British Journal of Haematology, 2000, **110**, 813–818

In vitro drug resistance profiles of adult versus childhood acute lymphoblastic leukaemia

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Received 8 January 2000; accepted for publication 25 April 2000

Summary. The difference in the current cure rates between adult and childhood acute lymphoblastic leukaemia (ALL) may be caused by differences in drug resistance. Earlier studies showed that *in vitro* cellular drug resistance is a strong independent adverse risk factor in childhood ALL. Knowledge about cellular drug resistance in adult ALL is still limited. The present study compared the *in vitro* drug resistance profiles of 23 adult ALL patients with that of 395 childhood ALL patients. The lymphoblasts were tested by the MTT assay. The group of adult ALL samples was significantly more resistant to cytosine arabinoside, L-asparaginase, daunorubicin, dexamethasone and prednisolone. The resistance ratio (RR) was highest for prednisolone (31·7-fold) followed by dexamethasone (6·9-fold), L-asparaginase (6·1-fold), cytosine arabinoside (2·9-fold), daunorubicin (2·5-fold) and vincristine (2·2-fold). Lymphoblasts from adult patients were not more resistant to mercaptopurine, thioguanine, 4-HOO-ifosfamide, mitoxantrone and teniposide. There were no significant differences in drug resistance between adult T-cell (T-) ALL ($n = 11$) and adult common/pre-B-cell (B-) ALL ($n = 10$). Additionally, adult T-ALL did not differ from childhood T-ALL ($n = 69$). There were significant differences between adult common/pre-B-ALL and childhood common/pre-B-ALL ($n = 310$) for prednisolone (RR = 302, $P = 0{\cdot}008$), dexamethasone (RR = 20·9, $P = 0{\cdot}017$) and daunorubicin (RR = 2·7, $P = 0{\cdot}009$). Lymphoblasts from adults proved to be relatively resistant to drugs commonly used in therapy. This might contribute to the difference in outcome between children and adults with ALL.

Keywords: ALL, drug resistance, MTT assay, children, adults.

Childhood leukaemias represent 8% of the leukaemias affecting people of all ages (Pui, 1996). Acute lymphoblastic leukaemia (ALL) constitutes about 80–85% of childhood leukaemias, but only constitutes about 20% of acute leukaemias in adults (Pui, 1996; Larson *et al*, 1998). The rate of complete remission ranges is now 97–99% in children and 75–90% in adults (Pui & Evans, 1998). Nearly 75% of children are currently cured compared with only about 30% of adults with ALL (Pui & Evans, 1998).

Clinical features associated with poor prognosis are often found in adult ALL patients. These include a higher fraction [up to 62% in T-cell (T-) ALL] of patients presenting with a high white blood cell count (more than 30×10^9/l), a high proportion of lymphoblasts expressing myeloid antigens (in about 25% of the cases), the absence of the CD10 antigen (in half of the patients) and a slow induction of remission with initial intensive therapies (Parentesis, 1997; Hoelzer, 1998; Larson *et al*, 1998). A majority of these ancillary clinical characteristics are associated with the high-risk immunophenotypic, karyotypic and molecular genetic features. ALL in adults often shows unfavourable cytogenetic characteristics, as illustrated by the significantly higher proportion of patients with the t(9;22) Philadelphia (Ph) chromosome that, together with the expression of p190 and p210 proteins, is associated with extremely poor prognosis in 25–30% of adults and in 3–5% of children (Laneuville *et al*, 1992; Parentesis, 1997). Hyperdiploidy, an important favourable prognostic factor, is seen in approximately 25% of children and in only 5% of adults (Parentesis, 1997). TEL–AML1 fusion occurs in about 22% of childhood ALL cases and in up to 3–4% in adult ALL (Rubnitz *et al*, 1999). Even for the same genetic subtypes of ALL, survival

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rates are lower in adults than in children (Pui & Evans, 1998). Adults younger than 30 years of age have a better prognosis than those 30–59 years old, who, in turn, have a better prognosis than patients who are 60 years of age or older (Cortes & Kantarijan, 1995; Laport & Larson, 1997; Chessels *et al*, 1998).

