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Decreased spermatogonial quantity in prepubertal boys with leukaemia treated with alkylating agents

Leukemia (2017) **31,** 1460–1463; doi:10.1038/leu.2017.76

Cancer therapies are known to cause fertility impairment as a long-term treatment side effect. Although postpubertal male cancer patients have the possibility to cryopreserve semen before gonadotoxic treatment, prepubertal boys do not yet produce spermatozoa.[1] Therefore, proposed fertility-preservation strategies for these boys are cryopreservation of testicular tissue followed by autotransplantation of spermatogonial stem cells, tissue grafting or *in vitro* maturation.[1–3] Numerous animal studies, including those of non-human primates, have proven the successful accelerated recovery of spermatogenesis following the application of fertility-restoration techniques. Hence, at this moment, testicular tissue cryopreservation is offered to patients as a part of clinical research programs, while the fertility-restoration strategy remains experimental.[3]

Male patients with leukaemia who are at a high risk of infertility include those receiving allogenic haematopoietic stem cell transplantation, while conventional chemotherapy is associated with a low risk of infertility.[4,5] Therefore, the majority of patients with haematological malignancies do not meet the criteria for testicular tissue cryopreservation at the time of the original diagnosis. However, disease response or relapse may result in patients undergoing potentially sterilizing therapy regimens. This means that many patients with leukaemia have already received chemotherapy before testicular tissue cryopreservation is offered.[1] The potential effect of previous chemotherapy must be taken into account when testis biopsy and storage are considered for these patients.

In order to optimize the cryobanking of prepubertal testicular tissue, more information on the effects of cancer therapies on spermatogonial quantities is required. Recently, reference values concerning spermatogonial quantity in human testes throughout healthy prepuberty were established.[6] In the present study, these reference values were used to evaluate the effects of leukaemia treatment on spermatogonial quantity and hence spermatogenic recovery in prepubertal boys with acute lymphoblastic leukaemia.

The study material consisted of testis samples from prepubertal boys with acute lymphoblastic leukaemia who had undergone routine bilateral/unilateral testicular biopsy to examine possible testicular leukaemia at the cessation of antileukaemia therapy at University Central Hospitals in Helsinki and Turku between 1979 and 1995. The Research Ethics Committees of Helsinki University Hospital (No 192/13/03/03/2013) and Turku University Hospital (DNR 1905-32/300/05) approved the study. Altogether, 37 boys were identified through hospital records and enrolled in the study. Eighteen of these boys had earlier been included in a study to evaluate the expression of spermatogonial markers during leukaemia therapy, as previously described.[7] Testicular biopsies were performed at the end of the treatment, during the last days of the per oral maintenance therapy. Four of the 37 boys were subjected to two biopsies—one at the end of induction therapy (50 days after diagnosis) and a second biopsy at the end of the treatment (Figure 1). Antileukaemia therapy was carried out as described earlier.[8,9] Briefly, 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. Alkylating agents were included in the therapy for 21 patients. Alkylating agent exposures, mean cumulative cyclophosphamide equivalent doses (CEDs)[10] and cumulative doses of anthracyclines using conversion factor 1 for doxorubicin and 0.833 for daunorubicin are presented in Table 1. Leukaemic infiltration was detected in four samples (Figure 1a). These four patients experienced testicular relapse and underwent a multidrug chemotherapy regimen together with testicular irradiation at a dose of 24 Gy. An additional testis biopsy was available for analysis after irradiation from one patient. Of the 37 boys, 23 became long-term survivors.

