who had received doses of cisplatin/alkylating agents with 'high risk' of azoospermia in combination with RT, 64% (95% CI 35–87) developed azoospermia. In those treated with doses of cisplatin/alkylating agents shown to result in 'high risk' of azoospermia, but no RT, the proportion of men with azoospermia was 80% (95% CI 28–99%). Among CCS who received cisplatin/alkylating agents below the reported threshold values for azoospermia but also were treated with RT, 33% (95% CI, 15–57) developed azoospermia. Among CCS treated with cisplatin/alkylating agents below the 'high risk' threshold values and no RT, 5.3% (95% CI 0.13–26) developed azoospermia.
Mathiesen *et al.* (2020)	98	*At HSCT* Median 9.7 (0.4–16.9)	Median 28.1 (18.5–47.0)	Median 18.3 (7.7–34.6)	Myeloablative allogeneic HSCT 6 treatment groups according to their cumulative therapy: (1) chemotherapy only, (2) low-dose testicular RT including TBI 2 Gy, TLI 6 Gy and TBI with gonadal shielding, (3) TBI without shielding, (4) TBI plus additional CNS RT, 5) TBI plus additional testicular RT, (6) TBI plus additional CNS and additional testicular RT. HSCT with TBI 71%, with CT only 24%, with CT + 4–6 Gy TBI/TLI 4% CED 5.2 (0–28.7) g/m^2 Additional CRT or Testicular irradiation 14%	72	30 participants had sperm in their ejaculate (31%), 42 (43%) had azoospermia without testosterone substitution, and 24 (24%) with testosterone substitution. All patients treated with >12 Gy had azoospermia, all but 1 patient treated with >16 Gy were receiving testosterone replacement. CT only (n = 23), a higher cumulative CED was associated with increased risk of azoospermia (OR for each 1 g/m^2 increase in CED 1.34, 95% CI 1.01–2.15). All patients treated with more than ~10 g/m^2 had azoospermia All those treated with less than ~6 g/m^2 had detectable sperm.

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Nurmio *et al.* (2009)	23	5.7 ± 2.9	21 ± 1.5	17.0 ± 1.9	The high-risk patients and the patient with secondary ALL received a high cumulative dose of cyclophosphamide, which is higher than that used in the modern protocols. The patients considered being at standard risk received the treatment that is comparable to the current protocols. In addition, four patients in the high-risk group received prophylactic cerebral RT (24 Gy), but spinal RT was not used. Patients experiencing testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial irradiation at a dose of 24 Gy.	6	N = 3 at standard risk treatment All had normal mean sperm counts N = 3 at high-risk therapy One patient showed total recovery of spermatogenesis 2 patients were oligo/azoospermic.
van den Berg *et al.* (2004)	76	Group 1: Median 10.8 (5–14.3) Group 2: Median 11.7 (3.8–15.2) Group 3: Median 13 (5–17.2)	NR	Group 1: Median: 16.3 (2–24.2) Group 2: Median 12.3 (4.9–15.6) Group 3: Median 5.8 (0.6–11.3)	Group 1: n = 13; MOPP without RT Group 2: n = 10; ABVD group Group 3: n = 10; ABVD-MOPP group	13	*Group 1:* 1/10: normozoospermia 1/10 oligozoospermia 8/10 azoospermia *Group 2:* only one patient had a semen analysis and it appeared normal *Group 3:* Semen analysis done in 2 patients, one normal and one azoospermic.
van Beek *et al.* (2007)	56	Median 11.4 (3.7–15.9)	Median 27 (17.7–42.6)	Median 15.5 (5.6–30.2)	Adriamycin/epirubicin, bleomycin, vinblastine, dacarbazine) with or without MOPP divided into three groups: no MOPP (n = 16) 3–4 MOPP (n = 14) ≥6 MOPP (n = 26)	21	The median sperm concentrations were significantly lower in MOPP+ patients when compared with MOPP−-patients. 9/17 had azoospermia (53%), 1/17 (6%) had oligozoospermia (<20 × 10^6/ml) and 3/17 (18%) had severe oligozoospermia (<5 × 10^6/ml) MOPP+ patients. 4/17 (23%) MOPP+ patients showed normospermia; of which three were treated with three MOPP cycles and one was treated with six MOPP cycles.
Poganitsch-Korhonen *et al.* (2017)	37	Non-alkylating: 5.3 ± 2.7 (1.1–11.3) Alkylating: 7.3 ± 5.2 (1.7–16.1)	Non-alkylating: 22.9 ± 5.6 (18.0–32.0) Alkylating: 22.6 ± 5.0 (18.0–29.0)	Non-alkylating: 15.2 ± 6.0 (9.0–25.2) Alkylating: 10.5 ± 7.5 (0.9–19.2)	Antimetabolites, vinca-alkaloids and anthracyclines 15 also received prophylactic cerebral RT 21 also received alkylating agents (CED 7.0 ± 3.8 (3.0–16.0)) Four patients also underwent a multidrug chemotherapy regimen together with testicular irradiation at a dose of 24 Gy.	17	*Non-alkylating* (n = 10) Total sperm count: 173.2 × 10^6/ml ± 175.0 × 10^6/ml (0.0–485.0) Motile sperm: 43% ± 25 (0–83) Azoospermia: 1 (10%) *Alkylating* (n = 7) Total sperm count: 50.1 × 10^6/ml ± 60.5 (0.0–150.0) Motile sperm: 38% ± 26 (0–79) Azoospermia: 1 (14%)

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Siimes *et al.* (1993)	41	Median 7.5 (1–16)	18–27	*After diagnosis* Median 15.2 (4–25)	All 41 patients had received intravenous vincristine, and oral prednisone, 6-mercaptopurine, and methotrexate. In addition, asparaginase (n = 33), cyclophosphamide (n = 23), adriamycin (n = 21), and cytosine arabinosine (n = 9) had been used. In 32 patients, intravenous infusions of high-dose methotrexate were used in combination with intrathecal methotrexate N = 17 had received cranial RT of 20–24 Gy without other RT CT ± CRT No information about exposures for those with sperm analysis	18	3/18 had azoospermia and 7/18 had oligozoospermia. No significant association was observed between the sperm count and the preceding treatment.
Lähteenmäki *et al.* (2008)	25	Median 8.5 (0.9–15.9)	Median 20.5 (15.6–31.2)	*After diagnosis* Median 14.5 (2.1–26.1)	N = 11: cyclophosphamide N = 3: MOPP or MOPP/ABVD or ABVD N = 1 cisplatin N = 1 TBI N = 8 CNS RT N = 3 local abdominal RT N = 1 neck and mediastinum RT	23	The median of semen volume was 3.5 (1.0–7.6) ml, sperm concentration 35.5 (0–273) × 10^6/ml, total sperm count 124.3 (0–625) × 10^6/ml, and percentage of motile sperm 56 (0–86)%. Eight patients (35%) had sperm concentration less than 20 × 10^6/ml, and three (13%) were azoospermic (sperm count 0). Absence of motile sperm was noticed in five men (22%). Azoospermic patients had treatment with either MOPP, high-dose cyclophosphamide (>7 g/m^2), or testicular irradiation.
Heikens *et al.* (1996)	19	Median 11 (5–15)	Part 1: Median 19 (16–27)	Part 1: Median 10 (6–14) Part 2: Median 14 (13–20)	All patients were treated with six courses of MOPP chemotherapy. RT was given as adjuvant treatment in eight patients with large lymph node tumours; six received irradiation above the diaphragm, and two were irradiated below the diaphragm (20 Gy on the para-aortal and splenic regions, respectively, and 25 Gy on the inguinal region).	19	A normal sperm count was found in only one patient. Three patients had moderate oligozoospermia, 3 had severe oligozoospermia, and 12 were azoospermic (including all 4 males treated during puberty). In patients in which spermatozoa were seen, including the patient with a normal sperm count, sperm motility was always decreased. In seven patients from whom serial samples of semen could be studied up to 20 years after treatment, no recovery of spermatogenesis was found.
Beaud *et al.* (2019)	13	Mean 12.8 ± 1.3	Mean 27.8 ± 1.6	Mean 13.4 ± 2.3	Vinca alkaloids (mean dose 31.45 ± 18.6 mg/m^2) Alkylating CT (mean dose 4084 ± 1036.6 mg/m^2) 6 patients also received RT (mean dose 241.1 ± 65.1 mg/m^2)	13	Three samples were azoospermic and two were oligozoospermic. A significant negative correlation between sperm count and the cumulative dose of alkylating agent. No other drugs correlated negatively with sperm count.
Relander *et al.* (2000)	77	Median 11 (0.8–17)	Median 23.6 (18.6–38.5)	*After diagnosis* 13.2 (3.5–22.8)	41/77 (55%) patients had received only local treatment being surgery in 16, RT in 6, and a combination of surgery and RT in 19 patients. One had CT only and 35 had CT + local therapy	54	**Testicular volume** / **Normo spermia** / **Oligozoo spermia** / **Azoo spermia** ≥20 ml: 16 / 3 / 1 ≥15, <20 ml: 3 / 1 ≥10, <15 ml: 12 / 4 / 2 <10 ml: 3 / 3 / 6 Total: 34 / 11 / 9 Normozoospermia was seen only in patients treated with <10 g/m^2 except for 1 patient in whom the testicles were also irradiated. There was a significant negative correlation between total dose of cyclophosphamide and sperm count (correlation coefficient –0.28, $P = 0.04$).

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Watson *et al.* (1985)	30	Median 9.4 (2.9–17.3)	Median 22 (17–29.5)	Median 12.8 (6.7–15.8)	Patients who had been treated with cyclophosphamide for childhood nephrotic syndrome	30	Of the 30 patients, four were azoospermic, 9 oligozoospermic (sperm count $<20 \times 10^6$/ml), and 17 normospermic (sperm count $\geq 20 \times 10^6$/ml.) A significant inverse correlation was evident between sperm density and cyclophosphamide dosage in terms of duration of treatment and total dosage. 13 patients had undergone a semen analysis 5.5–9 years previously, 9 remained in the same categories (4 normospermic, 3 azoospermic, and 2 oligozoospermic), but 4 who had had a low or, in one case, no sperm count before were found to be normospermic after an average additional follow up of 7.2 years.
Shafford *et al.* (1993)	40	Median 10.4 (4.3–15.9)	Median 23 (16.7–30)	Median 12.5 (6–20)	N = 7: CT alone N = 16: CT + RT above diaphragm N = 1: CT + RT below diaphragm N = 4: CT + RT above and below diaphragm N = 7: RT alone above diaphragm N = 4: RT alone below diaphragm N = 1: RT alone above and below diaphragm	14	*Patients that received CT* 11/12 patients having semen analysis were azoospermic after a median of 6 courses of ChIVPP and one severely oligozoospermic after 4 courses of ChIVPP and 6 courses of ABVD. *Patients that only received RT* Only 1 patient with RT above diaphragm had semen analysis and was normozoospermic. 3 patients received 3500 cGy to an inverted Y field, of which 1 had semen analysis and was severely oligozoospermic.
Delgouffe *et al.* (2023)	12	Median 5.8 (neonatal–15.1)	Median 22.4 (18.1–28.3)	Median 12.3 (2.3–21.0)	HSCT (n = 7): MAC, 1/7 with TBI CT/RT (n = 5) 41%	12	8/12: ongoing spermatogenesis with production of spermatozoa: 3/12 patients with normozoospermia, 5 with oligozoospermia (3 severe and 2 moderate). 4/12: confirmed azoospermia of which 3 received conditioning treatment prior to HSCT (note: 2 were overweight and 1 presented with Sertoli cell-only syndrome at the time of banking.) 4/12 Azoospermia: 2/2 Sickle cell disease, 2/3 ALL with TBI and CT conditioning.
Lee *et al.* (2025)	228	Median 6.86 (0.5–20.2)	Median 19.7 (6.8–44.2)	Median 12 (5.1–33.7)	Patients having HSCT Malignant group: n = 157 Non-malignant group: n = 71 Conditioning: TBI (12 Gy): n = 81 Busulfan (16–20 mg/kg): n = 103 RIC: n = 14 Cyclophosphamide (200 mg/kg) + ATG: n = 16 Thoraco-abdominal RT (5 Gy)/cyclophosphamide (20 mg/kg): n = 6 No conditioning: n = 7 Missing: n = 1	5	5 men who had semen analysis after TBI had azoospermia, as did 3 of 6 who had been conditioned with Busulfan and Cyclophosphamide; the other 3 had oligozoospermia. One of 6 (16.7%) males conditioned with cyclophosphamide alone and 3/4 (75%) conditioned with RIC had impaired spermatogenesis.

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Kenney *et al.* (2001)	17	Median 12 (4–19)	Median 25 (16–34)	Median 12 (5–22)	All patients received vincristine, actinomycin D, and cyclophosphamide, and 8 patients also received doxorubicin. The median total dose of cyclophosphamide was 20.5 g/m^2 (range 4.7–31.9 g/m^2). 1 patient received bleomycin at the time of initial therapy. 11 patients received radiation as part of their initial planned therapy (6 to the head/neck, 3 to an extremity, 1 to the chest, and 1 to the lumbar spine).	17	Of the 17 patients, only 2 had a normal sperm count (11.8%), 5 patients had oligozoospermia (29.4%), and 10 patients had azoospermia (58.8%). None of the 10 patients treated prior to the onset of puberty had normal sperm counts. All 15 men who received >7.5 g/m^2 of cyclophosphamide had abnormal semen analysis and all the men who received >25 g/m^2 of cyclophosphamide were azoospermic (5/5 patients).
Kruseová *et al.* (2021)	143	Median 13.7 (0.1–19.1)	Median 23.6 (14.9–40.3)	Median 11.6 (5.1–32.0)	Compared five chemotherapeutic groups: antitumour antibiotics, alkylating agents, topoisomerase and mitotic inhibitors, platinum-based agents and antimetabolites. 34 patients also underwent RT (26 patients underwent abdominal RT with a median dose 24.8 Gy (range 15–40 Gy), eight patients underwent cranial RT with a median dose 40.2 Gy (range 12–55.6 Gy), and three patients underwent cranial + spinal irradiation 25 Gy).	143	Only 35% of survivors had normal semen analysis compared to 73.5% of the healthy controls. The highest risk for abnormal semen analysis was observed after procarbazine ($P < 0.0001$) and cyclophosphamide ($P < 0.018$) treatments. The lowest risk for abnormal semen analysis (higher number of normal semen analysis) was observed after methotrexate ($P < 0.0001$), cytosine arabinoside ($P < 0.002$), daunorubicin ($P < 0.004$), asparaginase ($P < 0.004$), 6-thioguanine ($P < 0.006$), and doxorubicin ($P < 0.043$) treatments. The highest risk for semen abnormalities was associated with survivors treated with alkylating agents (OR = 3.595, $P < 0.008$), and the lowest risk was associated with those treated with antitumour antibiotics (OR = 0.253, $P < 0.027$). The mean CED values in survivors with aspermia (12.6 g/m^2) were significantly higher than those in survivors with oligozoospermia (5.3 g/m^2) ($P < 0.01$) and normozoospermia (4.5 g/m^2) ($P < 0.01$).
Jaffe *et al.* (1988)	27	Median 12 (5–16)	NR	*After diagnosis* Median 11 (5–26)	Radiation therapy was administered to all patients with Hodgkin's disease and in six, the radiation field included the inguinal or paraaortic nodes. Seven patients received 2–6 cycles of MOPP CT (nitrogen mustard, oncovin, prednisone, and procarbazine) and five, COPP (cyclophosphamide, oncovin, prednisone, and procarbazine) or chlorambucil. Among the remaining patients, 10 received RT (five to the inguinal or pelvic nodes) and seven, an alkylating agent (cyclophosphamide, nitrogen mustard, or chlorambucil). One leukaemia patient with testicular relapse received RT to the gonads (2400 rad). Four patients received Adriamycin.	23	4 were oligozoospermic 14 were azoospermic *CT alone:* One patient who received 30 g of cyclophosphamide (21.4 g/m^2) and another 68 mg of phenylalanine mustard (45 mg/m^2) had normal reproductive capacity (fathered children). In contrast, two patients, one who received 32 g of cyclophosphamide (29.0 g/m^2) and another 55 g (37.9 g/m^2) were sterile. Two patients who received smaller quantities of cyclophosphamide 3.45 g (2.76 g/m^2) and 9.5 g (5.0 g/m^2) in combination with procarbazine were sterile and of 'questionable fertility', respectively. *RT:* The estimated gonadal scatter radiation dose ranged from 8 to 414 rad. One patient who received 106 rad had normal reproductive capacity, whereas another with 9 1 rad was sterile. A patient who received 146 rad had hypospermia, and another with 140 rad was sterile. *CT + RT:* Sterile patients generally received larger scatter doses of RT; among these, four also received alkylating agents and procarbazine, and two received alkylating agents.

