Good practice recommendations on fertility preservation in child and adolescent males receiving gonadotoxic therapies†

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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 five years. It is expected that this will continue to rise as the number of centres offering the procedure increases and the availability becomes more widespread.

However, there is no clear consensus on the best clinical practice for testicular tissue cryopreservation from the perspective of cryopreservation approach, tissue quality control, clinical assessment and follow-up for patients who are having tissue stored. This Good Practice Recommendations paper 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; from setting up a fertility preservation program, determining who is eligible and counselling, to the practical aspects of the testicular tissue biopsy and cryopreservation.

Methodology

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 paper. 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.

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. XX comments from XX reviewers were received and 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

1. Fertility preservation programme

1.1 What clinical expertise is required to start a program?

Evidence

Several of the identified studies point to the importance of a multi-disciplinary team for providing a fertility preservation (FP) programme (Anazodo et al., 2019, Carlson et al., 2017, Crespi et al., 2021, Goossens et al., 2020, Joshi et al., 2021, Keim-Malpass et al., 2018, Kim et al., 2014, Loren et al., 2013b, Ludemann et al., 2023, Moravek et al., 2019, Norton and Wright, 2020, Oktay et al., 2018, Picton et al., 2015, Sadri-Ardekani et al., 2016, Sehring et al., 2021, Stern and Agresta, 2019). 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 (Goossens et al., 2020, Wyns et al., 2015) 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 an 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 (Anazodo et al., 2019, Joshi et al., 2021, Keim-Malpass et al., 2018, Kim et al., 2014, Ludemann et al., 2023, Moravek et al., 2019, Sehring et al., 2021).

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 (Joshi et al., 2021, Moravek et al., 2019, Stern and Agresta, 2019).

Recommendation

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

To optimise this multi-disciplinary care pathway, the addition of an ethicist/geneticist, and psychologist is advised.

1.2 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) to process the tissue, a tissue bank (or place to store cryopreserved tissue), operating theatre, clinical facilities providing care to patients receiving therapies, and funding. This should be provided in accordance with local and national regulations.

2. Who is eligible?

2.1 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 ten years (Table 1). The results of another 11 studies with a median follow-up of less than ten years are summarised in **Supplementary Table S4**.

173 **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-
174 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/m²)** / **Non-CRT (n=82)** / **CRT (n=91)** >0 to <4 / 10 (12%) / 7 (8%) ≥4 to 8 / 18 (22%) / 17 (19%) ≥8 to 12 / 50 (61%) / 50 (55%) ≥12 / 4 (5%) / 17 (18%) CT ± CRT	173	Risk of azoospermia or oligospermia 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 oligospermia 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 to <12 g/m², and CED ≥12 g/m² compared to CED >0 to <4 g/m². 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/m² compared to CED >0 to <4 g/m².
(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/m² 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.	42	12/42 males presented with azoospermia 7/42 were oligospermic 23/42 were normospermic The proportion of azoospermia increased with treatment burden.

					<table><tr><td></td><td>Other regimen, total dose cyclophosphamide >6 g/m²</td></tr><tr><td>HL</td><td>HDT with TBI and high-dose cyclophosphamide as conditioning regimen. HDT with BEAM as conditioning regimen. MVPP or LVPP ≥4 courses</td></tr></table>		
(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, testicular irradiation [any dose], or hypothalamic–pituitary irradiation (≥40 Gy)	214	Azoospermia was identified in 53/214 (25%), oligospermia in 59/214 (28%), normospermia in 102/214 (48%). Mean CED was 10.83 g/m² (± 7.27) for those with azoospermia, 8.48 g/m² (±4.26) for those with oligospermia, and 6.63 g/m² (±3.58) for those with normospermia. Of the 35 patients with a CED of < 4 g/m², 31 (89%) were normospermic. CED and sperm concentration were negatively correlated (r=–0·37, p<0·0001).
(Korhonen et al., 2024)	255	Median 6.1 (3.2–11.4)	Median 27 (25–30)	Median 21 (15–23)	<table><tr><td>**CED** (g/m²) +/- CRT</td><td>(n=253)</td></tr><tr><td><4</td><td>116 (46%)</td></tr><tr><td>≥4 to <12</td><td>63 (25%)</td></tr><tr><td>≥12</td><td>74 (29%)</td></tr></table> <table><tr><td>**Testicular RT** (Gy)</td><td>(n=253)</td></tr><tr><td>>0 to <1</td><td>71 (28%)</td></tr><tr><td>≥1 to <10</td><td>7 (3%)</td></tr><tr><td>≥10</td><td>71 (28%)</td></tr></table> <table><tr><td>**HSCT**</td><td>(n=254)</td></tr><tr><td>No</td><td>202(80%)</td></tr><tr><td>Yes</td><td>52(20%)</td></tr></table>	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/m² 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: 6Gy, n=1 Testicular RT: 24 (10-24) Gy Cyclophosphamide: 6.9 (1.2-29.0) g/m²	47	0–10 g/m² 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/m² of cyclophosphamide (n=2).
(Romerius et al., 2011)	129	Median 10 (0.10-17)	Median 29 (20-46)	Median 19 (4-36)	<table><tr><td>**Treatment**</td><td>**Number**</td></tr><tr><td>Brain surgery</td><td>16</td></tr></table>	129	18% of childhood cancer survivors were azoospermic. Those treated with chemotherapy only, as well as

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<table>
<tr><td>Other surgery</td><td>16</td></tr>
<tr><td>CT alone</td><td>35</td></tr>
<tr><td>RT to testes</td><td>1</td></tr>
<tr><td>RT alone (not testes)</td><td>13</td></tr>
<tr><td>CT+RT</td><td>48</td></tr>
</table>

CT previously shown to imply a ‘high risk’ of azoospermia
<table>
<tr><th>Agent</th><th>Cumulative dose</th></tr>
<tr><td>Carmustine</td><td>1 g/m²</td></tr>
<tr><td>Lomustine</td><td>500 mg/m²</td></tr>
<tr><td>Chlorambucil</td><td>1.4 g/m²</td></tr>
<tr><td>Cisplatin</td><td>500 mg/m²</td></tr>
<tr><td>Cyclophosphamide</td><td>19 g/m²</td></tr>
<tr><td>Melphalan</td><td>140 mg/m²</td></tr>
<tr><td>Procarbazine</td><td>4 g/m²</td></tr>
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<td></td>
<td>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.</td>
</tr>
<tr>
<td>(Mathiesen et al., 2020)</td>
<td>98</td>
<td>At HSCT
Median 9.7
(0.4-16.9)</td>
<td>Median 28.1
(18.5-47.0)</td>
<td>Median 18.3
(7.7-34.6)</td>
<td>Myeloablative allogeneic HSCT
6 treatment groups according to their cumulative therapy: (1) chemotherapy only,
(2) low-dose testicular irradiation 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.
HSCT with TBI 71%, with CT only 24%, with CT + 4-6Gy TBI/TLI 4%
CED 5.2 (0-28.7) g/m²
Additional CRT or Testicular irradiation 14%</td>
<td>72</td>
<td>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/m² increase in CED 1.34; 95% CI 1.01 - 2.15).
All patients treated with more than ~10 g/m² had azoospermia,
All those treated with less than ~6 g/m² had detectable sperm.</td>
</tr>
<tr>
<td>(Nurmio et al., 2009)</td>
<td>23</td>
<td>5.7±2.9</td>
<td>21±1.5</td>
<td>17.0±1.9</td>
<td>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 irradiation (24 Gy), but spinal</td>
<td>6</td>
<td>N=3 at standard risk treatment
All had normal mean sperm counts
N=3 at high-risk therapy
One showed total recovery of spermatogenesis
2 were oligo/azoospermic</td>
</tr>
</table>

					irradiation was not used. Patients experiencing testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial irradiation at a dose of 24 Gy.		
(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 3 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 oligozoospermia (<20 x 10^6 /ml) < (6%) and 3/17 severe oligozoospermia (<5 x 10^6/ ml) (18%) MOPP+ patients. 4/17MOPP+ patients (23%) showed normospermia; of which 3 were treated with 3 MOPP cycles and 1 was treated with 6 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)) 4 patients also underwent a multidrug chemotherapy regimen together with testicular irradiation at a dose of 24 Gy.	17	<u>Non-Alkylating (n=10)</u> Total sperm count: 173.2 x 10^6 /ml ± 175.0 x 10^6 /ml (0.0–485.0) Motile sperm: 43% ±25 (0–83) Azoospermia: 1 (10%) <u>Alkylating (n=7)</u> Total sperm count: 50.1 x 10^6 /ml ± 60.5 (0.0–150.0) Motile sperm: 38% ±26 (0–79) Azoospermia: 1 (14%)
(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 irradiation of 20-24 Gy without other RT	18	3/18 had azoospermia and 7/18 had oligozoospermia. No significant association was observed between the sperm count and the preceding treatment.

					CT ± CRT No information about exposures for those with sperm analysis		
(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) x 10^6/mL, total sperm count 124.3 (0–625) x 10^6/mL, and percentage of motile sperm 56 (0–86)%. Eight patients (35%) had sperm concentration less than 20 x 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 (>7g/m²), 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 6 courses of MOPP chemotherapy. RT was given as adjuvant treatment in 8 patients with large lymph node tumours; 6 received irradiation above the diaphragm, and 2 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 oligospermia, 3 had severe oligospermia, 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 7 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/m²) Alkylating CT (mean dose 4084±1036.6 mg/m²) 6 patients also received RT (mean dose 241.1±65.1 mg/m²)	13	3 samples were azoospermic and 2 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** / **Oligo 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/m² 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).
(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 oligospermic (sperm count <20x 10^6/ml), and 17 normospermic (sperm count ≥20x 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 oligospermic), 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 oligospermic 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 3,500 cGy to an inverted Y field, of which 1 had semen analysis and was severely oligospermic.
(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., 2024)	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	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

					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 (5Gy)/cyclophosphamide (20 mg/kg): n=6 - No conditioning: n=7 Missing: n=1		oligospermia. One of 6 (16.7%) males conditioned with cyclophosphamide alone and 3/4 (75%) conditioned with RIC had impaired spermatogenesis,
(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/m² (range, 4.7– 31.9 g/m²). 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 oligospermia (29.4%), and 10 patients had azoospermia (58.8%). None of the ten patients treated prior to the onset of puberty had normal sperm counts. All 15 men who received > 7.5 g/m² of cyclophosphamide had abnormal semen analysis and all the men who received > 25 g/m² 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: antitumor antibiotics, alkylating agents, topoisomerase and mitotic inhibitors, platinum-based agents and antimetabolites. 34 patients also underwent RT (26 patients underwent abdominal irradiation with a median dose 24.8 Gy (range, 15–40 Gy), eight patients underwent cranial irradiation 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 antitumor antibiotics (OR = 0.253, $p < 0.027$). The mean CED values in survivors with aspermia (12.6 g/ m²) were significantly higher than those in survivors with oligozoospermia (5.3 g/m²) ($p < 0.01$) and normozoospermia (4.5 g/m²) ($p < 0.01$)
(Jaffe et al., 1988)	27	Median 12 (5-16)	NR	*After diagnosis*	Radiation therapy was administered to all patients with Hodgkin's Disease and in six, the radiation field	23	4 were oligospermic 14 were azoospermic

				Median 11 (5-26)	included the inguinal or paraaortic nodes. Seven patients received 2-6 cycles of MOPP chemotherapy (nitrogen mustard, oncovin, prednisone and procarbazine) and five, COPP (cyclophosphamide, oncovin, prednisone, and procarbazine) or chlorambucil. Among the remaining patients, ten received radiation therapy (five to the inguinal or pelvic nodes) and seven, an alkylating agent (cyclophosphamide, nitrogen mustard, or chlorambucil). One leukaemia patient with testicular relapse received radiation to the gonads (2,400 rad). Four patients received Adriamycin.		*CT alone*: One patient who received 30 g of cyclophosphamide (21.4 g/m²) and another 68 mg of phenylalanine mustard (45 mg/m²) had normal reproductive capacity (fathered children). In contrast, two patients, one who received 32 g of cyclophosphamide (29.0 g/m²) and another 55 g (37.9 g/m²) were sterile. Two patients who received smaller quantities of cyclophosphamide 3.45 g (2.76 g/m²) and 9.5 g (5.0 g/m²) 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 radiation therapy; among these, four also received alkylating agents and procarbazine, and two received alkylating agents
(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 15-44 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)	15	The 3 boys receiving RT alone were all oligospermic. 10/12 boys treated with MOPP with or without RT were azoospermic.

					CT alone: n=7; MOPP/ABVD for six cycles-16, PAVe for six cycles-3. VBM for six cycles1, ABVD for six cycles		
(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 oligospermia, 5 had severe oligospermia, 8 (40%) were azoospermic In group 2, 4/5 patients who received additional inverted Y radiotherapy 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 leukemia; **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**: hematopoietic 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.

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) and the risk azoospermia. Whilst there is some variability in findings between studies, CED <4 g/m^2 is usually associated with normospermia in adulthood, while exposure to >8 g/m^2 is associated with a significantly increased risk of oligospermia and >10g/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 the high doses of alkylating agents (Korhonen et al., 2024). Radiotherapy to the pelvis or direct radiotherapy to the testis results in a significant risk of subsequent azoospermia with limited options for recovery.

Thirteen studies were identified that reported on the value of follicle stimulating hormone (FSH) or Inhibin B in predicting azoospermia (Table 2a) and an additional 28 studies that describe FSH levels in relation to gonadotoxic treatment received during childhood (Table 2b). The majority of studies that included semen analysis showed an association between raised FSH and impaired semen parameters (Table 2a). 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 summarised in Supplementary Table S5.

200 **Table 2a**: Studies reporting on the predictive value of FSH and Inhibin B for predicting azoospermia in childhood cancer survivors, arranged in descending order
201 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 hormone level determination	Effect
(Korhonen et al., 2024)	255	Median 6.1 (3.2–11.4)	Median 27 (25–30)	Median 21 (15–23)	**CED** (g/m²) +/- CRT (n=253) <4: 116 (46%) ≥4 to <12: 63 (25%) ≥12: 74 (29%) **Testicular RT** (Gy) (n=253) >0 to <1: 71 (28%) ≥1 to <10: 7 (3%) ≥10: 71 (28%) **HSCT** (n=254) No: 202(80%) Yes: 52(20%)	74	AUCs for identifying patients with azoospermia were 0.93 for FSH (optimal cut-off at 12.0 IU/L, n=74) and 0.94 for inhibin B (optimal cut-off at 44 ng/L, n=43) measured 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/m²	47	The AUC of the ROC curves to predict fertility was 0.70 for FSH, and 0.63 for inhibin B. Cutoff levels of 2.5 IU/L for FSH, and 180 ng/L for inhibin B. All showed 80% sensitivity in identifying patients who had fathered a child, and the false-positive rate for FSH was 30% and for inhibin B 40%.
(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	129	FSH = 10.9 IU/L had 96% sensitivity and 96% specificity to predict azoospermia. 66% (95% CI 47–81) with subnormal Inhibin B levels and 50% (95% CI 35–67) with elevated FSH levels were azoospermic.

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td><table><tr><th>Agent</th><th>Cumulative dose</th></tr><tr><td>Carmustine</td><td>1 g/m²</td></tr><tr><td>Lomustine</td><td>500 mg/m²</td></tr><tr><td>Chlorambucil</td><td>1.4 g/m²</td></tr><tr><td>Cisplatin</td><td>500 mg/m²</td></tr><tr><td>Cyclophosphamide</td><td>19 g/m²</td></tr><tr><td>Melphalan</td><td>140 mg/m²</td></tr><tr><td>Procarbazine</td><td>4 g/m²</td></tr></table></td><td></td><td></td></tr>
<tr><td>(Mathiesen et al., 2020)</td><td>98</td><td>At HSCT
Median 9.7 (0.4-16.9)</td><td>Median 28.1 (18.5-47.0)</td><td>Median 18.3 (7.7-34.6)</td><td>Myeloablative allogeneic HSCT
6 treatment groups according to their cumulative therapy:
(1) chemotherapy only,
(2) low-dose testicular irradiation 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.</td><td>72</td><td>Inhibin B was the best surrogate marker of azoospermia (AUC 0.91; 95% CI 0.85 - 0.98; 90% sensitivity and 83% specificity), compared with FSH and testicular volume.</td></tr>
<tr><td>(van Beek et al., 2007)</td><td>56</td><td>Median 11.4 (3.7–15.9)</td><td>Median 27 (17.7-42.6)</td><td>Median 15.5 (5.6-30.2)</td><td>Adriamycin/epirubicin, bleomycin, vinblastine, dacarbazine) with or without MOPP
divided into 3 groups:
- no MOPP (n=16)
- 3-4 MOPP (n=14)
- ≥6 MOPP (n=26)</td><td>56</td><td>FSH increased, and inhibin B levels and sperm concentration decreased significantly with an increasing number of MOPP cycles.</td></tr>
<tr><td>(Brignardello et al., 2016)</td><td>199</td><td><table><tr><th>Age</th><th>No (%)</th></tr><tr><td>0-4</td><td>45 (22.6%)</td></tr><tr><td>5-10</td><td>57 (28.6%)</td></tr><tr><td>≥10</td><td>97 (48.7%)</td></tr></table></td><td>NR</td><td>Median 14.01 (IQR 10.1-17.8)</td><td>Referred to (Brignardello et al., 2013) for treatment details:<table><tr><th>Treatment</th><th>Number</th></tr><tr><td>Any RT</td><td>199 (64.2)</td></tr><tr><td>TBI</td><td>40 (12.9)</td></tr><tr><td>Cranial RT</td><td>74 (23.9%)</td></tr><tr><td>CT</td><td>294 (94.8%)</td></tr><tr><td>HSCT</td><td>74 (23.9%)</td></tr><tr><td>Surgery</td><td>115 (37.1%)</td></tr></table></td><td>194</td><td>Impaired spermatogenesis was diagnosed in 68 patients (34.17 %); this diagnosis was confirmed in all 41 patients in whom semen analysis was performed.
Among 33 patients previously treated with TBI, none had normal gonadal function, 17 had impaired spermatogenesis.</td></tr>
<tr><td>(Kenney et al., 2001)</td><td>17</td><td>Median 12 (4-19)</td><td>Median 25 (16-34)</td><td>Median 12 (5-22)</td><td>All patients received vincristine, actinomycin D, and cyclophosphamide, and 8 patients also</td><td>16</td><td>All ten patients with an elevated baseline FSH level had abnormal sperm counts.</td></tr>
</table>

					received doxorubicin. The median total dose of cyclophosphamide was 20.5 g/m² (range, 4.7–31.9 g/m²). 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)		However, 3/10 azoospermic men (30%) and 2/5 oligospermic men (40%) had a normal baseline FSH. Thus, normal FSH did not appear to be predictive of a normal sperm count.
(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)	We compared five chemotherapeutic groups: antitumor antibiotics, alkylating agents, topoisomerase and mitotic inhibitors, platinum-based agents and antimetabolites. 34 patients also underwent RT (26 patients underwent abdominal irradiation with a median dose 24.8 Gy (range, 15–40 Gy), eight patients underwent cranial irradiation with a median dose 40.2 Gy (range, 12– 55.6 Gy), and three patients underwent cranial + spinal irradiation 25 Gy)	126	Survivors with abnormal semen analysis had increased levels of FSH with time since diagnosis ($p < 0.0001$)
(Jaffe et al., 1988)	27	Median 12 (5-16)	NR	*After diagnosis* Median 11 (5-26)	RT was administered to all patients with Hodgkin's Disease and in six, the radiation field included the inguinal or para-aortic nodes. Seven patients received 2-6 cycles of MOPP and five, COPP or chlorambucil. Among the remaining patients, ten 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 radiation to the gonads (2,400 rad). Four patients received Adriamycin.	23	Higher FSH levels were associated with sterility; however, the range of values overlapped those detected in men with normal fertility
(Green et al., 2013)	275	**Age** / **No (%)** >0-<5 / 109 (39.6%) 5-<10 / 72 (26.2%) 10-<15 / 61 (22.2%)	Median 30.5 (19.7-59.1)	≥ 10	From (Green et al., 2014); same study cohort): CT with alkylating agent, testicular irradiation [any dose], or hypothalamic–pituitary irradiation (≥40 Gy)	238	82/105 (78.1) azoospermic males had FSH levels >11.5 mIU/mL. 126 (74.1%) males with oligospermia or normal sperm counts had a FSH levels of ≤11.5 mIU/mL. The threshold of 11.5 mIU/mL for FSH had a specificity of 74.1% and PPV 65.1% AUC-ROC: 0.83

