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Original Article

Andrology

Semen analysis and reproductive hormones in boys with classical Hodgkin lymphoma treated according to the EuroNet-PHL-C2 protocol

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ABSTRACT

STUDY QUESTION: What is the impact of the EuroNet-PHL-C2 treatment for boys with classical Hodgkin lymphoma (cHL) on semen parameters?

SUMMARY ANSWER: More than half of the patients (52%, n = 16/31) had oligozoospermia or azoospermia at 2 years from cHL diagnosis; particularly boys treated for advanced-stage cHL had low sperm counts and motility.

WHAT IS KNOWN ALREADY: Chemotherapy and radiotherapy to the inguinal region or testes can impair spermatogenesis and result in reduced fertility. The EuroNet-PHL-C2 trial aims to minimize radiotherapy in standard childhood cHL treatment, by intensifying chemotherapy. The present study aims to assess the (gonadotoxic) impact of this treatment protocol on semen parameters and reproductive hormones in boys aged ≤18 years.

STUDY DESIGN, SIZE, DURATION: This international, prospective, multi-centre cohort study was an add-on study to the randomized phase-3 EuroNet-PHL-C2 trial, where the efficacy of standard cHL treatment with OEPA-COPDAC-28 (OEPA: vincristine, etoposide, prednisone, and doxorubicin; COPDAC-28: cyclophosphamide, vincristine, prednisone, and dacarbazine) was compared to intensified OEPA-DECOPDAC-21 chemotherapy (DECOPDAC-21: COPDAC with additional doxorubicin and etoposide and 25% more cyclophosphamide). Patients were recruited between January 2017 and September 2021.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Eligibility criteria included male patients, diagnosed with classical HL before or at the age of 18 years, and treated according to the EuroNet-PHL-C2 protocol in any of the 18 participating sites in the Netherlands, Germany, Belgium, Czech Republic, and Austria. Sperm parameters (sperm concentration, progressive motility, sperm volume, and calculated total motile sperm count) were assessed at diagnosis and 2 years after diagnosis in (post)pubertal boys. Laboratory measurements (serum follicle-stimulating hormone (FSH) and inhibin B) were performed in samples drawn at diagnosis, during treatment (2–3 times), and at 2 years post-diagnosis, and (age-adjusted) analyses were conducted separately for pre-pubertal and (post)pubertal boys. Outcomes were compared between the treatment levels (TL1, TL2, and TL3) and consolidation treatment schemes (COPDAC-28 and DECOPDAC-21).

MAIN RESULTS AND THE ROLE OF CHANCE: In total, 101 boys were included in the present analysis: 73 were (post)pubertal (median age 15.4 years, (IQR 14.4; 16.6), 10 TL1, 29 TL2, 34 TL3, 62% of TL2/3 patients received COPDAC-28) and 28 boys were pre-pubertal (median age 9.6 years (IQR 6.6; 11.4), 4 TL1, 7 TL2, 17 TL3, 38% of TL2/3 patients received COPDAC-28). The study included six boys who had received pelvic radiotherapy; none were irradiated in the inguinal or testicular area. At diagnosis, 48 (post)pubertal boys delivered semen for cryopreservation; 19 (40%) semen samples were oligospermic and 4 (8%) were azoospermic. Low sperm concentration

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(<15 mil/ml) appeared to be related to the HL disease itself, with a higher prevalence in boys who presented with B symptoms (76% vs 26%, aOR 2.3 (95% CI 1.0; 3.8), $P = 0.001$) compared to those without such symptoms. At 2 -years post-diagnosis, 31 boys provided semen samples for analysis, of whom 12 (39%) boys had oligozoospermia and 4 (13%) had azoospermia, while 22 boys (71%) had low total motile sperm counts (TMSC) (<20 mil). Specifically, the eight boys in the TL3 group treated with DECOPDAC-21 consolidation had low sperm counts and low progressive motility after 2 years (i.e. median sperm count 1.4 mil/ml (IQR <0.1; 5.3), n = 7 (88%), low sperm concentration, low median progressive motility 16.5% (IQR 0.0; 51.2), respectively). Age-adjusted serum FSH levels were significantly raised and inhibin B levels (and inhibin B:FSH ratios) were decreased during chemotherapy in (post)pubertal boys, with subsequent normalization in 80% (for FSH) and 60% (for inhibin B) of boys after 2 years. Only 4 out of the 14 (post)pubertal boys (29%) with low sperm concentrations after 2 years had elevated FSH (>7.6 IU/l), while 7 (50%) had low inhibin B levels (<100 ng/l). In pre-pubertal boys, reproductive hormones were low overall and remained relatively stable during chemotherapy.

LIMITATIONS, REASONS FOR CAUTION: The present analyses included sperm and laboratory measurements up to 2 years post-diagnosis. Long-term reproductive outcomes and potential recovery of spermatogenesis remain unknown, while recovery was reported up to 5- or even 10-year post-chemotherapy in previous studies.
Boys who were pre-pubertal at diagnosis were still too young and/or physically not able to deliver semen after 2 years and we could not assess a potential difference in gonadotoxicity according to pubertal state at the time of treatment. Overall, the statistical power of the analyses on sperm concentration and quality after 2 years was limited.

WIDER IMPLICATIONS OF THE FINDINGS: Results of the semen analyses conducted among the 31 boys who had provided a semen sample at 2 years post-treatment were generally poor. However, additional long-term and adequately powered data are crucial to assess the potential recovery and clinical impact on fertility. The participating boys will be invited to deliver a semen sample after 5 years. Until these data become available, benefits of intensified chemotherapy in cHL treatment to reduce radiotherapy and lower risk for development of secondary tumours should be carefully weighed against potentially increased risk of other late effects, such as diminished fertility due to the increased chemotherapy burden. Boys with newly diagnosed cHL should be encouraged to deliver sperm for cryopreservation whenever possible. However, patients and clinicians should also realize that the overall state of disease and inflammatory milieu of cHL can negatively affect sperm quality and thereby reduce chance of successful fertility preservation. Furthermore, the measurement of FSH and inhibin B appears to be of low value in predicting low sperm quality at two years from cHL treatment.

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Introduction

Childhood Hodgkin lymphoma (HL) is a haematological malignancy known for its incidence peak during puberty (Hjalgrim and Jarrett, 2020; Brice *et al.*, 2021). The disease is nowadays highly curable with an overall event-free survival of ∼90% (Mauz-Körholz *et al.*, 2022). Nevertheless, treatment for HL is associated with a risk of late effects including second malignancies and impaired fertility (Schaapveld *et al.*, 2015; Drechsel *et al.*, 2023). Chemotherapy and radiotherapy may damage differentiating spermatogonia and result in loss of sperm cell production in males. Recovery potential depends on the survival of the spermatogonial stem cells within the testis (Weinbauer *et al.*, 2010; Goossens *et al.*, 2020). Infertility becomes definite if the spermatogonial stem cells are fully depleted. Especially alkylating agents and pelvic irradiation have been associated with (definite) gonadal damage, in a dose-dependent manner (Brougham *et al.*, 2003; Romerius *et al.*, 2010; Meistrich, 2013; Drechsel *et al.*, 2023). In addition, the HL disease itself may also affect fertility, as impaired sperm parameters are often observed at time of diagnosis (Drechsel *et al.*, 2023). Although the impact of HL on spermatogenesis is presumed to be temporary, potentially related to a general inflammatory status, the exact underlying mechanisms and duration of these effects currently remain unknown.