(continued)

Table 1. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients with semen analysis	Effect
Zaletel *et al.* (2010)	64	Median 13 (3–16)	Median 21 (13–34)	Median 10 (4–27)	CT + RT: n = 49 RT: n = 10 CT: n = 5 CT: MOPP, MOPP-ABV, MOPP/ABVD, LOPP, COPP(A), and OPPA RT: (n = 59), n = 27 (19 boys, 8 girls) had RT above the diaphragm with 20–40 (median 30) Gy, N = 17 (8 boys and 9 girls) RT to the upper abdomen with 24–49 (median 30) Gy and N = 15 (11 boys, 4 girls) RT to the pelvis with 22–45 (median 30) Gy	6	Semen analyses were performed in 6 of 24 (25%) males with primary hypogonadism and all were azoospermic.
Ortin *et al.* (1990)	20	Median 14 (8–15)	NR	Median 10 (3–15)	RT alone: n = 3; the delivered dose at the midplane of the pelvis ranged from 15 to 44 Gy. Based on previously published studies using this technique. the testicular dose is reduced to less than 3% of the midplane tumour dose when a testicular shield is routinely used. RT+CT: n = 5; min 6 cycles of MOPP and pelvic RT (20–44 Gy) CT alone: n = 7; MOPP/ABVD for six cycles-16, PAVe for six cycles-3. VBM for six cycles1, ABVD for six cycles.	15	The 3 boys receiving RT alone were all oligozoospermic. 10/12 boys treated with MOPP with or without RT were azoospermic.
Ben Arush *et al.* (2000)	26	Group 1: Median 13.7 (2.1–16.4) Group 2: Median 8.8 (2.3–15.2)	Group 1: Median 22.0 (14.8–19.3) Group 2: Median 20.8 (16.0–29.0)	Group 1: Median 8.0 (4.0–17.3) Group 2: Median 10.7 (7.2–18.7)	Group 1: n = 12 CT: MOPP or MOPP/ABVD Group 2: n = 8 CT: COM, COMP, LSA_2L_2, 'NCI protocol' 5 patients also received RT, median dose 2320 Gy (1550–4000 Gy) with testicular shielding.	20	4 patients (20%) had normal sperm counts, 3 patients had oligozoospermia, 5 had severe oligozoospermia, 8 (40%) were azoospermic. In group 2, 4/5 patients who received additional inverted Y RT were azoospermic and 1 patient, who received a dose of only 16.5 Gy, had a sperm count of 3 000 000 sp/ml.

ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine; ALL: acute lymphoblastic leukaemia; ATG: anti-thymocyte globulin; BEAM: carmustine, etoposide, cytarabine, melphalan; BFM: Berlin-Frankfurt-Münster protocol; CCS: childhood cancer survivor; CED: cyclophosphamide equivalent dose; ChlVPP: chlorambucil, vinblastine, procarbazine, prednisone; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; CI: confidence interval; CNS: central nervous system; COM(P): cyclophosphamide, vincristine, methotrexate, (prednisone); COP: cyclophosphamide, vincristine, prednisone; COPP: cyclophosphamide, doxorubicin, procarbazine, prednisone; CPM: cyclophosphamide; CRT: cranial radio therapy; CT: chemotherapy; EBVP: epirubicin, bleomycin, vinblastine, prednisone; HDT: high-dose chemotherapy with autologous stem cell support; HSCT: haematopoietic stem cell transplant; LOPP/LVPP: vinblastine, chlorambucil, procarbazine, prednisone; LSA_2L_2: cyclophosphamide, vincristine, doxorubicin, asparaginase, thioguanine, methotrexate, 6-mercaptopurine; MAC: myeloablative conditioning; MOPP/MVPP: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; MTX: methotrexate; NCI protocol: methotrexate, cyclophosphamide, doxorubicin, prednisone; NHL: non-Hodgkin lymphoma; NR: not reported; OEPA: doxorubicin, etoposide, prednisone, vincristine; OPPA: doxorubicin, procarbazine, prednisone, vincristine; OR: odds ratio; PAVe: procarbazine, alkeran, velban; RIC: reduced intensity conditioning; RR: risk ratio; RT: radiotherapy; TBI: total body irradiation; TLI: total lymphoid irradiation; VBM: velban, bleomycin, methotrexate.

negative association between direct testicular exposure to radiotherapy and paternity at doses exceeding 4 Gy (Wasilewski-Masker *et al.*, 2014), ≥1 Gy (Korhonen *et al.*, 2025), or ≥7.5 Gy (Green *et al.*, 2010). Haematopoietic stem cell transplant (HSCT) was also associated with a reduced chance of paternity (Korhonen *et al.*, 2023). Conflicting results are seen for associations between alkylating agent exposure and paternity, with some studies showing reduced paternity in alkylating agent exposed childhood cancer survivors compared to those who had not received alkylators. However, increasing cumulative alkylating agent exposure as measured by alkylating agent dose score (AAD) or CED was associated with a reduced chance of siring a pregnancy (Green *et al.*, 2010; Chow *et al.*, 2016; Korhonen *et al.*, 2023, 2025; Wasilewski-Masker *et al.*, 2014; Korhonen *et al.*, 2025). When interpreting paternity data, it is important to consider that there are many factors in addition to testicular damage that will have an impact, including physical, social and psychological factors, as well as the age and fertility status of the partner.

Recommendations

Patients facing gonadotoxic treatment of less than 4 g/m^2 CED doses without additional gonadotoxic treatments are at low risk of infertility as a result of their gonadotoxic treatment, and therefore are not recommended to have a testicular biopsy for fertility preservation.

For patients facing gonadotoxic treatment equivalent to 4–8 g/m^2 CED, a testicular biopsy for fertility preservation can be considered, especially with increasing CED, provided that the general health of the patient allows such procedure. The lack of evidence quantifying the risk of azoospermia must be acknowledged.

For patients facing gonadotoxic treatment equivalent to >8 g/m^2 CED, a testicular biopsy for fertility preservation should be considered, especially with increasing CED, provided that the general health of the patient allows such procedure. The potential for delayed spontaneous spermatogenic recovery should be acknowledged.

Myeloablative conditioning treatment for bone marrow transplants and direct radiation of the gonads have a significant risk of infertility and a testicular biopsy for fertility preservation should be considered.

Patients receiving scattered radiation to the testis of less than 1 Gy alone are not recommended to have a testicular biopsy for fertility preservation.

Which patients are eligible after previous exposure?

Evidence

In total, 17 studies have reported on histological evidence of testicular damage after gonadotoxic treatment for malignant (Table 3) or non-malignant disease (Table 4). Overall, there was a strong and consistent reduction in spermatogonial number in testicular tissues exposed to alkylating agents, compared to controls who had received non-alkylating chemotherapy or unexposed controls. Age-adjusted spermatogonia counts have demonstrated a negative correlation with increasing cumulative exposure to alkylating agents, with exposures exceeding a CED of 4 g/m^2 leading to significant depletion of spermatogonia (Table 3). Reduced spermatogonial numbers have also been observed in boys with severe haematological conditions, such as sickle cell disease, regardless of hydroxyurea therapy, and those with Fanconi anaemia, even prior to undergoing gonadotoxic HSCT therapy (Table 4).

Recommendations

Ideally, testicular biopsy in eligible patients (see recommendations 2.1) should be performed before gonadotoxic treatment is started as prior gonadotoxic treatment may have reduced spermatogonial numbers.

Prior gonadotoxic treatment is not a contra-indication for testicular tissue cryopreservation in eligible patients, although the chance of future sperm production might be reduced. Minimizing alkylating agent exposure before cryopreservation is recommended. There is no minimum time period between treatment cycles of any chemotherapy and testicular tissue cryopreservation.

Boys with severe benign haematological disorders due to receive haematopoietic stem cell transplantation are eligible for testicular tissue cryopreservation after appropriate counselling regarding the potentially decreased spermatogonial numbers.

Further research is necessary to determine the advisability of testicular tissue freezing for patients with disorders associated with hereditary cancers and a significantly reduced spermatogonial pool (e.g. Fanconi anaemia).

What are the contra-indications to testicular tissue cryopreservation?

Evidence

No study could be retrieved from literature investigating contra-indications to testicular biopsy in the prepubertal population.

While testicular tissue cryopreservation may be considered, it is crucial to conduct a thorough patient evaluation, including an assessment of medical fitness and potential risks. Patient-related factors could influence the decision to offer FP. Exclusion criteria for testicular biopsy that have been proposed in research studies on FP include patients with a high bleeding and/or infection risk (Stukenborg *et al.*, 2018; Barraud-Lange *et al.*, 2024), patients who are determined to be medically inappropriate or unstable to undergo FP, patients who are actively participating in a phase I clinical trial, patients having already undergone a form of fertility intervention, patients having a treatment plan with goal of palliative care only or less than 20% expected survival (Sax *et al.*, 2022), and patients having an underlying testicular abnormality or pathology (Uijldert *et al.*, 2017).

Recommendation

In patients from whom viable sperm (not exposed to chemotherapy) can be obtained, regardless of the collection method, testicular tissue cryopreservation is not recommended. Patient- or disease-related factors should be considered in the decision to offer testicular tissue cryopreservation.

Counselling

Who should receive counselling?

Evidence

Parents of boys diagnosed with cancer and alive at the time of the study were invited to complete two questionnaires, of which 365 responded (Sadri-Ardekani *et al.*, 2013). The questionnaires assessed parental attitudes toward fertility preservation options, considering varying levels of infertility risk and success rates of fertility restoration. The study concluded that counselling on the risks of infertility due to cancer treatment should be universally provided, as parental decision-making appeared largely

Table 2. Studies reporting on the effects of gonadotoxic therapy on paternity, as a direct measure of subfertility, arranged in descending order of median follow-up duration.

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients investigated	Effect
Kitlinski *et al.* (2023)	1 159 959	<15	NR	(<15, ≥15 and <24, or ≥24 years	Fathers with a history of cancer were divided into 8 categories based on the information regarding cancer localization. (1) skin cancer (ICD-7: 140.0–140.9, 190.0–191.9); (2) prostate cancer (ICD-7: 177.0–177.9); (3) testicular cancer (ICD-7: 178.0–178.9); (4) digestive, respiratory, and urogenital tract cancers (ICD-7: 141.0–163.9, 179.0–181.9); (5) central nervous system and eye cancers (ICD-7: 192.0–193.1); (6) soft tissue and bone cancers (ICD-7: 193.3, 193.8, 193.9, 196.0–197.9); (7) haematological and lymphatic cancers (ICD-7: 200.0–207.9); and (8) all other cancer diagnoses (ICD-7: 164.0–164.9, 170.1, 170.2, 194.0–194.9, 195.0–195.9, 199.1–199.9).	861	Among childhood cancer survivors, 6% conceived by assisted reproduction, compared to 3% for controls (aOR 3.52, 95% CI 2.52–4.93; $P < 0.001$ for ICSI). When compared to the general population, CCS were all more likely to father a child through ART using donated spermatozoa (aOR 8.84, 95% CI 4.41–17.7; $P < 0.001$).
Reinmuth *et al.* (2013)	618	Median 10 (0–15)	Median 30 (19–43)	*After diagnosis* Median 22 (4–28)	**Treatment** / **Percentage of patients** Pelvic/spinal RT 4.1% *Cyclophosphamide* 67.6% Ifosfamide 19.4% Etoposide 17.0% Carboplatin 1.9% Cisplatin 12.8%	234	Of 59 male study participants who received cumulative cyclophosphamide doses >5 g/m^2 (but none more than 9.6 g/m^2), 11 reported that their partners became pregnant from them. Of 85 male childhood cancer survivors who had received doses of ifosfamide >42 g/m^2, eight reported that their partners became pregnant from them. Infertile men had significantly received RT of the pelvic region more often as compared to fertile/probably fertile men
Haavisto *et al.* (2024)	1212	Median 7 (0–17)	Median 29 (19–40)	Median 21 (1–37)	**Treatment** / **Number (%)** CT 887 (73.5%) Any local RT 262 (21.6%) Local cranial RT 141 (11.6%) Local abdominal RT 20 (1.7%) HSCT with or without TBI 99 (8.2%)	1212	**Intensity of therapy** / **Biological children** Minimally invasive 44/118 (37.3%) Moderately invasive 199/613 (32.5%) Very intensive 79/312 (25.3%) Most intensive 20/169 (11.8%) CT 233/887 (26.3%) Any local RT 71/262 (27.1%) Local cranial RT 38/141 (27.0%) Local abdominal RT 7/20 (35.0%) HSCT with or without TBI 9/99 (9.1%)
Jahnukainen *et al.* (2011)	75	Median 5 (1–15)	Median 29 (26–38)	Median 20 (11–30)	*Cumulative values* CRT: 24 (18–48) Gy Spinal RT: 6 Gy, n = 1 Testicular RT: 24 (10–24) Gy Cyclophosphamide: 6.9 (1.2–29.0)	47	None of the survivors treated with a >20 g/m^2 cumulative dose of cyclophosphamide or with testicular irradiation had fathered a child; they were potentially sterile. Testicular size and FSH were shown to be better than inhibin B in predicting fertility.

(continued)

Table 2. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients investigated	Effect
Mathiesen *et al.* (2020)	98	*At HSCT* Median 9.7 (0.4–16.9)	Median 28.1 (18.5–47.0)	Median 18.3 (7.7–34.6)	Myeloablative allogeneic HSCT 6 treatment groups according to their cumulative therapy: 1. CT only, 2. Low-dose testicular RT including TBI 2 Gy, TLI 6 Gy and TBI with gonadal shielding, 3. TBI without shielding, 4. TBI plus additional CNS irradiation, 5. TBI plus additional testicular irradiation, 6. TBI plus additional CNS and additional testicular irradiation.	24	Of 24 patients who had attempted to conceive, 9 men sired 21 pregnancies. None of the survivors treated for ALL sired pregnancies. Of the 6 men who reportedly fathered children, 4 had been treated with TBI without gonadal shielding, and spermatogenesis was confirmed in 3 of these 4 men at the time of the study.
Tromp *et al.* (2011)	565	Median 7.8 (0.0–17.8)	Median 21.0 (18.0–46.0)	Median 15.0 (5.0–39.0)	Combination of CT and surgery for 172 survivors (30.4%). Almost 90% of the population received chemotherapy; only nine survivors (2.4%) were treated with a chemotherapeutic agent other than an alkylating agent, vinca-alkaloid or antimetabolite. TBI	73	During the follow-up period, 73 men reported that their partner had become pregnant: 120 conceptions resulted in 103 live births and 14 miscarriages. 56 (77%) were able to achieve conception naturally.
Relander *et al.* (2000)	77	Median 11 (0.8–17)	Median 23.6 (18.6–38.5)	*After diagnosis* 13.2 (3.5–22.8)	41/77 (55%) patients had received only local treatment including surgery in 16, RT in 6, and a combination of surgery and RT in 19 patients. One had CT only and 35 had CT + local therapy.	10	Ten patients had fathered children (n = 4; 1–3 children). A semen analysis was done in 9 of these patients and showed normozoospermia in 7 and oligozoospermia in 2 (one severe). Four of the patients with children had testicles <15 ml.
Jaffe *et al.* (1988)	27	Median 12 (5–16)	NR	*After diagnosis* Median 11 (5–26)	RT was administered to all patients with HD and in six, the radiation field included the inguinal or paraaortic nodes. N = 7: 2–6 cycles of MOPP N = 5: COPP or chlorambucil. N = 10: RT (five to the inguinal or pelvic nodes) n = 7: CT with alkylating agent (cyclophosphamide, nitrogen mustard, or chlorambucil). N == 1: patient with testicular relapse received radiation to the gonads (2400 rad). N = 4: Adriamycin.	27	6 males fathered children, of which 3 had 2 children.
Sylvest *et al.* (2021)	9353	**Age** / **Number (%)** 0–4 / 852 (9.1%) 5–9 / 603 (6.5%) 10–14 / 708 (7.6%) 15–19 / 1540 (16.5%) 20–24 / 2474 (26.5%) 25–29 / 3176 (34.0%)	NR	Cancer group: Median 9.7 Control group: Median 10.3	NR	9353	3404 (36%) males with a previous cancer diagnosis became fathers during the study period compared to 42% in the age-matched group. Men surviving CNS cancer had the lowest HR of fatherhood compared with the age-matched comparison group (HR 0.67, 95%CI 0.57–0.79), followed by survivors of haematological cancers (HR 0.90, 95% CI 0.81–1.01), while the highest chance of fatherhood was among survivors of solid cancers (HR 1.16, 95%CI 1.12–1.20), with a slightly increased chance compared with undiagnosed males.