		15-<20: 32 (11.6%) ≥20: 1 (0.4%)					
(Ortin et al., 1990)	20	Median 14 (10-15)	NR	Median 8.5 (1-10)	RT alone: n=3; the delivered dose at the midplane of the pelvis ranged from 15-44 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=3; min 6 cycles of MOPP and pelvic RT (20-44 Gy) CT alone: n=4; MOPP/ABVD for six cycles-16, PAVe for six cycles-3. VBM for six cycles1, ABVD for six cycles	10	No correlation was seen between serum gonadotropin levels and sterility. Four of seven boys who had abnormal sperm counts had consistently elevated FSH levels, However, one boy who had an elevation of FSH subsequently fathered two children.
(Hobbie et al., 2005)	11	Median 13 (6-19)	NR	Median 6.5 (1.5-21)	CT: COPP/ABV hybrid total cyclophosphamide doses of 2.4–3.6 g/m²	11	5/9 infertile males had normal FSH levels. There was no association between fertility status and gonadotropin status (p = 0.49).
(Dhabhar et al., 1993)	26	Median 12 (4-15)	Median 17 (15-23)	Median 6 (2.3-11)	16 patients received 6 cycles of COPP and 4 patients received COPP/ABVD. 2 patients had 10 and 9 cycles of COPP, respectively. 4 patients received MOPP/ABVD. 14 patients received RT supradiaphragmatic (2000-4000 cGy) The cumulative dose of cyclophosphamide, procarbazine and adriamycin varied from 3-10 g (median 7.2g), 4.5-20 g (median 9g) and 120-240 mg (median 150 mg), respectively.	23	16 patients with follow-up of ≥6 years with azoospermia showed increased levels of FSH. 10 patients had normal FSH.

ABV: adriamycin, bleomycin, vinblastine; **ABVD**: doxorubicin, bleomycin, vinblastine, dacarbazine; **AUC**: area under the curve; **CI**: confidence interval; **CNS**: central nervous system; **COPP**: cyclophosphamide, doxorubicin, procarbazine, prednisone; **CRT**: cranial radio therapy; **CT**: chemotherapy; **FSH**: follicle stimulating hormone; **HL**: Hodgkin lymphoma; **HSCT**: hematopoietic stem cell transplant; **IQR**: inter-quartile range; **MOPP**: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; **NR**: not reported; **PAVe**: procarbazine, alkeran, velban; **PPV**: positive predictive value; **ROC**: receiver operating characteristic; **RT**: radiotherapy; **TBI**: total body irradiation; **TLI**: total lymphoid irradiation; **VBM**: velban, bleomycin, methotrexate.

206 **Table 2b**: Studies reporting on FSH levels in relation to gonadotoxic treatment received in childhood cancer survivors, arranged in descending order of median
207 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 hormone level determination	Effect
(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/m² 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/m² HL: HDT with TBI and high-dose cyclophosphamide as conditioning regimen; HDT with BEAM as conditioning regimen; MVPP or LVPP ≥4 courses	64	20/64 (31%) had FSH levels above age-adjusted limits. FSH increased significantly with treatment burden. No significant difference in FSH for males treated before or after puberty
(Utriainen et al., 2019)	20	Median 1.6 (0.2-3.6)	Median 21.7 (15.9-30.1)	Median 19 (13-27)	Induction CT with Cyclophosphamide, vincristine with or without cisplatin and doxorubicin Local RT in 14/20 TBI + CT in 10/20	9	All 9 males had high FSH levels.

					Combination CT with Eto+carbo+tiotepa/melphalam/other		
(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 RT at a dose of 24 Gy.	11	N=8 with standard risk treatment levels of FSH (3.2±0.5 IU/L) and inhibin-B (225±38 ng/L) were comparable to values among healthy Finnish young men. N=3 after 'high-risk' therapy. Two had normal gonadotropin levels, one had increased levels.
(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	33	*Group 1:* 3/13 had normal FSH, 11/13 had increased FSH levels *Group 2*: All 10 had normal FSH *Group 3*: 7/10 had normal FSH 3/10 had elevated FSH levels
(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 3 groups: - no MOPP (n=16) - 3-4 MOPP (n=14) - ≥6 MOPP (n=26)	56	Median FSH values were significantly higher in MOPP+ patients when compared with MOPP- patients ($P < 0.01$), who all had normal to marginally increased FSH levels. Median inhibin B levels were significantly lower in MOPP+ patients when compared with MOPP- patients ($P< 0.01$).
(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 chemotherapy 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. 11 survivors (4.6%) were treated with TBI	FSH: 488	FSH levels were raised in 161 men (33.0%). All survivors with TBI had elevated FSH levels. Multivariate logistic regression analysis identified a significantly higher risk of elevated FSH levels after use of procarbazine (OR 3.8; 95% CI 1.8 – 8.2), cyclophosphamide (OR 4.2; 95% CI 2.2 – 8.0), other alkylating agents (OR 2.1; 95% CI 1.1 – 4.0), vinca-alkaloids (OR 2.8; 95% CI 1.0 –7.3), pelvic/abdomen irradiation (OR 2.4; 95% CI 1.0 – 5.4)
(Siimes et al., 1993)	41	Median 7.5 (1-16)	18-27	*After diagnosis*	All 41 patients had received intravenous vincristine, and oral prednisone, 6-mercaptopurine, and	41	The only risk factor for abnormal serum FSH was cyclophosphamide, which was associated with

<table>
<tr><td></td><td></td><td></td><td></td><td>Median 15.2 (4-25)</td><td>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</td><td></td><td>increases of 8.2 (-0.5-16.9) IU/L (p= 0.065) in FSH.</td></tr>
<tr><td>(Brignardello et al., 2016)</td><td>199</td><td><table><tr><td>Age</td><td>No (%)</td></tr><tr><td>0-4</td><td>45 (22.6%)</td></tr><tr><td>5-10</td><td>57 (28.6%)</td></tr><tr><td>≥10</td><td>97 (48.7%)</td></tr></table></td><td>NR</td><td>Median 14.01 (IQR 10.1-17.8)</td><td>Referred to (Brignardello et al., 2013) for treatment details:<table><tr><th>Treatment</th><th>Number</th></tr><tr><td>Any RT</td><td>199 (64.2)</td></tr><tr><td>TBI</td><td>40 (12.9)</td></tr><tr><td>Cranial RT</td><td>74 (23.9%)</td></tr><tr><td>CT</td><td>294 (94.8%)</td></tr><tr><td>HSCT</td><td>74 (23.9%)</td></tr><tr><td>Surgery</td><td>115 (37.1%)</td></tr></table></td><td>194</td><td>102/194 (51.26 %) male childhood cancer survivors had normal gonadal function.
An extremely high rate of gonadal dysfunction (46/48) was also detected in patients who underwent HSCT.
The risk of gonadal dysfunction was higher in patients treated with radiotherapy (crude OR = 5.83; 95 % CI 2.95–11.52 and adjusted OR = 8.72; 95 % CI 3.94–19.30) and in patients exposed both to alkylating agents and to platinum-derived agents (adjusted OR = 9.22; 95 % CI 2.17–39)</td></tr>
<tr><td>(Lähteenmäki et al., 2008)</td><td>25</td><td>Median 8.5 (0.9-15.9)</td><td>Median 20.5 (15.6-31.2)</td><td>After diagnosis
Median 14.5 (2.1-26.1)</td><td>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</td><td>25</td><td>Abnormal FSH levels were found in 7 patients, of which 3 also had abnormal LH levels.
2 patients had abnormal LH with normal FSH levels.</td></tr>
<tr><td>(Heikens et al., 1996)</td><td>19</td><td>Median 11 (5-15)</td><td>Part 1: Median 19 (16-27)
Part 2: Median</td><td>Part 1: Median 10 (6-14)
Part 2: Median 14 (13-20)</td><td>All patients were treated with 6 courses of MOPP chemotherapy.
RT was given as adjuvant treatment in 8 patients with large lymph node tumours; 6 received irradiation above the diaphragm, and 2 were irradiated below the diaphragm (20 Gy on the para-aortic and splenic regions, respectively, and 25 Gy on the inguinal region)</td><td>19</td><td>4 patients had normal basal levels of FSH. In 15 patients, basal FSH levels were above the normal range.
Follow-up hormone measurements were available for 16 patients. Mean FSH levels increased significantly over time (P < 0.001)</td></tr>
<tr><td>(Beaud et al., 2019)</td><td>13</td><td>Mean 12.8±1.3</td><td>Mean 27.8±1.6</td><td>Mean 13.4±2.3</td><td>Vinca alkaloids (mean dose 31.45±18.6 mg/m²)
Alkylating CT (mean dose 4084±1036.6 mg/m²)
6 patients also received RT (mean dose 241.1±65.1 mg/m²)</td><td>13</td><td>No differences were observed in the mean FSH between healthy controls and childhood cancer survivors and, further, between the controls and the two subgroups of childhood cancer survivors (pre-pubertal and pubertal)</td></tr>
</table>

(Relander et al., 2000)	77	Median 11 (0.8-17)	Median 23.6 (18.6-38.5)	*After diagnosis* Median 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.	66	62 patients had completed normal pubertal development, whereas 4 had Tanner staged at 3/5. One of them had received testicular irradiation; in the remaining 3 the finding could not be explained. FSH was within the normal range in 57 patients (88%). Nine patients had an increased FSH.
(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	Both basal and peak FSH concentrations were significantly raised in the oligospermic and azoospermic patients compared with those in the control group, as was the peak FSH concentration in the normospermic group
(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	40	*Patients that received CT* 26/28 of patients have elevated FSH levels. *Patients that only received RT* 7/7 patients with RT above diaphragm all have normal FSH. 3 patients received 3,500 cGy to an inverted Y field, all have elevated FSH levels. 2 patients received 3,500 cGy to the right groin. Both have normal FSH.
(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 CT/RT (n=5)	12	4/12 patients had high serum FSH levels 9/12 patients had low serum inhibin levels
(Lee et al., 2024)	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 (5Gy)/cyclophosphamide (20 mg/kg): n=6 - No conditioning: n=7 - Missing: n=1	117	Of 37 men who had received TBI +/- additional testicular RT, or therapeutic testicular RT without TBI (cumulative testicular doses of 12-36Gy), 33/37 had available gonadotrophin measurements; 32 /33 (97%) had elevated FSH. 24/27 males receiving 12 Gy TBI without testicular RT, 10/24 had elevated FSH
(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/m² (range, 4.7– 31.9 g/m²).	16	Ten of 15 patients (66.7%) had elevated basal FSH levels.

					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)		
(Ridola et al., 2009)	159	Group 1: Median 12 (0.5-20.7) Group 2: Median 9.8 (0-17.6)	Group 1: Median 22.5 (17.3-36.1) Group 2: Median 19.5 (17.5-28.6)	Group 1: Median 8.5 (5-16.5) Group 2: Median 12 (5.4-20.5)	Group 1: n=100; patients treated with ifosfamide, cumulative dose 18 to 60 g/m². Some patients received higher dose (relapse etc), 54 g/m² (range 18–114 g/m²) Group 2: n=59; patients treated with cyclophosphamide, median cumulative dose 8.3 g/m² (4.6 to 22.0 g/m²)	159	**Cumulative Cyclophosphamide dose** / **Patients with abnormal FSH** <9 g/m² — 21.4% (6/28) 9-11.9 g/m² — 53% (8/15) ≥12 g/m² — 87.5% (14/16) Total — 47.4% (28/59) **Cumulative Ifosfamide dose** / **Patients with abnormal FSH** <36 g/m² — 3.4% (1/29) 36-47.9 g/m² — 0% (0/11) ≥48 g/m² — 8.3% (5/60) Total — 6% (6/100)
(Zaletel et al., 2010)	64	Median 13 (3-16)	Median 21 (13-34)	Median 10 (4-27)	CT+RT: n=49 RT only: n=10 CT only: 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 - n=15 (11 boys, 4 girls) RT to the pelvis with 22-45 (median 30) Gy	40	All 24 had elevated FSH levels
(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	FSH levels were above normal values (1±14 U/L) in 10/20 patients (50%).
(Williams et al., 2008)	45	Median 11.8 (5.4-21.3)	Median 20.8 (16.0-29.3)	Median 9.7 (3.3-12.6)	32 males received a median dose of ifosfamide 92 g/m²	32	In the high-dose group, 8/26 had high FSH levels (>10 U/L)

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td>9 patients had also received cyclophosphamide 0.3–2.4 g/m² during RT
Patients were divided into two ifosfamide dose ranges, based on the bimodal distribution of doses: low-dose (<60 g/m², n=6) and high dose (>60 g/m², n=26).</td><td></td><td>FSH was significantly correlated with age at treatment (r=0.39, p=0.049)
No abnormal FSH levels were observed in the low dose group</td></tr>
<tr><td>(Servitzoglou et al., 2015)</td><td>171</td><td>Median 10.8 (2.1-17.3)</td><td>Median 21.1(17-30.4)</td><td>Median 9.3 (2-22.4)</td><td>For HL, children received combined RT (mantle field, subtotal nodal, or involved field RT) and CT, consisting of several MOPP cycles alone or in combination with ABVD or ABVP
More recently, patients received either VBVP cycles alone or VBVP combined with OPPA or in combination with COPP
For NHL, RT has been used for CNS prophylaxis or rarely for resistant mediastinal disease. CT consisted of COPAD cycles associated with lomustine (CCNU) or high-dose methotrexate, cytarabine, etoposide, asparaginase, 6-mercaptopurine, 6-thioguanine, or vinblastine.</td><td>171</td><td>42.1% (72/171) of survivors had abnormal FSH levels (≥10 IU/L).
Only 3 alkylating agents and their cumulative doses were associated with a higher FSH level: cyclophosphamide (P < .0001), CCNU (P = .002), and procarbazine (P < .0001). Older age at evaluation was associated with higher FSH but it was also associated with older treatment regimens and higher alkylating agent dose</td></tr>
<tr><td>(Aubier et al., 1989)</td><td>30</td><td>Median 9 (21mo-17)</td><td>NR</td><td>Median 9 (1-20)</td><td>CT with non-alkylating: 13%
CT with alkylating agents: 85%</td><td>9</td><td>All 9 males showed an increase over basal FSH values.</td></tr>
<tr><td>(Borgström et al., 2020)</td><td>14</td><td>Median 10.7 (1.5-14.5)</td><td>Median 18.3 (12.7-21)</td><td>Median 7.2 (5-13.7)
N=5 ≥ 10 years</td><td>N=10 were conditioned with TBI (4 fractions × 3 Gy, 12 Gy in 1 week),
N= 10 received ‘high dose’ busulfan, usually in combination with ‘high dose’ cyclophosphamide.</td><td>14</td><td>Hormone levels were repeatedly measured in 14 boys.
9/14 boys had FSH levels above the reference levels
Inhibin: very low in 3 boys, normal in 1 boy</td></tr>
<tr><td>(Kanbar et al., 2021)</td><td>114</td><td>At biopsy
7.5±4.1 years</td><td>20.6±2.3</td><td>7.1 ± 3.0</td><td>CT with an alkylating or alkylating-like agent (n=123)
70% CED >4 g/m², 54% CED >8 g/m²,16% CRT for those with sperm analysed
+ CT-RT (n=30)
BMT (n=41)</td><td>57</td><td>19/57 patients (33%) were found to have high FSH levels (20 ± 8.8 IU/l).
A multiple logistic regression model with high post-treatment FSH had an OR of 1.1 (95% CI 1.01–1.21; p= 0.03) for CED (referring to a 1 g/m² increase).</td></tr>
<tr><td>(Rafsanjani et al., 2007)</td><td>33</td><td>Median 9.1 (5-15)</td><td>Median 19.2 (17-29)</td><td>Median 7 (2-20)</td><td><table><tr><th>Therapy</th><th>Number (%)</th></tr><tr><td>MOPP/ABVD</td><td>23 (69.7%)</td></tr><tr><td>MOPP/ABVD+XRT</td><td>3 (9.1%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, prednisolone</td><td>1 (3%)</td></tr><tr><td>MOPP/AVBD+vinbastin, Leukeran</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+COPP/ABVE</td><td>1 (3%)</td></tr></table></td><td>33</td><td>The median level of FSH was 8 mIU/ml (range, 1-32), 6/33 cases were above normal.</td></tr>
</table>

					MOPP+splenectomy: 1 (3%) MOPP/ABVD+CCNU, VP16, MTX, CPA: 1 (3%) MOPP/ABVD+CCNU, VP16, MTX: 1 (3%) MOPP: 1 (3%)		
(Bordallo et al., 2004)	21	Median 10 (6-19) years	Median 18 (17-23)	≥ 2 years 3-11 years	C-MOPP/ABV hybrid program (cyclophosphamide, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, vinblastine) given in six or more cycles	21	6 (28.6%) patients from group A presented normal FSH basal levels.
(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/m²), procarbazine (50 mg/day for 2 days and 100 mg/day for 26 days) in combination with prednisone (30 mg/m²/day) and vincristine (1.5 mg/m²) and finally cyclophosphamide (1200 mg/m²). 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	FSH was within the normal range for all patients in group 1. Thirteen of 25 (52%) group B (CT ± RT−) patients and 5 of 6 group C (CT + RT+) patients had increased serum concentrations of FSH
(Felicetti et al., 2020)	196	**Age at diagnosis** / **No** 0-4: 37 5-9: 55 ≥10: 104	Median 24.35 (IQR 21.84-29.39)	≥ 5 years	**Treatment** / **No (%)** **RT** Any: 103 (52.6%) Abdominopelvic: 32 (16.3%) TBI: 21 (10.7%) Cranial: 13 (6.6%) **CT** Any: 196 (100%) Alkylating: 185 (94.4%) CED 0-4 g/m²: 104 (53.1%) CED 4-8 g/m²: 71 (36.22%) CED > 8g/m²: 21 (10.7%) HSCT: 50 (25.5%)	196	Spermatogenesis damage (FSH > 10 IU/L and inhibin B < 100pg/mL) was found in 58 out of 196 patients (29.6%), A greater exposure to alkylating agents was associated with a higher risk of spermatogenesis damage ($OR_{CED\ (per\ g/mg^2)}$ = 1.52, 95% CI 1.28-1.81), LCF ($OR_{CED(per\ 1\ g/mg^2)}$ = 1.34, 95% CI 1.03- 174).
(Braye et al., 2023)	59	Median 4.0 (0.0–15.4)	Median 12.9 (3.9-25.6)	Median 5.0 (1.0-13.0)	HR-C/R (n=25) CT-HSCT (n=34): MAC with or without 12 Gy TBI (26/34), RIC (5/34) or NMA (3/34).	59	Significantly more CT-HSCT patients showed low inhibin levels (<50 pg/mL) compared to HR-C/R patients (41% vs. 5%, p = .0130). Significantly more low inhibin levels (<50 pg/mL) were seen after MAC or 12 Gy TBI compared to NMA or RIC (70% vs. 0%, p = .0098).