Sperm quantity and quality are considered the best indicators of male fertility (Cooper *et al.*, 2010). In addition, in (post)pubertal boys and adult males, measurement of follicle-stimulating hormone (FSH) and inhibin B levels may be useful indirect and interdependent markers of spermatogenesis. FSH stimulates spermatogenesis via the Sertoli cells and inhibin B indirectly plays a role in FSH secretion via feedback mechanisms (Anderson *et al.*, 1997; Meachem *et al.*, 2001; Santi *et al.*, 2020). If spermatogenesis is impaired, FSH levels rise and inhibin B levels diminish (Franchimont *et al.*, 1972; Kelsey *et al.*, 2017). The ratio between inhibin B and FSH may be the most reliable reference to estimate fertility (Andersson *et al.*, 2004; Bordallo *et al.*, 2004; van Casteren *et al.*, 2008). In pre-pubertal boys, however, measurement of endocrine hormones such as FSH is of little value in a setting without a (HCG) stimulation test. Nevertheless, pre-pubertal inhibin B levels are still presumed to reflect the number and integrity of Sertoli cells, potentially offering a prediction of future reproductive ability (Raivio *et al.*, 2000; Andersson and Skakkebaek, 2001; Pierik *et al.*, 2003). Previous studies have observed high incidences of abnormal semen parameters and abnormal reproductive hormones in male childhood HL survivors, yet these studies have often included small patient numbers or studied treatment regimens that are no longer used (Drechsel *et al.*, 2023).

Nowadays, children with classical HL (cHL, ±95% of HL cases) in Europe are treated with vincristine, etoposide, prednisolone, and doxorubicin (OEPA) followed by consolidation treatment with cyclophosphamide, vincristine, prednisone, and dacarbazine (COPDAC) (Mauz-Körholz *et al.*, 2022). The number of administered chemotherapy courses and use of radiotherapy depends on the stage of disease and treatment response. All advanced-stage cHL patients are treated with alkylating agents at a dose equivalent to a CED-score (cyclophosphamide equivalent dose score) ≥4000 mg/m^2, which, according to the current PANCARE guideline, is associated with a high risk of infertility in males up to the age of 25 years old (Mulder *et al.*, 2021).

The current EuroNet-PHL-C2 trial aims to reduce the use of radiotherapy in standard cHL treatment in an effort to lower the risk of secondary malignancies, by administering intensified chemotherapy. In the intensified treatment-arm, patients receive DECOPDAC-21 consolidation courses, in which doxorubicin and

etoposide are added to COPDAC and the cumulative dose of (gonadotoxic) cyclophosphamide (thus CED-score of treatment) is increased by 25% (European Network-Paediatric Hodgkin Lymphoma Study Group (EuroNet-PHL), 2015). The present add-on study to the EuroNet-PHL-C2 trial was designed to prospectively evaluate the effect of the EuroNet-PHL-C2 protocol for childhood cHL on reproductive markers (i.e. sperm parameters and serum FSH, inhibin B levels and the inhibin B:FSH ratio) in newly diagnosed boys.

Materials and methods

Study design and study population

This international, multi-centre, observational prospective cohort study is an add-on study to the EuroNet-PHL-C2 trial (Clinicaltrials NCT02684708; EudraCT number 2012-004053-88). Inclusion criteria comprised a confirmed diagnosis of cHL in children up to the age of 18 years, scheduled for treatment according to the EuroNet-PHL-C2-protocol. Patients were recruited between January 2017 and September 2021 in 18 participating sites in the Netherlands (n = 5), Belgium (n = 6), Germany (n = 5), Austria (n = 1), and Czech Republic (n = 1). The present analysis included all participating boys, while the data of the participating girls have been described elsewhere (Drechsel *et al.*, 2024).

cHL treatment regimen

All patients received treatment for cHL according to the EuroNet-PHL-C2 protocol. A schematic description of the protocol is included in Fig. 1. In brief, the intensity of treatment depended on Ann Arbor stage of disease and presence of risk factors (bulky disease with tumour volume ≥200 ml, elevated erythrocyte sedimentation rate (ESR) ≥30 mm/h, E-lesions (extra nodal involvement)) and treatment response determined by PET-CT and MRI. Patients assigned to TL1 (treatment-level 1; early stage cHL) received two induction courses of OEPA followed by one course of COPDAC-28 consolidation treatment (in case of adequate treatment response) or involved-node radiotherapy (20 Gy) (in case of inadequate treatment response). Patients assigned to TL2 and TL3 (intermediate and advanced stages of cHL) received two courses of OEPA and were randomized to receive either standard COPDAC-28 or intensified DECOPDAC-21 consolidation chemotherapy (two courses in case of TL2 and four courses in case of TL3). Patients who declined randomization received standard COPDAC-28 consolidation. TL2 and TL3 patients with adequate response at time of early response assessment (ERA) had no indication for additional radiotherapy. TL2/TL3 patients with inadequate response at ERA underwent a late response assessment after chemotherapy (LRA). The COPDAC-28 treatment-arm received radiotherapy to all initially involved sites (20 Gy) with a boost (10 Gy) to LRA PET-positive sites. The DECOPDAC-21 treatment-arm only received radiotherapy to LRA PET-positive sites (30 Gy). The inclusion of the EuroNet-PHL-C2 trial ended in December 2020, leading to the cessation of randomization between COPDAC-28 and DECOPDAC-21 consolidation. The EuroNet-PHL-C2 protocol was adopted as standard cHL treatment protocol in Europe. Patients included after the end of accrual of the EuroNet-PHL-C2 trial received standard COPDAC-28 consolidation treatment, although in some cases (e.g. advanced-staged patients with inadequate response at ERA, where large radiotherapy fields should be applied), the treating paediatric oncologist could decide to give DECOPDAC-21 consolidation treatment. In the present fertility add-on study, we studied assigned treatment per-protocol.

Data collection and measurements

Data were collected at various time points, including: T0: at diagnosis, T1: after 2× OEPA, T1b: after 1× COPDAC-28 (only for TL1 patients), T2: after 2× COPDAC-28/DECOPDAC-21 (only for TL2/

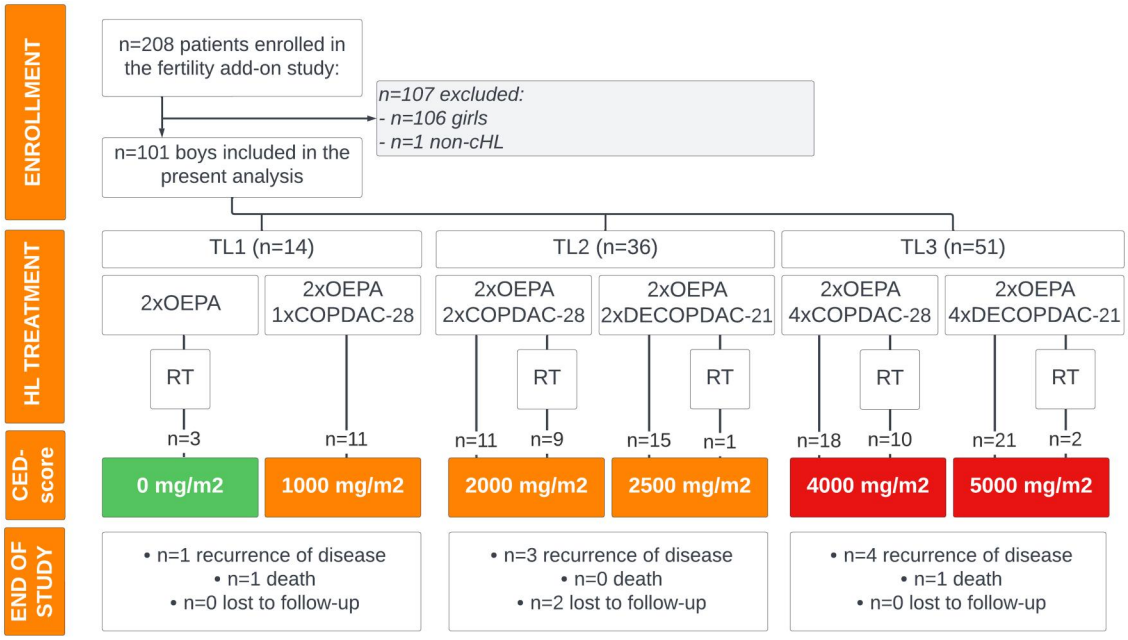

Figure 1. Study flow chart. Flow diagram of the fertility add-on study, depicting enrolment, assigned treatment according to the EuroNet-PHL-C2 protocol with corresponding CED-score, and follow-up/end of study for the present study. According to current PANCARE guidelines (Mulder *et al.*, 2021) treatment with a CED-score ≥4000 mg/m^2 should be considered high risk of infertility in boys. The depicted CED-score of high-risk treatment is coloured in red, low-risk in orange, and CED-score 0 g/m^2 is depicted in green. CED, cyclophosphamide equivalent dose; cHL, classical Hodgkin lymphoma; COPDAC-28, cyclophosphamide, vincristine, prednisone, and dacarbazine; DECOPDAC-21, doxorubicin, etoposide, cyclophosphamide, vincristine, prednisone, and dacarbazine; OEPA, vincristine, etoposide, prednisone, doxorubicin; OPPA, vincristine, procarbazine, prednisone, doxorubicin; TL, treatment level; RT, radiotherapy; n, number.