Testicular biopsy samples from the 37 patients were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin. In each case, blind analysis of germ cell numbers was performed in sections by an experienced examiner using a bright-field microscope (Labophot, Nikon, Tokyo, Japan) and spermatogonia per tubular cross-section (S/T) were counted. At least 25 tubular cross-sections per biopsy sample were evaluated to achieve result validity.[11] In 2 patients, 12.2 and 14.0 years of age treated with non-alkylating agents, tissue samples showed spermatocytes and spermatids in some tubules. However, the morphology did not allow us to calculate exact spermatogonial quantity; hence S/T data from these two cases were not included in this study. In all samples, the different cell types were identified on the basis of size, shape and location, according to Paniagua and Nistal.[12]

Adult testicular volume documented by pubertal maturation to Tanner stage 5, achievement of final height or age >18 years was available in connection with 23 patients (Table 1). The volume was measured using an orchidometer (Endocrine society, Washington DC, USA) or a ruler (Faber-Castell, Stein, Germany), taking the mean value if both testes were evaluated. Semen analysis was performed in 17 cases (Table 1) and consisted of evaluation of sperm motility (a+b) and total sperm count as described by WHO.[13] Patients were considered to be azoospermic when no spermatozoa were detected in the sample in any field of a double-chambered haemocytometer (Neubauer Improved, VWR International, Stockholm, Sweden) before or after centrifugation.[13]

Simple and multiple regression analysis, Student's unpaired *t*-test (normally distributed variables), Mann–Whitney *U*-test (non-normally distributed variables) and χ^2 test were used as appropriate to determine the relationships between spermatogonial quantity, treatment characteristics and gonadal function. Results were reported as mean ± s.d. and range. Analyses were performed using the IBM SPSS Statistics V22.0 software (IBM Corp, Armonk, NY, USA). STATA 14.2 (StataCorp, College Station, TX, USA) was used to explore associations in polynomial regression functions.

Results of the analysis on 37 testicular biopsy samples showed that numbers of S/T in samples from patients not exposed to alkylating agents (1.6 ± 0.8, $n = 19$, Table 1) were within the 95% confidence intervals of normative reference values[6] (Figure 1a), following a physiological increase until the age of 6–7 years and a plateau until the age of 11 years. In contrast, in samples from patients exposed to alkylating agents, a significantly lower mean S/T value (0.4 ± 0.5, $n = 16$, $P < 0.001$) was found compared with

Accepted article preview online 8 March 2017; advance online publication, 7 April 2017

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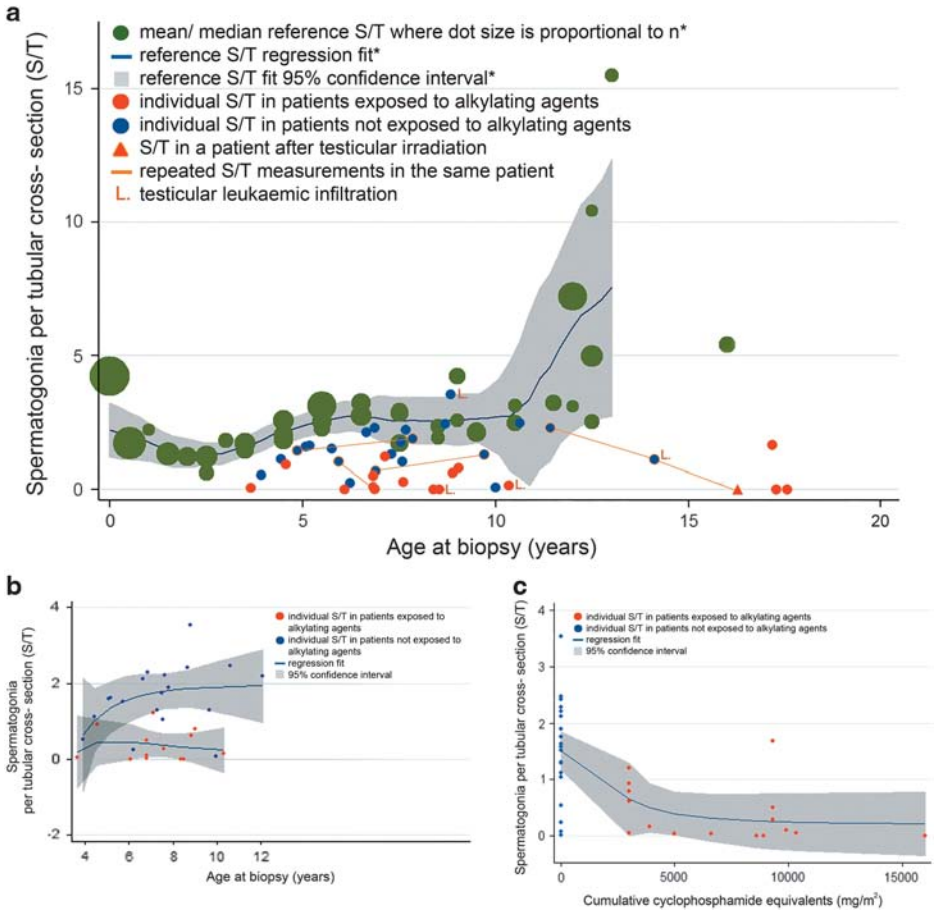