<table>
<tr><td>(Krawczuk-Rybak et al., 2009)</td><td>59</td><td>Group 1:
4.3±1.7
Group 2:
7.9±4.3</td><td>Group 1:
8.4 ±2.2
Group 2:
15.9±2.6</td><td>Group 1:
1.9±1.3
Group 2:
5.3±3.5</td><td>Protocols of the Polish Pediatric Leukemia/Lymphoma Study Group based (in standard-risk group) on BFM protocols of 1985, 1990, and 1995 (n = 2) or, in the high-risk group, COPPAon New York (NY) protocol (n = 7)
RT:
Group 1: n=8 (NY: 18 Gy, n=5, BFM: 12 Gy, n=3)
Group 2: n= 6: 18 Gy (2 NY and 4 BFM) and n=12 received 12 Gy (BFM)</td><td>59</td><td>Group 1: No statistically significant differences were found in the mean values of FSH compared to heathy controls.
No differences between irradiated and non-irradiated patients.
Group 2: 5 boys showed elevated (+2 SD) FSH levels. Four of them had received irradiation to the CNS (12 Gy).</td></tr>
<tr><td>(Mackie et al., 1996)</td><td>58</td><td>Median 12.2 (8.2-15.3).</td><td>NR</td><td>After diagnosis
Median 6 (2.5-11.1)</td><td>Combination CT was given for a recommended minimum of six courses (equivalent to 504 mg/m² chlorambucil and 8,400 mg/m² procarbazine) or a maximum of eight courses.</td><td>46</td><td>41/46 (89.1 %) subjects had elevated FSH levels (range 10.8-40.7 IU/L.).
No association was identified between raised FSH levels and age or pubertal status at time of receiving chemotherapy or time elapsed since treatment.</td></tr>
<tr><td>(Quigley et al., 1989)</td><td>45</td><td>Median 4.39 (1.23-12.35)</td><td>NR</td><td>Median 4.62 (2.35-8.97)</td><td>Cyclophosphamide: mean dose 4.8 g/m², cytarabine: mean dose 13.1 g/m²
asparaginase, daunorubicin, hydroxyurea, lomustine, methotrexate, prednisolone, thuiguanine, vincristine
Cranial RT: 24 Gy and intrathecal methotrexate</td><td>23</td><td>Plasma FSH level was elevated in 19/23 boys</td></tr>
<tr><td>(Brämswig et al., 1990)</td><td>75</td><td>12.44±2.1</td><td>17.24±2.19</td><td>4.3±1.87</td><td><table><tr><th>Treatment</th><th>HD I-IIA</th><th>HD II-IIIA</th><th>HD IIIB-IV</th></tr><tr><td>CT</td><td>2 OPPA</td><td>2OPPA/ 2 COPP</td><td>2 OPPA/ 4-6 COPP</td></tr><tr><td>Vincristine</td><td>4.5</td><td>10.5</td><td>13.5-16.5</td></tr><tr><td>Prednisone</td><td>1800</td><td>2360</td><td>2920-3480</td></tr><tr><td>Procarbazine</td><td>3000</td><td>5800</td><td>8600-11400</td></tr><tr><td>Adriamycin</td><td>160</td><td>160</td><td>160-160</td></tr><tr><td>Cyclophos-phamide</td><td></td><td>2000</td><td>4000-6000</td></tr></table></td><td>75</td><td>Basal and stimulated FSH levels (14.12 U/L and 25.09 U/L) were higher than controls (7.17 U/L and 9.42 U/L), indicating severe damage to the germinal epithelium.
With the intensification of chemotherapy, the incidence of pathologically elevated FSH levels increased.
The frequency of elevated FSH levels was higher in the middle or late pubertal group with a chronologic age of 18.21 ± 2.04 years</td></tr>
<tr><td>(Whitehead et al., 1982)</td><td>17</td><td>Median 11.2 (4.8-14.8)</td><td>NR</td><td>Median 3.8 (1-8)</td><td>CT: n=16
Combination CT with MOPP
(Mustine 68.6±15.9 mg/m²; Vincristine 21.6±4.3</td><td>15</td><td>All 4 prepubertal subjects had normal basal and peak gonadotrophin responses to LH-RH.</td></tr>
</table>

					mg/m²; prednisolone 4741.3±1330.5 mg/m²; procarbazine 11030.7±2815.8 mg/m²) RT: n=15 Neck or mantle RT: n=15; 2500-3000 cGy Abdominal RT: n=5; radiation dose to the testes was 100-300 cGy		4 subjects in early puberty, one had normal gonadotropin levels, 2 showed increased FSH one showed increasingly more abnormal gonadotropin levels with time. 10 were late pubertal or adult, 7/10 showed increased FSH levels.
(Hudson et al., 1993)	79	Median 14.6 (4.3-20.1)	NR	Median 3.75 (0.33-9)	COP regimen alternated monthly with the ABVD regimen, for a total of 12 months 2 weeks of prednisone RT for patients with stage IIB-IV disease The dose for nodal sites was 20 Gy at 1.5 Gy/fraction; the visceral dose was 15 to 20 Gy	8	Gonadotropin findings were within normal ranges in all 8 males screened.
(Green et al., 1981)	17	NR	Median 17.0 (9.6-24.4)	Median 3.6 (0.5-8.17)	CT: MOPP, CVPP, BOPP, ABVD, COPP, CCNU and vinblastine Pelvic RT (n=9); (557.7 rads (105-1090)) No pelvic RT (n=8)	17	*Pelvic RT and CT:* 6/9: elevated FSH level 3/9: normal gonadotropin levels *CT only:* 5/8: elevated FSH levels
(Ise et al., 1986)	46	Median 5.4 (0.08-13)	NR	N=8: Median 0.3 (0-0.7) N=4: Median 3 (2-4)	Vincristine, prednisolone, anthracycline, L-asparaginase, cytosine arabinoside, prophylactic skull irradiation 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	46	No abnormal basal FSH concentrations were observed.
(Ahmed et al., 1983)	10	Group 1: Median 10.8 (6.9-13.1) Group 2: Median 6.5 (2.2-14)	Group 1: Median 14.8 (12-17) Group 2: Median 16.4 (14-18.7)	*After CT completion* Median 2.95 (0.3-5)	Group 1: Cranial RT (1500 cGy) + cerebrospinal RT (3000 cGy) + vincristine (2.0 mg/m²; 3x/week) + adjuvant CT for 1-2 years (carmustine + vincristine, lomustine or procarbazine) Group 2: cranial RT (variable dose; max scatter to the gonad was calculated to be 45 cGy after 4 MV and 150 cGy after 300 kV RT) + cerebrospinal RT (2700 cGy) + vincristine (2.0 mg/m²; 3x/week)	10	Group 1: all had raised FSH concentrations Group 2: all had gonadotropin values within the normal adult range
(Wallace et al., 1989)	8	Median 12.6 (7.3-14.6)	Median 14.8 (10.3-22.6)	Median 2.6 (0.1-7.8)	All patients received CT containing cis-platinum, in combination with either adriamycin, HDMTX, vincristine, bleomycin, cyclophosphamide, dactinomycin or ifosfamide	8	3/8 showed normal gonadotropin levels. 1/8 had significantly elevated FSH levels.

(Garolla et al., 2006)	33	Group A: 7.13±3.11 Group B: 10.68±1.71	Group A: 26.5±3.5 Group B: 25.9±3.6	> 2 years	8 patients (group A) had received chemotherapy treatment in which the alkylating agent was cyclophosphamide (RMS 79 protocol), and 25 (group B) chemotherapy treatment in which alkylating drug was ifosfamide (18 patients with RMS 88 protocol, 5 with RMS 96 protocol and 2 with ISG/SSGI protocol).	33	Significant differences among the two groups of patients above all in terms of FSH (23.1 ± 15.6 in group A versus 8.8 ± 10.2 in group B, $p < 0.05$).
(Gerres et al., 1998)	46	14.9±1.5	17.2±1.6	1.95±1.18	RT: involved field irradiation with total radiation doses of 25 Gy in patients with Stages I-IIA disease and Stages IIB-IIIA disease and 20 Gy in patients with Stages IIIB-IV disease. CT: patients with Stages I-IIA HD received two courses of OEPA, and patients with Stages IIB-IIIA and IIIB-IV HD received two OEPA courses and two or four courses of COPP. The recommended cumulative doses (mg/m²) were different for each treatment group	46	Basal FSH levels were elevated when the patients received additional chemotherapy with two cycles of OEPA and two cycles of COPP (Group 2) or two cycles of OEPA and four cycles of COPP (Group 3).

ABV: adriamycin, bleomycin, vinblastine; **ABVD**: doxorubicin, bleomycin, vinblastine, dacarbazine; **ABVP**: Adriamycin, bleomycin, vincristine, prednisolone; **ALL**: acute lymphoblastic leukemia; **ATG**: anti-thymocyte globulin; **BEAM**: carmustine, etoposide, cytarabine, melphalan; **BFM**: Berlin-Frankfurt-Münster protocol; **BMT**: bone marrow transplant; **BOPP**: 1,3-bis (2-chloroethyl)-lnitrosourea, vincristine, procarbazine, and prednisone; **CCNU**: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; **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; **COPAD**: cyclophosphamide, oncovin, prednisone, adriamycin; **COPP(A)**: cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin); **CRT**: cranial radio therapy; **CT**: chemotherapy; **CVPP**: 1 -(2-chloroethyl)-3-cyclohexyl-1 -nitrosourea, vinblastine, procarbazine, and prednisone; **EBVP**: epirubicin, bleomycin, vinblastine, prednisone; **FSH**: follicle stimulating hormone; **GnRH**: gonadotropin releasing hormone; **HD**: Hodgkin's disease; **HDMTX**: high-dose methotrexate; **HDT**: high-dose chemotherapy with autologous stem cell support; **HL**: Hodgkin lymphoma; **HSCT**: hematopoietic stem cell transplant; **IQR**: inter-quartile range; **ISG/SSGI protocol**: high doses metotrexate, cisplatin, adriamicin, ifosfamide; **LHRH**: luteinising hormone releasing hormone; **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; **NMA**: non-myeloablative; **NR**: not reported; **NY protocol**: BFM protocol with higher dosages; **OEPA**: doxorubicin, etoposide, prednisone, vincristine; **OPPA**: doxorubicin, procarbazine, prednisone, vincristine; **OR**: odds ratio; **RIC**: reduced intensity conditioning; **RMS**: rhabdomyosarcoma; **RT**: radiotherapy; **SD**: standard deviation; **TBI**: total body irradiation; **VBVP**: vinblastine, bleomycin, etoposide and prednisone.

Four studies were identified that reported on the value of testicular volume in predicting azoospermia (Table 3a) and an additional 26 studies that describe testicular volume in relation to gonadotoxic treatment received during childhood (Table 3b). 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 (Table 3b). The only study with repeated testicular volume measurements (Korhonen et al., 2024) found normalization of adult testicular volume in survivors treated exclusively with chemotherapy, despite low volumes during puberty. This highlights the importance of extended follow-up to capture the recovery of testicular volume. The four studies that included semen analysis showed an association between testicular volume and semen parameters (Table 3a). An adult testicular volume (unilateral) of ≥17 ml (Jahnukainen et al., 2011) or ≥15 ml was predictive of non-azoospermia (Korhonen et al., 2024, Romerius et al., 2011), whilst a testicular volume of <12ml was predictive of azoospermia (Mathiesen et al., 2020).

234 **Table 3a**: Studies reporting on the predictive value of testicular volume for predicting azoospermia in childhood cancer survivors, arranged in descending order
235 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 reported testicular volumes	Effect
(Korhonen et al., 2024)	255	6.1 (3.2–11.4)	Median 27 (25–30)	Median 21 (15–23)	**CED** (g/m²) ± CRT: (n=253) <4: 116 (46%) ≥4 to <12: 63 (25%) ≥12: 74 (29%) **Testicular RT** (Gy): (n=253) >0 to <1: 71 (28%) ≥1 to <10: 7 (3%) ≥10: 71 (28%) **HSCT**: (n=254) No: 202(80%) Yes: 52(20%)	37	None of the 28 patients with testicular volume Z-score ≥-2 (≥15.6 mL) had azoospermia, whereas 6 of the 9 (67%) patients with testicular volume Z-score <-2 had azoospermia. In ROC curve analyses, AUCs to predict non-azoospermia were 0.91 for testicular volume Z-scores at age 16 years (optimal cut-off point at Z-score -4.2) and 1.0 in adulthood (optimal cut-off point at Z-score -2.5).
(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/m²	47	The AUC of the ROC curves to predict non azoospermic sample was 0.99 with a cut-off for testicular size of 17 ml. Sensitivity to identify non-azoospermic of 98% and a false-positive rate of 0%. The AUC of the ROC curves to predict paternity was 0.77 with a cut-off for testicular size of 23 ml. Sensitivity to identify patients who fathered a child of 80% and a false-positive rate of 30%.
(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	129	Total testicular volume (left+right) 24 ml had 70% sensitivity and 93% specificity to predict azoospermia. 61% (95% CI 39–80) of CCS with low total testicular volume (<24 mL) delivered azoospermic semen samples, giving an OR of 17 (95% CI 5.7–49) for azoospermia.

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<td>CT previously shown to imply a high risk of azoospermia
<table>
<tr><th>Agent</th><th>Cumulative dose</th></tr>
<tr><td>Carmustine</td><td>1 g/m²</td></tr>
<tr><td>Lomustine</td><td>500 mg/m²</td></tr>
<tr><td>Chlorambucil</td><td>1.4 g/m²</td></tr>
<tr><td>Cisplatin</td><td>500 mg/m²</td></tr>
<tr><td>Cyclophosphamide</td><td>19 g/m²</td></tr>
<tr><td>Melphalan</td><td>140 mg/m²</td></tr>
<tr><td>Procarbazine</td><td>4 g/m²</td></tr>
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<tr>
<td>(Mathiesen et al., 2020)</td>
<td>98</td>
<td>At HSCT
Median 9.7 (0.4-16.9)</td>
<td>Median 28.1 (18.5-47.0)</td>
<td>Median 18.3 (7.7-34.6)</td>
<td>Myeloablative allogeneic HSCT
6 treatment groups according to their cumulative therapy:
(1) chemotherapy only,
(2) low-dose testicular irradiation 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.</td>
<td>72</td>
<td>The AUC of the ROC curves to predict non-azoospermic sample was 0.83 for testicular size with a cut-off for testicular size of 15 ml. Sensitivity to identify non-azoospermic of 79% and 80% specificity.</td>
</tr>
</table>

AUC: area under the curve; **CCS**: childhood cancer survivor; **CI**: confidence interval; **CRT**: cranial radio therapy; **CT**: chemotherapy; **HSCT**: hematopoietic stem cell transplant; **OR**: odds ratio; **ROC**: receiver operating characteristic; **RT**: radiotherapy; **TBI**: total body irradiation; **TLI**: total lymphoid irradiation.

238 **Table 3b**: Studies reporting on testicular volume in relation to the gonadotoxic treatment received in childhood cancer survivors, arranged in descending order
239 of 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 reported testicular volumes	Effect
(Korhonen et al., 2024)	255	6.1 (3.2–11.4)	Median 27 (25–30)	Median 21 (15–23)	**CED** (g/m²) ± CRT (n=253) <4 116 (46%) ≥4 to <12 63 (25%) ≥12 74 (29%) **Testicular RT** (Gy) (n=253) >0 to <1 71 (28%) ≥1 to <10 7 (3%) ≥10 71 (28%) **HSCT** (n=254) No 202(80%) Yes 52(20%)	12y (n=38) 14y (n=57) 16y (n=63) 18y (n=105) Adult age (n=43, median age 27y)	Those exposed only to CT (alkylating and non-alkylating) showed a late testicular growth in adulthood. In contrast, no late testicular growth occurred in those exposed to testicular radiation ≥1 Gy (median dose 12 Gy). In multivariable analyses including testicular and pituitary RT dose, CED, age at diagnosis and time since therapy only testicular RT ≥1 Gy (median dose 12 Gy) had an association with reduced testicular volume Z-score (below -2 SD, <15.6 ml) in adulthood.
(Utriainen et al., 2019)	20	Median 1.6 (0.2-3.6)	Median 21.7 (15.9-30.1)	Median 19 (13-27)	Induction CT with Cyclophosphamide, vincristine with or without cisplatin and doxorubicin Local RT in 14/20 TBI + CT in 10/20 Combination CT with Eto + carbo + tiotepa/melphalam/other	9	All 4 males treated with TBI had atrophic testes (1-9 ml). 3/5 males not treated with TBI had testis volumes > 15 ml. The only male survivor with offspring had normal testis volume but high FSH and low inhibin B level. His HR-NBL treatment had included induction chemotherapy with cisplatin and etoposide, and melphalan as the high-dose therapy, p with a cumulative CED of 5.6 g/m². His sperm analysis showed oligoasthenozoo-spermia.
(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	11	N=8 with standard risk treatment Testicular size was comparable to values among healthy Finnish young men. N=3 after high-risk therapy Two had significantly reduced testicular size (6 and 7 ml).

					testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial RT at a dose of 24 Gy.		
(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	29	*Group 1:* volumes ranged from 5 ml to 22.5 ml, median volume was 13 ml *Group 2*: Testicular volumes ranged from 15 ml to 30 ml, median 25 ml *Group 3*: Testicular volume: median 21 ml, mean value 18.3 ml (range 5–30 ml)
(van Beek et al., 2007)	56	Median 11.4 (3.7–15.9)	Median 27 (17.7-42.6)	15.5 (5.6-30.2)	Adriamycin/epirubicin, bleomycin, vinblastine, dacarbazine) with or without MOPP (mechlorethamine, vincristine, prednisone, procarbazine) divided into 3 groups: no MOPP (n=16) 3-4 MOPP (n=14) ≥6 MOPP (n=26)	8	1/8 MOPP- and 16/26 MOPP+ patients had a testicular volume below the normal reference value (15-25 ml).
(Poganitsch-Korhonen et al., 2017)	37	Group 1: 5.3±2.7 Group 2: 7.3±5.2	Group 1: 22.9±5.6 Group 2: 22.6±5.0	Group 1: 15.2±6.0 Group 2: 10.5±7.5	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. Alkylating agents were included in the therapy for 21 patients Group 1: n=21; non-alkylating agents: anthracycline (55±61 mg/m²) Group 2: n=16; alkylating agents: cyclophosphamide 6.3±3.5 g/m² and carmustine 46.9±84.1 mg/m²; CED 7.0±3.8 g/m²	17	Mean adult testicular size (19±7 vs 20 ± 7 ml) and sperm quality did not differ between patients treated with and without the alkylating agents.
(Siimes et al., 1993)	41	Median 7.5 (1-16)	18-27	*After diagnosis* 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 irradiation of 20-24 Gy without other RT	41	Cranial irradiation was associated with a decrease in testicular size of 4.8 (0.3-9.2) ml (p= 0.036).
(Lähteenmäki et al., 2008)	25	Median 8.5 (0.9-15.9)	Median 20.5 (15.6-31.2)	*After diagnosis*	N=11: cyclophosphamide N=3: MOPP or MOPP/ABVD or ABVD	15	The median of testicular volume, measured by orchidometer, was 20 mL

