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ORIGINAL ARTICLE

The degree of myelosuppression during maintenance therapy of adolescents with B-lineage intermediate risk acute lymphoblastic leukemia predicts risk of relapse

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Drug doses, blood levels of drug metabolites and myelotoxicity during 6-mercaptopurine/methotrexate (MTX) maintenance therapy were registered for 59 adolescents (⩾10 years) and 176 non-adolescents (<10 years) with B-cell precursor acute lymphoblastic leukemia (ALL) and a white blood cell count (WBC) $<50 \times 10^9$/l at diagnosis. Event-free survival was lower for adolescents than non-adolescents ($pEFS_{12y}$:0.71 vs 0.83, $P=0.04$). For adolescents staying in remission, the mean WBC during maintenance therapy (mWBC) was related to age ($r_S=0.36$, $P=0.02$), which became nonsignificant for those who relapsed ($r_S=0.05$, $P=0.9$). The best-fit multivariate Cox regression model to predict risk of relapse included mWBC and thiopurine methyltransferase activity, which methylates mercaptopurine and reduces the intracellular availability of cytotoxic 6-thioguanine nucleotides (coefficient: 0.11, $P=0.02$). The correlation of mWBC to the risk of relapse was more pronounced for adolescents (coefficient = 0.65, $P=0.003$) than for non-adolescents (coefficient = 0.42, $P=0.04$). Adolescents had higher mean neutrophil counts ($P=0.002$) than non-adolescents, but received nonsignificantly lower mercaptopurine and MTX doses during maintenance therapy. Red blood cell MTX levels were significantly related to the dose of MTX among adolescents who stayed in remission ($r_S=0.38$, $P=0.02$), which was not the case for those who developed a relapse ($r_S=0.15$, $P=0.60$). Thus, compliance to maintenance therapy may influence the risk of relapse for adolescents with ALL.
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Introduction

A majority of the collaborative groups report that children above 10.0 years of age with acute lymphoblastic leukemia (ALL) have a worse prognosis than younger patients.[1–4] This may in part reflect that the older children more often have T-cell leukemia,[5] and that their leukemic clones more commonly harbor higher risk translocation such as t(9;22)(q34;q11) or those involving the *MLL* gene, and less frequently the prognostic favorable *ETV6/RUNX1*-translocations and high-hyperdiploid clones that are common in younger children.[6,7] Other, although less well explored, risk factors for relapse among adolescents could involve compliance to the treatment protocol, puberty-related changes in drug disposition, and changes in the bone marrow microenvironment.

To examine to which extent the actual compliance to the protocol recommendations for myelosuppression influences the risk of relapse, we retrospectively analyzed methotrexate (MTX)/6-mercaptopurine (6MP) maintenance therapy data from 59 adolescents and 176 non-adolescents, who participated in the Nordic Society of Pediatric Hematology and Oncology (NOPHO) ALL92 maintenance therapy study.[8] As part of that study, we registered all data on blood counts as well as on MTX and 6MP doses (approximately 30.000 data sets in total), and we repeatedly analyzed the erythrocyte levels of the cytotoxic metabolites of 6MP (that is, 6-thioguanine nucleotides, E-6TGN) and of MTX (that is, MTX polyglutamates, E-MTX) during therapy. The data indicate that failure to achieve the protocol target for myelosuppression during MTX/6MP maintenance therapy significantly increases the risk of relapse, and more so for adolescents than for the younger patients.

Patients and methods

Patients

The NOPHO ALL92 protocol for children 1.0–14.9 years of age with non-B-cell childhood ALL was opened in Denmark, Finland, Iceland, Norway and Sweden on 1 January 1992.[1,8] The risk group assignment was based on age and white blood cell count (WBC) at diagnosis (standard risk: age 2.0–9.9 years and WBC $<10.0 \times 10^9$/l; intermediate risk (IR): age 1.0–1.9 or ⩾10.0 years and/or WBC 10–49.9×10^9/l; higher risk (that is, high risk or very high risk): WBC $\geqslant 50.0 \times 10^9$/l) and the presence of one or more of the following higher risk features: T-lineage ALL, the presence of central nervous system or testicular leukemia, translocations t(9;22)(q34;q11) or t(4;11)(q21;q23), lymphomatous leukemia or mediastinal lymphoma, and a poor treatment response (⩾25% leukemic blasts in the bone marrow at day 15 or ⩾5% day 29).[1] In the NOPHO ALL92 protocol, patients who had higher risk features were assigned to the very high-risk treatment arm, if they were at least 5 years of age at diagnosis (because of the use of cranial irradiation in that protocol arm) and in addition had (i) T-cell disease is association with other higher risk features, (ii) central nervous system