TL3 patients), T3: after 2× COPDAC-28/DECOPDAC-21 (only for TL3 patients), and T4: 2 years post-diagnosis. Study participation and data collection ended prematurely in case of progression of disease, recurrence, death, or loss to follow-up.

The primary outcomes of the present study included quantitative and qualitative sperm parameters from semen samples collected 2 years after diagnosis (in (post)pubertal boys). Secondary outcomes comprised sperm parameters at diagnosis (also compared to baseline semen) and measured serum levels of FSH, inhibin B, and the inhibin B:FSH ratio over time (separately analysed in pre- and (post)pubertal boys).

Semen analysis

All (post)pubertal boys were invited to deliver semen at diagnosis and 2 years post-diagnosis. Boys were instructed to maintain a period of abstinence of minimal 3–5 days prior to sperm collection. Actual duration of abstinence was not recorded in the CRF (case report form). All semen analyses were performed in specialized laboratories on site, in accordance with the WHO-criteria and the Björndahl *et al.* (2016) checklist (Cooper *et al.*, 2010). Reported results included semen volume (ml), concentration (million/ml), progressive motility (A + B%), and the calculated TMSC (in million, obtained by multiplying sperm concentration × volume × progressive motility divided by 100%). The WHO reference values for human semen characteristics were applied to define abnormal semen parameters (Cooper *et al.*, 2010). A sperm volume of <1.5 ml was defined as hypospermia and a progressive sperm motility <32% was defined as asthenozoospermia. A sperm count ≥15 million/ml was considered normozoospermic (also referred to as 'normal sperm count'), 5–15 million/ml oligospermic, 0–5 million/ml severe oligospermic, and if there were no spermatozoa in the ejaculate the sample was classified as azoospermic. All azoospermic and (severe) oligospermic sperm counts were labelled as 'abnormal'. A calculated TMSC <20 million was considered 'low'.

The collected semen samples at diagnosis were used for cryopreservation after determining semen parameters. Of note, the fertility add-on study was observational, with semen cryopreservation provided and conducted as part of standard care practice. If multiple semen samples were provided in effort to store semen, results of the sample with the highest sperm concentration were used for the study. Attempts and results of TESE (testicular sperm extraction) were separately registered in the CRF. If boys underwent testicular biopsy for the purpose of preserving testicular tissue (potentially as part of another trial, unrelated to the fertility add-on study), the procedure was noted in the CRF, but no additional (tissue) reports were available.

Blood sampling

Upon approval of the patient/parent, blood samples were drawn at each checkup. Samples were stored at the local sites at (minimal) −20°C. All available samples were transported on dry-ice and analysed in the laboratory of Amsterdam UMC, location AMC in May 2023. The lower limit of quantitation (LLOQ) for FSH was 0.1 IU/l with an intra-assay variation of <3.5% and inter-assay variation of <4.8% over the whole concentration range (Alinity I, Abbott Diagnostics). Serum inhibin B levels were analysed using the Gen II Inhibin B Elisa (Beckman Coulter), with an LLOQ 10 ng/l and an inter-assay variations <10% over the whole concentration range.

Patient characteristics and treatment data

Information on age (y), height (m), weight (kg), Tanner stage (G: genital and P: pubic hair) and testicular volume (measured by the attending physician using Prader orchidometer) were obtained during regular checkup visits, using a predesigned CRF. A testicular volume of ≥4 ml (or in case of missing: Tanner-G ≥ 2) was used to define the onset of puberty. Additional details about the cHL staging, administered treatments, and recurrences or secondary malignancies were collected from the central EuroNet-PHL-C2 database (or retrieved from medical files for those patients included after the end of accrual of the EuroNet-PHL-C2 study in December 2020). B symptoms at diagnosis included drenching night sweats, unexplained fever of >38.5°C and/or ≥10% weight loss in the previous 6 months. Active tumour sites within the porta hepatis, splenic hilum, mesenteric, upper/lower para-aortic, iliac, and inguinal areas were referred to as 'infradiaphragmatic'. The cumulative alkylating agent exposure for each TL and treatment-arm was estimated by calculating the CED-score; see Fig. 1 (Green *et al.*, 2014).

Statistical analysis

Measured continuous values of semen parameters were categorized/dichotomized, using the previously mentioned WHO reference values for human semen characteristics (Cooper *et al.*, 2010).

Continuous and categorical sperm parameters at diagnosis and 2 years post-diagnosis were compared between treatment-arms (TL-stages and COPDAC-28/DECOPDAC-21 consolidation schemes) using chi-square or Fisher's exact (categorical variables) or Mann–Whitney *U*-tests (continuous variables). Additionally, we conducted a descriptive comparison of semen concentration before and after treatment in the subset of boys who had provided semen samples at both time points.

Analyses of laboratory measurements were separately conducted in pre- and (post)pubertal boys. Inhibin B:FSH ratios were calculated by dividing serum inhibin B by serum FSH. FSH levels that were reported as '<0.1' were replaced by 0.1 (n = 3) and inhibin B levels '<10 μg/l' were replaced by 10 (n = 2) to enable statistical analysis. For (post)pubertal boys, FSH levels above 7.6 IU/l were considered elevated and inhibin levels below 100 ng/l were considered low (Schoor *et al.*, 2002; Brignardello *et al.*, 2016; Felicetti *et al.*, 2020). For pre-pubertal boys, abnormal reproductive hormone levels were defined as FSH above 5 IU/l and inhibin B below 50 ng/l (Crofton *et al.*, 2002; Kelsey *et al.*, 2016).

Median serum markers and number of patients with elevated FSH or low inhibin B were compared in unadjusted analyses between the treatment arms at the different time points using chi-square/Fisher's exact test or Mann–Whitney *U*-test. Furthermore, linear mixed models analyses, adjusted for age at time of sampling, were used to assess laboratory results over time (checkups T0 up to T4). A random intercept at patient level was used to control for the dependency of subsequent observations within the same patient. Potential differences in outcomes between treatment levels (TL1 vs TL2 vs TL3) and treatment-schemes (TL2/3 COPDAC-28 vs TL2/3 DECOPDAC-21) were assessed by adding these variables as covariates to the models.

Differences in laboratory results between (post)pubertal boys with normal or abnormal sperm concentrations were assessed among those who provided both serum and semen samples during either T0 or T4 checkups, using chi-square/Fisher's exact test or Mann–Whitney *U*-test. Furthermore, the impact of B symptoms on sperm parameters and laboratory measurements at diagnosis were evaluated by comparing all outcomes between (post)pubertal boys who presented with and without B symptoms in age-adjusted linear (continuous outcomes) and logistic (dichotomous outcomes) regression analyses. Results were

respectively presented as adjusted difference (B-coefficient) or odds ratio (OR) with their corresponding 95% CI.