				14.5 (2.1-26.1)	N=1 cisplatin N=1 TBI N=8 CNS RT N=3 local abdominal RT N=1 neck and mediastinum RT		(range = 12–40). For one patient (ID 8) the volume had diminished during the 10-year follow-up
(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	66	21 patients had received cyclophosphamide to a median cumulative dose of 9 g/m² (range 0.8–31.8 g/m²). The median testicular volume was 11 ml (1–34 ml).
(Shafford et al., 1993)	40	Median 10.4 (4.3-15.9)	Median 23 (16.7-30)	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	39	*Patients that received CT* 27 patients had a median testicular volume of 11 ml (5-25 ml), of which 17 have testicular volumes of ≤ 12 ml. *Patients that only received RT* 7/7 patients with RT above diaphragm all have normal testicular volume (>15 ml) 3 patients received 3,500 cGy to an inverted Y field, all have small testes (≤ 12 ml.). 2 patients received 3,500 cGy to the right groin, of which one has small testes.
(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 Non-conditioning chemo- and/or radiotherapy (n=5)	12	9/12 participants had small testicular volumes below the reference limit of 15.2 ml. In the 5 patients who underwent a hemi-orchiectomy, the volume of the biopsied testis was 1–5 ml smaller than the contralateral testis.
(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/m² (range, 4.7– 31.9 g/m²). 1 patient received bleomycin at the time of initial therapy. 11 patients received RT 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)	15	Testicular volume was 15 cm³ in 5 of 15 patients, a finding that is consistent with a lack of development or regression of testicular tissue.
(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 para-aortic nodes. Seven patients received 2-6 cycles of MOPP and five, COPP or chlorambucil. Among the remaining	27	Each of nine men with normal or questionable fertility, in whom testicular size was available, had a combined testicular volume (left + right) equal to or

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td>patients, ten received radiation therapy (five to the inguinal or pelvic nodes) and seven, an alkylating agent (cyclophosphamide, nitrogen mustard, or chlorambucil). One leukemia patient with testicular relapse received radiation to the gonads (2,400 rad). Four patients received Adriamycin.</td><td></td><td>greater than 30 ml. Four of 14 men who were sterile also had a testicular volume exceeding 30 ml, and the remaining ten a volume less than 30 ml.</td></tr>
<tr><td>(Williams et al., 2008)</td><td>45</td><td>Median 11.8 (5.4-21.3)</td><td>Median 20.8 (16.0-29.3)</td><td>Median 9.7 (3.3-12.6)</td><td>32 males received a median dose of ifosfamide 92 g/m² and 9 patients had also received cyclophosphamide 0.3–2.4 g/m² during RT.
Patients were divided into two ifosfamide dose ranges, based on the bimodal distribution of doses: low-dose (<60 g/m², n=6) and high dose (>60 g/m², n=26).</td><td>32</td><td>27 males had testicular volumes of >12 ml with a median of 20 ml (range 5–25 ml). 5 males had testicular volumes < 12 ml and although there was no overall correlation between testicular size and ifosfamide dose (p=0.23) and no significant difference in testicular size between the ‘low-‘ and ‘high dose’ groups (p=0.32), all the males with small (<12 ml) testes were in the ‘high dose’ group having received a median dose of ifosfamide of 96 g/m² (range 94–114.5 g/m²). The male with 5ml testes had an elevated FSH and was azoospermic on sperm analysis.</td></tr>
<tr><td>(Servitzoglou et al., 2015)</td><td>171</td><td>Median 10.8 (2.1-17.3)</td><td>Median 21.1(17-30.4)</td><td>Median 9.3 (2-22.4)</td><td>For HL, children received combined RT (mantle field, subtotal nodal, or involved field RT) and CT, consisting of several MOPP cycles alone or in combination with ABVD or ABVP
More recently, patients received either VBVP cycles alone or VBVP combined with OPPA or in combination with COPP.
For NHL, RT has been used for CNS prophylaxis or rarely for resistant mediastinal disease. CT consisted of COPAD cycles associated with lomustine (CCNU) or high-dose methotrexate, cytarabine, etoposide, asparaginase, 6-mercaptopurine, 6-thioguanine, or vinblastine.</td><td>156</td><td>Testicular volume was less than 8 ml in 24% of patients</td></tr>
<tr><td>(Müller et al., 1996)</td><td>54</td><td>Median 14 (3-17)</td><td>Median 21 (19-34)</td><td>Median 8 (1-18)</td><td>24/33 male patients received alkylating agents
<table><tr><th>Alkylating agent</th><th>N (dose g/m², median, range)</th></tr><tr><td>Cyclophosphamide</td><td>21 [4.0 (1.5-26.0)]</td></tr><tr><td>Ifosphamide</td><td>3 [63 (12-72)]</td></tr><tr><td>CCNU</td><td>1 (0.8)</td></tr><tr><td>Procarbazine</td><td>10 [6.5 (3-29.2)]</td></tr></table></td><td>33</td><td>Median testicular volumes were significantly lower in childhood cancer survivors compared to healthy controls, measured both by ultrasound and Prader orchidometer.</td></tr>
</table>

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td>25/33 male patients received RT
Total dose: 3600 (2000-5600 cGy)
Gonadal dose: 5 (2-50/-2400 cGy)</td><td></td><td></td></tr>
<tr><td>(Rafsanjani et al., 2007)</td><td>33</td><td>Median 9.1 (5-15)</td><td>Median 19.2 (17-29)</td><td>Median 7 (2-20)</td><td><table><tr><th>Therapy</th><th>Number (%)</th></tr><tr><td>MOPP/ABVD</td><td>23 (69.7%)</td></tr><tr><td>MOPP/ABVD+RT</td><td>3 (9.1%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, prednisolone</td><td>1 (3%)</td></tr><tr><td>MOPP/AVBD+vinbastin, Leukeran</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+COPP/ABVE</td><td>1 (3%)</td></tr><tr><td>MOPP+splenectomy</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX, CPM</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX</td><td>1 (3%)</td></tr><tr><td>MOPP</td><td>1 (3%)</td></tr></table></td><td>33</td><td>Testicular size was slightly less than the normal limit for all study participants (sexual maturation rate [SMR] Tanner 4). The mean testis volume was 17.5 ml (range, 14-20ml).</td></tr>
<tr><td>(Papadakis et al., 1999)</td><td>36</td><td>Median 13.0 (2.4-22.6)</td><td>Median 22.3 (15.1-32.5)</td><td>Median 6.8 (2.0-19.3)</td><td>CT: first doxorubicin (60-75 mg/m²), procarbazine (50 mg/day for 2 days and 100 mg/day for 26 days) in combination with prednisone (30 mg/m²/day) and vincristine (1.5 mg/m²) and finally cyclophosphamide (1200 mg/m²).
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</td><td>36</td><td>Testicular volume was decreased in 7 patients (41%), including 1 of 3 group A (RT−) patients, 4 of 11 group B (CT ± RT−) patients, and 2 of 3 group C (CT + RT+) patients</td></tr>
<tr><td>(Bordallo et al., 2004)</td><td>21</td><td>10 (6-19) years</td><td>Group 1: 18 (17-23)</td><td>≥ 2 years
3-11 years</td><td>C-MOPP/ABV hybrid program given in six or more cycles.</td><td>21</td><td>The median of the testicular volume of the patients of group A was lower than that of group B (15 vs 20 ml, ρ = 0.001)</td></tr>
<tr><td>(Braye et al., 2023)</td><td>59</td><td>4.0 (0.0–15.4)</td><td>12.9 (3.9-25.6)</td><td>5.0 (1.0-13.0)</td><td>Patients receiving high-risk CT and/or RT (n=25).
CT-HSCT (n=34): MAC with or without 12 Gy TBI (26/34), RIC (5/34) or NMA (3/34).</td><td>35</td><td>Significantly more CT-HSCT patients had small testicular volumes compared to HR-C/R patients (36% vs. 5%, p = .0278).</td></tr>
<tr><td>(Krawczuk-Rybak et al., 2009)</td><td>59</td><td>Group 1: 4.3±1.7
Group 2: 7.9±4.3</td><td>Group 1: 8.4 ±2.2
Group 2: 15.9±2.6</td><td>Group 1: 1.9±1.3
Group 2: 5.3±3.5</td><td>Protocols of the Polish Pediatric Leukemia/Lymphoma Study Group based (in standard-risk group) on BFM protocols of 1985, 1990, and 1995 (n = 2) or, in the high-risk group, on New York (NY) protocol (n = 7)
RT:
Group 1: n=8 (NY: 18 Gy, n=5, BFM: 12 Gy, n=3)
Group 2: n= 6: 18 Gy (2 NY and 4 BFM) and n=12 received 12 Gy (BFM)</td><td>59</td><td>Group 1: The mean testicular volume was lower than in healthy boys (14.8 ± 5.1 vs. 18.5 ± 4.7 mL).

Group 2: Nine out of 32 males in Tanner stage III–V had median testicular volumes < 12 ml.</td></tr>
</table>

(Mackie et al., 1996)	58	12.2 (8.2-15.3).	NR	*After diagnosis* 6 (2.5-11.1)	Combination chemotherapy was given for a recommended minimum of six courses (equivalent to 504 mg/m² chlorambucil and 8,400 mg/m² procarbazine) or a maximum of eight courses.	25	All had small testes (<15 ml)
(Quigley et al., 1989)	45	4.39 (1.23-12.35)	NR	4.62 (2.35-8.97)	Cyclophosphamide: mean dose 4.8 g/m², cytarabine: mean dose 13.1 g/m². Asparaginase, daunorubicin, hydroxyurea, lomustine, methotrexate, prednisolone, thuiguanine, vincristine. Cranial irradiation: 24 Gy and intrathecal methotrexate.	13	All 13 pubertal boys had small testes, 1.19-2.18 SD below the normal mean testicular size for their pubic-hair stage.
(Whitehead et al., 1982)	17	Median 11.2 (4.8-14.8)	NR	3.8 (1-8)	CT: n=16 Combination CT with MOPP (Mustine 68.6±15.9 mg/m²; Vincristine 21.6±4.3 mg/m²; prednisolone 4741.3±1330.5 mg/m²; procarbazine 11030.7±2815.8 mg/m²). RT: n=15 Neck or mantle RT: n=15; 2500-3000 cGy. Abdominal RT: n=5; radiation dose to the testes was 100-300 cGy.	15	4/5 adult men had small testes (<15 ml) and all 5 late pubertal males had small testes (mean 7 ml) in comparison with the degree of pubertal development. Only one developed gynaecomastia and this was slight.
(Ahmed et al., 1983)	17	Group 1: 10.8 (6.9-13.1) Group 2: 6.5 (2.2-14)	Group 1: 14.8 (12-17) Group 2: 16.4 (14-18.7)	*After CT completion* Group 1: 2.95 (0.3-5)	Group 1: Cranial RT (1500 cGy) + cerebrospinal RT (3000 cGy) + vincristine (2.0 mg/m²; 3x/week) + adjuvant CT for 1-2 years (carmustine+vincristine, lomustine or procarbazine). Group 2: cranial RT (variable dose; max scatter to the gonad was calculated to be 45 cGy after 4 MV and 150 cGy after 300 kV RT) + cerebrospinal RT (2700 cGy) + vincristine (2.0 mg/m² ; 3x/week).	10	Group 1: all had small testes Group 2: all had normal adult-size testes
(Garolla et al., 2006)	33	Group A: 7.13±3.11 Group B: 10.68±1.71	Group A: 26.5±3.5 Group B: 25.9±3.6	> 2 years	8 patients (group A) had received chemotherapy treatment in which the alkylating agent was cyclophosphamide (RMS 79 protocol), and 25 (group B) chemotherapy treatment in which alkylating drug was ifosfamide (18 patients with RMS 88 protocol, 5 with RMS 96 protocol and 2 with ISG/SSGI protocol).	33	Significant reduction of testicular mean volume in patients in group A compared with patients in group B (left side 5.3 ± 3.1 mL and 11.3 ± 3.5 mL respectively, P < 0.001; right side 6.1 ± 2.7 mL and 12.4 ± 3.9 mL, respectively, P < 0.001).

ABV: adriamycin, bleomycin, vinblastine; **ABVD**: doxorubicin, bleomycin, vinblastine, dacarbazine; **ABVP**: Adriamycin, bleomycin, vincristine, prednisolone; **ALL**: acute lymphoblastic leukemia; **BFM**: Berlin-Frankfurt-Münster protocol; **CCNU**: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; **CED**: cyclophosphamide equivalent dose; **CNS**: central nervous system; **COPAD**: cyclophosphamide, oncovin, prednisone, adriamycin; **COPP(A)**: cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin); **CPM**: cyclophosphamide; **CT**: chemotherapy; **FSH**: follicle stimulating hormone; **HL**: Hodgkin lymphoma; **HR-NBL**: high-risk neuroblastoma; **HSCT**: hematopoietic stem cell transplant; **ISG/SSGI protocol**: high doses metotrexate, cisplatin, adriamicin, ifosfamide; **MAC**: myeloablative conditioning; **MOPP/MVPP**: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; **NHL**: non-Hodgkin lymphoma; **NMA**: non-myeloablative; **NR**: not reported; **NY protocol**: BFM protocol with higher dosages; **OEPA**: doxorubicin, etoposide, prednisone, vincristine; **OPPA**: doxorubicin, procarbazine, prednisone, vincristine; **RIC**: reduced intensity conditioning; **RMS**: rhabdomyosarcoma; **RT**: radiotherapy; **SD**: standard deviation; **TBI**: total body irradiation; **VBVP**: vinblastine, bleomycin, etoposide and prednisone; **VP16**: vincristine, platinol.

A total of 17 studies that reported on the association between gonadotoxic therapy and paternity are summarised in Table 4. Several studies report a reduced paternity in childhood cancer survivors compared to healthy controls. Overall, there was an association between direct testicular exposure to radiotherapy and paternity at doses exceeding 4 Gy (Wasilewski-Masker et al., 2014), >6Gy (Korhonen et al., 2023) or ≥7.5Gy (Green et al., 2010). Hematopoietic 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 (Chow et al., 2016, Green et al., 2010, Korhonen et al., 2023, Wasilewski-Masker et al., 2014). When interpreting paternity data it is important to consider that there are many factors that will impact in addition to testicular damage, including physical, social and psychological factors.

260 **Table 4**: Studies reporting on the effects of gonadotoxic therapy on paternity, as a direct measure of subfertility, arranged in descending order of median follow-
261 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)	1159959	<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. (i) skin cancer (ICD-7: 140.0-140.9, 190.0-191.9); (ii) prostate cancer (ICD-7: 177.0-177.9); (iii) testicular cancer (ICD-7: 178.0-178.9); (iv) digestive, respiratory, and urogenital tract cancers (ICD-7: 141.0-163.9, 179.0-181.9); (v) central nervous system and eye cancers (ICD-7: 192.0-193.1); (vi) soft tissue and bone cancers (ICD-7: 193.3, 193.8, 193.9, 196.0-197.9); (vii) hematological and lymphatic cancers (ICD-7: 200.0-207.9); and (viii) 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/m² (but none more than 9.6 g/m²), eleven reported that their partners became pregnant from them. Of 85 male childhood cancer survivors who had received doses of ifosfamide >42 g/m², 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%)	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%)

					HSCT with or without TBI: 99 (8.2%)		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: 6Gy, 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.
(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 chemotherapy 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 sperm test was made 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)	Radiation therapy was administered to all patients with HD and in six, the radiation field included the inguinal or paraaortic nodes. Seven patients received 2-6 cycles of MOPP and five, COPP or chlorambucil. Among the remaining patients, ten received RT (five to the inguinal or pelvic nodes) and seven, an alkylating agent (cyclophosphamide, nitrogen mustard, or	27	6 males fathered children, of which 3 had 2 children.

					chlorambucil). One leukaemia patient with testicular relapse received radiation to the gonads (2,400 rad). Four patients received Adriamycin.		
(Sylvest et al., 2021)	9353	<table><tr><th>Age</th><th>Number (%)</th></tr><tr><td>0-4</td><td>852 (9.1%)</td></tr><tr><td>5-9</td><td>603 (6.5%)</td></tr><tr><td>10-14</td><td>708 (7.6%)</td></tr><tr><td>15-19</td><td>1540 (16.5%)</td></tr><tr><td>20-24</td><td>2474 (26.5%)</td></tr><tr><td>25-29</td><td>3176 (34.0%)</td></tr></table>	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. <table><tr><th>Age</th><th>Adjusted HR</th></tr><tr><td>0-4</td><td>0.66 (95% CI 0.55-0.79)</td></tr><tr><td>5-9</td><td>0.64 (95% CI 0.53–0.78)</td></tr><tr><td>10-14</td><td>0.61 (95% CI 0.51–0.72)</td></tr></table>
(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/m²), procarbazine (50 mg/day for 2 days and 100 mg/day for 26 days) in combination with prednisone (30 mg/m²/day) and vincristine (1.5 mg/m²) and finally cyclophosphamide (1200 mg/m²) 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 group A (RT−) patients fathered three normal children. Additionally, one group B (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 radiation 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 radiation dose of more than 7.5 Gy were less likely to obtain a pregnancy compared with those who did not receive testicular radiation (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 to 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/m²; HR 0.48; 95% CI 0.26 - 0.87) or third tertile (7.0 to 58.7 g/m²; 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 to 143.8 g/m²; 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.
(Reulen et al., 2009)	10483	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: methotrexate, 150 to 12,347 mg/m² (median, 4,520 mg/m²) (12 patients); 6-mercaptopurine, 2.5 to 130.4 g/m² (median, 99.0 g/m²) (10 patients); vincristine, 6 to 76 mg/m² (median, 54 mg/m²) (12 patients); daunorubicin, 180 to 950 mg/m² (two patients); cyclophosphamide, 1.1 to 17.2 g/m² (2 patients); BCNU, 233 to 270 mg/m² (2 patients); and 1-asparaghase, 30,000 to 360,000 U/m² (median, 150,000 U/m²) (7 patients)	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 liveborn 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