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Table 1 Clinical and pharmacological characteristics of adolescents and younger patients

Age	*1.00–9.99 years*	*10.00–14.99 years*
Number	176	59
Girls/boys	88/88	35/24
WBC at diagnosis (median)	17	5
t(12;21), HeH/other/N-M	11/39/29/97	2/12/15/30
Average dose 6MP (median)	57.2	52.1
Average dose MTX (median)	14.2	13.9
TPMT activity (median)	18.5	17.6
mWBC (median; 50% range)	3.1	3.3
mANC (median; 50% range)	1.9	2.2
mE-6TGN (median; 50% range)	156	191
mE-MTX (median; 50% range)	5.5	6.7
BM/CNS/other relapse	26/1/1	13/1/1

Abbreviations: 6MP, 6-Mecaptopurine; 6TGN, 6-thioguanine nucleotides; ANC, absolute neutrophil count; BM, bone marrow including combined relapse; CNS, isolated central nervous system relapses; E (as prefix), erythrocyte level; HeH, high hyperdiploid (>50 chromosomes); m (as prefix), time-weighted mean; MTX, Methotrexate; N-M, normal or missing; TPMT, thiopurine methyltransferase; WBC, white blood cell count at diagnosis.

leukemia, (iii) lymphomatous leukemia and/or (iv) higher risk ALL at diagnosis and $\geqslant$25% leukemic blasts in the bone marrow at day 15 or $\geqslant$5% day 29.[9] All the remaining patients with higher risk features were assigned to the high-risk treatment arm. Patients with very high-risk ALL were not eligible for the randomized NOPHO ALL92 MTX/6MP maintenance therapy study.[8] Of the 538 patients that entered the ALL92 maintenance therapy study, 62 were above 10.0 years of age at diagnosis, and are in this report classified as adolescents (Table 1). Of these 62 patients, only three had high-risk ALL (including one T-ALL). Of these three high-risk ALL patients, one developed a second malignant neoplasm, whereas the other two patients are in first remission. As the remaining 59 patients all had IR–ALL (because of their age at diagnosis), we only included adolescent ($N=59$) and non-adolescent children ($N=176$) with IR–ALL in this study. None of the 235 patients had Down's syndrome. The 235 study patients were all included in the previous publication of the NOPHO ALL92 trial, but have not previously been analyzed separately.[8]

Cytogenetics

Only G-band karyotyping was mandatory in the NOPHO ALL92 protocol. However, many leukemic samples were examined by fluorescent *in situ* hybridization, reverse transcriptase PCR, comparative genomic hybridization, spectral karyotyping and/or DNA-index by flow cytometry.[10] All cytogenetic results are scrutinized annually by the NOPHO cytogenetic working group and described according to International System for Human Cytogenetic Nomenclature (ISCN) 1995.[11]

Therapy

IR–ALL induction and consolidation therapy have previously been described in detail.[1] During induction therapy, all patients received Prednisolone (60 mg/m^2/day on days 1–36, then tapered), weekly Vincristine (2.0 mg/m^2 six times, maximum 2.0 mg), Doxorubicin (40 mg/m^2 three times), Erwinia asparaginase (30 000 IU/m^2 daily on days 37–46), and intrathecal MTX on four occasions. Subsequently, patients received two doses of Cyclophosphamide (1000 mg/m^2 two times, 4 weeks apart) with low-dose Cytarabine (75 mg/m^2 daily for two 4-day periods after each Cyclophosphamide dose) and oral 6MP. This was followed by oral 6MP (25 mg/m^2/day) with four courses of high-dose MTX at 2 weeks intervals. Delayed intensification consisted of dexamethasone (10 mg/m^2/day for 3 weeks, then tapered), weekly Vincristine (2.0 mg/m^2 four times), weekly Daunorubicin (30 mg/m^2/day four times) and Erwinia asparaginase (30 000 IU/m^2 four times) followed by Cyclophosphamide 1000 mg/m^2, low-dose Cytarabine and 6-Thioguanine. Maintenance therapy with starting 6MP doses of 75 mg/m^2/day and MTX doses of 20 mg/m^2/week was initiated at treatment week 32 and continued until 2 years from diagnosis. During the first year of maintenance therapy, the patients received alternate pulses of either (i) Vincristine (2.0 mg/m^2 once) and Prednisolone (60 mg/m^2/day for 1 week) or (ii) high-dose MTX at 4 week intervals until five courses of high-dose MTX had been given. The high-dose MTX courses were given as 5 g/m^2 24-h infusions with intrathecal MTX (age-adjusted doses) and Leucovorin rescue from hour 36 and continued at 6-h intervals until p-MTX was $<$200 nM.[12]