(continued)

Table 2. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients investigated	Effect	
							Age	Adjusted HR
							0–4	0.66 (95% CI 0.55–0.79)
							5–9	0.64 (95% CI 0.53–0.78)
							10–14	0.61 (95% CI 0.51–0.72)
Papadakis *et al.* (1999)	36	Median 13.0 (2.4–22.6)	Median 22.3 (15.1–32.5)	Median 6.8 (2.0–19.3)	CT: first doxorubicin (60–75 mg/m^2), procarbazine (50 mg/day for 2 days and 100 mg/day for 26 days) in combination with prednisone (30 mg/m^2/day) and vincristine (1.5 mg/m^2) and finally cyclophosphamide (1200 mg/m^2) RT: 24 or 36 Gy following the first 3 cycles CT or 24 Gy after 6 cycles CT. Group 1: n = 13; only RT, not involving the pelvis Group 2: n = 40; CT ± RT, not involving the pelvis Group 3: n = 12; CT+RT involving the pelvis	36	Two patients who did not receive RT fathered three normal children. Additionally, one (CT ± RT−) patient's partner conceived but the pregnancy ended in a spontaneous abortion.	
Green *et al.* (2010)	6224	<21 years	NR	≥5 years	Summed alkylating agent dose 0: n = 2270 1: n = 483 2: n = 570 3: n = 724 4: n = 234 5: n = 138 6–11: n = 163	941	Participants who received testicular RT at a dose ≤7.5 Gy were not less likely to obtain a pregnancy compared with patients who received no testicular radiation (HR 1.62; 95% CI 0.39–6.71). Those who received a testicular RT dose of more than 7.5 Gy were less likely to obtain a pregnancy compared with those who did not receive testicular RT (HR 0.12; 95% CI 0.02–0.64). Those who had a summed AAD score of 2 (HR 0.67; 95% CI 0.51–0.88), 3 (HR 0.48; 95% CI 0.36–0.65), 4 (HR 0.34; 95% CI 0.22–0.52), 5 (HR 0.38; 95% CI 0.22–0.66), or 6–11 (HR 0.16; 95% CI 0.08–0.32) were also less likely to ever sire a pregnancy compared with those who did not receive any alkylating agents. Participants who received a cumulative procarbazine dose in the second tertile (4.2–7.0 g/m^2; HR 0.48; 95% CI 0.26–0.87) or third tertile (7.0–58.7 g/m^2; HR 0.17; 95% CI 0.07–0.41) were less likely to obtain a pregnancy compared with those who did not receive procarbazine. Similarly, those exposed to a cumulative cyclophosphamide dose in the third tertile (9.36–143.8 g/m^2; HR 0.42; 95% CI 0.31–0.57) were less likely to ever obtain a pregnancy compared with those who did not receive cyclophosphamide.	

(continued)

Table 2. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients investigated	Effect
Reulen *et al.* (2009)	10 483	NR	NR	≥5 years	No RT. RT other than to the brain or abdomen, RT to the brain. RT to the abdomen	3244	No significant variation in the ORs of any adverse pregnancy outcome by cancer type, exposure to chemotherapy, brain irradiation, or abdominal irradiation.
Green *et al.* (1989)	39	Median 9.8 (3.8–15.6)	NR	>5 years from diagnosis	The ranges of cumulative dose for the seven agents were: N = 12: methotrexate, 150–12 347 mg/m^2 (median, 4520 mg/m^2); N = 10: 6-mercaptopurine, 2.5–130.4 g/m^2 (median, 99.0 g/m^2); N = 12: vincristine, 6–76 mg/m^2 (median, 54 mg/m^2); N = 2: daunorubicin, 180–950 mg/m^2; N = 2: cyclophosphamide, 1.1–17.2 g/m^2; N = 2: BCNU, 233–270 mg/m^2; and n = 7: 1-asparaghase, 30 000–360 000 U/m^2 (median 150 000 U/m^2)	NR	Spouses of 4 men with childhood ALL reported 10 pregnancies. There was one spontaneous abortion. One infant was stillborn after obstetric complications during a delivery complicated by shoulder dystocia. There were eight live-born infants.
Green and Hall (1988)	48	Median 14.9 (5.1–19.9)	Single men: Median 23.1 (18.1–40.1) Married men: Median 31.1 (20.5–42.1)	>5 years from diagnosis	NR	48	Pregnancies were reported by the spouses of seven of the married male patients who were not known to be severely oligo- or azoospermic. These women, six of whom were spouses of male patients treated only with radiation therapy that did not include the abdomen-or pelvis and one of whom was the spouse of a male patient treated with supradiaphragmatic irradiation and combination chemotherapy, reported 14 pregnancies, of which three were in gestation, two aborted spontaneously, and nine resulted in the birth of full-term infants.
Korhonen *et al.* (2023)	252	Mean 6.2 (IQR 3.2–11.4)	37.6 ± 7.6	>5 years from diagnosis (6–42 years)	**Treatment / Number / Dose** CED (g/m^2) / 176 / 9.9 (4.1–16.5) DIE (mg/m^2) / 144 / 240 (120–350) Pituitary RT / 183 / Cumulative pituitary RT dose (Gy) / / 12 (0.53–24) Testicular RT / 148 / Cumulative testicular RT (Gy) / / 2.9 (0.13–12) HSCT / 52 /	252	OR of ever fathering a child after childhood cancer treatment **Treatment / OR (95 % CI)** **Treatment intensity** Least-moderate / Reference Very / 0.94 (0.51–1.72) Most / 0.47 (0.21–1.04) **CED (g/m^2)** 0 / Reference >0 –<4 / 1.29 (0.55–3.01) 4–15 / 0.64 (0.33–1.28) >15 / 0.60 (0.27–1.33)

(continued)

Table 2. Continued

Reference	Total no. of patients	Age at diagnosis (years)	Age at evaluation (years)	Follow-up period (years)	Type of gonadotoxic treatment	No. of patients investigated	Effect
							DIE (mg/m^2)
							0 Reference <250 1.30 (0.65–2.60) ≥250 0.79 (0.40–1.57)
							Cumulative pituitary RT dose (Gy)
							0 Reference >0 –<10 0.99 (0.47–2.10) ≥10 –<24 0.79 (0.33–1.87) ≥24 0.48 (0.22–1.02)
							Cumulative testicular RT dose (Gy)
							0 Reference >0 –<1 0.88 (0.47–1.67) 1–6 0.59 (0.10–3.63) >6 0.19 (0.08–0.48) No Reference Yes 0.33 (0.11–0.85)
Chow *et al.* (2016)	10 938	<5 n = 2085 5–9 n = 1254 10–14 n = 1287 15–20 n = 1014	NR	5 years since initial diagnosis or age 15 years, whichever was later.	Lower CED: <4897 mg/m^2 Middle CED: 4897–9638 mg/m^2 Upper CED: ≥9639 mg/m^2	4149	Male participants who received cumulative doses of cyclophosphamide, ifosfamide, and procarbazine in the upper tertiles (≥7.4 g/m^2, ≥53 g/m^2, and ≥5.1 g/m^2, respectively) reported a significantly decreased likelihood of pregnancy compared with those not exposed to each drug. Cyclophosphamide doses of 5.6 g/m^2 or higher (median cutoff point) were associated with a reduced likelihood of pregnancy. High cisplatin doses (upper tertile ≥488 mg/m^2) were also significantly associated with a decreased likelihood of pregnancy in male survivors. For alkylating drugs, higher CED were significantly associated with a decreased likelihood of male survivors obtaining a pregnancy.
Madanat *et al.* (2008)	6071	0–14 years at diagnosis	NR	≥9 months after diagnosis	NR	1476	The cumulative probability of having a first child was clearly lower in cancer survivors than in siblings. The relative probability of parenthood was RR 0.51, 95% CI 0.46–0.57 in the childhood age group. In the paediatric diagnostic age-group, the lowest relative probabilities of parenthood were observed for in the CNS tumour and HL groups. The least reduced relative probabilities were in the NHL and soft-tissue sarcoma groups.

(continued)

Table 2. Continued

Reference	Total no. of patients	Age at diagnosis (years)		Age at evaluation (years)		Follow-up period (years)	Type of gonadotoxic treatment		No. of patients investigated	Effect
Wasilewski-Masker *et al.* (2014)	701	**Age**	**Number**	**Age**	**Number**	NR	**Characteristic**	**N = 701**	290	Infertility was 24% among survivors younger than 30 years of age and increased to greater than 40% among those older than 30 years of age. AAD ≥3, surgical excision of any organ of the genital tract, and testicular radiation dose ≥4 Gy were all statistically significant independent risk factors for infertility. An AAD ≥3 (RR 2.13, 95% CI 1.69–2.68) was associated with a high risk for infertility versus an AAD.
		0–4	69/191	20–29	12/50		**AAD score in first 5 years after diagnosis**			
		5–9	63/163	30–39	142/337		0	75/269 (27.9%)		
		10–14	91/186	40–49	121/282		1	17/70 (24.3%)		
		15+	67/161	50+	15/32		2	38/102 (37.3%)		
							3	65/103 (63.1%)		
							4	26/39 (66.7%)		
							≥5	27/37 (73.0%)		
							Unknown	42/81 (51.9%)		
							Testicular RT in first 5 years after diagnosis			
							None	85/231 (36.8%)		
							<4 Gy	166/397 (41.8%)		
							≥4 Gy	19/23 (82.6%)		
							Unknown	20/50 (40.0%)		
							TBI in first 5 years after diagnosis			
							Yes	2/2 (100%)		
							No	264/640 (41.3%)		
							Unknown	24/59 (40.7%)		

AAD: alkylating agent dose score; ALL: acute lymphoblastic leukemia; aOR: adjusted Odds ratio; CCS: childhood cancer survivor; CED: cyclophosphamide equivalent dose; CI: confidence interval; CNS: central nervous system; COPP(A): cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin); CRT: cranial radio therapy; CT: chemotherapy; DIE: cumulative doxorubicin isotoxic dose; FSH: follicle stimulating hormone; HD: Hodgkin's disease; HL: Hodgkin lymphoma; HR: hazards ratio; HSCT: haematopoietic stem cell transplant; ICD: International Classification of Diseases; MOPP/MVPP: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; NR: not reported; OR: odds ratio; RR: risk ratio; RT: radiotherapy; TBI: total body irradiation.

Table 3. Histological evidence of testicular damage after exposure to gonadotoxic therapy.

Reference	Total no. of patients	Age at biopsy (years)	Type of gonadotoxic treatment	Number of biopsies assessed	Effect
Barraud-Lange *et al.* (2024)	350	7.1 (0.6–17.1)	274/350 (78%) boys were exposed to CT before biopsy, of which 165 were exposed to an alkylating agent (47%)	302	237 of the 302 boys were exposed to CT before cryopreservation. The risk of absence of spermatogonia in testicular tissue samples was significantly higher (4-fold) in boys previously exposed to alkylating agent (OR 4.28, 95% CI 1.22–14.98).
Feraille *et al.* (2023)	79	6.7 (0.5–16)	68 patients received alkylating CT: -Alkylating (n = 58): mean CED 7.3 ± 7.3 g/m^2 (0.8–37.6 g/m^2) -Carboplatin cumulative dose (n = 10): 1.6 ± 0.7 g/m^2 (0.8–3.2 g/m^2) 11 patients received non-alkylating CT	79	The number and percentage of Sertoli cells per seminiferous tubule expressing the proliferation marker PCNA were positively correlated with the delay between the last chemotherapy course and TTF. The TFI and S/T decreased significantly with an increasing CED. However, no difference in TFI and S/T was seen between patients who received carboplatin alone and patients who received no alkylating agent.
Moussaoui *et al.* (2022)	35	8.5 ± 5.1	CT before biopsy: n = 19; of which alkylating CT: n = 16 Average CED exposure: 5.5 g/m^2 (±3.4, range 2–15.6 g/m^2). Untreated patients: n = 16 The primary indication for TTC was conditioning for HSCT in 25 patients (71.4%)	35	The median number of spermatogonia per tubule cross-section was 2 (range 0–6). In patients having received alkylating chemotherapy prior to TTC, the median number of spermatogonia was significantly lower than in patients who had not yet received alkylating (0.5 with a range 0–4, and 2.75 with a range 0–6 respectively).
Funke *et al.* (2021)	79	7.82 (0.1–17.49)	Non-treated controls (n = 20) Non-treated cancer patients (n = 12) Non-alkylating (n = 25) Alkylating (n = 22): 6610.91 ± 3512.35 mg/m^2	79	The Z-scores for S/T values in the non-treated samples (−2.08 ± 2.20) and samples treated with non-alkylating agents (−1.90 ± 2.60) were comparable within ±3 SD of the reference mean value but differed significantly from samples exposed to alkylating agents (−12.14 ± 9.20). The Z-scores for S/T were correlated with increasing cumulative exposure to alkylating agents ($r = 0.7020$, $P < 0.0001$). An S/T Z-score less than −3 showed good diagnostic value (AUC 0.93; 95% CI 0.86–0.99) when identifying cancer patients exposed to any alkylating chemotherapy. An S/T Z-score less than −7 identified patients exposed to CED ≥ 4 g/m^2 (AUC 0.96; 95% CI 0.90–1.01), and was associated with a significantly depleted spermatogonial pool.
Medrano *et al.* (2021)	56	Non-treated: 6.89 ± 4.54 Weakly affected: 7.11 ± 4.16 Severely affected: 6.94 ± 4.30	28 patients had been exposed to CT before testicular biopsy 28 patients were not exposed to CT before biopsy.	56	2 groups identified: patients with higher overall Z-score values in all studied variables (weakly affected group; n = 9) and a group with lower overall Z-score values in all studied variables (severely affected group; n = 19). Regression analysis identified seven drugs associated with this altered histologic phenotype: cyclophosphamide and ifosfamide, cytarabine and asparaginase, were associated with a worse histologic phenotype, whereas the daunorubicin and idarubicin, and 6-mercaptopurine seemed to be associated with the weakly affected phenotype.
Valli-Pulaski *et al.* (2019)	189	7.9 ± 5.0	N = 74 had already started CT; Of which n = 30 non-alkylating CT And n = 44 alkylating CT, average CED 2.8 ± 1.7 g/m^2 (0.5–7 g/m^2) N = 115 not yet exposed to CT	189	A previous exposure to non-alkylating or alkylating chemotherapy did not impact the number of (UTF1+ or DDX4+) spermatogonia per tubule cross section compared with patients that did not have a previous exposure.

(continued)

Table 3. Continued

Reference	Total no. of patients	Age at biopsy (years)	Type of gonadotoxic treatment	Number of biopsies assessed	Effect
Stukenborg *et al.* (2018)	32	*At biopsy* Non-Alkylating 6.6 ± 4.8 Alkylating 7.3 ± 3.7 Hydroxyurea 7.9 ± 3.6	Non-alkylating (n = 7) Alkylating (n = 6): CED 5.5 ± 3.0 g/m^2, cyclosphophamide: 5.1 ± 3.2 g/m^2 Hydroxyurea (n = 6): 24.5 ± 2.7 mg/kg No CT (n = 12) Controls (n = 14)	32	Mean S/T in samples from cancer patients exposed to non-alkylating agents (1.7 ± 1.0, n = 8) and biobank controls (4.1 ± 4.6, n = 14) did not exhibit significant differences in S/T numbers. Samples from cancer patients exposed to alkylating agents, exhibited a lower mean S/T value (0.2 ± 0.3, n = 6) compared with samples from patients treated with non-alkylating agents ($P = 0.003$) or biobank controls ($P < 0.001$).
Ho *et al.* (2017)	44	N = 33 prepubertal 0.3–11.3 N = 3 pubertal 12.7-16.8	BMT: n = 12 Malignancy: n = 30 7 patients were exposed to CT before biopsy	44	All patients with sperm identified were pubertal with testicular size >10 ml and Tanner staging 3+. One patient had prior 'low risk' gonadotoxic therapy (vincristine, daunorubicin, methotrexate, cytarabine, asparaginase, prednisolone) for ALL. All other boys in whom sperm were found were chemotherapy naïve. A 13.6-year-old patient who received cyclophosphamide 2 months before testicular biopsy did not have any sperm found despite a testicular volume of 12 ml.
Poganitsch-Korhonen *et al.* (2017)	37	Group 1: 5.3 ± 2.7 Group 2: 7.3 ± 5.2	The therapy involved the use of antimetabolites, vinca-alkaloids and anthracyclines. Of the 37 patients, 15 received prophylactic cerebral RT (18–24 Gy), but spinal RT was not used. Group 1: n = 21; non-alkylating agents: anthracycline (55 ± 61 mg/m^2) Group 2: n = 16; alkylating agents: cyclophosphamide 6.3 ± 3.5 g/m^2 and carmustine 46.9 ± 84.1 mg/m^2; CED 7.0 ± 3.8 g/m^2	37	Numbers of S/T in samples from patients not exposed to alkylating agents (1.6 ± 0.8, n = 19) were within the 95% CI of normative reference values. A significantly lower mean S/T value (0.4 ± 0.5, n = 16) was found in samples from patients exposed to alkylating agents. Testicular RT resulted in complete depletion of spermatogonia. Regression analysis showed that cumulative CEDs >4 g/m^2 led to S/T values close to zero.
Van Saen *et al.* (2015)	20	Median 8.5 (1–15)	Group 1: untreated cancer patients (n = 7) Group 2: hydroxyurea (n = 6) Group 3: CT (n = 7) VP16, mitoxantrone, araC, cisplatin, carboplatin, cyclophosphamide, vincristine, HDMTX, daunorubicine, asparaginase, Adriamycin, ifosfamide, methotrexate, idarubicine, daunoxome, 6-thioguanine	20	No significant difference in the germ cell marker protein distribution (UCHLI, OCT 4) across the different groups. Sertoli cell marker protein distribution (AMH, AR, SOX9, INHA α). Normal Sertoli cell expression for INHA α and SOX9 in the Sertoli cells was observed in all groups.
Nurmio *et al.* (2009)	23	5.7 ± 2.9	The 'high risk' patients and the patient with secondary ALL received a high cumulative dose of cyclophosphamide, which is higher than that used in the modern protocols. The patients considered being at standard risk received the treatment that is comparable to the current protocols. In addition, four patients in the 'high risk' group received prophylactic cerebral RT (24 Gy), but spinal RT was not used. Patients experiencing testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial irradiation at a dose of 24 Gy.	28	After induction therapy, 90% of the seminiferous cords contained MAGE A4+ and 20% OCT4+ or CD9+ spermatogonia. The number of MAGE A4+ (differentiated) spermatogonia per cross-section of seminiferous cord reduced by 50% after standard risk therapy of ALL. The number of OCT4+ and CD9+ (germ stem-like) cells were not significantly influenced by standard risk therapy of ALL. After therapy for high-risk or secondary ALL, only 20% of the seminiferous cords contained MAGE A4+ spermatogonia, none contained CD9+ germ cells, and only a few individual OCT4+ cells were detected. No cells expressing spermatogonial markers were detected in the two samples obtained after 'high risk' therapy involving cyclophosphamide.