Poor prognosis of ALL in adults might be explained by drug resistance mechanisms that are probably multifactorial. Overexpression of transmembrane transporter molecules, including P-glycoprotein or the multidrug resistance-related protein, may be an important mechanism of drug resistance in patients with high-risk ALL (Arceci, 1993). Expression of P-glycoprotein has been associated with a lower success of remission induction and a higher relapse rate in adults with ALL (Goasguen *et al*, 1993). These results, however, were not confirmed by others (Wattle *et al*, 1995). Significant differences were also observed in methotrexate metabolism between cases of childhood and adult ALL, and blasts from adults exhibit decreased polyglutamylation and accumulation of methotrexate (Goker *et al*, 1993). A study including a small number of patients showed that lymphoblasts from adults with ALL demonstrated a higher degree of *in vitro* corticosteroid resistance than ALL blasts from children (Maung *et al*, 1995).

Several studies showed that cellular drug resistance is a strong independent adverse risk factor in childhood ALL (Pieters *et al*, 1991, 1998; Hongo *et al*, 1997; Kaspers *et al*, 1997,1998; Asselin *et al*, 1999). In addition, differences in the above-mentioned factors such as age, immunophenotype and DNA ploidy are related to differences in drug resistance (Pieters *et al*, 1993, 1998; Kaspers *et al*, 1995). The knowledge about cellular drug resistance in adult ALL is limited. In this study, we compared the *in vitro* drug resistance profile for 14 drugs in 23 adults with ALL with that of a group of 395 children.

PATIENTS AND METHODS

Patients and leukaemic cell samples. We tested 23 adults with ALL and compared the results with our reference data on the group of 395 childhood ALL. Bone marrow cells from 23 adults (12 men, 11 women) and 395 children (228 boys, 167 girls) with ALL at initial diagnosis were successfully tested. The median age was 32 years (range 16–51 years) for adults and 5 years 2 months (range 2 months–16 years) for children. Results of *in vitro* drug resistance in 395 children have been published previously (Pieters *et al*, 1998); here, they serve as a comparison group for adults. Eleven adult patients had T-ALL, 10 patients had common/pre-B-cell (B-) ALL and two patients had pro-B-ALL [$CD19^+$, $HLA\text{-}DR^+$, $CD10^-$, cytoplasmic μ $chain^-$ ($c\mu^-$) and surface $immunoglobulin^-$ (sIg^-)]. Three hundred and ten children had common/pre-B-ALL, 15 children had pro-B-ALL and 69 children had T-ALL, in one case the phenotype was unknown. Mature B-ALL cells characterized by $CD10^-/c\mu^-/sIg^+$ were excluded from the study. T-lineage ALL was characterized by the presence of two T-line markers.

Cytogenetic data: in the group of children, 24 out of 76 (31·5%) tested subjects were TEL–AML1 positive, 39 out of 240 (16·2%) showed DNA index $\geq 1{\cdot}16$ and 33 out of 127 (26·0%) had hyperdiploidy ≥ 50 chromosomes. Respective values for children with common/pre-B phenotype were 36·9%, 20·4% and 32·0%. In the group of adults, none of six successfully tested patients showed hyperdiploidy, and two out of 15 patients were positive for Ph chromosome, including two out of six common ALL.

For 10 out of 23 adult patients, samples were tested after cryopreservation. This procedure does not alter drug sensitivity in childhood ALL (Kaspers *et al*, 1994a). A comparison was made between the same specimen before and after cryopreservation in eight adult patients for a total number of 4–14 drugs for each patient. We did not observe any changes in drug resistance before and after cryopreservation in two out of two precursor B-ALL patients and in three out of six T-ALL patients (Spearman's rho = 0·875, $P = 0{\cdot}001$) However, in three T-ALL patients, the resistance was higher after cryopreservation exceeding one dilution step for prednisolone, vincristine and L-asparaginase (in all three patients), etoposide (VP-16; in two patients), mitoxantrone, daunorubicin and dexamethasone (in one patient). For further analysis, results obtained on cryopreserved samples of precursor-B-ALL only were used and there were no T-ALL adult patients included in this study who were tested from cryopreserved samples only. For calculation, the priority was given to data obtained on fresh samples.