Maintenance therapy randomization in NOPHO ALL92

In total, 538 patients were randomized to two different dose-adjustment strategies of whom 235 patients had IR–ALL.[8] This includes >97% of all eligible patients during the study period. In the control group, the dosing of oral 6MP and MTX was targeted to a WBC of 1.5–3.5 $\times 10^9$/l, and the doses were recommended to be reduced to 50% at a WBC $<1.5\times10^9$/l and interrupted at a WBC $<1.0\times10^9$/l and/or a thrombocyte count $<100\times10^9$/l. Therapy was reinitiated, when blood counts were rising and WBC $\geqslant1.5\times10^9$/l. Blood counts were measured at an average interval of 1–2 weeks. If the WBC was $>3.5\times10^9$/l, the protocol recommended upward dose adjustments of MTX and/or 6MP, until the WBC was within the target range. In the pharmacology group, the doses of oral 6MP and oral MTX were adjusted according to WBC and thrombocyte counts similar to the control group. In addition and unless the WBC was $<1.5\times10^9$/l, the doses of 6MP and/or MTX were to be increased in steps of 20%, if E-6TGN*MTX was <1350 (nmol/mmol Hb)^2 and the treating physician regarded such upward dose adjustments to be tolerable. At least once a month, blood samples were to be sent for E-6TGN/MTX analyses at the Laboratory for Pediatric Oncology (Bonkolab), Rigshospitalet, Copenhagen.[13,14]

Thiopurine methyltransferase activity

Interindividual variations in response to thiopurine therapy are influenced by genetically determined polymorphisms in the activity of the enzyme thiopurine methyltransferase (TPMT)[15,16] that methylates 6MP and some of its metabolites and thus competes with the formation of 6TGN. In this study, TPMT activity measurements were available for 52 adolescents (88%) and 155 non-adolescents (88%), and it was measured 1–5 times during maintenance therapy, as described earlier.[17] For patients with more than one TPMT activity measurement, an arithmetic mean TPMT activity was calculated. All TPMT phenotype assays were performed at least 8 weeks after the most recent blood transfusion. The TPMT activity was not revealed to the physicians, while the patients were on therapy.

Statistics

Non-parametric methods were applied to compare the distribution of parameters between subgroups and to compare the correlation between parameters (r_S = Spearman's correlation coefficient) that did not fulfill requirements for parametric testing.[18] The average dose of MTX and 6MP given to a patient was calculated as the cumulative total prescribed dose divided by the duration of maintenance therapy. The mean WBC, absolute neutrophil count, and E-6TGN and E-MTX levels during maintenance therapy was calculated as weighted means (prefix m) using as weight the interval between the sample in question and the next blood sample. Cox proportional hazard backward regression analyses were performed, and the likelihood-ratio test was applied to test for differences in outcome.[19,20] Covariates were excluded from the models at a significance level of 0.10. Wherein relevant, the covariates were analyzed as time-dependent continuous parameters with recalculations of the weighted means of these variables every time a patient failed using as weight the interval between the sample in question and the next blood sample.[21] Survival analyses were carried out with a basic time scale defined by the date of diagnosis with delayed entry of patients at the start of their maintenance therapy. As events in the event-free survival (EFS) analyses, we included death in remission, relapse or the diagnosis of a second malignant neoplasm, whichever occurred first. Patients who died in first remission or developed a second cancer were censored at the time of these events in the analyses of relapse risk factors. The Kaplan–Meier method was applied for estimation of remission duration and for the generation of survival curves.[22] Subgroups were compared with the log-rank test, stratified when needed.[23] Two-sided *P*-values <0.05 were regarded as being significant. Survival analyses were performed using the SAS statistical software. SAS Institute Inc., 100 SAS Campus Drive, Cary, NC, USA.