(continued)

Table 3. Continued

Reference	Total no. of patients	Age at biopsy (years)	Type of gonadotoxic treatment	Number of biopsies assessed	Effect
Quigley et al. (1989)	45	4.39 (1.23–12.35)	Cyclophosphamide: mean dose 4.8 g/m^2, cytarabine: mean dose 13.1 g/m^2, asparaginase, daunorubicin, hydroxyurea, lomustine, methotrexate, prednisolone, thuiguanine, vincristine. Cranial irradiation: 24 Gy and intrathecal methotrexate	24	All 24 biopsy specimens were abnormal. In 13 patients, a total absence of germ cells was observed. In 11 patients, germ cells were markedly depleted.
Ise et al. (1986)	46	5.4 (0.08–13)	Vincristine, prednisolone, anthracycline, L-asparaginase, cytosine arabinoside, prophylactic skull RT and 5 intrathecal doses of methotrexate. Remission was maintained with daily 6-mercaptopurine, weekly methotrexate and vincristine, prednisolone, cyclophosphamide, Adriamycin or cytosine arabinoside every 2 or 3 months	34	No apparent relationship was found between the TFI and the cumulative dosage of chemotherapeutic agents, except for cyclophosphamide.
Müller et al. (1985)	10	(0–15)	Vincristine, prednisone, 6-mercaptopurine, methotrexate and asparaginase together with intrathecal methotrexate. n = 3 received daunorubicin, n = 1 adriamycin and n = 2 cyclophosphamide and cytosine arabinoside n = 6 received either cranial or craniospinal RT, n = 1 mediastinal RT.	10	The germinal epithelium of 2/10 boys matured partially. Cyclophosphamide and cytosine arabinoside had serious adverse effects on male germ cells

ALL: acute lymphoblastic leukaemia; AMH: anti-Müllerian hormone; AR: androgen receptor; AUC: area under the curve; BMT: bone marrow transplant; CD9: Cell surface glycoprotein; CED: cyclophosphamide equivalent dose; CI: confidence interval; CT: chemotherapy; DDX4: anti-DEAD-box helicase 4; HDMTX: high-dose methotrexate; INH: inhibin; HSCT: haematopoietic stem cell transplant; MAGE A4: melanoma-associated antigen 4; OCT4: Octamer-binding transcription factor 4; PCNA: Proliferating cell nuclear antigen; SD: standard deviation; SOX9: sex-determining region Y-box 9; S/T: Number of spermatogonia per seminiferous tubule cross-section; TFI: Tubular fertility index; TTC: testicular tissue cryopreservation; TTF: testicular tissue freezing; UCHLI: ubiquitin carboxyl-terminal esterase L1; UTF1: Undifferentiated embryonic cell transcription factor 1; VP16: vincristine, platinol.

unaffected by factors such as educational level, religious beliefs, financial status, or the presence of other children. Many parents want to preserve their son's fertility even if the risk of becoming infertile or the chances of fertility restoration are low.

A questionnaire was sent to a cohort of 290 patients and their parents/guardians referred for FP with testicular tissue freezing (TTF), and 120 questionnaires were recovered (Wyns et al., 2015). The results showed that most boys aged >12 years considered the information to be clear (72%), complete (80%), and understandable (90.9%). However, only 33.3% of boys aged <12 years were able to comprehend the information, the youngest being 11 years old.

Recommendation

Counselling on fertility risk of patients should be provided to both patients and care-givers (parents or legal guardian). This counselling should be age-appropriate.

When should counselling begin?

Evidence

In a systematic review, including 80 articles, recommendations for improving oncofertility discussions with adolescents are discussed (Barlevy et al., 2017). There was a general agreement that ideally, these discussions should take place before the treatment, at the time of diagnosis. Also, due to the potential risk of infertility, FP procedures should preferably take place before gonadotoxic treatment starts. A growing trend was found in recommendations to have several oncofertility discussions: before, during and after treatment.

Recommendation

Counselling about fertility risk and options for fertility preservation should be given at least verbally at the time of the diagnosis to ensure a clear understanding of the clinical implications.

Further counselling may be required, particularly if the prognosis or treatment plan is changing.

Who should deliver counselling?

Evidence

In a systematic review, including 80 articles, recommendations for improving oncofertility discussions with adolescents are discussed (Barlevy et al., 2017). In most of the articles, a comprehensive team approach is taken to initiate oncofertility discussions. These teams can include paediatric oncologists, haematologists, surgeons, paediatricians, gynaecologists, urologists, reproductive endocrinologists, nurses, psychologists, social workers, and bioethicists. In some papers, it was suggested that a specific team member should be responsible for these discussions, and some believe nurses would best take up this role.

A survey-based study among 120 families and paediatric patients demonstrated a significant increase in participant knowledge and perceived understanding after viewing educational videos on FP (Hanna et al., 2023). Post-test comprehension scores were significantly improved for all participants and all subgroups. These results suggest that video based educational tools may help to reduce barriers to FP.

In a small study, including 18 oncology nurses, semi-structured interviews were performed regarding their discussions of FP with adult male patients with cancer (Zhang et al.,

Table 4. Histological evidence of underlying testicular pathology in benign haematological diseases prior to gonadotoxic therapy exposure.

Reference	Total no. of patients	Age at biopsy (years)	Type of gonadotoxic treatment	Number of biopsies assessed	Effect
Lahtinen *et al.* (2024)	43	Mean 6.9 (range 0.4–15.9)	Aplastic anaemia (AA), bone marrow failure syndrome (BMFS), immunodeficiency (IMMUNO), myelodysplastic syndrome (MDS)/myeloproliferative neoplasia (MPN) HSCT patients undergoing testicular biopsy before HSCT, not exposed to CT except for 1 treated for ALL 2 years prior.	43	Altogether 49% (21/43) of patients had S/T Z-score value less than −3 SD, which was considered as the threshold for normal range. 8 patients (8/43, 19%) had S/T Z-score value less than −7 SD which is considered as severely depleted spermatogonial pool. Patients in AA/BMFS group had a median S/T Z-score of −4.0 (range −20.0–1.8). Patients in the IMMUNO group had a median S/T Z-score of −1.9 (range −19.3–2.6). Patients in the MDS/MPN group had a median S/T Z-score of −2.7 (range −20.5–0.0). All three patients with Fanconi anaemia had severely depleted spermatogonial numbers with very low S/T Z-score (range −20.0-−14.4). Fertility index (FI) values had large variation in each group. Median FI Z-score was −6.1 (range −14.1–7.4) in the AA/BMFS group, −2.9 (range −12.0–5.1) in the IMMUNO group, and −4.2 (range −15.5–1.5) in the MDS/MPN group. The proportion of patients with FI Z-score within the normal range was 33% (3/9), 52% (11/21) and 38% (5/13) respectively.
Benninghoven-Frey *et al.* (2022)	29	7.1 (2.8–15.1)	Sickle cell disease (SCD) patients having hydroxyurea (HU) therapy, undergoing testicular biopsy before HSCT.	29	N = 17 scored below previously published reference values of S/T (Z-score ≤ 3), but only four were devoid of spermatogonia. There was no correlation between spermatogonial numbers and HU dose or exposure time. Association was identified between reduced spermatogonial numbers and younger age at the initiation of HU.
Gille *et al.* (2021)	30	10.1 (5.8–15)	30 patients with SCD, 13 had not been exposed to HU, 17 patients had HU at a median dose of 22.0 mg/kg/day for a median of 36.0 months.	48	The spermatogonial pool was not statistically different between patients exposed and not exposed to HU: S/T ratio 2.5 ± 3.3 vs. 1.7 ± 0.6, respectively; SCO 42 ± 21 vs. 38 ± 16%, respectively, The spermatogonial quantity in SCD patients was lower than in healthy boys. There was no correlation between the duration of the transfusion therapy and the spermatogonial count ($r = -0.15$).
Ho *et al.* (2017)	189	7.9 ± 5.0	N = 74 had already started CT; Of which n = 30 non-alkylating CT And n = 44 alkylating CT, average CED 2.8 ± 1.7 g/m^2 (0.5–7 g/m^2). N = 115 not yet exposed to CT N = 15 SCD N = 15 thalassemia	189	Among the five patients with no germ cells, three were receiving treatments for SCD and thalassemia that are known to impact fertility.
Stukenborg *et al.* (2018)	32	*At biopsy* Non-Alkylating 6.6 ± 4.8 Alkylating 7.3 ± 3.7 Hydroxyurea 7.9 ± 3.6	Non-alkylating (n = 7) Alkylating (n = 6): CED 5.5 ± 3.0 g/m^2, cyclosphophamide: 5.1 ± 3.2 g/m^2 Hydroxyurea (n = 6): 24.5 ± 2.7 mg/kg No CT (n = 12) Controls (n = 14)	32	Five boys with SCD aged 4–7 and 13 years had a totally depleted spermatogonial pool, while the remaining 11.5-year-old boy had low spermatogonial quantity compared to normal values present in the control material. All boys with SCD had received hydroxyurea.

AA: aplastic anaemia; ALL: acute lymphoblastic leukaemia; BMFS: bone marrow failure syndrome; CED: cyclophosphamide equivalent dosing; CT: chemotherapy; FI: fertility index; HSCT: haematopoietic stem cell transplant; HU: hydroxyurea; MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasms; S/T: number of spermatogonia per seminiferous tubule cross-section; SCD: sickle cell disease; SD: standard deviation.

2023). The nurses had a positive attitude toward FP, but most had no practical role in routinely informing male patients of their options. Discussion of FP was outside their scope of practice. Therefore, local fertility nurses should be given new training regarding FP.

In a cross-sectional study, men above 18 years old with a cancer diagnosis within the last 10 years were eligible to participate, of whom 72 completed the survey part of the study (Ehrbar *et al.*, 2022). Participants rated experienced professionals as supportive. They would use an additional support tool too.

In a retrospective study, fertility consults were compared before and after hiring a full-time fertility navigator. A fertility navigator can have a variety of backgrounds/training levels, playing a critical role within multidisciplinary teams as counsellor, patient advocate and FP coordinator (Wright *et al.*, 2022). Overall, the number of fertility consults increased by more than threefold after hiring a fertility navigator, especially among female and long-time follow-up patients.

A survey was circulated among nurses in applicable care settings. Fifty-two nurses participated in the survey (Keim-Malpass *et al.*, 2018). Many nurses expressed the perception that fertility preservation counselling was important, but it was outside the scope of their practice to perform this education.

An online survey was distributed among oncology nurses and completed by 421 (Krouwel *et al.*, 2017). Less than half of oncology nurses were comfortable discussing fertility issues. The vast majority reported limited knowledge about FP options, but did feel responsible for addressing FP, in cooperation with the oncologist.

A survey was distributed among physicians who provided daily medical care to cancer patients, of whom 412 participated (Takeuchi *et al.*, 2017). Physicians who understood the importance and responsibility for supporting fertility issues were more likely to discuss such issues with cancer patients.

Recommendation

Counselling on fertility risk and fertility preservation is an inter-disciplinary team effort. A designated, experienced person taking up the role of counsellor, navigating the inter-disciplinary team communication, can improve the quality of counselling.

What should counselling include?

Evidence

In a systematic review, including 80 articles, recommendations for improving oncofertility discussions with adolescents are discussed (Barlevy *et al.*, 2017). Various forms of communication should be used and oncofertility discussion should include: possible cancer treatment-related effects on fertility; general education regarding fertility and sexuality; individual preferences regarding participation in decision-making; individual concerns and values regarding future parenting; fertility assessment; FP options, risks, benefits, success rates, and related financial costs and/or assistance programs; alternatives to FP; and plans for any cryopreserved biological materials in the event of patient death.

In a previous systematic review, including 24 studies (8 qualitative and 16 quantitative), experiences with fertility preservation were presented (Tschudin and Bitzer, 2009). Counselling should consider the patient's individual background and context, be provided in a timely, clear, transparent and accurate manner, and address the patient's emotional needs. Medical oncologists and fertility specialists ranked higher than other health professionals for adequately addressing concerns about fertility and an individual consultation with a fertility specialist was preferred over other various types of information sources (decision aids, leaflets and internet). The perceived relevance of fertility preservation seems to depend on factors such as the stage of life at cancer diagnosis. Parental support is therefore important and required regarding this issue. Provision of information by health professionals as well as patient and parental recall of having been informed seems to be selective. Men who had been informed about the potential for cancer-related infertility and men who chose to bank sperm scored higher on knowledge regarding this area. For younger patients, counselling and information around fertility were more important and females ranked counselling about fertility, reproductive problems and options for having children significantly higher than did males.

In a mixed-method randomized controlled study, adolescent and young adults (AYA) aged 15–30 years were allocated to either receive the written information resource (n = 13), or this resource in combination with a consultation with a health care provider (n = 10) (Allison *et al.*, 2023). A 60-page written resource designed to provide adolescent and young adults with age-appropriate information about oncofertility information was not associated with improved psychological well-being. Participants receiving the augmented intervention became more nervous/fearful about fertility treatment. With regards to timing, this intervention may be more helpful "for somebody going through it initially" (i.e. diagnosis/treatment).

The impact of fertility counselling was investigated with a survey in 51 parents and 7 adolescent patients before undergoing HSCT (Barnbrock *et al.*, 2023). For 44 of 49 parents, the counselling was understandable for themselves and their child, the duration was judged sufficient by 42 of 47 parents, and 87.5% were able to ask their questions during counselling. Six parents who were unable to ask their questions named emotional overload (3 parents), the presence of the child (1 parent) and unspecific issues (2 parents) as reasons. Of the participants, 17.8%, would have preferred counselling without a child. The most common suggestion for improvement (7 parents) was providing written information material, more appointments (5 parents), more time for counselling (4 parents), and more time for reflection (5 parents). A subgroup of participants received multiple fertility counselling sessions and 68.4% of parents reported that they had become more involved with the topic since the first consultation. The opinion about fertility was unchanged in 68.4% of participants, though familiarity with the counselling did not facilitate the decision-making in 72.6%. Parents who had a history of previous fertility counselling session(s) were found to be more satisfied with the counselling and interventions than parents who were counselled for the first time.

The satisfaction with fertility preservation discussions was investigated in a survey of 34 parents/caregivers and 34 adolescent cancer patients (Shin *et al.*, 2022). Among the caregivers, 47% (15 parents) who attended two or more discussions reported improved quality of information and communication. Only 47% (14 patients) of patients indicated that after the first discussion session, they understood the concept of fertility preservation sufficiently to make informed decisions regarding the initiation of a fertility preservation procedure.

In a cross-sectional study, men above 18 years old with a cancer diagnosis within the last 10 years were eligible to participate, of whom 72 completed the survey part of the study (Ehrbar *et al.*, 2022). Most participants agreed that general topics about reproductive health and sexuality did not need to be discussed immediately before cancer therapy but it was helpful to know these

discussions could be revisited later. The conclusion of the study was that most participants would value an additional support tool that contains not only information about fertility preservation, but also about sexuality, virility and consequences for partners, as well as experience reports from other patients.

In a study, 77 adolescent and young adult cancer patients aged 10–25 years were invited for FP discussions, of whom 34 agreed to participate. Patients and their families participated in FP discussions and processes before the survey and were contacted after completing all FP procedures. Medical professionals participating in the discussions were one or more paediatric oncologists, gynaecologists, resident paediatricians or gynaecologists or nurse practitioners. Patients and their families were provided a basic information sheet that included amongst other things the risks and benefits of FP methods and an overview of how FP is performed (Shin *et al.*, 2022). Most discussions (n = 25, 76%) occurred solely through verbal communication, without the use of memos or notes. Respondents reported an improved understanding of FP and better communication and information quality if they participated in more than one discussion session. The caregivers who were provided with FP additional communication tools (e.g. pamphlets, notes, internet sources) were more satisfied with the quality of the information they received compared to those who were only provided verbal information.