Statistical analyses were performed using IBM SPSS Statistics version 28.0 (IBM Corp., 2021). *P*-values <0.05 were considered statistically significant.

Ethical approval

This study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects. The trial was approved by local ethical committees in each participating country. All patients (≥12 years old) and their parents/guardians (of patients <16 years old) provided written informed consent.

Results

Included boys

101 boys were included in the present analysis; see the study flowchart in Fig. 1. Baseline data are included in Table 1. A total of 73 boys (72%) were (post)pubertal with median age at diagnosis of 15.4 years (IQR 14.4; 16.6). Ten boys (14%) had early stage cHL and were assigned to TL1. The remaining 63 (post)pubertal boys were diagnosed with intermediate (n = 29 TL2, 59% COPDAC-28) or advanced stage (n = 34 TL3, 65% COPDAC-28) of cHL. There were 31 (42.5%) (post)pubertal boys who had B symptoms at the time of cHL diagnosis (n = 22/31 (71%) TL3). Among the 28 pre-pubertal boys (28%), median age at diagnosis was 9.6 years (IQR 6.6; 11.4). Four pre-pubertal boys were assigned to TL1, 7 to TL2 (43% COPDAC-28), and 17 to TL3 (35% COPDAC-28). The CED-score of assigned chemotherapy varied between 0 and 5000 mg/m^2 (Fig. 1). In total, 25 (25%) boys received radiotherapy after chemotherapy of whom six were irradiated in the pelvic area (n = 3 TL2-COPDAC-28, n = 3 TL3-COPDAC-28) with target dose 19.8 Gy. However, none of the boys received radiotherapy to the inguinal area, and estimated radiotherapy dose to the testis was less than 0.5–1 Gy for all boys. Median duration of follow-up was 24.0 months (IQR 22.0; 25.0) at time of the analysis. Two (2%) patients died and eight boys (8%) experienced a recurrence of disease during follow-up. One boy was diagnosed with a secondary malignancy (1%) and two (2%) patients were lost to follow-up.

Semen analysis in (post)pubertal boys

At diagnosis

There were 48 boys (66% of (post)pubertal boys) who delivered semen for cryopreservation before chemotherapy, with a median age of 16.0 years (IQR 15.4; 17.0) at time of semen collection. None of the boys underwent a TESE procedure. The youngest boy who successfully delivered semen was 13.6 years old. Results on semen volume, sperm concentration, progressive motility, and TMSC are reported in Table 2. Overall, 48% of semen samples (n = 23) were abnormal in terms of concentration (<15 mil/ml).

Table 1. Baseline characteristics of included (post)pubertal and pre-pubertal boys.

	(Post)pubertal boys[a] (n = 73)	Pre-pubertal boys (n = 28)
Age at diagnosis (years), median (IQR)	15.4 [14.4; 16.6]	9.6 [6.6; 11.4]
Ann Arbor stage of disease		
1	1 (1.4%)	0 (0.0%)
2	33 (45.2%)	7 (25.0%)
3	22 (30.1%)	11 (39.3%)
4	17 (23.3%)	10 (35.7%)
B symptoms[b]	31 (42.5%)	12 (42.9%)
ESR ≥ 30 mm/h	44 (61.1%)	17 (60.7%)
Bulky disease[c]	36 (50.7%)	8 (28.6%)
Involved tumour sites in infradiaphragmatic region[d]	23 (31.5%)	17 (60.7%)
Assigned treatment level and consolidation scheme		
TL1	10 (13.7%)	4 (14.3%)
TL2, COPDAC-28	17 (23.3%)	3 (10.7%)
TL2, DECOPDAC-21	12 (16.4%)	4 (14.3%)
TL3, COPDAC-28	22 (30.1%)	6 (21.4%)
TL3, DECOPDAC-21	12 (16.4%)	11 (39.3%)
Radiotherapy		
Received radiotherapy	18 (24.7%)	7 (25.0%)
Assigned but not given	2 (2.7%)	1 (3.6%)
Ended study participation before end of treatment	1 (1.4%)	0 (0.0%)
Pelvic radiotherapy[e]	4 (5.5%)	2 (7.1%)
Median follow-up, months (IQR)	24.0 [23.0; 28.0]	24.5 [22.0; 26.2]
End of study		
Recurrence	5 (6.8%)	3 (10.7%)
Secondary malignancy	1 (1.4%)	0 (0.0%)
Death	2 (2.7%)	0 (0.0%)
Lost to follow-up	2 (2.7%)	0 (0.0%)

Results are presented as median (IQR) or number (%).
Assigned treatment was according to the EuroNet-PHL-C2 protocol. TL1 patients receive 2× OEPA induction followed by either 1× COPDAC-28 or involved node radiotherapy depending on adequate or inadequate treatment-response.TL2/TL3 patients are randomized between the COPDAC-28 and DECOPDAC-21 treatment-arm and receive 2× OEPA induction followed by 2× (TL2) or 4× (TL3) (DE)COPDAC consolidation. Indication for radiotherapy depends on treatment response and treatment-arm.
OEPA, vincristine, etoposide, prednisone, doxorubicin; COPDAC-28, cyclophosphamide, vincristine, prednisone, and dacarbazine; DECOPDAC-21, doxorubicin, etoposide, cyclophosphamide, vincristine, prednisone, and dacarbazine; ESR, erythrocyte sedimentation rate; IQR, interquartile range; TL, treatment level.
[a] (Post)pubertal is defined as testicular volume (measured by Prader orchidometer) ≥4 ml or tanner stage (genital) >1.
[b] B symptoms, i.e. unexplained fever >38.5°C, weight loss of 10% during the past 6 months and drenching night sweats.
[c] Bulky disease is defined as contiguous tumour volume of at least 200 ml.
[d] Including tumour sites in the porta hepatis, splenic hilum, mesenteric, upper para-aortic, lower para-aortic, iliac, and inguinal area.
[e] All patients who received pelvic radiotherapy were within the COPDAC-28 treatment-arm and target dose was 19.8 Gy. None of the boys were irradiated in the inguinal or testicular area, expected radiation directed towards the testes was <0.5–1 Gy.

More specifically, 4 boys (8%) had azoospermia, 11 (23%) had severe oligozoospermia, and 8 (17%) had oligozoospermia. In total, 22 boys (46%) had asthenozoospermia and 26 (54%) boys had semen volume <1.5 ml. The sperm concentration was statistically significantly lower in the 21 boys who presented with B symptoms compared to those (n = 27) who had no B symptoms at diagnosis (median 7.6 mil/ml (IQR 1.0; 13.6) vs 30.0 mil/ml (IQR 10.9; 68.5), adjusted difference −27.4 (95% CI −52.5; −2.3), $P = 0.03$, and the OR for having an abnormal sperm concentration was 2.3 (95% CI 1.0; 3.8), $P = 0.001$), see Supplementary Table S1.

Of note, five boys (i.e. 1 (post)pubertal and 4 pre-pubertal) underwent testicular biopsy to preserve testicular tissue in a research setting prior to starting cHL treatment.

At 2 years post-diagnosis

At 2 years post-diagnosis, 31 boys collected semen samples for analysis; 16 (52%) had abnormal sperm concentrations, i.e. 4 (13%) had azoospermia, 6 (19%) had severe oligozoospermia, and 6 (19%) had oligozoospermia (see Table 2). The group-median TMSC was low (12.3 million) (IQR 0.4; 33.9) and below reference values in 22 boys (71%). Boys in higher TL-stages had lower sperm concentrations when compared to patients with less advanced cHL (TL3 vs TL2, $P = 0.07$). All three TL1 staged patients who delivered semen during follow-up had normozoospermia, while 36% (n = 4/11) of the TL2-stage and 71% (n = 12/17) of the TL3-stage boys had abnormal concentrations; see Supplementary Table S2.