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>pregnancies, of which three were in gestation, two aborted spontaneously, and nine resulted in the birth of full-term infants.</td></tr>
<tr><td>(Korhonen et al., 2023)</td><td>252</td><td>Mean 6.2 (IQR 3.2-11.4)</td><td>37.6±7.6</td><td>>5 years from diagnosis (6-42 years)</td><td><table><tr><th>Treatment</th><th>Number</th><th>Dose</th></tr><tr><td>CED (g/m²)</td><td>176</td><td>9.9 (4.1-16.5)</td></tr><tr><td>DIE (mg/m²)</td><td>144</td><td>240 (120-350)</td></tr><tr><td>Pituitary RT</td><td>183</td><td></td></tr><tr><td>Cumulative pituitary RT dose (Gy)</td><td></td><td>12 (0.53-24)</td></tr><tr><td>Testicular RT</td><td>148</td><td></td></tr><tr><td>Cumulative testicular RT (Gy)</td><td></td><td>2.9 (0.13-12)</td></tr><tr><td>HSCT</td><td>52</td><td></td></tr></table></td><td>252</td><td>OR of ever fathering a child after childhood cancer treatment<table><tr><th>Treatment</th><th>OR (95 % CI)</th></tr><tr><td colspan="2">Treatment intensity</td></tr><tr><td>Least-moderate</td><td>Reference</td></tr><tr><td>Very</td><td>0.94 (0.51-1.72)</td></tr><tr><td>Most</td><td>0.47 (0.21-1.04)</td></tr><tr><td colspan="2">CED (g/m²)</td></tr><tr><td>0</td><td>Reference</td></tr><tr><td>>0 to <4</td><td>1.29 (0.55-3.01)</td></tr><tr><td>4 to 15</td><td>0.64 (0.33-1.28)</td></tr><tr><td>> 15</td><td>0.60 (0.27-1.33)</td></tr><tr><td colspan="2">DIE (mg/m²)</td></tr><tr><td>0</td><td>Reference</td></tr><tr><td><250</td><td>1.30 (0.65-2.60)</td></tr><tr><td>≥ 250</td><td>0.79 (0.40-1.57)</td></tr><tr><td colspan="2">Cumulative pituitary RT dose (Gy)</td></tr><tr><td>0</td><td>Reference</td></tr><tr><td>>0 to <10</td><td>0.99 (0.47-2.10)</td></tr><tr><td>≥10 to <24</td><td>0.79 (0.33-1.87)</td></tr><tr><td>≥24</td><td>0.48 (0.22-1.02)</td></tr><tr><td colspan="2">Cumulative testicular RT dose (Gy)</td></tr><tr><td>0</td><td>Reference</td></tr><tr><td>>0 to <1</td><td>0.88 (0.47-1.67)</td></tr><tr><td>1 to 6</td><td>0.59 (0.10-3.63)</td></tr><tr><td>> 6</td><td>0.19 (0.08-0.48)</td></tr><tr><td colspan="2">HSCT</td></tr><tr><td>No</td><td>Reference</td></tr><tr><td>Yes</td><td>0.33 (0.11-0.85)</td></tr></table></td></tr>
<tr><td>(Chow et al., 2016)</td><td>10938</td><td><5 n=2085
5-9 n=1254</td><td>NR</td><td>5 years since initial diagnosis or</td><td>Lower CED: <4897 mg/m²
Middle CED: 4897-9638 mg/m²
Upper CED: ≥9639 mg/m²</td><td>4149</td><td>Male participants who received cumulative doses of cyclophosphamide, ifosfamide, and procarbazine in the upper tertiles (≥7.4 g/m², ≥53</td></tr>
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<tr>
<td></td>
<td></td>
<td>10-14 n=1287
15-20 n=1014</td>
<td></td>
<td>age 15 years, whichever was later.</td>
<td></td>
<td></td>
<td>g/m², and ≥5.1 g/m², respectively) reported a significantly decreased likelihood of pregnancy compared with those not exposed to each drug. Cyclophosphamide doses of 5.6 g/m² or higher (median cutoff point) were associated with a reduced likelihood of pregnancy. High cisplatin doses (upper tertile ≥488 mg/m²) 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.</td>
</tr>
<tr>
<td>(Madanat et al., 2008)</td>
<td>6071</td>
<td>0-14 years at diagnosis</td>
<td>NR</td>
<td>≥ 9 months after diagnosis</td>
<td>NR</td>
<td>1476</td>
<td>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.</td>
</tr>
<tr>
<td>(Wasilewski-Masker et al., 2014)</td>
<td>701</td>
<td>
<table>
<tr><th>Age</th><th>Number</th></tr>
<tr><td>0-4</td><td>69/191</td></tr>
<tr><td>5-9</td><td>63/163</td></tr>
<tr><td>10-14</td><td>91/186</td></tr>
<tr><td>15+</td><td>67/161</td></tr>
</table>
</td>
<td>
<table>
<tr><th>Age</th><th>Number</th></tr>
<tr><td>20-29</td><td>12/50</td></tr>
<tr><td>30-39</td><td>142/337</td></tr>
<tr><td>40-49</td><td>121/282</td></tr>
<tr><td>50+</td><td>15/32</td></tr>
</table>
</td>
<td>NR</td>
<td>
<table>
<tr><th>Characteristic</th><th>N=701</th></tr>
<tr><td colspan="2">AAD score in first 5y after diagnosis</td></tr>
<tr><td>0</td><td>75/269 (27.9%)</td></tr>
<tr><td>1</td><td>17/70 (24.3%)</td></tr>
<tr><td>2</td><td>38/102 (37.3%)</td></tr>
<tr><td>3</td><td>65/103 (63.1%)</td></tr>
<tr><td>4</td><td>26/39 (66.7%)</td></tr>
<tr><td>≥5</td><td>27/37 (73.0%)</td></tr>
<tr><td>Unknown</td><td>42/81 (51.9%)</td></tr>
<tr><td colspan="2">Testicular RT in first 5y after diagnosis</td></tr>
<tr><td>None</td><td>85/231 (36.8%)</td></tr>
<tr><td><4 Gy</td><td>166/397 (41.8%)</td></tr>
<tr><td>≥4 Gy</td><td>19/23 (82.6%)</td></tr>
<tr><td>Unknown</td><td>20/50 (40.0%)</td></tr>
<tr><td colspan="2">TBI in first 5y after diagnosis</td></tr>
<tr><td>Yes</td><td>2/2 (100%)</td></tr>
<tr><td>No</td><td>264/640 (41.3%)</td></tr>
<tr><td>Unknown</td><td>24/59 (40.7%)</td></tr>
</table>
</td>
<td>290</td>
<td>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 ≥ 4Gy 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.</td>
</tr>
</table>

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**: hematopoietic 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.

Recommendation

Patients facing gonadotoxic treatment of less than 4 g/m² 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/m² 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/m² 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.

2.2 Previous exposure

Evidence

In total 17 studies have reported on histological evidence of testicular damage after gonadotoxic treatment for malignant (Table 5a) or non-malignant disease (Table 5b). 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 4g/m² leading to significant depletion of spermatogonia (Table 5a). Reduced spermatogonial numbers have also been observed in boys with severe haematological conditions, such as sickle cell disease and Fanconi anaemia, prior to undergoing gonadotoxic HSCT therapy (Table 5b).

292 **Table 5a**: 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/m² (0.8–37.6 g/m²) -Carboplatin cumulative dose (n=10): 1.6±0.7 g/m² (0.8–3.2 g/m²) 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/m² (± 3.4, range 2–15.6 g/m²). 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/m²	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<.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 ≥ 4g/m² (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	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

		Severely affected: 6.94±4.30			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/m² (0.5-7 g/m²) 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.
(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/m², cyclosphophamide: 5.1 ± 3.2 g/m² 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 two 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 irradiation (18–24 Gy), but spinal irradiation was not used. Group 1: n=21; non-alkylating agents: anthracycline (55±61 mg/m²) Group 2: n=16; alkylating agents: cyclophosphamide 6.3±3.5 g/m² and carmustine 46.9±84.1 mg/m²; CED 7.0±3.8 g/m²	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 irradiation resulted in complete depletion of spermatogonia. Regression analysis showed that cumulative CEDs >4g/m² 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. Interstitial SOX9 expression was observed in the more mature prepubertal patients in all groups, except for the untreated cancer patients group.
(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	28	After induction therapy, 90% of the seminiferous cords contained MAGE A4+ and 20% OCT4+ or CD9+ spermatogonia. The number of MAGE A4+ spermatogonia per cross-section of seminiferous cord

			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 irradiation (24 Gy), but spinal irradiation was not used. Patients experiencing testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial irradiation at a dose of 24 Gy.		reduced by 50% after standard risk therapy of ALL. The number of OCT4+ and CD9+ 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.
(Quigley et al., 1989)	45	4.39 (1.23-12.35)	Cyclophosphamide: mean dose 4.8 g/m², cytarabine: mean dose 13.1 g/m², asparaginase, daunorubicin, hydroxyurea, lomustine, methotrexate, prednisolone, thuiguanine, vincristine. Cranial irradiation: 24 Gy and intrathecal methotrexate	24	All 24 biopsy specimens were abnormal. N= 13 with total absence of germ cells N=11 germ cells were markedly depleted
(Ise et al., 1986)	46	5.4 (0.08-13)	Vincristine, prednisolone, anthracycline, L-asparaginase, cytosine arabinoside, prophylactic skull irradiation 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 cranio-spinal irradiation, n=1 mediastinal irradiation.	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**: hematopoietic 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.

298 **Table 5b**: 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 from −20.0 to 1.8). Patients in the IMMUNO group had a median S/T Z-score of −1.9 (range from −19.3 to 2.6). Patients in the MDS/MPN group had a median S/T Z-score of −2.7 (range from −20.5 to 0.0). All three patients with Fanconi anaemia had severely depleted spermatogonial numbers with very low S/T Z-score (range from−20.0 to −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/m² (0.5-7 g/m²). N=115 not yet exposed to CT N=15 SCD	189	Among the five patients with no germ cells, three were receiving treatments for SCD and thalassemia that are known to impact fertility.

			N=15 thalassemia		
(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/m², cyclosphophamide: 5.1 ± 3.2 g/m² 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**: Hematopoietic 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.

Recommendation

Ideally, testicular biopsy in eligible patients (refer to 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.

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

Experimental fertility preservation methods may be inadvisable for patients with Fanconi anaemia.

2.3 Contra-indications

Evidence

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

There are no specific contra-indications for testicular tissue cryopreservation. 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 a high bleeding and/or infection risk (Barraud-Lange et al., 2024, Stukenborg et al., 2018), patients that are determined to be medically inappropriate or unstable to undergo FP, patients who are actively participating in a phase I trial, or who have already undergone a form of fertility intervention, have a treatment plan with goal of palliative care only or less than 20% expected survival (Sax et al., 2022), or have an underlying testicular abnormality or pathology (Uijldert et al., 2017).

Recommendation

In patients able to produce sperm, 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.

3. Counselling

3.1 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). All parents should be counselled about the risks of infertility due to cancer treatment, because 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.

3.2 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.

In a cross-sectional study, men above 18 years old with a cancer diagnosis within the last 10 years were eligible to participate, of which 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.

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.

3.3 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 parents 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., 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 which 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. 52 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 which 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.

3.4 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; as well as 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 are 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 males.

In a mixed-method randomised 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, 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. 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.

In a cross-sectional study, men above 18 years old with a cancer diagnosis within the last 10 years were eligible to participate, of which 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. 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, consequences for partners, and experience reports from other patients.

In a study, 77 AYA cancer patients aged 10-25 years were invited for FP discussions, of which 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. 113 patients were 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. 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.

Recommendation

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.

This information should be provided verbally, as well as written.

4. Biopsy procedure

4.1 Which type of surgery?

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 summarised in Table 6.

Table 6: 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 testicular volume	2-4mm^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	from1–2 × 2–3 mm to 5 x 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 × 5mm <25% testicular volume	3–5 mm^3	NR	Paediatric surgeon (n=17) Paediatric urologist (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., 2018)	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
(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., 2008)	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 x 2–7 x 9–10 mm	~1–4 mm^3	NR	NR

NR: not reported.

Recommendation

It is considered good practice to perform a unilateral, conventional open testicular biopsy under general 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.

4.2 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, 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.

Recommendation

Surgery should be performed by a paediatric surgeon and/or urologist with training, 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.

5. Transport of the tissue

5.1 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 (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 hrs, 3 hrs, 5 hrs, and overnight, samples were evaluated by light microscopy and electron microscopy (Feng and Holstein, 1990). Spermatogonia were well preserved for 2hrs in Ringer's solution or Macrodex, and up to 5 hrs in solution of 0.9% NaCl, Dextran or 1640 Medium. Based on light and electron microscopy, it was

549 determined that Leydig cells were not well preserved for more than 1 hr, and this time could be realized
550 only in 1640 Medium at 4°C.

551 In an animal study, testicular tissue from four immature male rhesus monkeys was used to compare
552 fresh xenografting with 24h in ice-cold Leibovitz-L15 medium before xenografting (Jahnukainen et al.,
553 2007). Three months after xenografting, no effect was seen of a 24-h delay in grafting of fresh and
554 cooled tissue fragments on graft survival (51% vs. 52% recovery). Five months after xenografting, 73%
555 (35/48) of immediately implanted grafts and 79% (38/48) of grafts which were implanted with a 24-h
556 delay were recovered. The graft weight after delayed xenografting was significantly higher compared
557 to the immediately grafted group. Five months after grafting B spermatogonia were recorded in 12%
558 of grafts after delayed grafting and in 8% of grafts after immediate xenografting.

559 Conclusion

560 **Media that can be used for short-term storage or transport of testicular tissue include DMEM/F12 (3**
561 **days) or Leibovitz L15 (24 hours) medium. These data should be interpreted with caution, because the**
562 **human data are derived from adult tissue and observations might deviate with prepubertal tissue.**

563 ## 5.2 What is the acceptable duration for testicular tissue transport/short-term storage?

564 Evidence

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

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

579 Conclusion

580 **Testicular tissue can be transported or stored in DMEM for up to three days without altering cell**
581 **survival, tissue structure, number of spermatogonia and Sertoli cell morphology. These data should be**
582 **interpreted with caution, because they are derived from adult tissue and observations might deviate**
583 **with prepubertal tissue.**

5.3 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 4/16 centres, the remaining 12/16 centres aim for a shipment temperature of 0–8 °C (Duffin et al., 2024). Shipment temperature is controlled in 8/16 centres, with six of these eight 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 three-day storage at different temperatures: refrigerator (4°C), room temperature and 37°C (Faes and Goossens, 2017). No significant change in viability was noted at the different storage temperatures. Tubular morphology of the tissue was also 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 hr at room temperature and also well preserved overnight at 4°C. Sertoli cells were unaffected for about 5 hrs.

In an animal study, testicular tissue from four immature male rhesus monkeys was used to compare fresh xenografting with 24h 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% after immediate grafting.

Conclusion

Testicular tissue can be effectively transported or stored for short-term at 4°C. These data should be interpreted with caution, because the human data are derived from adult tissue and observations might deviate with prepubertal tissue.

5.4 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 mm^3, 15 mm^3, 50 mm^3 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 mm^3 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 mm^3, 15 mm^3; 50 mm^3 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

Testicular tissue can be transported or stored for short-term in sizes of 50 or 80 mm^3. These data should be interpreted with caution, because they are derived from adult tissue and observations might deviate with prepubertal tissue.

5.5 Overall recommendation

Testicular tissue can be transported or stored in DMEM/F12 for up to three days or in Leibovitz L15 (24 hours) medium for 24 hours, 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 minimised, as no functional data are available on how transport or short-term storage may affect subsequent SSC function or spermatogenesis.

6. Quality control for testicular tissue cryopreservation

6.1 Which quality controls are required for testicular tissue cryopreservation?

Given the lack of directly relevant studies, we recommend testicular tissue cryopreservation should be undertaken according to the Commission Directive 2004/23/EC and the regulation on Standards of quality and safety for substances of Human origin intended for human application (SOHO regulation), unless more stringent local regulations are in place.

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 one 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 ≤ 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 5 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, we recommend serology for bloodborne pathogens should be undertaken according to the Commission Directive 2006/14/EC Annex II, unless more stringent local regulations are in place.

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 2 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 alterations was scored as 0 and the worst score for alterations was 5. The nuclear, epithelial and global lesional scores increased significantly after thawing ($p < 0.001$). The percentage of intact seminiferous tubules decreased after thawing ($p=0.0105$). 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 showing 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. 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.

6.2 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.

7. Histology

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 include DDX4 (VASA) and melanoma-associated antigen 4 (MAGE-A4).

Table 7: 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
(Lahtinen et al., 2024)	HE/PAS	MAGE A4 , DDX4	/	/	At least 25 round tubular cross sections
(Barraud-Lange et al., 2024)	HE	/	/	/	All seminiferous tubules present in the section
(Tholeti et al., 2024)	HE	DDX4	/	/	minimum of 25 seminiferous tubules per cross section per sample
(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
(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 were counted
(Heckmann et al., 2018)	HE	MAGE A4	/	/	
(Medrano et al., 2018)	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., 2008)	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; **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.

Recommendation

Histological assessment at the time of cryopreservation is preferably performed as individual samples likely 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, as spermatogonia per tubular cross section or positive tubular cross sections, 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.

8. Cancer markers

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

In an international survey it was reported that of the 12 centres that specifically perform histological quality assessment of the testicular tissue, only 3 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 peri-pubertal 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 who had specific chromosomal rearrangements identified on 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 demonstrate 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 hematologic 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.

Recommendation

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.

9. Cryopreservation protocol

9.1 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 6. This is 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).

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 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 6 mm^3 have been used in human research studies. This is in line with comparative studies with animal testicular fragments that are best preserved after slow freezing with a size around 5 mm^3.

9.2 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 (CPA; 15/16), with one centre using ethylene glycol. 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% human serum albumin (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 hours after culture, reflected these results. The slow freezing method CSF 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 by microsurgical testicular sperm extraction from 10 patients with normal spermatogenesis, uncontrolled slow freezing (USF; using DMEM/F12, 0.15M 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 ethylene glycol or 2: 4.2 M DMSO, 5.4 M ethylene glycol; for the low and high concentration, respectively). Either the tissue was submerged in CPA or the CPA was injected in the seminiferous tubules (Han et al., 2021). Histology and immunohistochemistry showed the best results, in terms of maintenance of seminiferous tubule structure and low apoptosis, with USC 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 0.1M 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.

In 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 ethylene glycol, 0.1 mol/l sucrose and 10 mg/ml HSA with phosphate buffered saline (PBS) supplemented with 1.5 mol/l ethylene glycol, 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.

In an experimental study, using 16 non-frozen and 34 frozen testicular tissue samples of 16 infertile men, comparing 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) 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 in DMSO frozen fragments was equal to controls, while lower in PrOH-frozen fragments.

In an animal study, using testis tissue or cells from 4 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). 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 24h cryopreservation (controlled slow freezing with cooling rates of 0.5°C/min) without CPA or with 1.4M Ethylene Glycol or 1.4M DMSO or 0.7M DMSO (Jahnukainen et al., 2007). After xenografting, grafts from fresh tissue showed good survival and spermatogenic induction to spermatocytes. Cryopreservation in 1.4 M DMSO also allowed grafts to initiate spermatogenesis. In contrast, 0.7 M DMSO and ethylene glycol, which 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 0.1M 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.

9.3 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 test, respectively. This indicates that sperm can be better cryopreserved in minced tissue suspension than using freezing protocols designed for whole testis tissue.

Conclusion

The cited paper used a preservation protocol aimed at preserving sperm.

Cryopreservation of whole testicular tissue is preferred to preserve spermatogonia, while preserving sperm in testicular tissue might need a separate protocol and cryosolution.

9.4 Which technique 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, 1.5M DMSO, 0.15 sucrose and 10mg/ml HSA; 2: CSF with a gradual cooling program in Hanks balanced salt solution, 1.5M DMSO, 0.1M sucrose and 10mg/ml HSA; 3: vitrification in gradual concentration increasing steps to DMEM/F12, 2.1M DMSO, 2.7M ethylene glycol 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, number of MAGE-A4 spermatogonia and Vimentin positive cells.