For a multi-centre survey, newly diagnosed female and male patients aged 13 years and older, treated with any regimen including chemotherapy or radiation, were invited to participate. There were 113 patients enrolled in the study (Korte *et al.*, 2020). Most participants reported having received education regarding the risk for infertility and FP prior to cancer treatment. Almost half of the participants felt that they were not sufficiently informed to make a decision of their own. Three months after first completing the questionnaire, knowledge about fertility had increased, suggesting that participants have been made aware of this topic by the study and may have searched for further information or have talked to healthcare providers or parents. Those who do receive information use FP more often.

In a qualitative study, 290 prepubertal boys and adolescents aged between 12 and 18 years were eligible to join the study, and 120 questionnaires were completed (Wyns *et al.*, 2015). The content of information provided to patients and parents appeared to positively impact on the decision to preserve fertility. Pressure from doctors to reduce the delay between diagnosis and cancer treatment increased the number of refusals. Thus, discussions about FP should aim to provide full and understandable information and place the emphasis on the future as positive decisional factors.

Recommendations

Counselling should include discussion of the treatments the patients will receive and the risk to their fertility.

The information should also include the critical points to make an informed decision on fertility preservation, i.e. estimated level of risk for infertility, risk of complications from FP and current experimental options and risks for fertility restoration. At the time of consent, discussions should also include the use or disposal of testicular specimens.

This information should be provided verbally, as well as written, and should enable the patient and parents/caregivers to give informed consent. Repeated counselling sessions may help enhance understanding.

What psychological support is required during follow-up?

While numerous guidelines on fertility preservation advocate for timely discussion and psychological support including assistance in decision-making of reproductive-aged and adolescent cancer patients, there is no evidence on how to provide psychological support to address emotional distress in the paediatric population.

It was reported in several studies that fertility counselling referrals are being underutilized, resulting in patients with cancer missing out on a consult with a fertility specialist, increasing the likelihood of decision regret. High-quality information, delivered in an effective manner, minimizes the risk of misinformation and lack of understanding, ultimately reducing the risk of retrospective regret (Kuntz *et al.*, 2024).

Recommendation

Provision of psychological support should be considered for patients and their family to aid decision-making process, support a high-quality decision, consistent with personal values and beliefs, and alleviate distress during the decision-making process.

Biopsy procedure

Which type of surgery should be used?

Evidence

No study could be retrieved from literature comparing different testicular biopsy techniques in the prepubertal population.

A recent international survey reported, with regards to the surgical procedure, that all participating centres perform unilateral biopsies, and 6/16 centres sometimes perform bilateral biopsies. Half of the centres surveyed report collecting 21–30% of the testis during a unilateral biopsy. In those patients who are in the transition phase of puberty or with established puberty, 10/16 centres combine the biopsy for preservation of spermatogonia with an initial attempt at testicular sperm extraction with isolation of sperm, performed in theatre (3/10) or during tissue processing (7/10) (Duffin *et al.*, 2024).

Details from the surgical procedure for testicular tissue biopsy from research studies are summarized in Table 5.

Recommendations

It is considered good practice to perform a unilateral, conventional open testicular biopsy under general anaesthesia, ideally in combination with another surgical intervention planned as part of the therapy (e.g. port catheter implantation, bone marrow sampling, etc.) in order to minimize the burden of further anaesthesia.

There may be a group of patients who have reached mid-puberty but unable to provide an ejaculate, where testicular sperm retrieval may be attempted first, followed by a biopsy for testicular tissue cryopreservation if no sperm are identified. This can be performed during the same operating theatre session.

Who should perform the surgery?

Evidence

In a prospective cohort study, the establishment of an experimental testicular tissue banking system was detailed (Sadri-Ardekani *et al.*, 2016). Multiple specialties were involved in this banking process including paediatric oncology, male reproductive medicine and surgery (urology), paediatric surgery,

Table 5. Details of the surgical procedure for testicular tissue biopsy from research studies.

Reference	No. of patients	Tanner stage	Type of surgery	Uni- or bilateral	With other procedure	Size of biopsy	Size of fragment for cryopreservation	Sperm retrieval attempt	Biopsy performed by:
Barraud-Lange *et al.* (2024)	377	NR	Open testicular biopsy	Both	Yes	1/3 for unilateral 1/4 for bilateral	NR	NR	NR
Braye *et al.* (2023)	39	NR	Open testicular biopsy	Unilateral	Yes	Orchiectomy hemi-orchiectomy or small biopsy (10–25% of the testis	NR	NR	Urologist
Feraille *et al.* (2023)	79	I (n = 64) II-III (n = 7) IV-V (n = 8)	Open testicular biopsy	Bilateral (n = 63) Unilateral (n = 16)	NR	NR	NR	No	NR
Benninghoven-Frey *et al.* (2022)	29	NR	Open testicular biopsy	Unilateral	NR	<20% of testicular volume	NR	NR	NR
Moussaoui *et al.* (2022)	35	I (n = 24) II–IV (n = 9) V (n = 2)	Open testicular biopsy	Unilateral	N = 23 (65.7%)	57 mm^3 (24–120 mm^3)	1–2 mm^3	NR	Paediatric surgeon
Kanbar *et al.* (2021)	139	I (n = 122) II (n = 8) III (n = 6) IV (n = 3)	Testicular biopsy	Unilateral	Yes	<5% of the total testicu-lar volume	2–4 mm^3	In patients ≥10 years (n = 19)	NR
Borgström *et al.* (2020)	21	I (n = 14) II (n = 2) III–IV (n = 4) V (n = 1)	Open testicular biopsy	Both (10/10)	yes	From 1–2 × 2–3 mm to 5 × 5 mm	NR	Yes (n = 1)	Urologist
Corkum *et al.* (2019)	23	I (n = 18) II (n = 3) ≥III (n = 2)	Testicular wedge biopsy	Unilateral	N = 16 (70%)	~10 mm × 5 mm <25% testicular volume	3–5 mm^3	NR	Paediatric sur-geon (n = 17) Paediatric urolo-gist (n = 6)
Valli-Pulaski *et al.* (2019)	189	NR	Orchidectomy or testicular biopsy	Unilateral	NR	20% of testicular volume	NR	NR	NR
Heckmann *et al.* (2018)	39	NR	Testicular biopsy	Unilateral	NR	NR	NR	NR	NR
Medrano *et al.* (2021)	4	NR	Testicular biopsy	NR	NR	<10% of testicular volume	5–6 mm^3	NR	NR
Ming *et al.* (2018)	34	NR	Open testicular biopsy	Unilateral	N = 29 (85.3%)	<30% of testicular volume	NR	NR	Paediatric urologist
Stukenborg *et al.* (2018)	46	I-II	Open testicular biopsy	Unilateral	NR	<20% of testicular volume	NR	NR	NR
Ho *et al.* (2017)	44	I–II (n = 33) ≥III (n = 11)	NR	NR	Yes	Average of 4.1% of testicular volume	2–5 mm slices with 1–3 mm thickness	NR	NR

(continued)

Table 5. Continued

Reference	No. of patients	Tanner stage	Type of surgery	Uni- or bilateral	With other procedure	Size of biopsy	Size of fragment for cryopreservation	Sperm retrieval attempt	Biopsy performed by:
Uijldert *et al.* (2017)	78	NR	Open testicular biopsy	unilateral	Yes (100%)	<50% of testicular volume <1 ml	NR	NR	NR
Pietzak *et al.* (2015)	34	NR	Open testicular biopsy	NR	Yes (100%)	NR	NR	NR	NR
Ginsberg *et al.* (2014)	48	NR	Testicular biopsy	NR	Yes (100%)	On average 80 mm^3	NR	NR	Urologist
Babayev *et al.* (2013)	9	2 ± 1	Testicular biopsy	Unilateral	N = 2 (22.2%)	~15% of testicular volume	NR	NR	NR
Van Saen *et al.* (2012)	7	NR	Testicular biopsy	Unilateral	No	NR	6 mm^3	NR	NR
Curaba *et al.* (2011)	2	I (n = 1) II (n = 1)	NR	Unilateral	Yes	<5% of the testicular volume	~3 mm^3	NR	NR
Wyns *et al.* (2011)	62	I (n = 52) Peripubertal (n = 10)	Testicular biopsy	unilateral	Yes	<5% of the total testicular volume	2–4 mm^3	Yes	NR
Ginsberg *et al.* (2010)	14	NR	Open testicular biopsy	NR	Yes (100%)	~80 mm^3	NR	NR	Urologist
Wyns *et al.* (2007)	5	I–II (n = 3) III (n = 2)	NR	Unilateral	NR	<5% of the total testicular volume	2–4 mm^3	NR	NR
Keros *et al.* (2007)	5	NR	Open testicular biopsy	NR	Yes (100%)	1–2 × 2–7 × 9–10 mm	~1–4 mm^3	NR	NR

NR: not reported.

paediatric urology, clinical and laboratory pathology, microbiology, and reproductive biology. In their study testicular biopsy is a safe and feasible procedure and it can be performed by any surgeon properly trained in paediatric surgical technique after a brief extra training.

Recommendations

Surgery should be performed by a trained surgeon (typically a paediatric surgeon or urologist), according to local regulations.

Children should have testicular examination prior to surgery and the surgeon should identify other anatomical abnormalities at the time of biopsy.

Transport of the tissue

Which culture medium should be used for testicular tissue transport/short-term storage?

Evidence

In an experimental study, human testicular tissue samples from five adults undergoing vasectomy reversal with normal tubular structure was used to test four different transport media: DMEM/F12, DMEM/F12 + 20% human serum albumin (HSA), DMEM/F12 + 50% HSA, and pure HSA medium. After 3 days, the fragments were digested for cell viability measurement or evaluated by histology and immunohistochemistry for the spermatogonial marker Melanoma-associated antigen 4 (MAGE A4) (Faes and Goossens, 2016). There was no significant difference in viability between fresh control tissue and the four experimental conditions. The structure of the tissue deteriorated with increasing HSA concentrations: no significant changes compared to controls were found with DMEM/F12 and 20% HSA media, however, significant changes were seen with 50% and 100% HSA media (average scores 2.75 ± 0.19 vs. 2.16 ± 0.12, and 1.51 ± 0.18, respectively). Sertoli cells also exhibited morphological changes with increasing HSA concentrations: no significant changes were seen compared to fresh control tissue with DMEM/F12 media, however, 20%, 50%, and 100% HSA media induced significant changes in the Sertoli cells (2.69 ± 0.40, 1.91 ± 0.28, 1.49 ± 0.25, 1.01 ± 0.13 for control, 20% HSA, 50% HSA, and 100% HSA, respectively). The average number of spermatogonia per mm^2 was significantly lower in tissue kept in 100% HSA medium compared to fresh control tissue (374.55 ± 68.51, 372.40 ± 99.01, 327.74 ± 70.20, 249.92 ± 87.53, 133.08 ± 73.92 for control, DMEM/F12, 20% HSA, 50% HSA, and 100% HSA, respectively). No significant changes were found in the number of apoptotic cells with the different media.

In an experimental study, human testicular tissue from five adults who underwent orchidectomy prior to hormone therapy for carcinoma of the prostate was used to test five different media (Ringer's solution, 0.9% NaCl solution, Macrodex 4.5% RL, RPMI 1640 Medium with L-Glutamine and 25 mM Hepes, Dextran solution 40 with 0.9% NaCl). After 15′, 20′, 30′, 45′, 60′, 90′, 2 h, 3 h, 5 h, and overnight, samples were evaluated by light microscopy and electron microscopy (Feng and Holstein, 1990). Spermatogonia were well preserved for 2 h in Ringer's solution or Macrodex, and up to 5 h in solution of 0.9% NaCl, Dextran or 1640 Medium. Based on light and electron microscopy, it was determined that Leydig cells were not well preserved for more than 1 h, except for 1640 Medium at 4°C.

In an animal study, testicular tissue from four immature male rhesus monkeys was used to compare fresh xenografting with 24 h in ice-cold Leibovitz-L15 medium before xenografting (Jahnukainen *et al.*, 2007). Three months after xenografting, no effect was seen of a 24-h delay in grafting of fresh and cooled tissue fragments on graft survival (51% vs. 52% recovery). Five months after xenografting, 73% (35/48) of immediately implanted grafts and 79% (38/48) of grafts which were implanted with a 24-h delay were recovered. The graft weight after delayed xenografting was significantly higher compared to the immediately grafted group. Five months after grafting B spermatogonia were recorded in 12% of grafts after delayed grafting and in 8% of grafts after immediate xenografting.

Conclusion

Testicular tissue can best be stored or transported in DMEM/F12 (3 days) or Leibovitz L15 (24 h) medium. However, it should be kept in mind that the human data are derived from adult tissue and observations might deviate with prepubertal tissue.

What is the acceptable duration for testicular tissue transport/short-term storage?

Evidence

From the international survey, testicular tissues need to be transported outside the centre in 10/16 centres (Duffin *et al.*, 2024). The target maximum time from collection to cryopreservation is 24 h in all centres.

In an experimental study, human testicular tissue samples from four adults undergoing vasectomy reversal with normal tubular structure was used to define the maximal storage period at refrigerator temperature (4°C) in which no important morphological or functional alterations occur. The fragments were cultured in DMEM/F12 for 3, 5, or 8 days, after which they were digested for cell viability measurement or evaluated by histology and immunohistochemistry for the spermatogonial marker MAGE A4 (Faes and Goossens, 2016). No significant change in viability was seen with increasing time in the refrigerator compared to fresh control tissue. Tissue morphology deteriorated significantly with prolonged storage, i.e. 5 and 8 days, in the refrigerator, compared with fresh tissue (2.65 ± 0.16, 1.82 ± 0.31, and 1.47 ± 0.18 respectively). Only after 8 days of storage in the refrigerator were significant changes seen in the Sertoli cell integrity compared to fresh (2.58 ± 0.59 vs. 1.19 ± 0.17). Prolonged storage in the refrigerator did not change the average number of spermatogonia per mm^2 or number of apoptotic cells.

Conclusion

Immature testicular tissue can be transported or stored in DMEM for up to 3 days without altering cell survival, tissue structure, number of spermatogonia, and Sertoli cell morphology. However, it should be kept in mind that these data are derived from adult tissue, and observations might deviate with prepubertal tissue.

What is the best temperature for testicular tissue transport/short-term storage?

Evidence

From the international survey, testicular tissue is transported at ambient temperature in 4 of 16 centres, while the remaining 12 of 16 centres aim for a shipment temperature of 0–8°C (Duffin *et al.*, 2024). Shipment temperature is controlled in 8 of 16 centres, with 6 of these 8 centres using a temperature logger for each sample.

In an experimental study, human testicular tissue samples from four adults undergoing vasectomy reversal with normal tubular structure was used to compare 3-day storage at different temperatures: refrigerator (4°C), room temperature and 37°C

(Faes and Goossens, 2017). The number of apoptotic cells increased significantly when the fragments were stored at 37°C. Tubular morphology of the tissue was not significantly altered by the storage temperature. Sertoli cells did not undergo significant morphological changes at the different storage temperatures compared to fresh control tissue. Similarly, no difference was found in the average number of spermatogonia per mm^2 or number of apoptotic cells.

In another experimental study, human testicular tissue from five adults who underwent orchidectomy prior to hormone therapy for carcinoma of the prostate was used to test different storage temperatures [room temperature and refrigerator (4°C)] (Feng and Holstein, 1990). Spermatogonia were well preserved up to 1–3 h at room temperature and also well preserved overnight at 4°C. Sertoli cells were unaffected for about 5 h.

In an animal study, testicular tissue from four immature male rhesus monkeys was used to compare fresh xenografting with 24 h at 4°C before xenografting (Jahnukainen *et al.*, 2007). Three months after xenografting, 51% (33/64) of the grafts were recovered. No effect was seen of a 24-h delay in grafting of fresh and cooled tissue fragments on graft survival (52% recovery, 14/27). Five months after xenografting, 73% (35/48) of immediately implanted grafts and 79% (38/48) of grafts which were implanted with a 24-h delay were recovered. The graft weight after delayed xenografting was significantly higher compared to the immediately grafted group. Five months after grafting 12% of the grafts contained B spermatogonia or spermatocytes after delayed grafting and 8% contained these after immediate grafting.

Conclusion

Immature testicular tissue can be effectively transported or stored for short-term at 4°C. However, it should be kept in mind that the human data are derived from adult tissue and observations might deviate with prepubertal tissue.

What is the optimal size for testicular tissue during transport/short-term storage?

Evidence

In an experimental study, human testicular tissue samples from five adults undergoing vasectomy reversal with normal tubular structure was used to compare different tissue sizes (6, 15, 50, and 80 mm^3) during transport or three-day storage (Faes and Goossens, 2017). No significant changes were found in viability between different tissue sizes. Tissue morphology was scored and compared on four parameters: structure of tubules, rupture of the basement membrane, swelling of tubular cells, and tubular cell loss. Tissue morphology was best conserved with increasing tissue size: tissue morphology was best preserved when stored as fragments of 50 or 80 mm^3 (2.05 ± 0.16, 2.18 ± 0.26, 2.30 ± 0.13, 2.38 ± 0.11 and 2.42 ± 0.07 for fresh, 6, 15; 50, and 80 mm^3). Compared to fresh tissue, no differences were found in Sertoli cell integrity, average number of spermatogonia per mm^2 or apoptotic cells.