The sperm concentration, progressive motility, volume, and TMSC after 2 years are depicted as cumulative distribution figures per treatment arm in Fig. 2. Median sperm concentration was lowest in the TL3-DECOPDAC-21 arm, when compared to the other TL2/TL3 treatment arms (median sperm concentration 1.4 mil/ml in TL3-DECOPDAC-21 vs 13.0 mil/ml TL3-COPDAC-28, 29.0 mil/ml TL2-DECOPDAC-21 and 20.0 mil/ml TL2-COPDAC-28, $P = 0.08$ when compared to TL3-COPDAC-28 patients, respectively). Similarly, the median progressive motility was 16.5% in TL3 staged boys treated with DECOPDAC-21, while the median motility ranged between 47.0% and 54.0% in the other TL2/3 treatment arms ($P = 0.25$ when compared to TL3-COPDAC-28); see Supplementary Table S2. Three out of the six boys who had received pelvic radiotherapy delivered semen after 2 years (n = 1 TL2-COPDAC-28, n = 2 TL3-COPDAC-28); one had a normal sperm concentration after treatment and the other two boys were azoospermic. Of the 31 boys who delivered semen at 2 years post-diagnosis, 28 had also delivered semen at diagnosis. A schematic comparison of their sperm concentration over time, including assigned TL-stage and received consolidation treatment, is included in Supplementary Fig. S1.

Serum FSH and inhibin B

(Post)pubertal boys

A total of 290 blood samples obtained from boys who were (post)pubertal at time of diagnosis were included in the analysis (n = 68 at diagnosis, n = 163 during treatment, n = 59 at 2 years post-diagnosis). The measured FSH and inhibin B levels are presented as boxplots in Fig. 3. Median FSH was 3.8 IU/l (IQR 1.6; 5.5) and inhibin B 167.0 ng/l (IQR 125.0; 217.0) at diagnosis. Ten boys (15%) had FSH >7.6 IU/l and eight (12%) had low serum inhibin B (<100 ng/l). There were no statistically significant differences in circulating FSH and inhibin B between the boys who presented with B symptoms and those who had no B symptoms at diagnosis (Supplementary Table S1).

During treatment, serum FSH gradually increased and serum inhibin B gradually decreased, especially among advanced-stage cHL patients (estimated effect after 2× OEPA FSH: 5.2 (95% CI 4.1; 6.4) and inhibin B: −39.8 (95% CI −56.4; −23.2), both $P < 0.001$, analyses were adjusted for age at time of sampling). Overall, 38 boys (57%) had elevated FSH and 36 (54%) had low inhibin B after completion of therapy; 57% of boys with serum values outside of normal references were TL3 staged patients.

After 2 years, serum FSH and inhibin B levels had often recovered to within normal reference limits (80% FSH recovery, 60% inhibin B recovery), and there were no statistically significant differences in median serum FSH and inhibin B when compared to pre-treatment values. Median FSH was 4.2 IU/l (IQR 2.7; 6.0, 9 boys (15%) high FSH) and inhibin B was 156.0 ng/l (IQR 108.0; 199.0, 13 (22%) boys had low inhibin B). Inhibin B levels and inhibin B:FSH ratio were significantly lower in TL3 patients, when compared to TL2 patients (inhibin B: 121.0 ng/l vs 182.5 ng/l, ratio:

Table 2. Semen analyses at diagnosis and 2 years post-diagnosis in (post)pubertal boys.

	Semen analysis	
	At diagnosis (n = 48)	**2 years post-diagnosis (n = 31)**
Age at time of collection, years (IQR)	16.0 [15.4; 17.0]	18.8 [18.0; 19.4]
Tanner-G, median (IQR)	V (IV-V)	V (V-V)
Mean testicular volume,[a] median (IQR)	20.0 [15.0; 22.8]	20.0 [18.8; 25.0]
Semen volume (ml)	1.4 [0.6; 2.0]	1.9 [1.3; 3.4]
Hypospermia (<1.5 ml)	26 (54.2%)	9 (29.0%)
Sperm concentration (mil/ml), median (IQR)	17.0 [2.9; 38.9]	13.0 [2.2; 35.7]
Normozoospermia (≥15 mil/ml)	25 (52.1%)	15 (48.4%)
Oligozoospermia (5–15 mil/ml)	8 (16.7%)	6 (19.4%)
Severe oligozoospermia (0–15 mil/ml)	11 (22.9%)	6 (19.4%)
Azoospermia (0 mil/ml)	4 (8.3%)	4 (12.9%)
Normal sperm concentration	25 (52.1%)	15 (48.4%)
Abnormal sperm concentration[b]	23 (47.9%)	16 (51.6%)
Progressive motility a + b (%), median (IQR)	32.0 [10.8; 49.2]	48.0 [13.5; 60.9]
Asthenozoospermia (<32%)	22 (45.8%)	11 (35.5%)
Total motile sperm count (TMSC) (mil), median (IQR)	7.8 [0.3; 28.7]	12.3 [0.4; 33.9]
Low TMSC (<20 mil)	31 (64.6%)	22 (71.0%)

IQR, interquartile range.
Applied references values for semen analysis are according to WHO criteria (Cooper et al., 2010).
[a] Testicular volume defined by Prader orchidometer.
[b] Abnormal sperm concentration includes azoospermia, severe oligozoospermia and oligozoospermia, i.e. <15 mil sperm cells/ml.
None of the boys who were pre-pubertal at diagnosis had delivered sperm at 2 years post-diagnosis.