Figure 1. Numbers of spermatogonia per transverse tubular cross-section (S/T) of leukaemic boys exposed to chemotherapy with (red dots) and without (blue dots) alkylating agents or irradiation (triangle). (**a**) S/T values after completion of induction therapy and after cessation of ALL therapy (red and blue dots) plotted on a meta-regression fit line of S/T reference values throughout healthy prepuberty*. Samples with leukaemic infiltration are marked L. (**b**) Regression analysis of S/T values among patients up to 12 years of age. Values of S/T among patients not exposed to alkylating agents (blue dots) show a gradual increase until the age of 6–7 years and a plateau thereafter, whereas S/T values in patients exposed to alkylating agents (red dots) display a continuous decline from the age of 4 to 5 years. (**c**) Values of S/T among patients treated with alkylating agents show a plateau at values close to zero with cumulative cyclophosphamide equivalent doses >4000 mg/m^2. *Modified from Masliukaite *et al.*[6] Copyright (2016), with permission from Elsevier. This article is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND) https://creativecommons.org/licenses/by-nc-nd/4.0/.

samples from patients treated with non-alkylating agents (Figure 1b). Testicular irradiation resulted in complete depletion of spermatogonia (Figure 1a).

Similarly, the tubular fertility index (TFI), the percentage of tubular cross-sections containing spermatogonia, was significantly lower among patients exposed to alkylating agents compared with the non-alkylating agent-exposed group ($19\% \pm 21$ and $59\% \pm 31$, $P < 0.001$), confirming the results of a previous report.[14] Regression analysis showed that cumulative CEDs >4000 mg/m^2 led to S/T values close to zero (Figure 1c), which is in concordance with the results of previous reports showing that long-term impairment of spermatogenesis and decreased fertility is more likely after cumulative CEDs >4000 mg/m^2.[10,15] Patients exposed to alkylating agents also received higher cumulative doses of anthracyclines (Table 1). However, multiple regression analysis confirmed that the cumulative dose of alkylating agents ($B = 0.000$, $P < 0.001$) but not the dose of anthracyclines ($B = 0.002$, $P = 0.113$) predicted spermatogonial quantity. The progression of spermatogenesis up to spermatid stage was recorded in only 3 of the 11 boys aged >9 years. This is in agreement with an earlier histological report that leukaemia therapy, even without the involvement of alkylating agents, delays the initiation of human spermatogenesis.[16] Although present in only a low number of cases ($n = 4$), leukaemic infiltration in the testicular tissue had no further impact on spermatogonial quantity (Figure 1a).

Among the 23 long-term survivors, at the age of 22.8 ± 5.1 (range 18–32) years and 13.1 ± 6.7 (range 0.9–21.2) years after testicular biopsy, mean adult testicular size and sperm quality did not differ between patients treated with and without the alkylating agents (Table 1). No correlations were observed between S/T or TFI and adult testicular size ($r = -0.081$, $P = 0.699$ and $r = -0.163$, $P = 0.465$, respectively), total sperm count ($r = 0.298$, $P = 0.281$ and $r = 0.309$, $P = 0.244$, respectively) or sperm motility ($r = 0.222$, $P = 0.426$ and $r = 0.343$, $P = 0.193$, respectively).