Conclusion

Immature testicular tissue can be transported or stored for short-term in sizes of 50 or 80 mm^3. However, it should be kept in mind that these data are derived from adult tissue and observations might deviate with prepubertal tissue.

Overall recommendations

Immature testicular tissue can be transported or stored in DMEM/F12 for up to 3 days or in Leibovitz L15 medium for 24 h, at 4°C in fragments of up to 80 mm^3.

When possible, transport time and short-term storage of prepubertal testicular tissue should be minimized, as no functional data are available on how transport or short-term storage may affect subsequent spermatogonial stem cell function or spermatogenesis.

Quality control for testicular tissue cryopreservation

Which quality controls are required for testicular tissue cryopreservation?

Given the lack of directly relevant studies, unless more stringent local regulations are in place, we recommend testicular tissue cryopreservation should be undertaken according to the Commission Directive 2004/23/EC. From 2027, guidance published on the EU SoHO platform should be followed.

Microbiology

Evidence

A total of 121 samples were obtained across ten different biobank facilities in 2015 (Bajerski *et al.*, 2020). The longest time of continuous usage of liquid nitrogen (LN) storage without intermittent cleaning tanks amounted to 30 years; the shortest usage interval was less than 1 year. Most of the tanks had not been cleaned on a regular basis to avoid potential damage of the stored biological materials during the transfer to another LN storage tank (Bajerski *et al.*, 2020). Bacterial cell counts in both, negative controls as well as in the LN samples were low and at $\leq 10^2$ cells per ml LN. In contrast to the LN phase, cell numbers in samples from ice layers covering inner surfaces of storage tanks were up to 100 times higher (up to 10^4 cells per ml ice). In a generalized linear model using Gaussian distribution, the institute, storage phase, surrounding condition, number of openings, and the usage time predicted the presence of cells [Akaike information criterion (AIC) = 96.3] and gene copies (AIC = 168). Cells and gene copies increased with storage time and number of openings. The numbers of bacteria were lower in rooms with air supply and exhaust but higher in the debris samples and in tanks, where the material is stored in the LN phase. Over 20% also tested positive for 16S rRNA genes of Mycoplasma. However, Mycoplasma-DNA was only detected at very low abundances, accounting for up 1–3% of the 16S rRNA gene copies and freely occurring Mycoplasma cells were not detected in this study. Fungal internal transcribed spacer (ITS) sequences were present in 19 ice and debris samples from five institutes and predominately occurred in tanks containing mixed materials stored, as well as when stored in the gaseous nitrogen phase. The bacterial species richness determined on the genus level for individual LN storage tank samples typically stayed below 300 sequence variants. Higher values were only determined in five individual samples.

Recommendation

Samples should be stored in gas phase nitrogen or in liquid phase nitrogen, provided that measures are in place to avoid cross-contamination (high-security vials or sealing of samples).

Serology
Evidence

In a retrospective review of 23 testicular tissue cryopreservation cases, an infectious disease panel was obtained in compliance with long-term tissue storage facility regulations (Corkum et al., 2019).

Recommendation

Given the lack of directly relevant studies, unless more stringent local regulations are in place, we recommend testicular tissue cryopreservation should be undertaken according to the Commission Directive 2006/14/EC Annex II. From 2027, guidance published on the EU SoHO platform should be followed.

Morphology
Evidence

To assess the quality of testicular tissues stored for fertility preservation, a total of 15/16 centres perform histological (13/15) or immunohistochemical (10/15) analyses in order to assess germ cell counts and spermatogenesis and/or presence of malignant infiltration (Duffin et al., 2024).

In an experimental study, different markers for spermatogonia on histological analysis were compared on testicular samples from 24 (pre)pubertal boys with cancer, archived histologic samples of 35 prepubertal boys with acute lymphoblastic leukaemia (ALL) and 20 testicular biobank samples (Funke et al., 2021). Published quantitative histologic data was used to generate Z-scores for the number of spermatogonia per seminiferous tubule cross-section (S/T) and fertility index (FI) reference means to control for developmental variation. A significant correlation was found between the FI and Z-scores for S/T. The curves for Haematoxylin & Eosin/Periodic Acid Schiff (HE/PAS) stain, MAGE A4 and DDX4 immunostain were superimposable, meaning that the classification properties of the three measures were virtually identical. No difference in the mean Z-score values for S/T and FI were detected when the three different stains were compared in the nontreated tissue samples. S/T Z-scores were shown to enable the quantification of genetic and cancer treatment effects across tissue samples and to provide a method for estimating the quality of individual patient samples, taking into account developmental variation.

A study of 87 patients from two prospective cohorts of patients examined the impact of freeze-thawing tissue obtained prior to potentially sterilizing oncological treatment (Rives-Feraille et al., 2022). The integrity and structural changes of fresh and frozen-thawed testicular fragments were evaluated semi-quantitatively: a total absence of nuclear and epithelial alterations was scored as 0 and the worst score for alterations was 5 for both factors. The nuclear, epithelial and global lesional scores increased significantly after thawing ($P < 0.001$). The percentage of intact seminiferous tubules decreased after thawing ($P = 0.01$). Approximately 52.1 ± 24.8% of frozen-thawed seminiferous tubules were considered morphologically normal. In the remaining 47.9% of tubules, morphological alterations were slight and the morphology was considered to be generally well preserved (both nuclear and epithelial scores ≤1.5). The global lesional score of cryodamage in the different centres participating in the study was below 1.5. The spermatogonia concentration, the number of spermatogonia per total area or tubular area, and the number of spermatogonia per tubule did not vary significantly after thawing compared with fresh tissue. The number and the percentage of Sertoli cells and spermatogonia expressing PCNA per tubule were comparable in fresh and frozen-thawed testicular tissues.

In a case series, the preservation and proliferation capacity of residual spermatogonia and Sertoli cells after cryopreservation and grafting (grafting was used as an assay to assess tissue quality after freezing to validate the cryopreservation procedure) was evaluated (Wyns et al., 2007). For this purpose, testicular tissue from 11 prepubertal boys was cryopreserved and xenografted for 21 days. All boys were undergoing unilateral orchidopexy for cryptorchidism. In fresh tissue and corresponding cryopreserved and grafted tissue, MAGE A4 and vimentin were used to clearly identify spermatogonia and Sertoli cells, respectively, and Ki67 to mark proliferative cells. Well-preserved integrity of the tubules in 82.19 ± 16.46% of sections of frozen tissue indicated good morphology after cryopreservation and grafting, similar to the 93.38 ± 6.00% observed in fresh control tissue samples. The number of Sertoli cells identified was similar, ranging from 41.8 ± 2.61 per tubule in fresh tissue to 47.1 ± 1.20 per tubule in frozen-grafted tissue. Furthermore, 32% of spermatogonia continued to proliferate after freezing and grafting, compared to 17.8% in fresh tissue. For Sertoli cells, no proliferative activity was detected in fresh tissue, but Ki67 expression was observed in 5.1% of these cells after freezing and grafting.

Recommendation

The generation of reference values (e.g. Z-scores) of spermatogonia quantity is necessary for controlling developmental variation across tissue samples, ensuring evaluation of individual patient sample quality.

Should testicular cryopreservation be performed in ISO Class 5 clean rooms?
Evidence

No study could be retrieved from literature investigating the need to perform testicular cryopreservation in ISO Class 5 clean rooms.

All critical protocols are performed in a certified clean room. Moreover, protocols were performed using validated equipment and clinical-grade reagents and supplies according to cGMP guidelines. Documentation was followed for quality assurance/quality control and compliance with quality standards and regulations (Pacchiarotti et al., 2013).

Recommendation

From 2027, testicular tissue cryopreservation should be performed in a safety class environment according to the guide to the quality and safety of tissues and cells for Human application (EDQM), unless more stringent local regulations are in place.

Histological analysis

Should part of the tissue be sent for histological analysis at the time of testicular cryopreservation?
Evidence

In an international survey, it was reported that to assess the quality of testicular tissues stored for fertility preservation, a total of 15/16 centres perform histological (13/15) or immunohistochemical (10/15) analyses in order to assess germ cell counts and spermatogenesis and/or presence of malignant infiltration (Duffin et al., 2024). Immunohistochemical spermatogonial markers used in assessments included DDX4 (VASA) and MAGE A4.

Table 6. Overview of histological markers used for the identification of germ cells and somatic cells in testicular biopsy samples.

Reference	Morphology	Immunostain for germ cells	Immunostain for somatic cells	Proliferation/ cell death	Method of quantification
Barraud-Lange *et al.* (2024)	HE	/	/	/	All seminiferous tubules present in the section
Lahtinen *et al.* (2024)	HE/PAS	MAGE A4, DDX4	/	/	At least 25 round tubular cross sections
Tholeti *et al.* (2024)	HE	DDX4	/	/	Minimum of 25 seminiferous tubules per cross section per sample
Feraille (2023)	HES	MAGE A4	/	PCNA	The number of spermatogonia per tubule was counted in 10 tubule sections.
Masliukaite *et al.* (2023)	Haematoxylin	MAGE A4	/	/	≥30 cross-sections of seminiferous tubules per sample
Benninghoven-Frey *et al.* (2022)	NR	MAGE A4	/	/	At least 25 round tubular cross sections
Moussaoui *et al.* (2022)	HE	SALL4	/	/	20 seminiferous tubule cross sections
Rives-Feraille (2022)	HES	MAGE A4	/	PCNA	The number of spermatogonia per tubule was counted in 10 tubule sections.
Funke *et al.* (2021)	HE/PAS	MAGE A4, DDX4	/	/	
Kanbar *et al.* (2021)	HE	MAGE A4	/	/	
Medrano *et al.* (2021)	HE	UTF1, DDX4, UCHLI, SALL4, PLZF	vimentin, SOX9	Ki67	
Borgström *et al.* (2020)	HE	PLAP	Inhibin, vimentin, Cam 5.2, CD34	/	
Portela *et al.* (2020)	Haematoxylin	MAGE A4, UTF1	/	/	One full section for each marker
Valli-Pulaski *et al.* (2019)	HE, PAS	UTF1, DDX4	/	/	At least 40 seminiferous tubule cross sections
Heckmann *et al.* (2018)	HE	MAGE A4	/	/	
Medrano *et al.* (2021)	HE	TUNEL, UTF1, c-kit, DDX4, SYCP3	SOX9, vimentin	TUNEL	Up to 100 tubule cross-sections
Stukenborg *et al.* (2018)	PAS	DDX4	/	/	At least 25 round tubular cross sections
Pietzak *et al.* (2015)	Toluene blue	/	/	/	At least 50 tubular cross sections
Van Saen *et al.* (2015)	HE	MAGE A4, SSEA4, UCHLI	inhibin, AMH, AR, SOX9		
Curaba *et al.* (2011)	HE	MAGE A4	/	Ki67	The number of spermatogonia was not quantified
Wyns *et al.* (2011)	HE	MAGE A4	/	/	
Wyns *et al.* (2007)	HE	MAGE A4, LDH-C, 4D4 anti-proacrosin	3β-HSD	Ki67, caspase-3	
Keros *et al.* (2007)	HE	MAGE A4	vimentin, CD-34	/	1–3 sections
Kvist *et al.* (2006)	PAS, Mayer's haematoxylin	c-kit	/	/	20 cross-sectioned tubules
Funke *et al.* (2021)	PAS	DDX4, MAGE A4	/	/	At least 25 round tubular cross sections
Lahtinen et al. (2024)	PAS	MAGE A4	/	/	At least 25 round tubular cross sections

3β-HSD: 3-beta (ß)-hydroxysteroid dehydrogenase; AR: androgen receptor; AMH: Anti-Müllerian hormone; c-kit: receptor tyrosine kinase; DDX4: anti-DEAD-box helicase 4; HE: Haematoxylin & eosin; HES: haematoxylin-eosin-saffron; LDH-c: lactate dehydrogenase C; MAGE A4: Melanoma-associated antigen 4; NR: not reported; PAS: Periodic Acid Schiff; PLAP: placental alkaline phosphatase; PLZF: promyelocytic leukaemia zinc finger; SALL4: Sal-like protein 4; SOX9: sex-determining region Y-box 9; SSEA4: stage-specific embryonic antigen 4; SYCP: synaptonemal complex protein 3; TUNEL: terminal nucleotidyl transferase-mediated dUTP-biotin nick end-labelling; UCHLI: ubiquitin carboxyl-terminal esterase L1; UTF1: Undifferentiated embryonic cell transcription factor 1.

Details from the histological analysis on testicular tissue from research studies are summarized in Table 6.

Recommendations

Histological assessment at the time of cryopreservation is preferably performed as individual samples are likely to have distinct fertility potential. This analysis should take account of the limited material obtained, especially in younger patients. Personalized counselling and decisions regarding future use of the tissue should be based on these analyses.

High heterogeneity among patient groups, makes histological evaluation essential. Structural integrity and presence of germ cells should be assessed using histological and immunohistochemical staining with validated germ cell markers such as MAGE A4 or DDX4, and quantification of spermatogonia using validated methods, before or after cryopreservation.

Immunostaining of additional somatic markers as well as staining for apoptosis (e.g. TUNEL) and proliferation (e.g. Ki-67) can be informative regarding tissue maturation and integrity.

Cancer markers

Should tumour marker assessment be performed on the tissue at the time of cryopreservation and/or after thawing?

Evidence

In an international survey it was reported that of the 12 centres that specifically perform histological quality assessment of the testicular tissue, only three do so for malignant markers in the case of a known malignant diagnosis at the time of TTC (Duffin *et al.*, 2024).

A retrospective cohort study included 54 pre- and peripubertal boys who were diagnosed with a haematological malignancy and who underwent a testicular biopsy for FP at the time of diagnosis before any gonadotoxic therapy (Kourta *et al.*, 2024). Formalin-fixed paraffin-embedded testicular tissue was available for 28 boys diagnosed either with ALL (n = 14) or lymphoma (n = 14) and was used to evaluate malignant cell contamination. H&E staining did not detect malignant cells. Using immunohistochemistry (IHC), contamination with cancerous cells using markers specific to the patient's disease was found in 10 of 28 boys, with a higher rate in patients diagnosed with ALL (57%, n = 8/14) compared with lymphoma (14%, n = 2/14) ($P < 0.05$). PCR showed contamination in three of 15 patients with specific chromosomal rearrangements in their bone marrow at the time of diagnosis; one of these patients had negative results from the IHC (Kourta *et al.*, 2024).

Similarly, in a retrospective chart review study, two patients undergoing testicular tissue cryopreservation for FP were found to have malignancy upon routine pathology of the testis biopsy out of 134 participants included in the study (McElhinney *et al.*, 2024). They demonstrated that there is a low rate of identifying malignancy in gonadal tissue biopsies taken from FP specimens even in patients with known malignancy. However, when malignancy was identified, it could alter the diagnosis and treatment plan significantly for patients.

For patients with haematologic malignancies, there is a risk that reimplanted tissue may be contaminated with cancer cells. Performing testicular tissue cryopreservation (TTC) once the patient has achieved a minimal residual disease state may limit this risk; however, at this time there is no widely accepted way to screen the testicular tissue for malignancy prior to reimplantation, and thus the risk persists (Close *et al.*, 2023).

It is also important to note that the part of the tissue evaluated is always different from that used for transplantation.

Recommendations

For patients with malignant disease, careful assessment of the cryopreserved testicular tissue for malignant infiltration using relevant tumour markers is required prior to re-transplantation. Molecular markers provide a more sensitive assessment of the tissue compared to conventional histology and immunohistochemistry.

Whilst assessment at the time of cryopreservation may be helpful for counselling families about the future use of the tissue, re-assessment may be required prior to re-transplantation as new methods for detecting malignant contamination may become available.

In the event that there are positive markers for malignancy, re-transplantation should be avoided due to the high risk of malignant contamination, in particular for haematological and metastatic malignancies.

Whilst negative testing for malignant contamination may significantly reduce the chance or re-introducing malignancy, patients must be counselled that a theoretical risk remains for the specific piece(s) of tissue that are re-transplanted.

Cryopreservation protocol

Cryopreservation should be performed in, or the tissue transported to, an appropriate environment (see section on quality control).

What are the approximate sizes (mm^3) of the testicular fragments at cryopreservation?

Evidence

No comparative studies could be retrieved from literature comparing different sizes of testicular fragments for cryopreservation.

The sizes of the testicular tissue fragments in research studies are listed in Table 5. This was confirmed by the data from the recent international survey, where the majority of centres reported cutting the testicular tissue in fragments of ≤5 mm^3 (Duffin *et al.*, 2024). Sizes of fragments up to 20 mm^3 have been cryopreserved in centres worldwide.