The protocol was approved by the ethical committee of Copenhagen (no. V.200.2080/91) as well as by the local ethical committees, and participants gave informed consent according to the Helsinki Declaration.

Results

The median follow-up time for the 188 patients who remained in first remission was 14.0 years (75% range: 12.6–15.5 years). At the end of the study, one patient had died in first complete remission (CR1), 43 patients had relapsed 0.9–12.0 years from diagnosis (median: 3.0 years), and three patients had developed a second malignant neoplasm.[24] The projected 12-year EFS ($pEFS_{12y}$) and overall survival (pOS_{12y}) of the 235 IR patients was $80 \pm 3\%$ and $88 \pm 2\%$, respectively. The 12-year EFS and overall survival of the 59 adolescents were inferior compared with that of the 175 younger children ($pEFS_{12y}$:0.71 ± 0.08 vs 0.83 ± 0.03, $P=0.04$; pOS_{12y}:0.77 ± 0.07 vs 0.92 ± 0.02, $P=0.003$) (Figure 1).

The increased relapse risk for adolescents stayed statistically significant when the analysis was stratified for cytogenetic subgroups (t(12;21)(*ETV6/RUNX1*)-translocation or high-hyperdiploidy vs other aberrations vs normal or missing karyotype; $P=0.04$).

With Cox multivariate regression analysis, we tested the effect on risk of relapse of gender, WBC and age at diagnosis, the average dose of MTX and 6MP, the TPMT activity, mWBC, mE-6TGN and mE-MTX. Only the mWBC during maintenance therapy and the TPMT activity (B (coefficient) = 0.11, $P=0.02$) were significantly and independently associated with the risk of relapse. The effect of mWBC on the risk of relapse was more pronounced for adolescents ($B=0.65$, $P=0.003$) than for the patients below 10.0 years of age ($B=0.42$, $P=0.04$), and homogeneity analysis have shown these coefficients for mWBC differed significantly between adolescents and non-adolescents ($P=0.01$). Among the patients below 10.0 years of age, the effect of mWBC on the risk of relapse did not differ significantly when the 50 patients who had a high-hyperdiploid karytotype or a t(12;21)(*ETV6/RUNX1*)-translocation were compared with the remaining 126 patients.

The 27 adolescents randomized to pharmacological dose adjustments did not differ significantly in their risk of relapse from the 32 adolescents in the control group (28 vs 25%, $P=0.89$).

For non-adolescents who stayed in remission, the mWBC during maintenance therapy was negatively related to the age of the patient ($r=-0.08$, $P=0.10$), whereas a positive correlation was observed for the adolescents ($r=0.36$, $P=0.02$) (Figure 2). This positive correlation between mWBC and age disappeared for adolescents who developed a relapse ($r=0.04$, $P=0.9$). Overall, the median mWBC of the 59 adolescents was 3.3×10^9/l. The 29 adolescents with mWBC $<3.3 \times 10^9$/l did not differ significantly from those with higher mWBC with respect to sex, age or WBC at diagnosis, or their average dose of

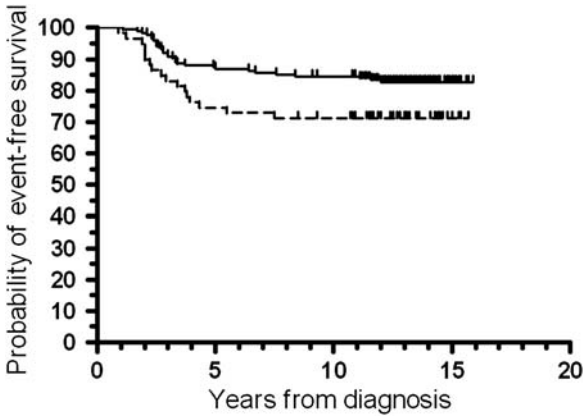

Figure 1 Probability of event-free survival (pEFS) for non-adolescents (1.0–9.9 years, upper curve, $N=176$) and adolescents (10.0–14.9 years, lower curve, $N=59$). pEFS at 12 years: 0.83 ± 0.03 vs 0.71 ± 0.08, $P=0.04$.