In an experimental study, straws with tissue fragments and cryoprotectant were brought directly in the nitrogen vapor, 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-hour 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 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, 0.15M 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 ethylene glycol or 4.2 M DMSO, 5.4 M Ethylene glycol for the low and high concentration, respectively (Han et al., 2021). Histology and ICH showed the best results with 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 0.7M DMSO) and vitrified-warmed (cryoprotectant included 7.5% ethyleneglycol 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 pre-pubertal 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), ethylene glycol (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.

9.5 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 (infection/disease et al., 2021).

9.6 Is tissue stored in liquid or vapour N2?

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 (infection/disease et al., 2021) and the Directive 2004/23/EC.

9.7 Overall recommendation

DMSO-based cryoprotectant combined with controlled slow freezing can be used for testicular tissue cryopreservation. Uncontrolled slow freezing protocols may be considered providing internal validation of post-thaw tissue quality has been performed. Cryopreservation of whole testicular tissue 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.

10. Follow-up

10.1 Does a testicular biopsy harm the testis?

Evidence

Short-term follow-up

The international survey reported that protocols to monitor for postoperative 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).

Thirty-nine 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.

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 hematoma (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 hematoma (n=2, 1 patient with blood clotting disorder) and severe pain with negative work-up (n=1).

Twenty 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 hematoma (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. Rate of infection was 2.5% and rate of postoperative bleeding was 1.3%.

In a multicentre case series, 34 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.

Forty-four 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 two weeks postoperatively.

Seventy-eight 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%). A total of 7/64 (10.9%) boys had intra-scrotal

haematomas at 1 month after surgery. In 5/64 (7.8%) boys these were only extra-testicular and in 2/64 (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).

52 prepubertal patients under 12 years of age and 10 patients between 12-16 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.

112 patients 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

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 5 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 2 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).

112 patients 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 eight 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.

10.2 What psychological support is required?

Evidence

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.

In a systematic review on fertility decision regret in AYA 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. In addition, it was reported in several studies that fertility counselling referrals are being underutilised, 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, minimises 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. This support should be adapted to meet the needs of paediatric and adolescent patients.

10.3 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

Specialist support with experience in counselling FP patients should continue during follow-up upon request to assist patients in decision-making with regards to future use or disposition 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 what is best for a patient given their individual clinical context, even when hard data are scarce. It is to be hoped that, in the coming years, studies will be published that can 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 hematopoietic stem cell transplant (HSCT) (myeloablative or reduced intensity) containing at least one alkylating agent or total body irradiation. High-risk therapy also included gonadal radiation exposure (direct or indirect) 4 Gy or higher in males (Close et al., 2023). It was recommended that males at high risk be offered testicular tissue cryopreservation. However, the evidence supporting this cut-off of 4g/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 4g/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 variability, age-related sensitivity to gonadotoxic treatments (Kanbar et al., 2021), and the potential for synergistic damage caused by combinations of therapeutic agents. 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). Many 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 (Korhonen et al., 2024, Mathiesen et al., 2020, Romerius et al., 2011). In contrast, high-dose 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 programmes 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 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 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/care givers.

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 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 information in writing is mandatory.

There is a general consensus in literature that a designated "fertility navigator" improved 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 risks of complications. It is important to consider the timing of the biopsy, so that it can be combined with other procedures requiring general anaesthesia. Lastly, it is clear from the literature that there is no consensus on the amount of testicular tissue that can 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 (Barraud-Lange et al., 2024, Funke et al., 2021, Moussaoui et al., 2022). Notably, exposures exceeding a CED of 4 g/m² are associated with substantial depletion of spermatogonia (Poganitsch-Korhonen et al., 2017). Ideally, testicular biopsy for fertility preservation should be performed prior to 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. The optimal slot for fertility preservation should be pointed out in future cancer therapy protocols to harmonize the service and increase healthcare providers‘ awareness.

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). Experimental fertility preservation strategies may therefore be contraindicated in individuals with Fanconi anaemia. Similarly, the availability of fertility preservation options may be restricted in other severe haematological disorders requiring HSCT (Benninghoven-Frey et al., 2022, Gille et al., 2021). These findings highlight the need to evaluate spermatogonial numbers to accurately assess the quality of individual patient samples and to guide informed 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 (Duffin et al., 2024, Kourta 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 standardised 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 24h culture of the tissue followed by immunohistochemistry (Keros et al., 2007). However, currently the data is missing to determine if 24h 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 the conduct of ethics, 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 program is validated in each laboratory, and that if changes are made to any step in the process, 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.

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1713

1714 # Supplementary Data S1 – Abbreviations

Abbreviation	Explanation
3β-HSD	3-beta (ß)-hydroxysteroid dehydrogenase
AA	Aplastic anaemia
AAD	Alkylating agent dose score
ABV	Adriamycin, bleomycin, vinblastine
ABVD	Doxorubicin, bleomycin, vinblastine, dacarbazine
ABVP	Adriamycin, bleomycin, vincristine, prednisolone
ACTHD	Adrenocorticotropic hormone deficiency
AIC	Akaike information criterion
ALL	Acute lymphoblastic leukaemia
AMH	Anti-Müllerian hormone
AR	Androgen receptor
ATG	Anti-thymocyte globulin
AUC	Area under the curve
AYA	Adolescent and young adults
BEAM	Carmustine, etoposide, cytarabine, melphalan
BFM	Berlin-Frankfurt-Münster protocol;
BMFS	Bone marrow failure syndrome
BMT	Bone marrow transplant
BOPP	1,3-bis (2-chloroethyl)-lnitrosourea, vincristine, procarbazine, and prednisone
c-kit	Receptor tyrosine kinase
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CCS	Childhood cancer survivor
CD9	Cell surface glycoprotein
CED	Cyclophosphamide equivalent dosing
ChIVPP	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
COPAD	Cyclophosphamide, oncovin, prednisone, adriamycin
COPP(A)	Cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin)
CPA	Cryoprotective agent
CPM	Cyclophosphamide
CRT	Cranial radio therapy
CSF	Controlled slow freezing
CT	Chemotherapy
CVPP	1 -(2-chloroethyl)-3-cyclohexyl-1 -nitrosourea, vinblastine, procarbazine, and prednisone
DDX4	anti-DEAD-box helicase 4
DIE	Cumulative doxorubicin isotoxic dose
EBVP	Epirubicin, bleomycin, vinblastine, prednisone
FI	Fertility index
FP	Fertility preservation

FSH	Follicle stimulating hormone
GHD	Growth hormone deficiency
GPP	Good practice point
Gy	Radiation dose, expressed as absorbed energy per unit mass of tissue
HD	Hodgkin's disease
HDMTX	High-dose methotrexate
HDT	High-dose chemotherapy with autologous stem cell support
HE	Haematoxylin & eosin
HL	Hodgkin's lymphoma
HR	Hazards ratio
HR-NBL	High-risk neuroblastoma
HSA	Human serum albumin
HSCT	Hematopoietic stem cell transplant
HU	Hydroxyurea
IHC	Immunohistochemistry
IQR	Inter-quartile range
ISG/SSGI protocol	High doses metotrexate, cisplatin, adriamicin, ifosfamide
KL	Klinefelter syndrome
LCF	Leydig cell failure
LDH-c	Lactate dehydrogenase C
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LN	Liquid nitrogen
LOPP/LVPP	Vinblastine, chlorambucil, procarbazine, prednisone
LSA_2L_2	Cyclophosphamide, vincristine, doxorubicin, asparaginase, thioguanine, methotrexate, 6-mercaptopurine
MAC	Myeloablative conditioning
MAGE A4	Melanoma-associated antigen 4
MD	Mean difference
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasms
MOPP/MVPP	Nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone
MTX	Methotrexate;
NCI protocol	Methotrexate, cyclophosphamide, doxorubicin, prednisone
NHL	Non-Hodgkin lymphoma;
NMA	Non-myeloablative
NR	Not reported;
NY protocol	BFM protocol with higher dosages;
OCT4	Octamer-binding transcription factor 4
OEPA	Doxorubicin, etoposide, prednisone, vincristine
OPPA	Doxorubicin, procarbazine, prednisone, vincristine;
OR	Odds ratio
PAS	Periodic Acid Schiff
PAVe	Procarbazine, alkeran, velban
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PLAP	Placental alkaline phosphatase
PLZF	Promyelocytic leukemia zinc finger
PPV	Positive predictive value
PVB	Cisplatin, vinblastine and bleomycin
RIC	Reduced intensity conditioning
RMS	Rhabdomyosarcoma
ROC	Receiver operating characteristic
RR	Risk ratio;
RT	Radiotherapy;
S/T	Number of spermatogonia per seminiferous tubule cross-section
SALL4	Sal-like protein 4
SCD	Sickle cell disease
SD	Standard deviation
SHBG	Sex hormone binding globulin
SIG	Special interest group
SOX9	Sex-determining region Y-box 9
SSEA4	Stage-specific embryonic antigen 4
SYCP	Synaptonemal complex protein 3
TBI	Total body irradiation
TFI	Tubular fertility index
TLI	Total lymphoid irradiation
TTC	Testicular tissue cryopreservation
TTF	Testicular tissue freezing
TUNEL	Terminal nucleotidyl transferase-mediated dUTP-biotin nick end-labelling
UCHLI	Ubiquitin carboxyl-terminal esterase L1
USF	Uncontrolled slow freezing
UTF1	Undifferentiated embryonic cell transcription factor 1
VAC	Vincristine, actinomycin, cyclophosphamide
VBM	Velban, bleomycin, methotrexate.
VBVP	Vinblastine, bleomycin, etoposide and prednisone
VP16	Vincristine, platinol

1715

Supplementary Data S2 – Overview of recommendations

	Recommendation	Quality of evidence
1.	**Fertility preservation programme**	
1.1	Testicular tissue cryopreservation in this patient population requires multi-disciplinary expertise. The team should include clinical expertise in gonadotoxic therapies, gonad surgery, laboratory expertise including pathology, testis tissue cryopreservation and reproduction/fertility in support of the treating physician.	GPP
	To optimise this multi-disciplinary care pathway, the addition of an ethicist/geneticist and psychologist is advised.	GPP
1.2	Testicular tissue cryopreservation in this patient population requires access to a sterile environment (laboratory or clean room) to process the tissue, a tissue bank (or place to store cryopreserved tissue), operating theatre, clinical facilities providing care to patients receiving therapies, and funding. This should be provided in accordance with local and national regulations.	GPP
2.	**Who is eligible**	
2.1	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.	GPP
	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.	⊕○○○
2.2	Ideally, testicular biopsy in eligible patients (refer to 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.	⊕○○○
	Boys with severe benign hematological disorders due to receive hematopoietic stem cell transplantation are eligible for testicular tissue cryopreservation after appropriate counselling regarding the potentially decreased spermatogonial numbers.	⊕○○○
	Experimental fertility preservation methods may be inadvisable for patients with Fanconi anaemia.	GPP
2.3	In patients able to produce sperm, 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.	GPP

3. Counselling		
3.1	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.	⊕○○○
3.2	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.	GPP
3.3	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.	⊕○○○
3.4	Counselling should include discussion of the treatments the patients will receive and the risk to their fertility.	GPP
	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.	GPP
	This information should be provided verbally, as well as written.	GPP
4. Biopsy procedure		
4.1	It is considered good practice to perform a unilateral, conventional open testicular biopsy under general anaesthesia.	GPP
	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.	GPP
4.2	Surgery should be performed by a paediatric surgeon and/or urologist with training, according to local regulations.	GPP
	Children should have testicular examination prior to surgery and the surgeon should identify other anatomical abnormalities at the time of biopsy.	GPP
5. Transport of the tissue		
	Testicular tissue can be transported or stored in DMEM/F12 for up to three days or in Leibovitz L15 (24 hours) medium for 24 hours, 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 minimised, as no functional data are available on how transport or short-term storage may affect subsequent SSC function or spermatogenesis.	GPP
6. Quality control		
6.1	Given the lack of directly relevant studies, we recommend testicular tissue cryopreservation should be undertaken according to the Commission Directive 2004/23/EC and the regulation on Standards of quality and safety for substances of Human origin intended for human application (SOHO regulation), unless more stringent local regulations are in place.	GPP
	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).	GPP
	Given the lack of directly relevant studies, we recommend serology for bloodborne pathogens should be undertaken according to the Commission Directive 2006/14/EC Annex II, unless more stringent local regulations are in place.	GPP
	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.	GPP

6.2	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.	GPP
7. Histology		
	Histological assessment at the time of cryopreservation is preferably performed as individual samples likely 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.	GPP
	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, as spermatogonia per tubular cross section or positive tubular cross sections, before or after cryopreservation.	GPP
	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.	GPP
8. Cancer markers		
	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.	GPP
	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.	GPP
	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.	GPP
	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.	GPP
9. Cryopreservation protocol		
	DMSO-based cryoprotectant combined with controlled slow freezing can be used for testicular tissue cryopreservation. Uncontrolled slow freezing protocols may be considered providing internal validation of post-thaw tissue quality has been performed. Cryopreservation of whole testicular tissue 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.	GPP
10. Follow-up		
10.1	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.	⊕○○○
10.2	Provision of psychological support should be considered for patients and their family. This support should be adapted to meet the needs of paediatric and adolescent patients.	⊕○○○

10.3	Specialist support with experience in counselling FP patients should continue during follow-up upon request to assist patients in decision-making with regards to future use or disposition of cryostored testicular tissue.	⊕○○○

GPP: good practice point, ⊕○○○: very low quality evidence.

Supplementary Data S3 – List of experts participating in the stakeholder review

Reviewer	Country	Participation on behalf of (if any)

Supplementary Data S4 – Studies reporting on semen analysis results after long-term follow-up (<10 years) of childhood cancer survivors.

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
(Williams et al., 2008)	45	Median 11.8 (5.4-21.3)	Median 20.8 (16.0-29.3)	Median 9.7 (3.3-12.6)	32 males received a median dose of ifosfamide 92 g/m². 9 patients had also received cyclophosphamide 0.3–2.4 g/m² during RT. Patients were divided into two ifosfamide dose ranges, based on the bimodal distribution of doses: low-dose (<60 g/m², n=6) and high dose (>60 g/m², n=26).	13	Sperm counts were obtained in 13 males with a median sperm count 11x10⁶/ml (range 0–125x10⁶/ml). 8/11 males in the 'high dose' group had low sperm counts <20x10⁶/ml, of whom 3 were azoospermic. Sperm counts were available in only 2 males in the 'low dose' group: both had sperm counts >20x10⁶/ml. FSH had a strong negative (r=0.80, p<0.001) and inhibin B a positive relationship (r=0.67, p=0.013) with sperm count.
(Aubier et al., 1989)	30	Median 9 (21mo-17)	NR	Median 9 (1-20)	CT with non-alkylating: 13% CT with alkylating agents: 85% 15 patients received cyclophosplamide (median dose 12 g/m², 2.6-29 g/m²), 10 patients received MOPP Other CT used: vincristine, dactinomycin, Adriamycin, cytarabine, daunomycin, mercaptopurine, asparaginase, procarbazine, mechlorethamine and fluorouracil.	22	Of the 13 children who received a dose > 9 g/m² cyclophosphamide, only two were found to have normal testicular function. These two patients did not otherwise differ from the rest of the group. 7/10 patients treated with MOPP had confirmed azoospermia 2 to 16 years after completing the chemotherapy Azoospermia 23/27 (74%)
(Müller et al., 1996)	54	Median 14 (3-17)	Median 21 (19-34)	Median 8 (1-18)	24/33 male patients received alkylating agents **Alkylating agent** / **N (dose g/m², median, range)** Cyclophosphamide: 21 [4.0 (1.5-26.0)] Ifosfamide: 3 [63 (12-72)] CCNU: 1 (0.8) Procarbazine: 10 [6.5 (3-29.2)] 25/33 male patients received RT	14	9/14 (64%) showed azoospermia 3/14 (21%) showed oligozoospermia 2/14 (14%) showed normozoospermia Azoospermic long-time survivors had been treated more often with alkylating agents and had received higher gonadal doses of RT when compared with normospermic patients. Differences between azoospermic and normospermic patients in regard to

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td>Total dose: 3600 (2000-5600 cGy)
Gonadal dose: 5 (2-50/-2400 cGy)</td><td></td><td>cumulative doses of other cytotoxic drugs were not detectable.</td></tr>
<tr><td>(Borgström et al., 2020)</td><td>14</td><td>Median 10.7 (1.5-14.5)</td><td>Median 18.3 (12.7-21)</td><td>Median 7.2 (5-13.7)
N=5 ≥ 10 years</td><td>N=10 were conditioned with TBI (4 fractions × 3 Gy, 12 Gy in 1 week),
N= 10 received ‘high dose’ busulfan, usually in combination with ‘high dose’ cyclophosphamide.
HSCT with TBI 1/6 17%, with CT 5/6 83%</td><td>6</td><td>6 boys provided a semen sample, 4-9 years after HSCT
4/6: azoospermia
2/6: few motile sperm</td></tr>
<tr><td>(Kanbar et al., 2021)</td><td>114</td><td>At biopsy
7.5±4.1 years</td><td>20.6±2.3</td><td>7.1±3.0</td><td>CT with an alkylating or alkylating-like agent (n=123); 70% CED >4 g/m^2, 54% CED >8 g/m^2,16% CRT for those with sperm analysed
CT-RT (n=30)
BMT (n=41)</td><td>27</td><td>27 patients provided a semen sample for analysis after a median of 6.5 (2.6–14) years from the end of their treatments.
14/27 (52%) had severely impaired semen parameters including 8 who were azoospermic.</td></tr>
<tr><td>(Rafsanjani et al., 2007)</td><td>33</td><td>Median 9.1 (5-15)</td><td>Median 19.2 (17-29)</td><td>Median 7 (2-20)</td><td><table><tr><th>Therapy</th><th>Number (%)</th></tr><tr><td>MOPP/ABVD</td><td>23 (69.7%)</td></tr><tr><td>MOPP/ABVD+RT</td><td>3 (9.1%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, prednisolone</td><td>1 (3%)</td></tr><tr><td>MOPP/AVBD+vinbastine, Leukeran</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+COPP/ABVE</td><td>1 (3%)</td></tr><tr><td>MOPP+splenectomy</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX, CPA</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX</td><td>1 (3%)</td></tr><tr><td>MOPP</td><td>1 (3%)</td></tr></table></td><td>33</td><td>Twenty-seven patients had azoospermia, 2 patients had severe oligospermia, and one case had a count of 6,000,000, and another case of 20,000,000.</td></tr>
<tr><td>(Bordallo et al., 2004)</td><td>21</td><td>Median 10 (6-19) years</td><td>Median 18 (17-23)</td><td>≥ 2 years
3-11 years</td><td>C-MOPP/ABV hybrid program (cyclophosphamide, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, vinblastine) given in six or more cycles</td><td>18</td><td>We found azoospermia in 11 males, severe oligospermia in 4 males, and normal sperm count in 3.
Only one patient had recovered fertility with normalization of sperm count 11 years following treatment.</td></tr>
<tr><td>(Hobbie et al., 2005)</td><td>11</td><td>Median 13 (6-19)</td><td>NR</td><td>Median 6.5 (1.5-21)</td><td>CT: COPP/ABV hybrid
total cyclophosphamide doses of 2.4–3.6 g/m²</td><td>11</td><td>9/11 subjects were categorized as infertile; 7 of 9 were azoospermic, 5/6 who received 2.4 g/m² of cyclophosphamide were infertile, with the one fertile male having received 0.4 g/m² rather than the planned 2.8 g/m² of procarbazine.
2/3 pre-pubertal males were azoospermic. The pre-pubertal male who was fertile only</td></tr>
</table>

