# Prognostic Value of Persistent Peripheral Blood and Bone Marrow Lymphoblasts on Day 15 of Therapy in Childhood Acute Lymphoblastic Leukemia as Detected by Flow Cytometry

ANNA JAWORSKA-POSADZY*, JAN STYCZYNSKI*, MALGORZATA KUBICKA, ROBERT DEBSKI, BEATA RAFINSKA-KURYLO, BEATA KOLODZIEJ, MONIKA POGORZALA and MARIUSZ WYSOCKI

*Department of Pediatric Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland*

**Abstract.** *Aim: The predictive value of residual disease measured by flow cytometry at day 15 of induction therapy was analyzed in 182 children treated for acute lymphoblastic leukemia (ALL). Materials and Methods: Peripheral blood (PB) and bone marrow were assessed for leukemia cells by morphology and flow cytometry at days 0, 8, and 15. Results: Absolute blast count (ABC) >200/μl in PB by day 15 assessed by flow cytometry predicted a lower probability of disease free survival (pDFS) (p=0.056). Patients with bone marrow lymphoblast (BML)>0.5% had a lower pDFS (p=0.002). Cumulative relapse incidence for patients with BML<0.5% was 8.9% vs. 47.1% (OR=4.6, p=0.036). In common/pre-B-ALL patients aged >10 years with BML>0.5%, pDFS value was significantly lower. In the multivariate analysis, the only significant factor with adverse prognostic value for pDFS was BML>0.5% (HR=5.3 p=0.030). Conclusion: BML>0.5% analyzed by flow cytometry at day 15 is possibly the strongest prognostic factor in pediatric ALL.*

The speed of blast clearance during therapy is a major prognostic factor of outcome in childhood acute lymphoblastic leukemia (ALL) (1-3). Blast count in the peripheral blood (PB) on day 8, or in the bone marrow on day 15 and day 33, have been widely used to deliver risk-directed therapy (4-6). Another approach to measure the speed of leukemia clearance is the detection of minimal residual disease (MRD) during induction therapy (7, 8), or at days 33 and 78 (9, 10). The key factor of clinical relevance for most centers is the availability of measurement of residual disease at an early time point with a low-cost method. Presence of leukemia blasts during the early phase of therapy can be assessed by microscopy and, preferably, by flow cytometry (FC) or polymerase chain reaction (PCR) of the rearrangements of immunoglobulin (Ig) or T-cell receptor (TCR) gene segments (2, 3). Since PCR methods are laborious and costly, and thus unavailable to most centers, FC which is the method with acceptable sensitivity and wide availability in many centers/countries for hematological and immunological investigations (2, 11). FC in detection of residual disease, although formerly less standardized, may represent an important technology, due to its speed and cheaper availability (1). This technology enables most treatment centers to identify a high proportion of children with ALL who have a good early treatment response or are at high risk of leukemia relapse (11, 12).

In order to estimate the predictive impact of early blast reduction parameters, we investigated the potential of PB and bone marrow (BM) findings at diagnosis (day 0) and early follow-up time points (day 8, day 15, and day 33). In this analysis, we focused on the prognostic impact of residual disease measured by flow cytometry at day 15 of induction therapy.

## Patients and Methods

A total of 182 children, including 98 males and 84 females, aged 1-19 years (median 5 years), consecutively diagnosed and treated for ALL between 1997 and 2010, were included in this study. Infants were excluded from this study. Median follow-up was 5.1 years (range, 0.5-13 years). Patients were recruited from the ALL-BFM-90 (n=78) (13) or ALL-IC-2002 (n=104) (2) protocols. Standard, intermediate-, and high-risk group (SRG, IRG, HRG) criteria were specifically characterized in each therapy protocol.

PB was assessed for leukemia cells by morphology at days 0, 8, 15 and 33, while BM was analyzed by morphology and flow cytometry at days 0 and 15. PB absolute blast count (ABC) at day 15 was analyzed as a categorical value for the following cut-off values: 500 blasts/μl, 200 blasts/μl, and 100 blasts/μl. Early BM response

*Both Authors contributed equally to this study.