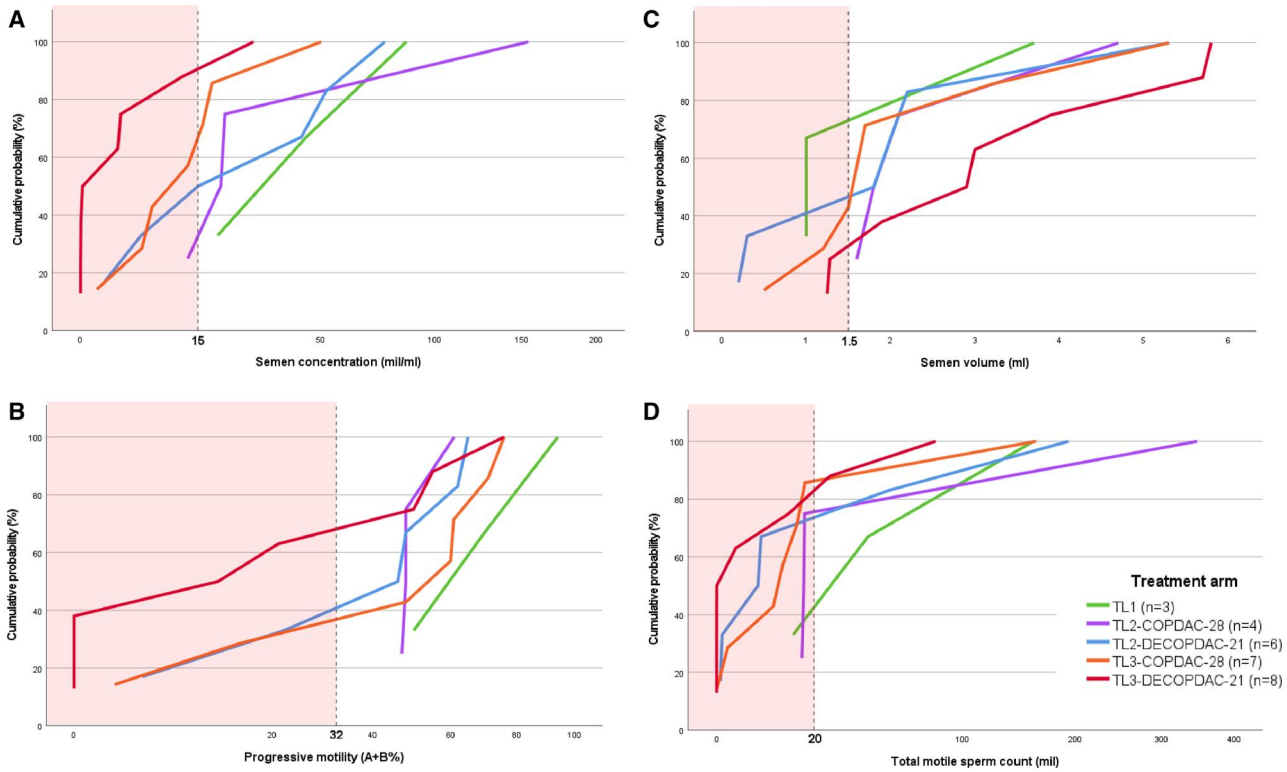

Figure 2. Sperm parameters at 2 years from childhood classical Hodgkin lymphoma diagnosis, according to assigned treatment level and consolidation treatment. Cumulative distribution figures depicting the cumulative proportion of patients with a specific (**A**) sperm concentration (**B**) progressive sperm motility (**C**) semen volume or (**D**) total motile sperm count (TMSC) at 2 years from Hodgkin lymphoma diagnosis. Vertical dashed lines indicate WHO reference cutoff values to define abnormal sperm parameters, and the red-marked areas highlight the abnormal range (Cooper *et al.*, 2010). TL, treatment level. Assigned treatment was according to the EuroNet-PHL-C2 protocol. TL1 patients received 2× OEPA±1× COPDAC-28 (CED-score = 0–1 g/m^2), TL2 patients received 2× OEPA + 2× (DE)COPDAC (CED-score = 2–2.5 g/m^2), TL3 patients received 2× OEPA + 4× (DE)COPDAC (CED-score 4–5 g/m^2). OEPA, vincristine, etoposide, prednisone, doxorubicin; COPDAC-28, cyclophosphamide, vincristine, prednisone, and dacarbazine; DECOPDAC-21, doxorubicin, etoposide, cyclophosphamide, vincristine, prednisone, and dacarbazine. Additional explanation on how to read a cumulative distribution figure: each of the cumulative distribution figures illustrates the relationship between the respective assessed sperm parameter on the x-axis and the cumulative probability on the y-axis. Each point on the curve represents the cumulative probability of observing a value less than or equal to the corresponding value on the x-axis. For instance, consider figure A (semen concentration): for a sperm concentration of 15 mil/ml on the x-axis, the cumulative probability is 90% for the red line (indicating the TL3-DECOPDAC-21 treatment arm). This implies that ~90% of the assessed sperm concentrations in this specific subgroup are ≤15 mil/ml.

26.0 vs 64.9, $P = 0.004$ and $P = 0.02$ according to the linear mixed models, respectively). The linear mixed models did not present any significant differences in reproductive hormones over time when comparing the COPDAC-28/DECOPDAC-21 treatment schemes; see Supplementary Table S3.

Pre-pubertal boys

The total number of included samples obtained from pre-pubertal boys was 123 (n = 26 at diagnosis, n = 72 during treatment, n = 25 at 2 years post-diagnosis). As expected, median serum FSH and inhibin B levels were relatively low at diagnosis (FSH: 0.6 IU/l (IQR 0.3; 1.0) and inhibin B: 88.5 ng/l (IQR 75.2; 123.2)). We observed a small and gradual increase in serum FSH and inhibin B levels during treatment and follow-up (estimated effect at 2 years post-treatment FSH: 1.5 (95% CI 0.7; 2.2) and inhibin B: 43.4 (95% CI 16.4; 58.7), both $P < 0.001$). After 2 years, median FSH was 1.8 IU/l (IQR 0.8; 3.1) and inhibin B was 126.0 ng/l (IQR 102.0; 179.0).

Serum reproductive markers in boys with normal or abnormal sperm concentration

Table 3 includes an overview of measured reproductive hormones in the (post)pubertal boys who delivered sperm at diagnosis (n = 45 boys of whom both semen and hormone levels were available) and after 2 years (n = 28 boys of whom both semen and hormone levels were available). At diagnosis, there were no statistically significant differences between the reproductive hormones of boys with a normal or abnormal sperm concentration. However, at 2 years from diagnosis, the 14 boys with a sperm concentration below 15 million had higher FSH (5.0 IU/l vs 3.1 IU/l, $P = 0.18$) and lower inhibin B (102.0 ng/l vs 163.5 ng/l, $P = 0.04$) levels, resulting in lower inhibin B:FSH ratios (24.7 vs 56.3, $P = 0.06$) compared to the 14 boys with normal sperm concentration. Nevertheless, 10 out of the 14 boys (71%) with oligozoo/azoospermia still had 'normal' FSH <7.6 IU/l, and 7 out of 14 (50%) had inhibin B levels within normal range.

Discussion

This study prospectively evaluated reproductive markers in boys with newly diagnosed cHL and treated according to the European EuroNet-PHL-C2 treatment protocol. More than half of the (post) pubertal boys (52%) who provided a semen sample had low semen concentrations and even more boys had low TMSC (71%) at 2 years from diagnosis. Serum FSH gradually increased, and inhibin B decreased during chemotherapy with subsequent normalization in most (post)pubertal boys (80% when looking at FSH recovery and 60% when looking at inhibin B recovery), while FSH

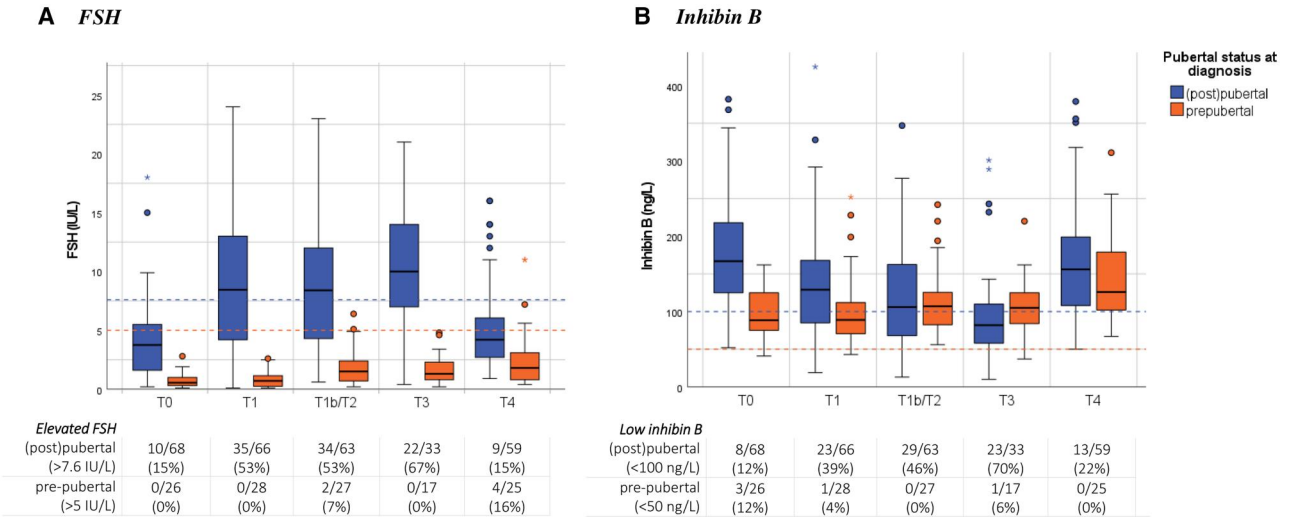

Figure 3. Serum FSH and inhibin B during treatment for childhood Hodgkin lymphoma up to 2 years follow-up in pre- and (post)pubertal boys. (**A**) FSH, B inhibin. (**B**) Boxplots depicting the distribution of uncorrected serum FSH or inhibin B from diagnosis up to 2 years post-diagnosis, including the median (centerline), interquartile range (end of the box), and range (end of the whiskers). Separate dots are outliers. Patients who were pre-pubertal at time of diagnosis are shown in orange, and boys who were (post)pubertal at diagnosis (definition testicular volume ≥4 ml or in case of missing Tanner-G ≥2) are shown in blue. The dashed lines in blue highlight applied cut-off scores to define normal (≤7.6 IU/l) or high FSH (>7.6 IU/l), respectively normal (≥100 ng/l) or low inhibin B (<100 ng/l) in (post)pubertal boys. The dashed lines in orange highlight applied cut-off scores to define normal (≤5 IU/l) or high FSH (>5 IU/l), respectively normal- (≥50 ng/l) or low inhibin B (<50 ng/l) in pre-pubertal boys. FSH, follicle-stimulating hormone; N, number. Timing sampling: T0, at diagnosis; T1, after 2× OEPA; T1b, after 1× COPDAC; T2, after 2×(DE)COPDAC; T3, after 4×(DE)COPDAC; T4, 2 years post-diagnosis.

and inhibin B levels were overall low and remained relatively stable in pre-pubertal boys.