Separation of mononuclear cells from bone marrow (BM) and peripheral blood (PB). Samples were diluted 1:1 or more with Roswell Park Memorial Institute (RPMI)-1640 medium (Dutch modification; Gibco, Uxbridge, UK). In case of the presence of small clots, the sample of BM or PB was first filtered through a cell strainer (70-μm nylon filter; Falcon, Franklin Lakes, New Jersey, USA) using RPMI-1640 to rinse off the strainer. Cells were separated on Ficoll gradient (Lymphoprep density; 1·077 g/ml; Nyegaard, Oslo, Norway) at 540 ***g*** for 20 min at room temperature. After centrifugation, cells were washed twice with RPMI-1640.

Frozen samples and thawing procedure. Samples of cryopreserved leukaemic cells were used. Cells were frozen in 10% DMSO (dimethylsulphoxide; Sigma Chemical, St. Louis, MO, USA) in culture medium (or RPMI-1640) containing 20% fetal calf serum (FCS) in a final volume of 2 ml and stored in closed cryovials (Sterillin, Stone, UK) in liquid nitrogen. The cryovials were rapidly thawed in a 37°C waterbath and the cell suspension was transferred to 18 ml RPMI-1640 containing 20% FCS. Isolation of cells was followed by two wash steps (380 ***g***, 10 min) in RPMI-1640 with 1% FCS. The recovery of cells, viability and the red blood cell (RBC)/white blood cell (WBC) ratio was calculated using 2% trypan blue in phosphate-buffered saline (PBS) and a cytospin stained with May–Grunwald–Giemsa (MGG) was made to check the percentage of blasts. If the percentage of blasts was less than 90%, the contamination with non-malignant cells was diminished with purification by 30 min incubation at 37°C with one or more of immunomagnetic polystyrene beads CD3, CD13, CD33, CD14, CD15 or immunoglobulin (Ig)M (Dynabeads M-450; Dynal, Oslo,

Table I. Concentration ranges of the drugs tested.

Drug	Concentration range (μg/ml)
Prednisolone (Prednisolone, Brocacef)	0·08–250
Dexamethasone (Dexamethasone, Brocacef)	0·0002–6
Vincristine (Oncovin, Lilly)	0·049–50
L-Asparaginase (Crasnitin, Bayer)	0·0032–10*
Daunorubicin (Cerubidine, Rhone-Poulenc)	0·002–2
Doxorubicin (Adriblastina, Farmitalia)	0·0078–8
Idarubicin (Zavedos, Farmitalia)	0·002–2
Mitoxantrone (Novantrone, Lederle)	0·001–1
Cytosine arabinoside (Cytosar, Upjohn)	0·0024–2·5
6-Mercaptopurine (Sigma, no. M7000)	15·6–500
6-Thioguanine (Sigma, no. A4882)	1·56–50
Vepesid (Vepesid, Bristol-Myers)	0·049–50
Teniposide (Vumon, Bristol-Myers)	0·003–8
4-HOO-Ifosfamide (Asta Medica)	0·1–100

*IU/ml.

Norway) (Kaspers *et al*, 1994a). If the RBC/WBC ratio $> 0{\cdot}5$, erythrocytes were lysed with ammonium chloride, followed by two wash steps.

Culture medium and culture suspension. The sample was finally resuspended in culture medium containing RPMI-1640, 20% heat-inactivated FCS, 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·125 μg/ml amphotericin B, 200 μg/ml gentamicin (Flow Laboratories, Irvine, UK), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma). The cell concentration for the MTT assay was finally adjusted to 2×10^6 vital cells/ml.

Drugs tested. Fourteen drugs were tested (Table I). Dexamethasone and prednisolone were dissolved in saline, 6-thioguanine and 6-mercaptopurine in 0·1 N NaOH, 4-HOO-ifosfamide in DMSO, daunorubicin and doxorubicin in distilled water. The others were obtained as solutions ready for use. All drugs were further diluted with RPMI-1640. Serial dilutions were prepared and dispensed into microculture plates. The concentrations of tested drugs are given in Table I.