*Correspondence to:* Jan Styczynski, MD, Ph.D., Department of Pediatric Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus University, ul. Curie-Sklodowskiej 9, 85-094 Bydgoszcz, Poland. Tel: +48 52 5854860, Fax: +48 52 5854867, e-mail: jstyczynski@cm.umk.pl

*Key Words:* Minimal residual disease, acute lymphoblastic leukemia, flow cytometry, children, early response.

Table I. *Overall treatment results with respect to therapy protocol.*

| | ALL-BFM-90 | ALL-IC-2002 | *p*-Value |
|---|---|---|---|
| Patients, n | 78 (41M, 37F) | 104 (57M, 47F) | |
| Age, years (median, range) | 5.3 (1.6-18.7) | 5.7 (1.1-19.9) | 0.616 |
| PGR/PPR | 75/3 | 97/7 | 0.519 |
| BM 15 (M1/2/3) | 64/10/2 | 43/2/3 | 0.690 |
| pDFS | 0.764±0.053 | 0.792±0.047 | 0.559 |
| Mean survival, years (95% CI) | 10.5 (9.5-11.7) | 6.3 (5.7-6.8) | |

PGR: Prednisolone good responder, PPR: prednisolone poor responder.

by day 15 was assessed as: M1 – blast percentage <5%, M2 – blast percentage 5-<25%, and M3 – blast percentage 25% or higher. Bone marrow lymphoblasts (BML) data assessed by flow cytometry at day 15 were analyzed for following cut-off values: >10% blasts, >1% blasts and >0.5% blasts.

Leukemia-associated immunophenotypes were studied at diagnosis by using the following monoclonal fluorochrome-conjugated antibody combinations: CD10, CD19, CD20, CD34, CD45, CD11a for B-cell precursor ALL; and TdT, CD2, sCD3, cyCD3, CD4, CD5, CD7, and CD8 for T-ALL. Antibodies were purchased from BD Biosciences (San Jose, CA, USA), DAKO/ DakoCytomation (Glostrup, Denmark), and Beckman/Coulter/ Immunotech (Marseille, France). At least two selected combinations were applied during follow-up for MRD detection for each patient. Analyses were performed on EPICS XL (Coulter, Miami, FL, USA) and Cytomics FC500 (Beckman-Coulter, Miami, FL, USA) flow cytometers.

According to blast immunophenotype, all patients were diagnosed as: pro-B (n=8), common/pre-B (n=150) and T-ALL (n=24). At day 15, blast morphology and PB smear (PB15) was assessed in 179 children, BM morphology (BM15) in 140 children and BML by flow cytometry in 91 children. Complete remission (CR) was defined as no physical signs of leukemia, BM with active hematopoiesis, and fewer than 5% leukemia blast cells as assessed by microscopy, and normal cerebrospinal fluid. Results of antileukemia therapy were presented as probability of disease-free survival (pDFS).

*Statistical analysis.* Baseline characteristics of all patients were summarized using descriptive statistics. Differences between groups were measured by chi-square test, Fisher exact test and Mann-Whiney *U*-test. The median survival of this patient group was estimated with a 95% confidence interval (CI). Kaplan-Meier curves were used to summarize DFS. Cox proportional hazards regression model was used to correlate each potential prognostic factor with a survival in univariate analysis. The factors that appeared to be important were then fitted together, and removed one at a time in a backward stepwise manner using the likelihood ratio test at a 0.05 level until all factors in the model were significant. A final check was made to ensure that no excluded factors would improve the fit.

## Results

Patients characteristics and overall treatment results are presented in Table I. No differences in pDFS between patients treated with specific therapy protocol were found. Blast immunophenotype had no significant impact on pDFS.

Table II. *pDFS for all patients with respect to BML at day 15.*

| Cut-off value for BML at day 15 | Below cut-off value | Above cut-off value | *p*-Value |
|---|---|---|---|
| 10% | 0.727±0.068 (n=71) | 0.533±0.248 (n=20) | 0.070 |
| 1% | 0.793±0.071 (n=40) | 0.551±0.107 (n=51) | 0.074 |
| 0.5% | 0.911±0.081 (n=28) | 0.529±0.093 (n=63) | 0.002 |

No difference in pDFS were observed between prednisolone good-responders and poor-responders: 0.756±0.044 *vs.* 0.788±0.134 ($p$=0.524); between children aged below 6 and older than 6 years: 0.751±0.061 *vs.* 0.761±0.049 ($p$=0.686), nor between children aged below 10 and older ones: 0.755±0.052 *vs.* 0.755±0.060 ($p$=0.537).