							received the 0.4 g/m² of procarbazine. There was no association between fertility status and prepubertal status at diagnosis (p = 1.00).
(Dhabhar et al., 1993)	26	Median 12 (4-15)	Median 17 (15-23)	Median 6 (2.3-11)	16 patients received 6 cycles of COPP and 4 patients received COPP/ABVD. 2 patients had 10 and 9 cycles of COPP, respectively. 4 patients received MOPP/ABVD. 14 patients received RT supradiaphragmatic (2000-4000 cGy) The cumulative dose of cyclophosphamide, procarbazine and adriamycin varied from 3-10 g (median 7.2g), 4.5-20 g (median 9 g) and 120-240 mg (median 150 mg), respectively.	18	All patients had azoospermia. Two patients had received only 3 cycles of COPP/MOPP followed by 3 cycles of ABVD and the remaining 16 patients had received 3 cycles or more of COPP/MOPP/ABVD.
(Whitehead et al., 1982)	17	Median 11.2 (4.8-14.8)	NR	Median 5.3 (2.4-8)	CT: n=16 Combination CT with MOPP (mustine 68.6±15.9 mg/m²; vincristine 21.6±4.3 mg/m²; prednisolone 4741.3±1330.5 mg/m²; procarbazine 11030.7±2815.8 mg/m²) RT: n=15 Neck or mantle RT: n=15; 2500-3000 cGy Abdominal RT: n=5; radiation dose to the testes was 100-300 cGy	6	All were azoospermic. 4/6 had only received combination CT, the remaining two had received both combination CT and small doses of testicular RT.
(Mackie et al., 1996)	58	Median 12.2 (8.2-15.3).	NR	*After diagnosis* Median 6 (2.5-11.1)	Combination CT was given for a recommended minimum of six courses (equivalent to 504 mg/m² chlorambucil and 8,400 mg/m² procarbazine) or a maximum of eight courses.	7	N=7, all displaying azoospermia. No association was seen between abnormal Leydig cell function and age at treatment, amount of chemotherapy received, or time of assessment from treatment.
(Garolla et al., 2006)	33	Group A: 7.13±3.11 Group B: 10.68±1.71	Group A: 26.5±3.5 Group B: 25.9±3.6	> 2 years	8 patients (group A) had received CT in which the alkylating agent was cyclophosphamide (RMS 79 protocol), and 25 (group B) CT in which alkylating drug was ifosfamide (18 patients with RMS 88 protocol, 5 with RMS 96 protocol and 2 with ISG/SSGI protocol).	33	Large reduction of mean sperm count in subjects of group A both in terms of sperm concentration and total sperm count (0.4 ± 0.7 mil/mL and 2.1 ± 4.4 million total number of sperm respectively). On the contrary, subjects of group B had a normal sperm count (46.8 ± 57.2 mil/mL and 91.3 ± 119.3 million total number of sperm).

ABV: adriamycin, bleomycin, vinblastine; **ABVD**: doxorubicin, bleomycin, vinblastine, dacarbazine; **BMT**: bone marrow transplant; **CCNU**: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; **CED**: cyclophosphamide equivalent dose; **COPP(A)**: cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin); **CPM**: Cyclophosphamide **CRT**: cranial radio therapy; **CT**: chemotherapy; **HSCT**: hematopoietic stem cell transplant; **ISG/SSGI protocol**: high doses metotrexate, cisplatin, adriamicin, ifosfamide; **MOPP/MVPP**: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; **MTX**: methotrexate; **RMS**: rhabdomyosarcoma; **RT**: radiotherapy; **TBI**: total body irradiation; **VP16**: Vincristine, platinol.

1730 # Supplementary Data S5 – Studies reporting effects on Leydig cell function of childhood cancer survivors.

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 hormone level determination	Effect
(Isaksson et al., 2018)	125	Median 9.6 (5.4-15.0)	Median 33.7 (30.2-40.1)	Median 24.3 (7.1)	**Treatment** / **No (%)** **CT** CED> 4g/m² — 10 (8%) **RT** Cranial — 12 (9.6%) Cranial + CT — 16 (13%) Testicular — 5 (4%) Other — 5% (4%) Other+CT — 23 (18%)	125	LH levels were significantly higher vs healthy controls (mean difference 1.1 IU/L, 95% CI 0.55; 1.6 IU/L). 26% was hypogonadal vs 14% of healthy controls (OR 2.1, 95% CI 1.1-4.1). Radiotherapy to testes increased the risk of developing hypogonadism (OR 28, 95% CI 2.9-279, p=0.004), as did chemotherapy in combination with radiotherapy to targets other than cranium or testes (OR 3.7, 95% CI 1.3-10 p=0.013), or cranial irradiation without chemotherapy (OR 4.4, 95% CI 1.1-18, p=0.038
(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/m² 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/m² HL — HDT with TBI and high-dose cyclophosphamide as	64	No significant differences of testosterone between the 3 treatment groups. A significant increase of levels of LH within normal range with increasing treatment toxicity.

					conditioning regimen HDT with BEAM as conditioning regimen MVPP or LVPP ≥4 courses		
(Chemaitilly et al., 2019)	1701	0-4.9; n=541 5-9.9; n=358 10-14.9; n=346 ≥15; n=271	Median 30.8 (18.1-63.8)	*After diagnosis* Median 22.0 (7.5-49.8)	CED 0 m^2; n=614 CED >0 to <4000 m^2; n=133 CED ≥4000 to <8000 m^2; n=269 CED ≥8000 to <12000 m^2; n=245 CED ≥12000 m^2; n=251 Missing: n=4	1516	Treatment-related risk factors for Leydig cell dysfunction (testosterone ≥250 ng/dl and LH>9.8 IU/L) included testicular radiotherapy at ≥ 12 Gy, CED's of ≥4g/m^2 and unilateral orchiectomy.
(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	Treatment with the ≤10 g/m^2 dose of cyclophosphamide was associated with decreased serum testosterone and calculated free-testosterone levels. No changes in serum LH levels were detected.
(Romerius et al., 2009)	144	Median 10 (0.10-17)	Median 29 (20-46)	Median 19 (4-36)	1) brain surgery, 2) surgery only (except brain surgery), 3) CT only (combined with surgery), 4) RT to the testes, 5) RT alone (combined with surgery), 6) both CT and RT (combined with surgery). The mean cranial irradiation dose was 38 Gy, and the mean dose of direct testicular irradiation was 21 Gy.	140	The age-adjusted free testosterone levels, but not the total testosterone levels, were found to be statistically significantly lower in CCS compared with controls (MD 0.038 nmol/L; 95% CI 0.017 - 0.059 nmol/L). Serum SHBG levels were also significantly higher in CCS (MD 3.6 nmol/L; 95% CI 1.2 - 6.0 nmol/L) and LH (mean difference 1.2 IU/L; 95% CI 0.72 - 1.7 IU/L). Biochemical hypogonadism (total testosterone <10 mmol/L and/or LH >10 IU/L), was more commonly detected among CCS (23%) than in controls (OR 6.7; 95% CI 2.7 - 17). Both CT only (OR 8.0; 95% CI 2.7 - 24) and the combination of CT- and RT (OR 6.5; 95% CI 2.3 - 19) were associated with an increased risk of hypogonadism. The proportion of biochemical hypogonadism in the men with a total testis volume greater than 24 ml was 13% (15/113), (OR 3.4; 95% CI 1.3 - 9.2), and in the 31 men with a total testis volume of 24 ml or less, the proportion of hypogonadism was 58% (18/31) (OR 31; 95% CI 11 - 92).
(Utriainen et al., 2019)	20	Median 1.6 (0.2-3.6)	Median 21.7 (15.9-30.1)	Median 19 (13-27)	Induction CT with Cyclophosphamide, vincristine with or without cisplatin and doxorubicin Local RT in 14/20	9	6/9 had gonadal failure with low testosterone. 3 males needed testosterone substitution.

					TBI + CT in 10/20 Combination CT with Eto+carbo+tiotepa/melphalam/other		
(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 irradiation 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 RT, (6) TBI plus additional CNS and additional testicular irradiation.	72	Inhibin B was the best surrogate marker of azoospermia (AUC, .91; 95% CI, 0.85 to 0.98; 90% sensitivity and 83% specificity) compared with FSH and testicular volume.
(van Casteren et al., 2009)	248	Median 5 (0-15)	Median 23 (18-41)	Median 18 (5-39)	Cyclophosphamide was part of the treatment protocol in 131 of the 248 survivors with a median dosage of 4.8 g/m² (range 0.25–32 g/m²). HL with MOPP or without procarbazine	221	145/221 had inhibin B values below 150 ng/L in contrast to 19/74 controls. Inhibin B levels showed a significant correlation with sperm concentration in both survivors (r=0.671, p=0.01) and controls (r=0.345, p=0.03).
(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 irradiation (24 Gy), but spinal RT was not used. Patients experiencing testicular relapse underwent a multidrug chemotherapy regimen together with testicular and cranial RT at a dose of 24 Gy.	11	N=8 with standard risk treatment levels of LH (4.2±0.9 IU/L) and testosterone (19±3 nmol/L), were comparable to values among healthy Finnish young men. N=3 after high-risk therapy Two had normal gonadotropin levels, one had increased levels. Testosterone was normal in all 3.
(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	33	*Group 1:* 4/13 had increased LH levels. 2/13 had decreased testosterone levels. *Group 2*: all had normal LH and testosterone levels. *Group 3*: 7/10 had normal LH and testosterone values. 3/10 had normal LH levels.

(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 (mechlorethamine, vincristine, prednisone, procarbazine) divided into 3 groups: no MOPP (n=16) 3-4 MOPP (n=14) ≥6 MOPP (n=26)	56	Median LH values were significantly higher in MOPP+ patients when compared with MOPP- patients (P < 0.01), who all had normal to marginally increased LH levels. Levels of SHBG were normal in all patients, whereas concentrations of testosterone and bioavailable testosterone were normal to marginally decreased and not different between MOPP+ and MOPP- patients. LH increased significantly with an increasing number of MOPP cycles.
(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 chemotherapy 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	LH: 489 Testosterone: 460	Only 14 survivors (2.9%) had elevated LH levels and 57 survivors (12.4%) had decreased testosterone levels.
(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	41	Patients 12 years or more of age at diagnosis had higher serum testosterone levels than the others by 5.5 (0.7-10.4) U/L (p= 0.026). The only risk factor for abnormal serum LH levels was cyclophosphamide, which was associated with increases of 3.9 (0.3-7.4) U/L (p= 0.036) in LH concentrations.
(Brignardello et al., 2016)	199	**Age** / **No (%)** 0-4 / 45 (22.6%) 5-10 / 57 (28.6%) ≥10 / 97 (48.7%)	NR	Median 14.01 (IQR 10.1-17.8)	Refer to (Brignardello et al., 2013) for treatment details: **Treatment** / **Number** Any RT / 199 (64.2) TBI / 40 (12.9) Cranial RT / 74 (23.9%) CT / 294 (94.8%) HSCT / 74 (23.9%) Surgery / 115 (37.1%)	194	102/194 (51.26 %) male CCS had normal gonadal function. Among 33 patients previously treated with TBI, none had normal gonadal function, 13 had primary hypogonadism, and 3 had central hypogonadism. An extremely high rate of gonadal dysfunction (46/48) was also detected in patients who underwent HSCT. The risk of gonadal dysfunction was higher in patients treated with radiotherapy (crude OR 5.83; 95 % CI 2.95–11.52 and adjusted OR 8.72; 95 % CI 3.94–19.30) and in patients

							exposed both to alkylating agents and to platinum-derived agents (adjusted OR 9.22; 95 % CI 2.17–39)
(Heikens et al., 1996)	19	Median 11 (5-15)	Part 1: Median 19 (16-27) Part 2: Median	Part 1: Median 10 (6-14) Part 2: Median 14 (13-20)	All patients were treated with 6 courses of MOPP chemotherapy. RT was given as adjuvant treatment in 8 patients with large lymph node tumours; 6 received irradiation above the diaphragm, and 2 were irradiated below the diaphragm (20 Gy on the para-aortic and splenic regions, respectively, and 25 Gy on the inguinal region)	19	1 patient had normal basal levels of testosterone, and LH, as well as a normal response of LH to GnRH). In 9 patients, serum testosterone concentrations were slightly decreased; in 4 of these patients, the decrease was accompanied by a raised LH. The serum LH level showed an exaggerated response to administration of GnRH in 3 patients, with normal basal values of testosterone and LH. There were no differences in testosterone levels or in basal or stimulated levels of LH between males treated before puberty and those treated during puberty. Follow-up hormone measurements were available for 16 patients. Testosterone and LH levels were not different between initial examination and follow-up.
(Relander et al., 2000)	77	Median 11 (0.8-17)	Median 23.6 (18.6-38.5)	*After diagnosis* Median 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.	66	All had normal testosterone values except for 1. 62 patients had completed normal pubertal development, whereas 4 had Tanner scores of 3/5. One of them had received testicular irradiation; in the remaining 3 the finding could not be explained. LH were within the normal range in 57 patients (88%). 2 of them had an increased LH.
(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	40	*Patients that received CT* 16/28 patients have elevated LH levels. Testosterone was measured in 25, all normal. *Patients that only received RT* 7/7 patients with RT above diaphragm all have normal LH and testosterone levels. 3 patients received 3,500 cGy to an inverted Y field, two have elevated LH levels. 2 patients received 3,500 cGy to the right groin. Both have normal LH and testosterone levels.

(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 CT/RT (n=5)	12	6/12 patients high serum LH levels All patients: normal testosterone
(Lee et al., 2024)	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 (5Gy)/cyclophosphamide (20 mg/kg): n=6 - No conditioning: n=7 Missing: n=1		Of 37 men who had received TBI +/- additional testicular RT, or therapeutic testicular RT without TBI (cumulative testicular doses of 12-36 Gy), 33/37 had available gonadotrophin measurements; 32 /33 (97%) had evidence of impaired spermatogenesis (raised FSH) with 11/33 (29.7%) having complete Leydig cell failure. Total testicular radiation dose impacted the degree of Leydig cell dysfunction. 24/27 males receiving 12Gy TBI without additional testicular irradiation had gonadotrophin levels available for evaluation; of these 24, 1 had normal gonadotrophin levels, 10 had isolated elevation of FSH, 5/24 (18.5%) had complete, and 8/24 (33.3%) compensated Leydig cell dysfunction. Two of 6 (33.3%) receiving 18Gy to the testes had complete Leydig cell failure and 3/6 (50%) compensated Leydig cell dysfunction, with no gonadotrophins available for the remaining patient. All 4 who received testicular exposure of ≥ 24Gy developed complete Leydig cell failure. In contrast, among those conditioned with myeloablative chemotherapy without testicular irradiation, only 18/36 (50%) with available gonadotrophin levels had impaired spermatogenesis; 3/40 (7.5%) had complete and 3/36 (8.3%) compensated Leydig cell dysfunction.
(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/m² (range, 4.7–31.9 g/m²). 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)	16	All patients had normal baseline testosterone levels. 6 of 15 patients (40%) had elevated baseline LH levels, and 13 of 14 patients (92.9%) had an increased LH response to GnRH stimulation.

(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)	We compared five chemotherapeutic groups: antitumor antibiotics, alkylating agents, topoisomerase and mitotic inhibitors, platinum-based agents and antimetabolites. 34 patients also underwent RT (26 patients underwent abdominal irradiation 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 RT 25 Gy)	126	LH levels increased with time in survivors with abnormal semen analysis ($p < 0.0001$)
(Hale et al., 1999)	73	Median 9.2 (1 day-18.3)	NR	Median 11.3 (5.1-26.5)	<table><tr><th>Treatment</th><th>Number (%)</th></tr><tr><td>CT+surgery</td><td>27 (37%)</td></tr><tr><td>RT+CT+sugery</td><td>21 (29%)</td></tr><tr><td>RT+surgery</td><td>8 (11%)</td></tr><tr><td>Surgery alone</td><td>17 (23%)</td></tr></table> RT: 25-30 Gy to abdomen and pelvis or 20 Gy to mediastinum and supraclavicular regions CT: until 1978: VAC with or without doxorubicin until 1988: VAC or PVB or both	26	None had delayed puberty or required testosterone replacement therapy
(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	40	Primary hypogonadism in 24/40 (60%) males. All of them but one had ≥ 6 cycles of CT containing alkylating agents and procarbazine containing CT in combination with RT (to the pelvis in 8), 2 had had pelvic RT only). 10 males also had elevated LH levels.
(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 para-aortic nodes. Seven patients received 2-6 cycles of MOPP chemotherapy and five, COPP (cyclophosphamide, oncovin, prednisone, and procarbazine) or chlorambucil. Among the remaining patients, ten received radiation therapy (five to the inguinal or pelvic nodes) and seven, an alkylating agent (cyclophosphamide, nitrogen mustard, or chlorambucil). One leukaemia patient with	23	Testosterone levels in sterile men did not differ from those with normal fertility. Higher LH levels were associated with sterility and diminished testicular volume; however, the range of values overlapped those detected in men with normal fertility and testicular size.

					testicular relapse received RT to the gonads (2,400 rad). Four patients received Adriamycin.		
(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	Testosterone, serum LH, oestradiol and prolactin were within normal range.
(Williams et al., 2008)	45	Median 11.8 (5.4-21.3)	Median 20.8 (16.0-29.3)	Median 9.7 (3.3-12.6)	32 males received a median dose of ifosfamide 92 g/m² 9 patients had also received cyclophosphamide 0.3–2.4 g/m² during RT Patients were divided into two ifosfamide dose ranges, based on the bimodal distribution of doses: low-dose (<60 g/m², n=6) and high dose (>60 g/m², n=26).	32	In the 'high dose' group, 8/26 had high FSH levels (>10 U/L), 13 had reduced inhibin B (<80 pg/ml), 2 had increased LH (>8.4 U/L) and 1 had decreased testosterone (<8 nmol/L). FSH was significantly correlated with age at treatment (r=0.39, p=0.049) but inhibin B showed no significant trend with age at treatment (r=-0.21, p=0.26). No abnormal values of LH, FSH or testosterone were observed in the 'low dose' group. One patient from this group had a low inhibin B but had nevertheless fathered a child.
(Servitzoglou et al., 2015)	171	Median 10.8 (2.1-17.3)	Median 21.1(17-30.4)	Median 9.3 (2-22.4)	For HL, children received combined RT (mantle field, subtotal nodal, or involved field RT) and CT, consisting of several MOPP cycles alone or in combination with ABVD or ABVP. More recently, patients received either VBVP cycles alone or VBVP combined with OPPA or in combination with COPP. For NHL, RT has been used for CNS prophylaxis or rarely for resistant mediastinal disease. CT consisted of COPAD cycles associated with lomustine (CCNU) or high-dose methotrexate, cytarabine, etoposide, asparaginase, 6-mercaptopurine, 6-thioguanine, or vinblastine.	171	8.9% (15/168) had abnormal LH levels (≥8 IU/L). Older age at evaluation was associated with higher LH levels, but it was also associated with older treatment regimens and higher alkylating agent dose
(Ortin et al., 1990)	20	Median 14 (10-15)	NR	Median 8.5 (1-10)	RT alone: n=3; the delivered dose at the midplane of the pelvis ranged from 15-44 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=3; min 6 cycles of MOPP and pelvic RT (20-44 Gy)	10	No correlation was seen between serum gonadotropin levels and sterility. Only four of ten azoospermic boys tested had abnormally elevated LH levels. However, one boy who had an elevation of both FSH and LH subsequently fathered two children.