In vitro drug resistance assay. The assay conditions were essentially the same as described previously (Kaspers *et al*, 1994a; Pieters *et al*, 1988,1989). Cell suspension (80 μl) containing 2×10^6 vital cells/ml was incubated with each drug concentration in 20 μl RPMI in duplicate wells of a 96-well round-bottomed microtitre plate (Greiner Labortechnik, Alphen aan den Rijn, The Netherlands). Six wells containing only cells in a drug-free medium served as controls for cell survival, whereas four wells containing only culture medium blanked the spectrophotometer. The outer wells of the microculture plate were filled with RPMI-1640 only. Plates were then wrapped in cling film and incubated for 4 d (96 h) at 37°C in humidified air containing 5% CO_2. After 4 d, 50 μg (10 μl of a solution of 5 mg/ml) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT; Sigma) was added to each well (final concentration 0·45 mg/ml); plates were shaken and incubated for another 6 h at 37°C. During exposure, yellow MTT was reduced into purple formazan by viable, but not dead, cells. The formazan crystals were dissolved with 100 μl of acidified (0·04 N HCl) 2-isopropanolol (Sigma) and the quantity of reduced product was measured by an enzyme-linked immunosorbent assay (ELISA) EL-312 microplate spectrophotometer at 562 nm (BioKinetics Reader, Bio-Tek Instruments, Winooski, VT, USA). The optical density (OD) at 562 nm is linearly related to the number of viable cells (Kaspers *et al*, 1994a). Cytospin slides from control wells, stained with MGG, were used to determine the percentage of blasts after 96 h incubation. Samples with more than 70% leukaemic cells in the control wells without drugs after 4 d of culture and with an OD greater than 0·050 arbitrary units (adjusted for blank values) were suitable for evaluation (Kaspers *et al*, 1995, 1997, 1998). The leukaemic cell survival was calculated by the equation: (OD drug well/mean OD control wells) × 100%. The OD of both control and tested wells were adjusted by OD of blank wells. The LC_{50}, the concentration of drugs that was lethal to 50% of the cells (in IU/ml for L-asparaginase and in μg/ml for other drugs), was used as a measure for the *in vitro* drug cytotoxicity in each sample (Kaspers *et al*, 1994a; Pieters *et al*, 1988, 1989).

Statistics. The non-parametric Wilcoxon matched pairs signed ranks and Spearman's rank correlation tests were used to compare LC_{50} values for fresh and cryopreserved samples. The Mann–Whitney *U*-test was used to compare drug resistance between groups. All tests were used for two-tailed testing at a level of significance of 0·05. Statistical calculations were performed with the program SPSS for Windows.

RESULTS

The group of adult ALL samples showed higher LC_{50} values for all 14 drugs than the childhood ALL samples (Table II). For cytosine arabinoside, L-asparaginase, daunorubicin, dexamethasone and prednisolone, the differences were statistically significantly different, and for doxorubicin, idarubicin and vepesid the results almost reached significance. The relative resistance ratios (RR) were calculated for each drug by dividing the median LC_{50} value of the adult ALL group by the median LC_{50} value of the child ALL group. The highest RR was found for prednisolone (31·7-fold) followed by dexamethasone (6·9-fold), L-asparaginase (6·1-fold), cytosine arabinoside (2·9-fold), daunorubicin (2·5-fold), vincristine (2·2-fold) and idarubicin (2·0-fold), although for the last two drugs significance was not reached. Lymphoblasts from adult patients did not differ significantly in sensitivity from childhood ALL for mercaptopurine, thioguanine, ifosfamide, mitoxantrone and teniposide.

Eleven adult patients were under 30 years and 12 were over 30 years of age. The resistance profiles of these two groups did not significantly differ with the exception of doxorubicin ($RR = 1{\cdot}49$, $P = 0{\cdot}049$), indicating that patients over 30 years were more resistant to this drug.

We also compared drug resistance profiles according to

Table II. Drug sensitivity profile: adult initial ALL vs. childhood initial ALL.