BM15 had no prognostic value for pDFS, neither for all patients: M1 *vs.* M2 *vs.* M3 (0.748±0.053 *vs.* 0.786±0.110 *vs.* 0.600±0.219, $p$=0.610), nor for M1/2 *vs.* M3 (0.758±0.045 *vs.* 0.600±0.219, $p$=0.320). PB15 ABC was assessed by flow cytometry. Common/pre-B patients with ABC>200/μl (n=9) had pDFS=0.438±0.315, while those with ABC<200/μl had pDFS=0.766±0.055 ($p$=0.056). Common/pre-B patients with ABC>500/μl (n=3) had pDFS=0 *vs.* 0.763±0.054 for patients with ABC<500/μl ($p$=0.124). No significant differences were found for T-ALL patients.

BML15 detected by flow cytometry showed that regardless of cut-off value for presence of BM residual disease (10%, 1% and 0.5% of blasts), a trend for worse pDFS was found for patients with higher BM blast percentage, and this reached statistical significance for patients at cut-off value of BML15=0.5% (Table II). For common/pre-B patients with residual disease >0.5%, a worse pDFS was observed, 0.506±0.101 *vs.* 0.905±0.065, for those with BML<0.5% ($p$=0.002) (Figure 1), while for T-ALL, no significant differences were found: 0.714±0.171 *vs.* 1.00.

Cumulative relapse incidence (CRI) for patients with BML15<0.5% was 8.9% and remained significantly lower than for patients with BML15>0.5% (47.1%, $p$=0.002). No differences in CRI values were observed between patients with BML15 of 0.5-10% and above 10% (Figure 2). In comparison to patients with BML15<0.5%, the relapse risk for patients with BML15>0.5% was 4.6 (odds ratio 95% CI=1.3-13; $p$=0.036).

In patients with common/pre-B-ALL, differences in pDFS which were dependent on BML15=0.5% varied with age, and the worse pDFS was found for children with BML15>0.5%. pDFS for patients aged >6 years with BML15>0.5% *vs.* BML15<0.5% reached 0.34±0.14 *vs.* 1.00 ($p$=0.015), while for patients <6 years: 0.64±0.12 *vs.* 0.89±0.08 ($p$=0.107), respectively. pDFS for patients aged >10 years with BML15>0.5 *vs.* BML15<0.5% was 0.20±0.17 *vs.* 1.00 ($p$=0.021), and for those <10 years: 0.61±0.11 *vs.* 0.89±0.07 ($p$=0.046) (Figure 3).