<table>
<tr><td></td><td></td><td></td><td></td><td></td><td>CT alone: n=4;
MOPP/ABVD for six cycles-16, PAVe for six cycles-3. VBM for six cycles1, ABVD for six cycles</td><td></td><td></td></tr>
<tr><td>(Aubier et al., 1989)</td><td>30</td><td>Median 9 (21mo-17)</td><td>NR</td><td>Median 9 (1-20)</td><td>CT with non-alkylating: 13%
CT with alkylating agents: 85%</td><td>9</td><td>LH levels were normal in all 9 patients tested</td></tr>
<tr><td>(Borgström et al., 2020)</td><td>14</td><td>Median 10.7 (1.5-14.5)</td><td>Median 18.3 (12.7-21)</td><td>Median 7.2 (5-13.7)
N=5 ≥ 10 years</td><td>N=10 were conditioned with TBI (4 fractions × 3 Gy, 12 Gy in 1 week),
N= 10 received ‘high dose’ busulfan, usually in combination with ‘high dose’ cyclophosphamide.</td><td>14</td><td>Hormone levels were repeatedly measured in 14 boys.
3/14 had LH levels above the reference levels (upper limits 12.5 U/L and 9.6 U/L, respectively).
2 boys were on testosterone replacement
3 boys had normal testosterone levels</td></tr>
<tr><td>(Rafsanjani et al., 2007)</td><td>33</td><td>Median 9.1 (5-15)</td><td>Median 19.2 (17-29)</td><td>Median 7 (2-20)</td><td><table><tr><th>Therapy</th><th>Number (%)</th></tr><tr><td>MOPP/ABVD</td><td>23 (69.7%)</td></tr><tr><td>MOPP/ABVD+RT</td><td>3 (9.1%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, prednisolone</td><td>1 (3%)</td></tr><tr><td>MOPP/AVBD+vinbastine, Leukeran</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+COPP/ABVE</td><td>1 (3%)</td></tr><tr><td>MOPP+splenectomy</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX, CPA</td><td>1 (3%)</td></tr><tr><td>MOPP/ABVD+CCNU, VP16, MTX</td><td>1 (3%)</td></tr><tr><td>MOPP</td><td>1 (3%)</td></tr></table></td><td>33</td><td>The median level of LH was 5 mIU/ml (range, 0.1-14), 6/33 were above normal. The median level of testosterone was 4.10 ng/ml (range, 0.1-14.10), 3/33 were below normal</td></tr>
<tr><td>(Bordallo et al., 2004)</td><td>21</td><td>Median 10 (6-19) years</td><td>Median 18 (17-23)</td><td>≥ 2 years
3-11 years</td><td>C-MOPP/ABV hybrid program (cyclophosphamide, vincristine, procarbazine, prednisone, doxorubicin, bleomycin, vinblastine) given in six or more cycles</td><td>21</td><td>Low normal levels of total testosterone in 3 (14.9%) patients from group A.
6 (28.6%) patients from group A presented normal</td></tr>
<tr><td>(Papadakis et al., 1999)</td><td>36</td><td>Median 13.0 (2.4-22.6)</td><td>Median 22.3 (15.1-32.5)</td><td>Median 6.8 (2.0-19.3)</td><td>CT: first doxorubicin (60-75 mg/m²), procarbazine (50 mg/day for 2 days and 100 mg/day for 26 days) in combination with prednisone (30 mg/m²/day) and vincristine (1.5 mg/m²) and finally cyclophosphamide (1200 mg/m²).
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</td><td>36</td><td>LH was within the normal range in 33 of 36 (91%) patients; specifically all group A (RT−) patients, 22 of 25 group B (CT ± RT−) patients, and 5 of 6 group C (CT + RT+) patients.
Testosterone was within the normal range in 29 of 33 (88%) patients, including 3 of 4 group A (RT−) patients, 24 of 25 group B (CT ± RT−) patients, and 2 of 4 group C (CT + RT+) patients.</td></tr>
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<tr><td></td><td></td><td></td><td></td><td></td><td>Group 3: n=12; CT+RT involving the pelvis</td><td></td><td></td></tr>
<tr><td>(Hobbie et al., 2005)</td><td>11</td><td>Median 13 (6-19)</td><td>NR</td><td>Median 6.5 (1.5-21)</td><td>CT: COPP/ABV hybrid total cyclophosphamide doses of 2.4–3.6 g/m²</td><td>11</td><td>5/9 infertile males had normal LH levels. There was no association between fertility status and gonadotropin status (p = 0.49). All had normal testosterone levels (10/11 available).</td></tr>
<tr><td>(Dhabhar et al., 1993)</td><td>26</td><td>Median 12 (4-15)</td><td>Median 17 (15-23)</td><td>Median 6 (2.3-11)</td><td>16 patients received 6 cycles of COPP and 4 patients received COPP/ABVD. 2 patients had 10 and 9 cycles of COPP, respectively. 4 patients received MOPP/ABVD.
14 patients received RT supradiaphragmatic (2000-4000 cGy).
The cumulative dose of cyclophosphamide, procarbazine and adriamycin varied from 3-10 g (median 7.2g), 4.5-20 g (median 9g) and 120-240 mg (median 150 mg), respectively.</td><td>23</td><td>16 patients with follow-up of ≥6 years with azoospermia showed increased levels of LH.</td></tr>
<tr><td>(Felicetti et al., 2020)</td><td>196</td><td><table><tr><th>Age at diagnosis</th><th>No</th></tr><tr><td>0-4</td><td>37</td></tr><tr><td>5-9</td><td>55</td></tr><tr><td>≥10</td><td>104</td></tr></table></td><td>Median 24.35 (IQR 21.84-29.39)</td><td>≥ 5 years</td><td><table><tr><th>Treatment</th><th>No (%)</th></tr><tr><td colspan="2">RT</td></tr><tr><td>Any</td><td>103 (52.6%)</td></tr><tr><td>Abdominopelvic</td><td>32 (16.3%)</td></tr><tr><td>TBI</td><td>21 (10.7%)</td></tr><tr><td>Cranial</td><td>13 (6.6%)</td></tr><tr><td colspan="2">CT</td></tr><tr><td>Any</td><td>196 (100%)</td></tr><tr><td>Alkylating</td><td>185 (94.4%)</td></tr><tr><td>CED 0-4 g/m²</td><td>104 (53.1%)</td></tr><tr><td>CED 4-8 g/m²</td><td>71 (36.22%)</td></tr><tr><td>CED > 8g/m²</td><td>21 (10.7%)</td></tr><tr><td>HSCT</td><td>50 (25.5%)</td></tr></table></td><td>196</td><td>18 (9.2%) were diagnosed with LCF (testosterone <300 ng/dl) and elevated gonadotropin levels).
All male hematologic malignancy survivors affected by LCF had received RT, mostly focused on abdominopelvic fields.
A greater exposure to alkylating agents was associated with a higher risk of LCF ($OR_{CED(per\ 1\ g/mg^2)}$ = 1.34, 95% CI, 1.03- 174).</td></tr>
<tr><td>(Krawczuk-Rybak et al., 2009)</td><td>59</td><td>Group 1: 4.3±1.7
Group 2: 7.9±4.3</td><td>Group 1: 8.4 ±2.2
Group 2: 15.9±2.6</td><td>Group 1: 1.9±1.3
Group 2: 5.3±3.5</td><td>Protocols of the Polish Pediatric Leukemia/Lymphoma Study Group based (in standard-risk group) on BFM protocols of 1985, 1990, and 1995 (n = 2) or, in the high-risk group, on New York (NY) protocol (n = 7)
RT:
Group 1: n=8 (NY: 18 Gy, n=5, BFM: 12 Gy, n=3)
Group 2: n= 6: 18 Gy (2 NY and 4 BFM) and n=12 received 12 Gy (BFM)</td><td>59</td><td>Group 1: No statistically significant differences were found in the mean values of LH or testosterone compared to heathy controls. No differences between irradiated and non-irradiated patients
Group 2: Four of them had received irradiation to the CNS (12 Gy). Two of the patients had abnormal LH values (more than + 2 SD) and 1 boy had a testosterone level less than −2 SD</td></tr>
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<tr>
<td>(Mackie et al., 1996)</td>
<td>58</td>
<td>Median 12.2 (8.2-15.3).</td>
<td>NR</td>
<td>After diagnosis
Median 6 (2.5-11.1)</td>
<td>Combination CT was given for a recommended minimum of six courses (equivalent to 504 mg/m^2 chlorambucil and 8,400 mg/m^2 procarbazine) or a maximum of eight courses.</td>
<td>46</td>
<td>5/41 subjects showed low testosterone levels.
10/41 showed raised LH levels (range 10.3-18 IU/L).</td>
</tr>
<tr>
<td>(Quigley et al., 1989)</td>
<td>45</td>
<td>Median 4.39 (1.23-12.35)</td>
<td>NR</td>
<td>Median 4.62 (2.35-8.97)</td>
<td>Cyclophosphamide: mean dose 4.8 g/m², cytarabine: mean dose 13.1 g/m². asparaginase, daunorubicin, hydroxyurea, lomustine, methotrexate, prednisolone, thuiguanine, vincristine.
Cranial irradiation: 24 Gy and intrathecal methotrexate.</td>
<td>23</td>
<td>Baseline plasma LH levels were elevated in 10/23 boys.</td>
</tr>
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<td>(Brämswig et al., 1990)</td>
<td>75</td>
<td>12.44±2.1</td>
<td>17.24±2.19</td>
<td>4.3±1.87</td>
<td>
<table>
<tr><th>Treatment</th><th>HD I-IIA</th><th>HD II-IIIA</th><th>HD IIIB-IV</th></tr>
<tr><td>CT</td><td>2 OPPA</td><td>2OPPA/ 2 COPP</td><td>2 OPPA/ 4-6 COPP</td></tr>
<tr><td>Vincristine</td><td>4.5</td><td>10.5</td><td>13.5-16.5</td></tr>
<tr><td>Prednisone</td><td>1800</td><td>2360</td><td>2920-3480</td></tr>
<tr><td>Procarbazine</td><td>3000</td><td>5800</td><td>8600-11400</td></tr>
<tr><td>Adriamycin</td><td>160</td><td>160</td><td>160-160</td></tr>
<tr><td>Cyclophos-phamide</td><td></td><td>2000</td><td>4000-6000</td></tr>
</table>
</td>
<td>75</td>
<td>Testosterone is 19.94 ± 8.71 nmol/l and above the value of the control group (10.96 ± 4.8 1 nmol/l). The mean basal and stimulated LH levels are also elevated, 8.66 U/L and 49.45 U/L, respectively.
With the intensification of CT the incidence of pathologically elevated basal LH levels rises.
The frequency of elevated LH levels is higher in the middle or late pubertal group with a chronologic age of 18.21 ± 2.04 years.</td>
</tr>
<tr>
<td>(Whitehead et al., 1982)</td>
<td>17</td>
<td>Median 11.2 (4.8-14.8)</td>
<td>NR</td>
<td>Median 3.8 (1-8)</td>
<td>CT: n=16
Combination CT with MOPP (mustine 68.6±15.9 mg/m²; vincristine 21.6±4.3 mg/m²; prednisolone 4741.3±1330.5 mg/m²; procarbazine 11030.7±2815.8 mg/m²)
RT: n=15
Neck or mantle RT: n=15; 2500-3000 cGy
Abdominal RT: n=5; radiation dose to the testes was 100-300 cGy</td>
<td>15</td>
<td>All 4 prepubertal subjects had normal basal and peak gonadotrophin responses to LH-RH.
4 subjects in early puberty, one showed increasingly more abnormal gonadotropin levels with time.
10 were late pubertal or adult, 6 showed increased basal or stimulated LH levels.
3/4 prepubertal patients showed subnormal testosterone response.
All late pubertal and adult males have basal testosterone levels within the normal range.</td>
</tr>
</table>

(Hudson et al., 1993)	79	Median 14.6 (4.3-20.1)	NR	Median 3.75 (0.33-9)	COP regimen alternated monthly with the ABVD regimen, for a total of 12 months Prednisone (2 weeks) of the first month in patients with B symptoms. RT for patients with stage IIB-IV disease. The dose for nodal sites was 20 Gy at 1.5 Gy/fraction; the visceral dose was 15 to 20 Gy	8	Gonadotropin findings were within normal ranges in all 8 males screened.
(Green et al., 1981)	17	NR	Median 17.0 (9.6-24.4)	Median 3.6 (0.5-8.17)	CT: MOPP, CVPP, BOPP, ABVD, COPP, CV-CCNU and vinblastine. Pelvic RT (n=9); (557.7 rads (105-1090) No pelvic irradiation (n=8)	17	*Pelvic irradiation and CT:* 2/9 elevated LH levels 3/9: normal gonadotropin levels *CT only:* All normal LH levels
(Ise et al., 1986)	46	Median 5.4 (0.08-13)	NR	N=8: Median 0.3 (0-0.7) year N=4: Median 3 (2-4) year	Vincristine, prednisolone, anthracycline, L-asparaginase, cytosine arabinoside, prophylactic skull irradiation 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.	46	Low basal serum testosterone concentrations were observed in the younger age group and higher in the older group. No abnormal basal LH concentrations were observed.
(Ahmed et al., 1983)	10	Group 1: Median 10.8 (6.9-13.1) Group 2: Median 6.5 (2.2-14)	Group 1: Median 14.8 (12-17) Group 2: Median 16.4 (14-18.7)	*After CT completion* Median 2.95 (0.3-5)	Group 1: cranial RT (1500 cGy) + cerebrospinal RT (3000 cGy) + vincristine (2.0 mg/m²; 3x/week) + adjuvant CT for 1-2 years (carmustine+vincristine, lomustine or procarbazine). Group 2: cranial RT (variable dose; max scatter to the gonad was calculated to be 45 cGy after 4 MV and 150 cGy after 300 kV RT) + cerebrospinal RT (2700 cGy) + vincristine (2.0 mg/m²; 3x/week).	10	Group 1: 2 had raised LH levels. Group 2: all had gonadotropin and testosterone values within the normal adult range.
(Wallace et al., 1989)	8	Median 12.6 (7.3-14.6)	Median 14.8 (10.3-22.6)	Median 2.6 (0.1-7.8)	All patients received CT containing cis-platinum, in combination with either adriamycin, HDMTX, vincristine, bleomycin, cyclophosphamide, dactinomycin or ifosfamide.	8	3/8 showed normal gonadotropin and testosterone levels. 1/8 had significantly elevated LH levels
(Garolla et al., 2006)	33	Group A: 7.13±3.11 Group B: 10.68±1.71	Group A: 26.5±3.5 Group B: 25.9±3.6	> 2 years	8 patients (group A) had received CT treatment in which the alkylating agent was cyclophosphamide (RMS 79 protocol), and 25 (group B) chemotherapy treatment in which alkylating drug was ifosfamide (18 patients	33	In group A, higher LH and lower testosterone plasma concentrations were found, not statistically different from group B.

					with RMS 88 protocol, 5 with RMS 96 protocol and 2 with ISG/SSGI protocol).		
(Gerres et al., 1998)	46	14.9±1.5	17.2±1.6	1.95±1.18	RT: involved field irradiation with total radiation doses of 25 Gy in patients with Stages I-IIA disease and Stages IIB-IIIA disease and 20 Gy in patients with Stages IIIB-IV disease. CT: patients with Stages I-IIA HD received two courses of OEPA, and patients with Stages IIB-IIIA and IIIB-IV HD received two OEPA courses and two or four courses of COPP. The recommended cumulative doses (mg/m²) were different for each treatment group	46	The mean testosterone values of 12.18 nmol/L (Tanner stages 3 and 4) and 15.10 nmol/L (Tanner stages 5 and 6) were greater than the mean values of each control group. The mean basal and stimulated values of LH were within the normal range in 7 boys with Tanner stages 3 and 4 and in 20 patients with Tanner stages 5 and 6.

ABV: adriamycin, bleomycin, vinblastine; **ABVD**: doxorubicin, bleomycin, vinblastine, dacarbazine; **ABVP**: Adriamycin, bleomycin, vincristine, prednisolone; **ALL**: acute lymphoblastic leukemia; **BEAM**: carmustine, etoposide, cytarabine, melphalan; **BFM**: Berlin-Frankfurt-Münster protocol; **BOPP**: 1,3-bis (2-chloroethyl)-lnitrosourea, vincristine, procarbazine, and prednisone; **CCNU**: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; **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; **COPAD**: cyclophosphamide, oncovin, prednisone, adriamycin; **COPP(A)**: cyclophosphamide, vincristine, procarbazine, prednisone, (doxorubicin); **CRT**: cranial radio therapy; **CT**: chemotherapy; **CVPP**: 1 -(2-chloroethyl)-3-cyclohexyl-1 -nitrosourea, vinblastine, procarbazine, and prednisone; **EBVP**: epirubicin, bleomycin, vinblastine, prednisone; **FSH**: follicle stimulating hormone; **GnRH**: gonadotropin releasing hormone; **HD**: Hodgkin's disease; **HDMTX**: high-dose methotrexate; **HDT**: high-dose chemotherapy with autologous stem cell support; **HL**: Hodgkin lymphoma; **HSCT**: hematopoietic stem cell transplant; **IQR**: inter-quartile range; **ISG/SSGI protocol**: high doses metotrexate, cisplatin, adriamicin, ifosfamide; **LH**: luteinising hormone; **LCF:** Leydig cell failure; **LHRH**: luteinising hormone releasing hormone; **LOPP/LVPP**: vinblastine, chlorambucil, procarbazine, prednisone; **LSA_2L_2**: cyclophosphamide, vincristine, doxorubicin, asparaginase, thioguanine, methotrexate, 6-mercaptopurine; **MAC**: ; **MD**: mean difference; **MOPP/MVPP**: nitrogen mustard, oncovin/vinblastine, procarbazine, prednisone; **MTX**: methotrexate; **NCI protocol**: methotrexate, cyclophosphamide, doxorubicin, prednisone; **NHL**: non-Hodgkin lymphoma; **NR**: not reported; **NY protocol**: BFM protocol with higher dosages; **OEPA**: doxorubicin, etoposide, prednisone, vincristine; **OPPA**: doxorubicin, procarbazine, prednisone, vincristine; **OR**: odds ratio; **PAVe**: Procarbazine, alkeran, velban; **PVB:** Cisplatin, vinblastine and bleomycin; **RMS**: rhabdomyosarcoma; **RT**: radiotherapy; **SHBG**: Sex hormone binding globulin; **TBI**: total body irradiation; **TLI**: Total lymphoid irradiation; **VAC**: Vincristine, dactinomycin, cyclophosphamide; **VBM**: Velban, bleomycin, methotrexate; **VP16**: Vincristine, platinol.