Similar to previous reports, low semen concentration and low motility were prevalent at time of cHL diagnosis (48% and 46%, respectively) (Drechsel *et al.*, 2023). We observed a clear correlation between impaired semen parameters and B symptoms at diagnosis, which are often present in advanced-stage cHL patients as an expression of inflammation. Serum FSH and Inhibin B were far less frequently affected at diagnosis and there was no clear correlation between abnormal semen and abnormal reproductive hormones at diagnosis, as has also been described previously in adult HL patients (Ragni *et al.*, 1985; Rueffer *et al.*, 2001; Sieniawski *et al.*, 2008). These results align with the hypothesis that the impaired health status and inflammatory status at time of cHL diagnosis can temporarily affect the sperm quality, but will probably not cause persisting gonadal damage (Tal *et al.*, 2000; Rueffer *et al.*, 2001; Laddaga *et al.*, 2021).

Moreover, the volume of the collected sperm samples at time of cHL diagnosis was also remarkably low (54% <1.5 ml, n = 26/48). Perhaps, the observed low sperm volume is related to the young age of the included boys, although median testicular volume was 20.0 ml (IQR 15.0; 22.8) among the included boys and many were at a Tanner-G stage V (56%). A previous large nationwide French cohort study demonstrated that sperm parameters, including ejaculate volume, significantly increased with age at time of cryopreservation (Daudin *et al.*, 2015). Still, the (cancer) disease itself could also have a negative impact on produced sperm volume at time of diagnosis. Three studies among young adolescents (mean age ± 25–26 years) observed significantly lower sperm volumes of samples collected from HL patients, when compared to patients with testicular cancer (Amirjannati *et al.*, 2011; Bizet *et al.*, 2012; Caponecchia *et al.*, 2016). Additionally, the reported median sperm volume was much higher in a large Danish cohort study including healthy males with median age of 19 years, when compared to the observed median sperm volume of collected samples at time of diagnosis in our cohort (2.7 ml (IQR 1.9; 3.7) vs 1.4 ml (IQR 0.6; 2.0), respectively) (Gaml-Sørensen *et al.*, 2024). Clinicians should realize that the presence of HL disease itself might have an adverse effect on sperm parameters and thereby affect the success rate of semen cryopreservation as well as number of stored straws. The storage of testicular tissue might offer a solution for fertility preservation in young, pre-pubertal and/or high-risk boys who are not able to collect semen. In most countries this procedure is currently only offered in research setting and is not yet considered standard of care (Mulder *et al.*, 2021). However, it is active field of research and this may change in the coming years, specifically since in a few countries ethical approval for immature testicular grafting is in place. The impact of disease on (pre-pubertal) testicular tissue is still relatively unknown, yet a recent study observed that the quantity of spermatogonia in pre-pubertal testicular tissue may also be affected by haematological disorders before treatment (Masliukaite *et al.*, 2023).

As expected, abnormal sperm parameters during follow-up appeared to be specifically present in boys assigned to the highest TL-stage with a higher chemotherapy burden, when compared to patients treated for less advanced cHL. Notwithstanding, 36% (n = 4/11) of TL2 patients also had low sperm counts at 2 years from diagnosis, while they received treatment with an estimated low-risk of infertility based on the PANCARE guidelines (i.e. CED-score <4000 mg/mg) (Mulder *et al.*, 2021). It remains unclear whether recovery of spermatogenesis will occur (and to what extent). cHL treatment lasted 3 to 6 months in most boys, hence the semen samples were collected at least 1.5 years after the last administration of chemotherapy. Based on previous reports, recovery of spermatogenesis could occur years after completion of chemotherapy (Howell and Shalet, 2005). Studies in childhood HL patients case-wise reported improved sperm counts even after 10–12 years after treatment, particularly among survivors with a relatively low exposure to alkylating agents (da Cunha *et al.*, 1984; Sy Ortin *et al.*, 1990). At present, we do not have the ability or tools to predict occurrence

Table 3. Circulating serum FSH and inhibin B in (post)pubertal boys with a 'normal' sperm concentration vs low sperm concentration at diagnosis and 2 years post-diagnosis.

	(Post)pubertal boys						
	Normal sperm concentration	Abnormal sperm concentration	Normal vs abnormal concentration (*P*-value)	Normozoo-spermia	Oligozoo-spermia	Severe oligozoo-spermia	Azoospermia
At diagnosis	*n* = 23	*n* = 22		*n* = 23	*n* = 7	*n* = 11	*n* = 4
Serum FSH (IU/l)	4.8 [3.0; 6.8]	4.3 [2.7; 5.8]	0.59	4.8 [3.0; 6.8]	4.3 [2.7; 5.3]	4.7 [3.4; 6.2]	3.5 [2.1; 5.2]
Elevated FSH (>7.6 IU/l)	6 (26.1%)	3 (13.6%)	0.46	6 (26.1%)	1 (14.3%)	1 (9.1%)	1 (25.0%)
Serum inhibin B (ng/l)	169.0 [125.0; 222.5]	170.0 [130.0; 204.8]	0.95	169.0 [125.0; 222.5]	165.0 [136.5; 239.0]	175.0 [125.5; 195.0]	181.5 [141.0; 208.5]
Low inhibin B (<100 ng/l)	2 (8.7%)	3 (13.6%)	0.66	2 (66.7%)	0 (0.0%)	2 (18.2%)	1 (25.0%)
Inhibin B:FSH ratio	33.4 [22.6; 70.5]	41.9 [24.4; 70.4]	0.91	33.4 [22.6; 70.5]	38.4 [30.6; 86.6]	37.6 [21.7; 49.1]	66.6 [38.1; 100.5]
After 2 years	*n* = 14	*n* = 14		*n* = 14	*n* = 5	*n* = 6	*n* = 3
Serum FSH (IU/l)	3.1 [2.7; 4.8]	5.0 [2.8; 7.7]	0.18	3.1 [2.7; 4.8]	3.2 [2.3; 5.1]	5.1 [4.6; 7.1]	16.0 [8.7; 16.0]
Elevated FSH (>7.6 IU/l)	0 (0.0%)	4 (28.6%)	0.10	0 (.%)	0 (.%)	2 (33.3%)	2 (66.7%)
Serum inhibin B (ng/l)	163.5 [145.0; 207.2]	102.0 [74.2; 177.8]	0.04	163.5 [145.0; 207.2]	147.0 [84.0; 159.0]	102.0 [77.8; 167.5]	54.0 [52.5; 186.0]
Low inhibin B (<100 ng/l)	0 (.%)	7 (50.0%)	0.006	0 (0.0%)	2 (40.0%)	3 (50.0%)	2 (67.0%)
Inhibin B:FSH ratio	56.3 [32.8; 69.4]	24.7 [11.1; 60.3]	0.06	56.3 [32.8; 69.4]	28.8 [23.1; 69.1]	19.6 [11.5; 34.2]	3.4 [3.3; 124.0]

Results are presented as median (IQR) and number (percentage). *P*-values were calculated by Mann–Whitney *U*-tests (continuous) or chi-square/Fisher's exact (categorical). Normal sperm concentration and normozoospermia refers to ≥15 mil sperm count; abnormal sperm concentration is defined as sperm count <15 mil/ml; oligozoospermia 5–15 mil/ml, severe oligozoospermia 0–5 mil/ml, and azoospermia 0 sperm cells (Cooper *et al.*, 2010).
FSH, follicle-stimulating hormone; vs, versus.

and timing of (long-term) recovery after cancer treatment. We did observe higher FSH- and lower inhibin B levels in patients with lower sperm concentration in our study, yet still 50% (n = 7/14) of boys with oligozoo/azoospermia had reproductive hormones within normal ranges at 2 years post-diagnosis. FSH and inhibin B levels reflect the early-phases of spermatogenesis, but are not considered predictive of future regeneration of spermatozoa (Khanehzad *et al.*, 2021). Participating boys of the fertility add-on study will be invited to deliver another blood and sperm sample for analysis 5-year post-diagnosis, and these results will be crucial to estimate lasting gonadotoxicity rates.