In an animal study, using goat testicles, fragments of 1, 5, and 9 mm^3 (n = 9 for each size) were obtained from each pair of testicles (Gomes *et al.*, 2023). Three fragments of each size were randomly separated for control (fresh fragments; n = 3), and the remaining six fragments of each size were cryopreserved by slow freezing (n = 3) or vitrification (n = 3). This procedure was repeated five times, totalling 45 fragments in each size. Both fresh and cryopreserved fragments were submitted for histomorphological evaluations. When comparing different fragment sizes within each cryopreservation method, fresh and vitrified, 1 mm^3 fragments showed significantly less alterations than 5 and 9 mm^3 fragments, while the 5 mm^3 fragments cryopreserved by slow freezing showed significantly less alterations than the 1 and 9 mm^3 fragments. In an animal study, using rat testicles, fragments of 1, 8, 18, and 27 mm^3 were obtained and cryopreserved (Wang *et al.*, 2022). After 30 days of grafting, the rates of recovery of 8 mm^3 (14/36) and 18 mm^3 (16/40) fragments were significantly higher than that for 1 mm^3 (8/31) and 27 mm^3 (10/40) respectively. Also, the seminiferous tubule integrity and number of spermatogonia was significantly lower for 27 mm^3 fragments compared to the other sizes.

In another animal study using rat testicles, fragments of 7.5 and 15 mg were cryopreserved (Travers *et al.*, 2011). Morphological alterations were more frequent, however not statistically significant, when a testicular tissue piece of 15 mg was frozen in comparison with 7.5 mg.

Conclusion

Although no comparative studies have been performed on human testicular tissue size at cryopreservation, cryopreserving testicular tissue fragment sizes up to 20 mm^3 have been used.

Which components should the cryosolution to freeze testicular tissue contain and at which concentration?

Evidence

Centres participating in the international survey reported largely using dimethyl sulphoxide (DMSO) as cryoprotectant agent (CPA; 15/16), with one centre using ethylene glycol (EG). Additional non-permeating CPA sucrose is used by some (8/15), and medium is supplemented with additional constituents, most commonly human serum albumin (HSA; 15/16 centres) (Duffin *et al.*, 2024).

In an experimental study, CPA TEST-yolk buffer (TYB) with 7.5% HSA was compared to 5% DMSO with 5% HSA and 8% DMSO with 20% HSA using a controlled slow freezing (CSF) method described for tissue CSF (Sanou *et al.*, 2022). Morphological damage was found to be increased in all those testis fragments cryopreserved in TYB-containing medium compared to DMSO-containing medium. The immunostaining of MAGE A4+ cells in these tissues, 24 h after culture, reflected these results. The CSF method with 5% or 8% DMSO as a CPA appears to be favourable for cryopreserving testicular tissue for future in vitro spermatogonial proliferation prior to transplantation as a fertility restoration treatment.

In a small experimental study, using adult testicular tissue retrieved during microsurgical testicular sperm extraction from 10 patients with normal spermatogenesis, uncontrolled slow freezing (USF; using DMEM/F12, 0.15 M sucrose and 10% HSA with either 1.5 or 2.1 M DMSO) was compared to vitrification (using DMEM/F12, 20% HSA and 0.5 M sucrose with 1: 2.1 M DMSO and 2.7 M EG or 2: 4.2 M DMSO, 5.4 M EG; for the low and high concentration, respectively) (Han *et al.*, 2021). Histology and immunohistochemistry showed the best results, in terms of maintenance of seminiferous tubule structure and low apoptosis, with USF and vitrification with high concentration of CPA.

In an experimental study, using 160 fragments from 14 adults undergoing vasectomy reversal, CSF with DMSO at a concentration of 0.7 or 1.5 M in the presence (+S) or absence (−S) of 0.1 M sucrose as CPA was compared to USF using either 0.7 or 1.5 M DMSO combined +S, solid-surface vitrification or direct cover vitrification (Baert *et al.*, 2013). The USF 1.5 M DMSO + S protocol proved to better prevent cell death and preserve seminiferous epithelial coherence, interstitial compartment integrity, spermatogonial proliferation and testicular cell ultrastructure than using CSF 0.7 M DMSO −S, CSF 0.7 M DMSO +S, CSF 1.5 M DMSO + S, USF 0.7 M DMSO + S, solid-surface vitrification or direct cover vitrification.

An experimental study, using testicular tissue from 11 biopsies from eight boys under six years of age with cryptorchidism, two CPAs were compared (Leibovitz L-15 medium supplemented with 1.5 mol/l EG, 0.1 mol/l sucrose and 10 mg/ml HSA with phosphate buffered saline (PBS) supplemented with 1.5 mol/l EG, 0.1 mol/l sucrose, and 10 mg/ml HSA), both in a slow freezing protocol (Kvist *et al.*, 2006). No differences were found between the two CPA protocols in terms of survival of spermatogonia or concentration of testosterone, and inhibin production.

An experimental study, using 16 non-frozen and 34 frozen testicular tissue samples of 16 infertile men, compared three CPA protocols (1: egg yolk-based medium containing 12% glycerol diluted 1:1 with Sperm Rinse-20 medium; 2: 1.5 mol/l 1,2-propanediol (PrOH) in PBS; 3: 0.7 mol/l DMSO in Hanks balanced salt solution), all with controlled slow freezing. After thawing, tissue was cultured for 12 days (Keros *et al.*, 2005). The tissue frozen with DMSO and to a lesser extent PrOH, maintained their cellular architecture well, while tubules frozen in glycerol were severely damaged. Testosterone secretion in medium from DMSO frozen fragments was equal to controls, while lower in PrOH-frozen fragments.

In an animal study, using testis tissue or cells from four prepubertal monkeys, conventional freezing media were compared to conventional media with additives (1.4 mol/l DMSO in 10% KnockOut™ Serum Replacement in Dulbecco's PBS combined with trehalose 200 mmol/l, hypotaurine 14 mmol/l, necrostatin-1 50 µmol/l or melatonin 100 µmol/l) (Jung *et al.*, 2020). The number of spermatogonia and proliferation is best preserved with slow freezing using 1.4 mol/l DMSO and 10% KnockOut™ Serum replacement with addition of 200 mmol/l trehalose.

In another animal study, using testicular tissue from four immature rhesus monkeys, fresh tissue was compared with 24 h cryopreservation (controlled slow freezing with cooling rates of 0.5°C/min) without CPA or with 1.4 M EG or 1.4 M DMSO or 0.7 M DMSO (Jahnukainen *et al.*, 2007). After xenografting, grafts from fresh tissue showed good survival and germ cell differentiation to spermatocytes. Cryopreservation in 1.4 M DMSO also allowed grafts to initiate spermatogenesis. In contrast, 0.7 M DMSO and EG, showed inferior protection.

Conclusion

Most studies showed that DMSO in a concentration of 1.4 or 1.5 M is the preferred cryoprotective agent. Addition of 0.1 M sucrose or 200 mmol/l trehalose might allow for lower DMSO concentrations to be used and may further improve viability of tissue and cells after cryopreservation. However, most studies performed cryopreservation on adult testicular tissue and caution is required in extrapolating this to prepubertal testis tissue. One study comparing different cryoprotectants in prepubertal testis fragments did not include DMSO as one of the CPAs.

Should there be a separate protocol for cryopreserving tissue that may contain sperm compared to tissue that does not contain sperm?

Evidence

In the international survey, it was reported that 7/16 centres use different cryopreservation protocols for testicular tissues containing spermatogonia and sperm (Duffin *et al.*, 2024).

In an experimental study, including samples from 14 adults undergoing ICSI, the quality of the testicular sperm was assessed after cryopreserving as whole testis tissue compared to minced tissue suspension in test-yolk buffer with (1:1) glycerol using a slow freezing protocol (Crabbé *et al.*, 1999). The mean sperm motility was decreased from 18.7% before freezing to 5.1% after freezing in the whole biopsy fraction, while the motility decrease from frozen minced testis was decreased to 10.1%. Furthermore, recovery and viability of sperm after Percoll was 4% and 33% in whole frozen testis biopsy and 10% and 56% in frozen minced testis, respectively. This indicates that sperm can be better

cryopreserved in minced tissue suspension than using freezing protocols designed for whole testis tissue.

In an animal study, mouse testicular tissue was cryopreserved as testicular cell suspension (TCS; n = 26) or as whole testicular tissue fragments (TT; n = 26) (Onofre *et al.*, 2020). After freezing–thawing and digestion of TT, cell recovery and viability were significantly higher when compared with freezing–thawing of TCS (cell recovery: $13.4 \times 10^4 \pm 7.2 \times 10^4$ vs. $8.2 \times 10^4 \pm 2.7 \times 10^4$; viability: 83.3% vs. 72.6%, respectively). After TT cryopreservation, cell recovery was similar to fresh controls ($14.1 \times 10^4 \pm 5.0 \times 10^4$). However, regardless of the method used, cryopreservation resulted in loss of viability compared with fresh controls. There was no significant difference in number of donor-derived offspring per litter between mice transplanted with frozen-thawed TT and fresh cells. However, mice transplanted with frozen-thawed TCS presented significantly less donor-derived offspring per litter compared to TT and fresh cells.

Conclusion

Cryopreservation of whole testicular tissue is preferred over cryopreservation of testicular cell suspension to preserve spermatogonia, while preserving sperm in testicular tissue might need a separate protocol and cryomedium.

Which rate for tissue freezing should be used?

Evidence

In a case series, using testicular tissue from four adult patients undergoing orchiectomy for various tumours, three cryopreservation methods for cryopreserving testicular tissue were compared, i.e. 1: USF in −80°C in DMEM/F12, 1.5 M DMSO, 0.15 sucrose, and 10 mg/ml HSA; 2: CSF with a gradual cooling program in Hanks balanced salt solution, 1.5 M DMSO, 0.1 M sucrose and 10 mg/ml HSA; 3: vitrification in gradual concentration increasing steps to DMEM/F12, 2.1 M DMSO, 2.7 M EG, and 20 mg/ml HSA straws were submerged in LN (Kabiri *et al.*, 2022). After thawing, no statistical differences were seen compared to fresh control samples for the number and architecture of testicular tubules or number of MAGE A4 spermatogonia.

In an experimental study, straws with tissue fragments and cryoprotectant were brought directly in the nitrogen vapour, or using a shorter method of controlled slow freezing originally designed for cryopreserving sperm (CSFS), the cryostraws were cooled with 0.5°C/min to 5°C followed by a cooling rate of 2°C/min until the samples reached 2°C (Sanou *et al.*, 2022). Finally, the samples were cooled until −80°C with a cooling rate of 10°C/min. No seeding was performed in this method. Morphology of the tissue was assessed after a 24-h culture compared to fresh tissue by MAGE A4 staining. Long-term cultures of isolated testicular cells from these tissues were assessed as well, including by spermatogonial stem cells (SSC) colony counts. Morphological damage was most pronounced in all CSFS frozen tissue. When testicular cells were isolated and cultured, cells frozen by CSFS and in nitrogen vapor of one patient did not survive long term culture.

In a small experimental study, using testicular tissue retrieved from 10 adult patients with normal spermatogenesis, freezing of testicular pieces by uncontrolled slow freezing (USF) using DMEM/F12, 0.15 M sucrose and 10% human serum albumin (HSA) with either 1.5 or 2.1 M DMSO combined was compared to vitrification using a cryosolution DMEM/F12, 20% HSA and 0.5 M sucrose with 2.1 M DMSO and 2.7 M EG or 4.2 M DMSO, 5.4 M EG for the low and high concentration, respectively (Han *et al.*, 2021). Assessment of histology and immunohistochemistry showed the best results were obtained for USF and vitrification with high concentration of CPA.

In an experimental study, using 160 fragments from 14 patients undergoing vasectomy reversal, CSF with DMSO at a concentration of 0.7 or 1.5 M in the presence (+S) or absence (−S) of sucrose as CPA was compared to USF using either 0.7 or 1.5 M DMSO combined with sucrose (+S), solid-surface vitrification or direct cover vitrification (Baert *et al.*, 2013). The USF 1.5 M DMSO + S protocol proved to better prevent cell death and preserve seminiferous epithelial coherence, interstitial compartment integrity, spermatogonial proliferation and testicular cell ultrastructure than cryopreservation using CSF with 0.7 M DMSO −S, CSF 0.7 M DMSO + S, CSF 1.5 M DMSO + S, USF 0.7 M DMSO + S, solid-surface vitrification, and direct cover vitrification.

Immature testicular tissue pieces from 10 patients aged 2–12 years were used in another study. Fragments of fresh tissue (serving as controls) and frozen-thawed (cryoprotectant included 0.7 M DMSO) and vitrified-warmed (cryoprotectant included 7.5% EG and 7.5% DMSO) testicular pieces were xenografted to the scrotum of nude mice for 6 months and compared to evaluate the cryopreservation protocol (Poels *et al.*, 2013). Seminiferous tubules showed good integrity after cryopreservation and xenografting for 6 months in all three groups. The recovery rate of spermatogonia was 3.4 ± 3.8, 4.1 ± 7.3 and 7.3 ± 6.3%, respectively, for fresh, slow-frozen and vitrified-warmed tissue after 6 months of xenografting. Double immunostaining with MAGE A4 and Ki67 revealed 4% (0–13.89), 5.5% (2.2–16.5), and 4.1% (0–16.4) of spermatogonia showing proliferative activity in fresh, slow-frozen and vitrified grafted tissue, respectively. No difference was observed between grafts.

In a small case series, using testicular tissue from two prepubertal boys (6 and 12 years of age) starting gonadotoxic treatment, cryopreservation protocols by vitrification and slow freezing were compared with fresh testicular tissue (Curaba *et al.*, 2011). Controlled slow freezing was performed with a cryosolution of DMSO (0.7 mol/l), sucrose and HSA (10 mg/ml), while for vitrification a cryosolution was used containing DMSO (2.8 mol/l), EG (2.8 mol/l) and HSA in MEM/glutamax medium. Tubular integrity was maintained similarly after vitrification and slow-freezing. MAGE A4 cells were present and proliferation (Ki-67 expression) was seen in the tubules, but the authors did not distinguish between Sertoli cell and spermatogonia proliferation.

Conclusion

While most studies found better results with uncontrolled or controlled slow freezing compared to vitrification, other studies have shown no difference between the slow freezing and vitrification and in some cases there are reports of better results with vitrification than slow freezing. These data should be interpreted with caution, because adult tissue was used in most studies and observations might deviate with prepubertal tissue.

Should straws or vials be used for testicular cryopreservation?

Evidence

No studies could be retrieved from literature comparing straws to vials for cryopreservation.

Conclusion

Whilst there is no evidence specifically relating to testicular tissue cryopreservation, differences in risk of infection may exist between different storage vessels, as discussed more in depth in the ESHRE guideline on medically assisted reproduction in

patients with a viral infection/disease (ESHRE guideline group on viral infection/disease 2021).

Is tissue stored in liquid or vapour nitrogen?

Evidence

No comparative studies could be retrieved from literature comparing liquid to vapour phase nitrogen for cryopreservation.

Conclusion

No studies compared storage in liquid versus vapour phase of liquid nitrogen. Whilst there may be differences in the viability of the tissue, vapour phase has the advantage of reducing the cross-contamination between samples, as discussed more in depth in the ESHRE guideline on medically assisted reproduction in patients with a viral infection/disease (ESHRE guideline group on viral infection/disease 2021) and the Directive 2004/23/EC.

Overall recommendations

DMSO-based cryoprotectant combined with controlled slow freezing can be used for testicular tissue cryopreservation. Uncontrolled slow freezing protocols may be considered. All cryopreservation protocols require internal validation of post-thaw tissue quality. Cryopreservation of testicular tissue fragments is preferred to preserve spermatogonia.

Where tissue potentially may contain sperm, it is recommended to analyse the testicular sample to determine if sperm is present. If sperm are identified, a protocol for sperm cryopreservation must be favoured over testicular tissue cryopreservation. If there are no sperm present, testicular tissue cryopreservation should be favoured. Alternatively, part of the tissue could be cryopreserved using a protocol aimed at preserving spermatogonia and the other part to preserve sperm.

Follow-up

Does a testicular biopsy harm the testis?

Short-term follow-up

Evidence

The international survey reported that protocols to monitor for post-operative complications are established in 12/16 centres, which report mean complication rates of 7.2% (median 1.9; range 0–70%). Specifically, wound infections were recorded in 0.7% (median 0.5%; range 0–2.6%) of all biopsied patients and bleeding requiring intervention in 0.1% (range 0–1.3%) of all biopsied patients (Duffin *et al.*, 2024).

A cohort of 39 prepubertal and pubertal males, without ongoing spermatogenesis, requiring treatment protocols with a high (≥80%) infertility risk, underwent testicular biopsy for fertility preservation (Braye *et al.*, 2023). No severe surgical complications related to the testicular biopsy procedure were recorded, whilst non-persistent pain was observed in some males.

Another cohort of 35 pre- and peripubertal boys (including 24 at stage Tanner 1), who were unable or unsuccessful in cryopreserving mature sperm and scheduled to undergo high-risk gonadotoxic treatment, underwent unilateral open biopsy under general anaesthesia. Reported adverse events were minor haematoma (n = 1) and minor wound dehiscence (n = 1) (Moussaoui *et al.*, 2022).

In a multicentre study, 139 pre- (n = 122) and pubertal (n = 17, Tanner 2–4) boys at risk of infertility, of which 10/139 had prior gonadotoxic therapy, underwent unilateral open testicular biopsy (Kanbar *et al.*, 2021). Three patients experienced short-term postoperative complications: intratesticular haematoma (n = 2, 1 patient with blood clotting disorder) and severe pain with negative work-up (n = 1).