	Median LC_{50} (25–75 percentiles, number of patients)			
	Adults	Children	RR	*P*-value
Prednisolone	40·0 (0·85 to > 250, 23)	1·26 (0·27–89·03, 350)	31·7	0·011
Dexamethasone	0·55 (0·11 to > 6, 20)	0·08 (0·028–3·54, 268)	6·9	0·008
Vincristine	1·67 (0·3–17·33, 21)	0·75 (0·3–2·95, 345)	2·2	0·331
L-Asparaginase	1·28 (0·13 to > 10, 21)	0·21 (0·0025–1·22, 329)	6·1	0·014
Daunorubicin	0·25 (0·1–0·54, 22)	0·10 (0·067–0·22, 359)	2·5	0·001
Doxorubicin	0·41 (0·25–0·93, 20)	0·31 (0·16–0·47, 226)	1·3	0·057
Idarubicin	0·08 (0·036–0·092, 19)	0·04 (0·02–0·09, 134)	2·0	0·086
Mitoxantrone	0·053 (0·011–0·23, 21)	0·049 (0·019–0·11, 234)	1·08	0·754
Cytosine arabinoside	1·39 (0·28 to > 2·5, 23)	0·47 (0·21–1·14, 296)	2·9	0·023
6-Mercaptopurine	107 (66·96–166·67, 19)	102 (50–250, 299)	1·05	0·718
6-Thioguanine	6·99 (4·3–10·82, 21)	6·00 (3·8–9·15, 311)	1·16	0·359
Vepesid	2·23 (0·88–7·57, 22)	1·45 (0·65–2·53, 107)	1·5	0·081
Teniposide	0·30 (0·2–1·42, 19)	0·27 (0·18–0·61, 252)	1·1	0·193
4-HOO-Ifosfamide	3·58 (1·16–5·25, 17)	3·24 (1·45–4·95, 167)	1·1	0·945

RR, median LC_{50} (adult)/median LC_{50} (children); LC_{50}, values given as IU/ml for L-asparaginase and μg/ml for other drugs; *P*-value, Mann–Whitney *U*-test.

immunophenotype of blasts. There were no significant differences in drug resistance between adult T-ALL ($n = 11$) and adult common/pre-B-ALL ($n = 10$). Adult T-ALL also did not differ significantly in drug resistance from childhood T-ALL ($n = 69$). There were differences between adult common/pre-B-ALL and childhood common/pre-B-ALL ($n = 310$) for prednisolone (RR = 302, $P = 0{\cdot}008$), dexamethasone (RR = 20·9, $P = 0{\cdot}017$) and daunorubicin (RR = 2·77, $P = 0{\cdot}009$) (Table III).

DISCUSSION

Failure to cure leukaemia is probably due to the failure of eradicating the leukaemic clone. Mechanisms of drug resistance acting before the drug reaches its target (pharmacological resistance) and after the drug interacts with its target (cellular resistance) are gaining greater importance in understanding treatment failures (Veerman *et al*, 1994; Minden *et al*, 1996). The results of the present *in vitro* study suggest that acute lymphoblastic leukaemia (ALL) in adults is a highly drug-resistant disease in comparison with childhood ALL.

Maung *et al* (1995), in a similar study including 16 adults, 32 children and six drugs, showed that lymphoblasts from adults demonstrated a significantly higher degree of *in vitro* resistance to prednisolone than those from children. He

Table III. Drug sensitivity profile: adult common/preB-ALL vs. childhood common/pre-B-ALL.

	Median LC50 (number of patients)			
	Adults	Children	RR	*P*-value
Prednisolone	145 (10)	0·48 (273)	302	0·008
Dexamethasone	1·40 (9)	0·067 (201)	20·9	0·017
Vincristine	0·44 (9)	0·67 (267)	0·65	0·257
L-Asparaginase	0·36 (9)	0·12 (255)	3·00	0·392
Daunorubicin	0·25 (10)	0·09 (282)	2·77	0·009
Doxorubicin	0·43 (9)	0·29 (175)	1·48	0·105
Idarubicin	0·085 (9)	0·030 (108)	2·83	0·118
Mitoxantrone	0·026 (10)	0·048 (182)	0·54	0·886
Cytosine arabinoside	0·44 (10)	0·44 (225)	1·00	0·945
6-Mercaptopurine	130 (8)	91 (230)	1·42	0·407
6-Thioguanine	5·72 (9)	5·84 (242)	0·97	0·749
Vepesid	2·22 (10)	1·36 (82)	1·63	0·093
Teniposide	0·29 (9)	0·25 (189)	1·16	0·398
4-HOO-Ifosfamide	2·76 (7)	2·86 (124)	0·96	0·976

RR, median LC_{50} (adult)/median LC_{50} (children); LC_{50}, values given as IU/ml for L-asparaginase and μg/ml for other drugs; *P*-value, Mann–Whitney *U*-test.

concluded that cellular glucocorticoid resistance may be a fundamental difference between adult and childhood ALL that may be caused by different biological aspects and may also explain the difference in prognosis. Our study confirms the problem of glucocorticoid resistance in adult ALL. In addition, we demonstrated resistance to cytosine arabinoside, L-asparaginase, daunorubicin and possibly other anthracyclines. On the other hand, adult ALL cells seem to be as sensitive as childhood ALL cells to 4-HOO-ifosfamide, mitoxantrone, teniposide and thiopurines.