There is an ongoing discussion whether a pre-pubertal/dormant state of the gonads may act as a protector against gonadotoxicity (Pennisi *et al.*, 1975; Sherins *et al.*, 1978; Bayle-Weisgerber *et al.*, 1984; Hobbie *et al.*, 2005; Zaletel *et al.*, 2010; Brignardello *et al.*, 2016; Drechsel *et al.*, 2023). We observed relatively low FSH and inhibin B levels throughout all assessments among the 28 included boys who were pre-pubertal at time of diagnosis, which is most likely the result of the pre-pubertal state itself, where overall circulating sex steroid levels are generally low (Miles *et al.*, 2015). The observed median inhibin B levels and small increase during the period of 2 years since diagnosis seems to correlate well with the normative model of serum inhibin B published by Kelsey *et al.* (2016). After 2 years, the boys were still too young and physically too immature (i.e. n = 12 still pre-pubertal, only 1 boy with Tanner 4, all testicular volume ≤12 ml) to deliver sperm for analysis. Therefore, it remains unclear whether cHL treatment affected spermatogonial stem cells and (future) spermatogenesis in these pre-pubertal boys. Larger studies, including long-term data are needed to assess the impact of gonadal development at diagnosis on the gonadotoxicity of treatment.

Potential benefits and risks of intensified chemotherapy to treat cHL should be carefully considered to determine superiority of the two treatment schemes of the EuroNet-PHL-C2 protocol. Any successful reduction in radiotherapy must be weighed against potentially more gonadotoxic chemotherapy, that also contains a higher cumulative dosage of anthracyclines (associated with other late effects such as cardiotoxicity and risk of (female) breast cancer) (Dempke *et al.*, 2023; Wang *et al.*, 2023). Based on the present analysis, advanced-stage cHL patients receiving DECOPDAC-21 could potentially be at a higher risk of impaired spermatogenesis after treatment, when compared to advanced-stage cHL patients receiving the COPDAC-28 consolidation arm of the EuroNet-PHL-C2 protocol. However, considering the limited power due to low numbers and short follow-up of this fertility study, we cannot draw any definite conclusions at the moment.

Strengths and limitations

The fertility add-on study is the first study to evaluate reproductive markers during and after treatment for childhood cHL in a longitudinal design. The results improve knowledge on the acute and late effects of treatment for cHL according to the EuroNet-PHL-C2 protocol in Europe, and the effect of disease itself, on sperm parameters, serum FSH and inhibin B. However, several limitations should be noted. Semen results were based on a single attempt (with some exceptions for cryopreservation at diagnosis) and could not be adjusted for abstinence period or incomplete semen collection. Semen samples were analysed in multiple laboratories, although all adhered to the WHO standard for semen analysis. However, there remains risk of inter-observer variation in the semen analyses, which also extends to the measurement of testicular volume using the Prader Orchidometer. Actually only 50% (n = 28/56) of boys from whom semen collection was expected (based on Tanner stage-G IV or V and/or testicular volume ≥15 ml) delivered semen at 2 years post-diagnosis. The patients could personally benefit from semen collection at diagnosis considering cryopreservation, while at follow-up there were no direct personal benefits, which may have affected participation rate. Moreover, testosterone and luteinizing hormone were not evaluated in the present study. However, the expected added value of measuring these hormones as reproductive markers is fairly limited as, based on previous studies, the hormones usually remain unaffected during chemotherapy and most boys who are treated for cancer during or before puberty

actually go through natural pubertal development (Müller, 2002; Stukenborg et al., 2018). The overall sample size and follow-up were limited. Therefore, due to a lack of power, we were unable to perform adjusted subgroup analyses on patients receiving pelvic radiotherapy and pre-pubertal boys at diagnosis.

Clinical implications and future research

The current study demonstrated an adverse effect of cHL treatment according to the EuroNet-PHL-C2 protocol on spermatogenesis in boys. Impaired sperm concentration and reduced progressive sperm motility were commonly observed in samples collected at 2 years from diagnosis. Nevertheless, samples of most boys contained at least some viable sperm cells and the potential for recovery remains uncertain. The measurement of FSH and inhibin B appears to be of low value to predict low sperm quality at 2 years from cHL treatment.

Boys with newly diagnosed cHL should be encouraged to deliver sperm for cryopreservation prior to treatment, whenever possible. The state of disease could negatively affect sperm quality, but boys should not be discouraged if the semen quality is impaired, because oligospermic samples can still be used for future IVF (*in vitro* fertilization) or ICSI (intracytoplasmic sperm injection) treatment. As long as there are vital sperm cells produced, there is a chance of having a biological child as a male cancer survivor. Although HL primarily occurs during or after puberty, young pre-pubertal boys who are unable to deliver semen for cryopreservation could also be confronted with the disease. According to the current PANCARE guidelines, testicular biopsy for cryopreservation can be offered to patients with substantial risk of infertility based on disease staging and planned treatment, as part of clinical trials or approved protocol (Mulder et al., 2021). Future studies should focus on potential recovery of spermatogenesis on the long term, but also evaluate clinical outcomes, including the desire for children, pregnancy rates (with or without assisted reproductive technology), and utilization of frozen semen samples and testicular tissue. In addition, the impact of new agents to treat HL (such as Brentuximab, Vedotin, Nivolumab, Pembrolizumab) on gonadal function should also be examined.

Summarizing conclusions

The current EuroNet-PHL-C2 protocol for childhood cHL impairs semen quality and quantity. Adverse semen parameters after 2 years were specifically present in boys treated for advanced stage cHL. However, additional long-term and adequately powered data are crucial to assess potential recovery of spermatogenesis and clinical impact on fertility. Serum FSH and inhibin B appear to be temporarily affected by chemotherapy with subsequent normalization after completion of treatment in most post-pubertal boys. In pre-pubertal boys, reproductive hormones were overall low and remained relatively stable during chemotherapy.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

Individual participant data that underlie this article cannot be shared publicly because of privacy. Pseudonymized data will be shared on reasonable request for an ethically approved study protocol, after compiling a data-sharing agreement.

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Authors' roles

M.A.V., A.B., G.J.L.K., W.H.W., E.v.D.-d.B., D.K., C.M.-K., M.C., A.U., L.R., and F.S.S. were responsible for the trial design and study-setup. D.H. and K.C.E.D. were also involved during the data collection. K.C.E.D. cleaned the data and performed the statistical analysis, supported by J.W.R.T., S.L.B., and M.A.V. K.C.E.D. wrote the first version of the manuscript. All authors were involved in data interpretation, critically reviewed the manuscript, and approved the submitted final version.

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Conflict of interest

C.M.-K., D.K., W.H.W., D.H., M.C., A.U., and A.B. were involved in the development of the EuroNet-PHL-C2 regimen. The other authors declare no potential conflict of interest.

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