In another study, 20 prepubertal (n = 14) and peripubertal (n = 6) boys at risk for future infertility and unable to provide a semen sample, underwent open testicular biopsy. Postoperative complications included epididymal infection (n = 1), local haematoma (n = 1); extra doses of pain-relieving drugs at day 1 were necessary for most boys (Borgström *et al.*, 2020).

In a large case series, 189 patients with an average age of 7.9 years underwent testicular tissue biopsy for fertility preservation (Valli-Pulaski *et al.*, 2019). No unanticipated adverse events were reported. The rate of infection was 2.5% and the rate of postoperative bleeding was 1.3%.

In a multicentre case series, 34 prepubertal and adolescent cancer patients (23/34 had prior chemotherapy) underwent open testicular tissue biopsies under general anaesthesia (Ming *et al.*, 2018). Overall, two (5.9%) patients had complications after biopsy: one experienced ipsilateral epididymo-orchitis and the other experienced ipsilateral torted appendix testis.

A cohort of 44 boys with a moderate to high risk of infertility (7/44 had prior chemotherapy) underwent open testicular biopsy under general anaesthesia (Ho *et al.*, 2017). Only one patient experienced complications of testicular biopsy, i.e. scrotal wound dehiscence 2 weeks postoperatively.

A cohort of 78 boys who needed gonadotoxic therapy underwent unilateral open microsurgical testicular biopsy under general anaesthesia (Uijldert *et al.*, 2017). Acute adverse effects up to 30 days post-biopsy included wound infections (n = 3/78; 3.8%). Seven of 64 (10.9%) boys had intra-scrotal haematomas at 1 month after surgery. In 5 of 64 (7.8%) boys, these were only extra-testicular and in 2 of 64 (3.1%) boys, the haematoma was intratesticular. Ultrasonography showed that all haematomas had resolved by 6 months.

In a case series, 48 prepubertal boys with cancer at high risk of infertility underwent open biopsy under general anaesthesia before the initiation of chemotherapy. Acute intra-operative and post-operative complications included infection (n = 1) and scrotal cellulitis (n = 1) (Ginsberg *et al.*, 2014).

In a small case series, nine patients, three with previous chemotherapy and six without, underwent open biopsy. No acute complications were reported from the biopsy procedure (Babayev *et al.*, 2013).

A cohort of 52 prepubertal patients, under 12 years of age and 10 patients between 12 and 16 years of age, underwent a testicular biopsy for fertility preservation (Wyns *et al.*, 2011). No complications occurred during or after tissue retrieval.

In a case series, 14 prepubertal boys with stage IV neuroblastoma, rhabdomyosarcoma, osteosarcoma or Ewing sarcoma underwent open biopsy under general anaesthesia before starting chemotherapy (Ginsberg *et al.*, 2010). None of the patients suffered from excessive pain, bleeding or infection during or up to 7 days after surgery.

In one more study, 112 boys with cryptorchidism underwent open testicular biopsy during orchidopexy (83 unilateral and 29 bilateral; not for the purpose of fertility preservation) (Patel *et al.*, 2005). None of the patients had evidence of testicular atrophy or any other abnormality, suggesting testicular damage related to the testis biopsy on ultrasound examination. None of the patients needed a repeat surgery for bleeding, were acutely treated for orchitis, developed sperm antibodies, or sustained loss of a testis secondary to bleeding or infection.

Long-term follow-up

Evidence

In the study by Braye *et al.* (2023), reporting on a follow-up period of 5.0 (1.0–13.0) years, no significantly different testicular volumes were recorded for males who underwent a testicular biopsy and those who did not (Braye *et al.*, 2023).

In a study by Delgouffe et al. (2023), reporting on 12 (of which 9 had FP) childhood cancer survivors with a follow-up period of 2.3–21.0 years, small testicular volumes below the reference limit of 15.2 ml were detected in the biopsied testicle for all 9 patients (Delgouffe et al., 2023). In the five patients who had half of a testis removed, the volume of the biopsied testis was 1–5 ml smaller than the contralateral testis. Testicular abnormalities were observed in only two cases: one presented with a discrete hydrocele in both testes and one had a slightly lobed biopsied testis, which may be due to scar formation.

Long-term follow-up of the patients in the study by Borgström et al. (2020) showed that most boys who underwent unilateral testicular biopsy had a similar testicular size (n = 4/6 survivors) to that of the contralateral testis at the last follow-up (Borgström et al., 2020). Among the seven surviving boys who had bilateral biopsies, one boy had equal testicular sizes, four had a small difference of 1 ml and two had a 2 ml difference between testes.

At 12 months after testicular biopsy, there was no significant impact of biopsy found on testicular growth in the case series by Uijldert et al., (2017). Very small fibrotic lesions, most likely related to the biopsy, were found at this stage in the testis of 4/55 boys. The remaining testis had no abnormalities (Uijldert et al., 2017).

A cohort of 112 boys with cryptorchidism underwent open testicular biopsy during orchidopexy (83 unilateral and 29 bilateral; not for the purpose of fertility preservation) (Patel et al., 2005). Long-term follow up showed testicular microlithiasis in 8 of 112 patients. None of the patients showed testicular atrophy or other abnormalities (scars, masses) at ultrasound.

Recommendation

Testicular sampling aimed at fertility preservation has low complication rates, and similar testicular growth between the biopsied and non-biopsied testis. It is recommended to collect long-term follow-up data on reproductive outcomes after testicular biopsy. Collection of standardized follow-up parameters (according to expert consensus) of procedures in a global registry should be encouraged for further progress in the field.

What psychological support is required?

Evidence

In a systematic review on fertility decision regret in adolescent and young adult cancer survivors, it was reported in multiple studies that psychologists play an important role in helping patients cope with their FP decisions, as well as preventing decision regret through discussions regarding treatment-related risk of infertility (Kuntz et al., 2024).

Recommendation

Provision of psychological support should be offered during follow-up for patients who did not have the chance to decide about FP options, or are facing emotional distress regarding fertility and fertility related concerns, to help deal with the distress of involuntary childlessness or to navigate and accept alternative family building options.

What counselling is required regarding the future use of cryopreserved testicular tissue?

Evidence

No studies could be retrieved from literature to answer this question.

Recommendation

Although the time and the content of the counselling still needs to be established, specialist support with experience in counselling FP patients should be made available during follow-up to assist patients in decision-making with regards to future use or disposal of cryostored testicular tissue.

Discussion

In these recommendations for good clinical practice, the ESHRE working group provided an overview of current available evidence. These recommendations are intended to complement previously published recommendations (Mulder et al., 2021). The recommendations provided are based on this evidence, with a clear acknowledgement of the lack of a robust evidence base to support some of them. However, it is the nature and requirement of clinical medicine to advise and support patient decision-making based on their individual clinical context, even when hard data are scarce. It is to be hoped that, in the coming years, studies will be published to provide a firmer basis for clinical recommendations and allow a revised consensus for the optimal management of fertility preservation in prepubertal males.

One of the most difficult topics for the working group was to define which patients are eligible for fertility preservation. A recent study evaluated gonadotoxic therapies in current treatment protocols for leukaemia and lymphoma, with conversion of exposure to alkylating agents to the CED. The study concluded that therapies associated with an increased likelihood of gonadal dysfunction and infertility in males include those with a CED exceeding 4 g/m^2 or any haematopoietic stem cell transplant (HSCT) (myeloablative or reduced intensity) containing at least one alkylating agent or total body irradiation. The Children's Oncology Group evaluated gonadotoxic therapies in current treatment protocols for leukaemia and lymphoma, with conversion of exposure to alkylating agents to CED. They concluded that therapies associated with an increased likelihood of gonadal dysfunction and infertility in males include those with a CED exceeding 4 g/m^2 or any haematopoietic stem cell transplant (HSCT) (myeloablative or reduced intensity) containing at least one alkylating agent or total body irradiation as well as total body irradiation and gonadal radiation exposure (direct or indirect) of 4 Gy or higher in males (Close et al., 2023), although no supporting evidence was provided. It was recommended that males at high risk be offered testicular tissue cryopreservation. However, the evidence supporting this cut-off of 4 g/m^2 in males is considered poor quality and many childhood cancer treatments reach these dosage levels. It is generally accepted that CED dosages of less than 4 g/m^2 are considered low risk, however, this does not automatically mean that dosages above 4 g/m^2 are high risk. With increasing CED and additional treatments, the risk of treatment-related infertility will increase. Defining a precise CED threshold for high risk of treatment-related infertility is challenging due to inter-individual variabilityto gonadotoxic treatments (Kanbar et al., 2021), and the potential for synergistic damage caused by combinations of therapeutic agents (Feraille et al., 2023). Furthermore, underlying medical conditions associated with paediatric cancer or severe haematological disorders may already compromise spermatogonial quantity prior to the initiation of gonadotoxic therapy, further increasing the risk of treatment-induced infertility (Lahtinen et al., 2024). Some alkylating chemotherapeutic agents have not been incorporated into the CED scoring framework, highlighting the need for

comprehensive longitudinal studies to accurately evaluate their gonadotoxic potential.

If the follow-up period is too brief and does not extend into adulthood, the opportunity to evaluate long-term spermatogenic recovery may be missed. This limitation can compromise the validity of clinical evidence used for risk stratification frameworks. This issue is particularly significant in studies with median follow-up durations of less than 10 years. Many boys who are not exposed to irradiation of the testes have good prospects for spermatogenetic recovery with extended follow up. Delayed spermatogenic recovery has been observed in chemotherapy-treated childhood cancer survivors, with peak sperm counts occurring 10–30 years post-therapy, suggesting reversible gonadotoxic effects even following high doses of alkylating agents (Romerius *et al.*, 2011; Mathiesen *et al.*, 2020; Korhonen *et al.*, 2025). In contrast, direct testicular radiation appears to cause irreversible damage, exceeding the regenerative capacity of the spermatogenic epithelium. These findings highlight the need for extended follow-up beyond childhood to fully assess long-term chemotherapy effects on spermatogenesis. The working group could not define any absolute contra-indications for testicular tissue cryopreservation in boys. Patient-related and treatment-related factors can impact suitability for testicular biopsy. It is however difficult to have definitive contra-indications, as most fertility preservation programs have defined their own set of contra-indications, which are often regulated locally.

There is a clear consensus on the need of counselling as an essential part of fertility preservation. In practice, however, there is still a lot of ambiguity on who should provide counselling, the information that should be provided, and the timing. It is important to inform the patient and the family that at this time, testicular tissue cryopreservation is considered an experimental fertility preservation technique, necessitating approval by an ethical review board. Counselling of the parents/caregivers is also an important part of the fertility preservation process. Many parents/caregivers want to preserve their son's fertility, even if the risk of treatment-related infertility is low and even if the prospects of fertility restoration are currently low. However, the decision to perform an invasive biopsy procedure is also the responsibility of the medical doctor, and therefore needs to be based on medical evidence of necessity and shared decision making with the patient and parents/caregivers.

Providing information about fertility preservation and testicular biopsy in a written format, such as a patient brochure or pamphlet can be very helpful, as patients and parents/caregivers may be overwhelmed with the amount of information to take in immediately after diagnosis. Of note, since testicular tissue cryopreservation for fertility preservation in boys is still an experimental procedure, providing written information is mandatory.

There is a general consensus in literature that a designated 'fertility navigator' can improve the uptake of fertility consults. One study reported that the number of fertility consults increased by more than threefold after hiring a fertility navigator, especially among female and long-time follow-up patients. Thus, a full-time fertility navigator may improve consult rates for paediatric patients at risk for infertility and positively impact access to fertility-related care from diagnosis to survivorship (Wright *et al.*, 2022).

From the literature, there is a consensus that an open testicular biopsy is currently the best technique for obtaining testicular tissue in prepubertal boys. This biopsy is preferably taken unilaterally. However, in some cases there is an increased risk of an already decreased number of spermatogonia in the testes (because of prior therapy or the urogenital history of the patient). The decision to perform a bilateral biopsy should be taken by the treating physician, taking into account the perceived benefit and the risk of complications. It is important to consider the timing of the biopsy, so that it can be combined with other procedures requiring general anaesthesia, where possible. Lastly, it is clear from the literature that there is no consensus on the amount of testicular tissue to be taken for fertility preservation.

Histological analysis has been extensively used to evaluate germ cell counts and spermatogenesis in testicular tissues stored for fertility preservation. A significant reduction in spermatogonial numbers has been observed in testicular tissues exposed to alkylating agents, with a negative correlation between cumulative exposure and spermatogonial count (Funke *et al.*, 2021; Moussaoui *et al.*, 2022; Barraud-Lange *et al.*, 2024). Notably, exposures exceeding a CED of 4 g/m^2 are associated with substantial depletion of spermatogonia (Poganitsch-Korhonen *et al.*, 2017). Ideally, testicular biopsy for fertility preservation should be performed prior to highly gonadotoxic treatment; however, this is not always feasible in paediatric oncology. Although prior gonadotoxic treatment is not considered a contraindication for testicular tissue cryopreservation in eligible patients, minimizing exposure to alkylating agents before cryopreservation is preferable. A longer interval between the initiation of gonadotoxic therapy and gonadal tissue cryopreservation has been shown to result in higher cumulative exposure to alkylating agents (Pampanini *et al.*, 2024). Promoting careful timing of cryopreservation in relation to treatment exposures is an option to limit alkylating agent exposure of cryopreserved tissue. Currently, no minimum time period between treatment cycles of any chemotherapy and testicular tissue cryopreservation can be defined. The optimal slot for fertility preservation should be pointed out in future cancer therapy protocols to harmonize the service and increase awareness among healthcare providers.

Paediatric patients with Fanconi anaemia have been reported to exhibit markedly reduced spermatogonial numbers, aligning with the infertility commonly observed in adults with this condition (Lahtinen *et al.*, 2024). Testicular tissue cryopreservation should be carefully considered in individuals with Fanconi anaemia. Similarly, the availability of fertility preservation options may be restricted in other severe haematological disorders requiring HSCT (Gille *et al.*, 2021; Benninghoven-Frey *et al.*, 2022). These findings highlight the need to evaluate spermatogonial numbers to accurately assess the quality of individual patient samples and to guide informed future fertility preservation decisions. This procedure should be limited to patients classified as high-risk, with comprehensive counselling to ensure that the child and the family are fully informed of the experimental nature and associated uncertainties of testicular tissue cryopreservation.

A major limitation of fertility preservation using cryopreserved testicular tissue is the risk of reintroducing malignant cells during autologous transplantation, particularly for patients with haematological malignancies (Kourta *et al.*, 2023; Duffin *et al.*, 2024). While achieving a minimal residual disease state before testicular tissue cryopreservation may reduce this risk, no reliable method currently exists to screen testicular tissue for malignant cell contamination prior to reimplantation, leaving the risk unresolved. Comprehensive counselling is essential to inform patients and families of the experimental nature and uncertainties of testicular tissue cryopreservation. A recent ORCHID-NET consortium report indicated that testicular tissue

has been cryopreserved for over 700 boys with acute leukaemia (Duffin *et al.*, 2024), but its use may be limited due to the lack of techniques to reliably exclude malignant contamination. Cryopreserving sufficient testicular tissue volumes for contamination analysis is crucial as more advanced detection methods may emerge.

For the transport and cryopreservation of the testicular tissue, the working group would like to point out the importance of validation of the cryopreservation procedures. Ideally, this includes determination of viability of the tissue after freezing. However, to date, there is no standardized method. Currently, we have three methods that can be used to determine viability of the testicular tissue after freezing: immunohistochemistry or cell membrane integrity and integrity of the tubules on histology, extended culture, and xenografting of the tissue. Keros *et al.* developed a system of survival analysis using 24 h culture of the tissue followed by immunohistochemistry (Keros *et al.*, 2007). However, currently the data is missing to determine if 24 h after thawing is the optimal duration of culture. Similarly, immunohistochemistry and histology may be affected by thawing of the tissue and prolonged culture may lead to a reduction in spermatogonia. Furthermore, it is currently unclear which are the best markers to use for survival analysis with immunohistochemistry. Xenografting is currently the only assay/tool that allows long-term evaluation needed for assessing the functionality of the tissue. However, rules to minimize reliance on animals must be followed according to ethical guidelines, and working with animals might not be possible for all researchers.

The lack of published evidence proved to be a challenge for the working group to provide definitive recommendations for each step of the transport and cryopreservation procedure. Therefore, it was decided to provide a conclusion of the data for each step of the cryopreservation procedure, and to provide a recommendation for the transport and cryopreservation procedure as a whole. Again, the working group would like to point out that it is important that the programme is validated in each laboratory, and that if changes are made to any step in the process, further validation is necessary.

In conclusion, progress has been made in developing testicular cryopreservation programmes for fertility preservation. These good practice recommendations provide a basis for establishing and developing a programme. It is anticipated that the recommendations will undergo further revisions as more evidence becomes available or any other approaches are established.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Authors' roles

RTM chaired the ESHRE FP in boys Working Group. NLC provided methodological support. All other authors contributed equally in writing the article, and all approved the final version for publication.

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