Although no correlation between TFI and total sperm count was recorded, TFI together with S/T are important factors to determine the risk of subfertility or infertility, as TFI reflects the distribution of spermatogonia over the testis irrespective of the total number of spermatogonia. From the patients who had testicular biopsy acquired after the treatment with TFI $< 50\%$ (not corrected with age-matched controls), 43% were normozoospermic (Supplementary Table 1), while from the patients with TFI $> 50\%$, 62.5% recovered normal germ cell function and were considered normozoospermic[13] at the median time of 12.4 years after stopping the treatment at the median age 20.5 years. Although our numbers are low, this trend is in agreement with a trend of better spermatogenic recovery for higher TFI cases described in

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Table 1. Descriptive statistics of testicular histology in boys who underwent testicular biopsy at the cessation of antileukaemia therapy and gonadal function measurements after follow-up

	n	*Exposure to non-alkylating agents, mean ± s.d. (range)*	n	*Exposure to alkylating agents, mean ± s.d. (range)*	P-*value*
Age at diagnosis, years	21	5.3 ± 2.7 (1.1–11.3)	16	7.3 ± 5.2 (1.7–16.1)	0.342
Age at biopsy, years	21	8.1 ± 2.8 (3.9–14.1)	16	9.1 ± 4.3 (3.6–17.5)	0.985
Exposure to cyclophosphamide (g/m^2)	21	0	16	6.3 ± 3.5 (3.0–16.0)	
Exposure to carmustine (mg/m^2)	21	0	16	46.9 ± 84.1 (0.0–210.0)	
CED (g/m^2)	21	0	16	7.0 ± 3.8 (3.0–16.0)	
Cumulative anthracycline dose (mg/m^2)	21	55 ± 61 (0–120)	16	239 ± 146 (0–450)	< 0.001
Spermatogonia/cross-section	19	1.6 ± 0.8 (0.0–3.5)	16	0.4 ± 0.5 (0.0–1.7)	< 0.001
Fertility index (%)	21	59 ± 31 (0–95)	16	19 ± 21 (0–63)	< 0.001
Proportion of SCO tubules (%)	21	37 ± 27 (0–96)	16	81 ± 21 (37–100)	< 0.001
Histological progression of spermatogenesis detected, *n* (%)	6[a]	2 (33%)	5[a]	1 (20%)	0.998
Age at follow-up (years)	15	22.9 ± 5.6 (18.0–32.0)	8	22.6 ± 5.0 (18.0–29.0)	0.828
Follow-up time from biopsy (years)	15	15.2 ± 6.0 (9.0–25.2)	8	10.5 ± 7.5 (0.9–19.2)	0.186
Adult testicular size (ml)	15	19.4 ± 7.4 (1.2–27.5)	8	19.5 ± 7.1 (7.3–27.0)	0.948
Total sperm count (10^6)	10	173.2 ± 175.0 (0.0–485.0)	7	50.1 ± 60.5 (0.0–150.0)	0.266
Motile sperm (a+b) (%)	10	43 ± 25 (0–83)	7	38 ± 26 (0–79)	0.745
Azoospermia detected, *n* (%)	10	1 (10%)	7	1(14%)	1.000

Abbreviations: CED, cumulative cyclophosphamide equivalent dose, SCO, Sertoli cell-only. [a]Boys aged > 9 years.

a previous report, where 45.5% of patients with TFI < 50 and 88.5% of patients with TFI > 50% recovered normal germ cell function at the median time 10.7 years after stopping the treatment at the median age 18.6 years. In the latter report, all patients with TFI < 50% and no development of spermatogenesis were treated with the alkylating agents, which is also the case in our cohort.