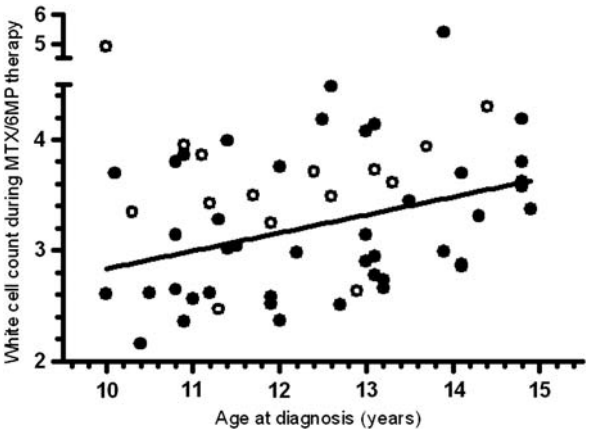

Figure 2 Scatterplot of the weighted mean WBC count during MTX/6MP maintenance therapy in relation to age of diagnosis for adolescents (10.0–14.9 years). A regression line is drawn for those who did not develop a relapse (closed dots, $r=0.36$, $P=0.02$). Open dots: patients with a relapse.

6MP and MTX during maintenance therapy. In contrast, adolescents and non-adolescents differed on several of the MTX/6MP dose-adjustment parameters (Table 1). Thus, adolescents had higher weighted mean WBC and absolute neutrophil count levels during maintenance therapy (median mWBC: 3.3 vs 3.1×10^9/l, $P=0.29$; median mANC: 2.2 vs 1.9×10^9/l, $P=0.002$) and they received on average moderately lower 6MP and MTX doses (median m6MP: 52.1 vs 57.2 mg/m^2, $P=0.33$; median mMTX: 13.9 vs 14.2 mg/m^2, $P=0.33$). In spite hereof, they had higher weighted mean E-6TGN and E-MTX levels (median mE-6TGN: 191 vs 156 nmol/mmol Hb, $P=0.01$; median mE-MTX: 6.7 vs 5.5×10^9/l, $P<0.001$). As the E-MTX level is significantly related to the dose of MTX,[25] we examined the correlation between the average dose of MTX and mE-MTX. Among the 40 adolescents who stayed in remission these two parameters were significantly correlated ($r_S=0.38$, $P=0.02$), whereas that was not the case for the 15 patients who developed a relapse ($r_S=0.15$, $P=0.60$). The Ponte di Legno group recently suggested a WBC target for MTX/6MP dose adjustments of 3.0×10^9/l.[26] The adolescents and non-adolescents in this study who had a mWBC $<3.0 \times 10^9$/l did not differ significantly in their EFS (0.91 ± 0.04 vs 0.86 ± 0.06, $P=0.57$), whereas for patients with a mWBC $\geqslant 3.0 \times 10^9$/l adolescents did significantly worse than non-adolescents (0.63 ± 0.08 vs 0.82 ± 0.04, $P=0.009$).

Discussion

The present data confirm the increased relapse rate for adolescents with ALL observed by most collaborative groups.[1–3] However, in this study adolescents with T-ALL, higher risk translocations, and/or WBC $\geqslant 50 \times 10^9$ l at diagnosis were excluded, and the pharmacological data and the significant association between the degree of myelosuppression and the risk of relapse among adolescents indicate that other factors than just leukemia biology has a role for the increased risk of treatment failure.