Analysis of phenotypes suggests that sensitivity of adult T-ALL is similar to adult common/pre-B-ALL and to childhood T-ALL, but adult common/pre-B-ALL is much more resistant than common/pre-B-ALL in children. This is in agreement with the fact that common/pre-B-ALL is not a favourable phenotype in adults. As prognosis in ALL decreases with increased age (Pui, 1996), we analysed the influence of age on cellular drug resistance within the adult group. Apart from doxorubicin, we did not find any statistically significant differences between age groups within adult patients, although patients over 30 years had high resistance ratios for glucocorticoids and mitoxantrone. This is in accordance with our earlier findings that cells from children aged >10 years are more resistant to glucocorticoids than cells from children <10 years of age (Pieters *et al*, 1998). In general, it is difficult to draw any conclusion, as the number of adult patients in our study is not high. As elderly patients tolerate treatment poorly (Chessells *et al*, 1998), pharmacokinetics and side-effects of chemotherapy may play an important role in the poorer outcome of elderly patients (Hoelzer, 1993a,b). Also, a higher frequency of adverse biological features, for example the incidence of Ph chromosome, may account for the poor treatment results in adults. However, predictive value of primary genetic abnormalities is not very high (Pui & Evans, 1998).

It has been hypothesized that 'good prognosis' ALL arises in lymphoid cells that are naturally 'poised' for apoptotic death and that chemotherapy is thus able to trigger programmed leukaemic cell death efficiently in these patients (Campana *et al*, 1993; Greaves, 1993; Fisher, 1994). Drug resistance in adult ALL may be associated with activation of antiapoptotic pathways as a consequence of aberrant growth signalling, resulting from the bypassing of normal growth factor signalling pathways in malignant cells (Baserga, 1994). Glucocorticoids, cytosine arabinoside and anthracyclines are thought to act on the proapoptotic pathway (Alnemri *et al*, 1992; Kaspers *et al*, 1994b; Den Boer *et al*, 1998; Savasan *et al*, 1998). Probably, the other drugs also have a proapoptotic mechanism of action, and apoptosis resistance may play a role in the resistance to therapy.

In ALL, the first goal of therapy is to induce complete remission with restoration of normal haematopoiesis. The induction regimen invariably includes a glucocorticoid (prednisolone or dexamethasone) and vincristine, as well as asparaginase and/or an anthracycline (Copelan & McGuire, 1995; Cortes & Kantarijan, 1995; Laport & Larson, 1997; Chessells *et al*, 1998). The therapy for ALL in adults has met with irregular success, often despite the use of treatment regimens similar to those used in childhood (Copelan & McGuire, 1995). Currently, there is no subgroup of ALL patients in which standard chemotherapy can be described as adequate and improved treatment is needed for all groups of patients (Linker, 1997). Leukaemic cells from adults proved to be highly resistant to drugs commonly used in induction therapy.

In conclusion, we have found that adults with ALL were more drug resistant than children. This might contribute to the difference in outcome between those age groups of patients with acute lymphoblastic leukaemia. However, the extent of resistance varied between individual drugs. These data could support the rational design of new treatment protocols.

ACKNOWLEDGMENTS

We acknowledge Nicole Ramakers-van Woerden, MD, for collecting all cytogenetic data and the cytogenetics laboratory, University Hospital Vrije Universiteit, Amsterdam, for performing cytogenetic analysis of adult patients. This study was supported financially by grants from The Dutch Cancer Society and Vrije Universiteit, Amsterdam, as well as a grant from NUFFIC (Netherlands organization for international cooperation in higher education, Den Haag). This work has been carried out in the research laboratory for the Department of Paediatric Haematology and Oncology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

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