Various studies in adults have shown that > 70% of cancer patients treated with low doses of alkylating agents (cumulative CED < 7500 mg/m^2) and 10–50% treated with high doses of alkylating agents recover spermatogenesis.[17,18] The fraction of patients recovering spermatogenesis increases gradually within 5–8 years after the end of treatment followed by a plateau. Case reports suggest that such recovery can take > 20 years, and severe delays in recovery process are expected if alkylating agent exposure is combined with irradiation.[4,19] Furthermore, one should take into account that recovery of spermatogenesis can only be detected from the onset of spermatogenesis at puberty. Longer follow-up time may therefore be needed for childhood cancer patients compared with adults. Similarly, a study reporting semen parameters in a large cohort (214 patients)[10] of childhood cancer survivors at the median time 21 years after stopping the treatment at the median age 29 years noted no or poor spermatogenic recovery (azoospermia or olizogoospermia in 25% and 28% of cases, respectively). The trend of impaired spermatogenesis was higher with cumulative CEDs > 4000 mg/m^2. Effectively, over half of childhood cancer survivors were shown to experience impaired spermatogenesis as an effect of the cancer treatment even after 20 years of follow-up. In the present study, patients treated with alkylating ($n = 7$) and non-alkylating agents ($n = 10$) showed no difference in total sperm count after a follow-up period of 9–19 years (Table 1 and Supplementary Figure S1). Although present results should be interpreted in the light of the small sample size, they provide confirmation that regeneration of spermatogenesis, given sufficient follow-up time, can be achieved as long as the spermatogonial pool is not completely depleted.[4,7,14] Nevertheless, an extensively prolonged waiting time for spermatogenesis recovery might compromise childbearing possibilities for some young couples, thus experimental procedures of testicular biopsy cryopreservation before gonadotoxic treatment[17] could be offered to young boys who are facing oncological treatments associated with a very high risk of infertility.

To our knowledge, this is the first report in which S/T throughout acute lymphoblastic leukaemia therapy in prepuberty has been investigated and related to follow-up measures of testicular function. The results demonstrate that leukaemia therapy involving alkylating agents results in long-term depletion of the spermatogonial pool in some long-term survivors, while spermatogonial quantity after therapy without alkylating agents remains within normative reference values for prepubertal boys. Therefore, in order to collect sufficient amounts of spermatogonial stem cells for fertility preservation as part of the clinical research programs, a testicular biopsy sample for this purpose needs to be acquired before initiation of alkylating agents for some boys who later will face potentially sterilizing cancer therapy, including allogenic haematopoietic stem cell transplantation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by grants from The Swedish Childhood Cancer Foundation, the Finnish Cancer Society, the Foundation for Pediatric Research, the Väre Foundation for Pediatric Cancer Research, the Academy of Finland, the Dutch Children Cancer free foundation KIKA86, Netherlands Organization for Health Research and Development (TAS-116003002) and EU-FP7-PEOPLE-2013-ITN 603568: 'Growsperm.'

AUTHOR CONTRIBUTIONS

MP-K, IM, KJ and J-BS designed the study; MP-K, MN, J-BS and PL carried out data collection; MvW and MP-K performed the statistical analyses; MP-K, KJ, J-BS, IM, AMMvP and MW analysed the data; MP-K, IM, KJ and J-BS wrote the initial draft of the manuscript. All authors contributed to interpretation of the results and revision of the paper.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

Response-adapted consolidation with bortezomib after ASCT improves progression-free survival in newly diagnosed multiple myeloma

Leukemia (2017) **31,** 1463–1466; doi:10.1038/leu.2017.83

Frontline induction chemotherapy followed by high-dose therapy (HDT) and autologous stem cell transplantation (ASCT) remains the standard treatment for newly diagnosed multiple myeloma (NDMM) in transplant-eligible patients.[1] However, progression-free survival (PFS) does often not exceed 2–3 years.[1] Thus, consolidation therapy designed to deepen and prolong responses to extend progression-free intervals is increasingly being explored.[2–4] We present a pre-specified single dataset analysis on the combined data of two randomized, controlled, open-label phase III studies of bortezomib consolidation. The objective of both similarly designed studies was to evaluate the efficacy of bortezomib consolidation after ASCT in NDMM patients aged (i) $\leqslant$60 years, of whom 71% had received a bortezomib-based induction regimen (VCD/VD; study

Accepted article preview online 15 March 2017; advance online publication, 18 April 2017