First, although nearly all collaborative ALL protocols recommend the doses of MTX and 6MP during maintenance therapy to be adjusted to a target WBC level,[26] few studies have actually examined to which extent this is achieved.[8,27–33] In general, the published studies support that low WBC levels during 6MP/MTX maintenance therapy is linked to a reduced risk of relapse. Furthermore, lack of physician compliance to dose titration has been associated with an increased risk of treatment failure.[31,34–36] This study emphasizes the association between the degree of myelosuppression and the chance of cure, and indicates that this is especially pronounced for adolescents. Furthermore, the lack of correlation between the age and mWBC for the patients that relapsed could indicate that the risk of relapse is related to the absolute mWBC levels rather than to the relative degree of myelosuppression with respect to the patients normal WBC levels.[37,38] This is important, because it indicates that although the *normal* mWBC may rise with age, the target for myelosuppression should be the same across age groups. In this respect it is noteworthy that adolescents with high WBC levels during maintenance therapy did not receive significantly higher 6MP or MTX doses than those with WBC within the target range, and that adolescents overall received lower doses than non-adolescents, although they in general had higher WBC levels. The reasons for this lack of physician compliance are unclear and calls for further exploration. However, one possible reason could be a higher incidence of toxicity among adolescents during maintenance therapy or earlier phases of treatment, which could reduce the physicians willingness to treatment intensification.[39]

Second, poor patient compliance to the prescribed oral doses of 6MP/MTX has been indicated as a risk factor for relapse.[40–42] Social, psychological and medical disorders may influence to which extent adolescents take their prescribed therapy,[43] and the problem is not trivial. Both electronic monitoring[40] and drug metabolite measurements[44] have indicated that 10–20% of childhood ALL patients intermittently fail to take their oral medication, with a few percent being consistently non-compliant, a problem that seems more frequent among adolescents.[45] During adolescence, parents may hand over responsibility for the oral medication to their child, but there is rarely available information on whether the patient or the parents were responsible for remembering and administering the oral medication. It is uncertain to which extent inferior patient compliance to the prescribed MTX/6MP doses influenced the inferior cure rate among adolescents in this study. However, the poor correlation between the prescribed MTX doses and the measured E-MTX levels among the adolescents who developed a relapse could indicate poor treatment compliance for such patients. Still, it cannot be excluded that adverse pharmacokinetic drug disposition rather than patient compliance determined both the increased risk of relapse and the lack of correlation between E-MTX and the dose of MTX.

Third, young children in general have a more rapid drug clearance than both infants and adolescents.[46] This age-related variation in drug disposition differs among the antileukemic agents. However, based on plasma and red blood cell drug level measurements, neither this nor previous studies indicate that the inferior outcome of adolescents with ALL reflects adverse bioavailability or pharmacokinetics of MTX and 6MP.[47,48] However, food habits may change during the transition phase from childhood to adolescence, and co-administration of food with oral 6MP/MTX may influence the bioavalability and/or pharmacokinetics of these agents.[49–52] However, the higher E-MTX and E-6TGN levels for the adolescents than for non-adolescents who stayed in remission does not support that age-related adverse pharmacokinetics for MTX and 6MP is a significant reason for the increased relapse rate among adolescents.

Finally, age-related changes in sex hormone levels could have influenced both the WBC levels during maintenance therapy and leukemic stem cell survival. Recent studies have shown that the efficacy of sex hormones (not least androgens) on bone marrow failure syndromes probably is mediated by increased telomerase activity.[53] Thus, the increased levels of androgens and estrogens during puberty could have stimulated both the normal bone marrow activity (as indicated by the significant positive correlation between mWBC during maintenance therapy and the age of the patient) and the survival of malignant stem cells. Future studies should explore whether the Tanner stage (and/or bone age) at the time of diagnosis of ALL or at the start of MTX/6MP maintenance therapy is related to the risk of relapse for adolescents.

Although the number of adolescents included in this study is limited, and the pharmacological and biological mechanism(s) behind the association between WBC levels and risk of relapse need further exploration, the data indicate that an increased relapse rate for adolescents may in part reflect inferior willingness and/or efforts of the patient, the family and/or the treating physician to increase the intensity of maintenance therapy.

Conflict of interest

The authors declare no conflict of interest.

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Authors Contribution

Kjeld Schmiegelow designed the study and performed the statistical analyses together with Susanne Rosthoej. Mats Heyman chairs the NOPHO Leukemia Registry and was together with Göran Gustafsson and the national representatives (Birgitte Lausen, Henrik Schrøder, Finn Wesenberg, Jon Kristinsson and Kim Vettenranta) responsible for collecting the data. Erik Forestier as chair of the NOPHO cytogenetic registry scrutinized all karyotypes. All authors approved the final paper.

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