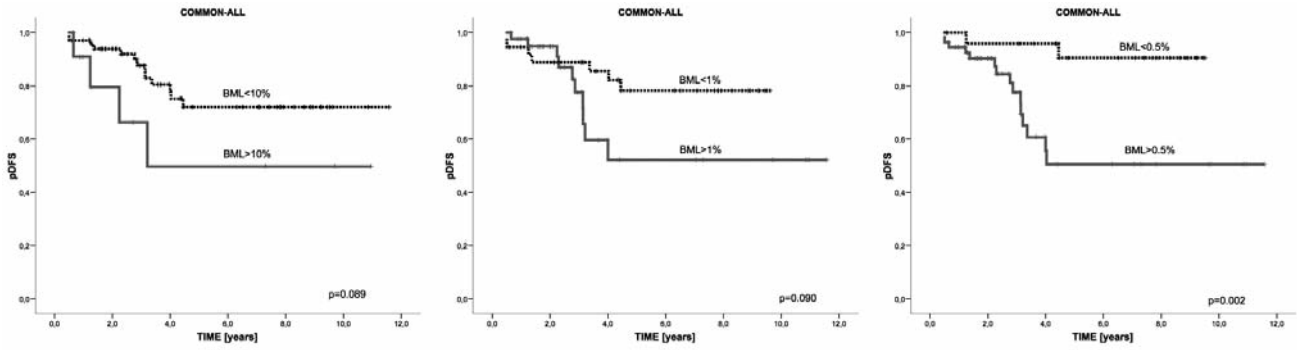


Figure 1. *pDFS for patients with common ALL with respect to BML at day 15.*

The only two factors with $p<0.1$ for pDFS in univariate analyses (PB15 ABC<200; BML15<0.5%) were fitted together in the multivariate analysis. For all patients, the only significant factor with adverse prognostic value for pDFS was BML15>0.5% (hazard ratio=5.3; 95% CI=1.2-23; $p=0.030$). The only significant factor with adverse prognostic value for pDFS for subgroup of patients with common/pre-B phenotype was BML15>0.5% (hazard ratio=5.5; 95% CI=1.2-24; $p=0.027$). No prognostic factor was found when patients were analyzed in subgroups determined by the two therapy protocols.

## Discussion

In this study, we have shown that FC analysis provided good information on MRD during induction therapy of ALL. We determined the favorable prognostic risk factor, based on the presence of <0.5% lymphoblasts in FC analysis in BM at day 15. With this factor, 30% of patients included in the analysis had an excellent outcome, pDFS with 91% with long-term follow-up.

With recently completed, risk-adapted treatment protocols for childhood ALL, most patients reached almost 80% of long-term remission (14-16). Further improvement is expected in better stratification of patients based on MRD studies. The optimal timing and method of MRD assessment are a matter of debate. Recently, several reports have been published dedicated to the role of FC studies of MRD during induction therapy of ALL.

Basso *et al*. in a study involving 815 patients with ALL, showed that MRD levels <0.1% measured by FC on day 15 of remission induction were a powerful prognostic factor that retained independent significance in a model including MRD levels determined by PCR amplification of antigen-receptor genes on days 33 and 78 from the start of remission induction (1). They also showed that classical risk factors such as blast immunophenotype, prednisolone response at day 8, or early BM response at day 15, had lower prognostic value with therapy protocol AIEOP-BFM-ALL 2000 (1).

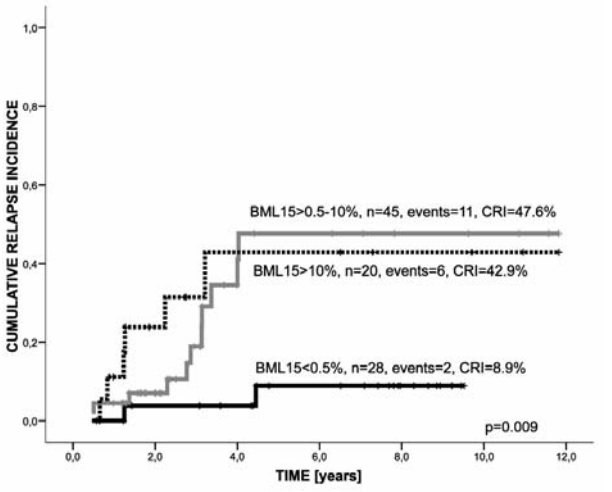


Figure 2. *Cumulative relapse incidence (CRI) in all patients with persisting blasts in bone marrow at day 15.*

Coustan-Smith *et al*., studied MRD by FC at day 19 in 248 patients and found that 46% were MRD negative (<0.01%). At this time point, patients could be segregated by these MRD results into two risk groups of almost equal size but with significantly different outcomes (11). Thus, the findings both of Basso *et al*. (1), and Coustan-Smith *et al*. (11) that MRD testing early in the treatment course can identify patients who are likely to have a superior treatment outcome and can possibly be spared from intensive chemotherapy might have an advantage, especially in resource-poor countries. Our study, performed in a country of central Europe, with relatively limited resources, corroborate results and conclusions of Italian and American research.

Studies of MRD at early time points such as day 15 can be performed proficiently for patients with simple and inexpensive combinations of antibodies (11). Moreover, the FC data can easily be standardized after being transferred *via* Internet to a central site for uniform analysis (17-19).

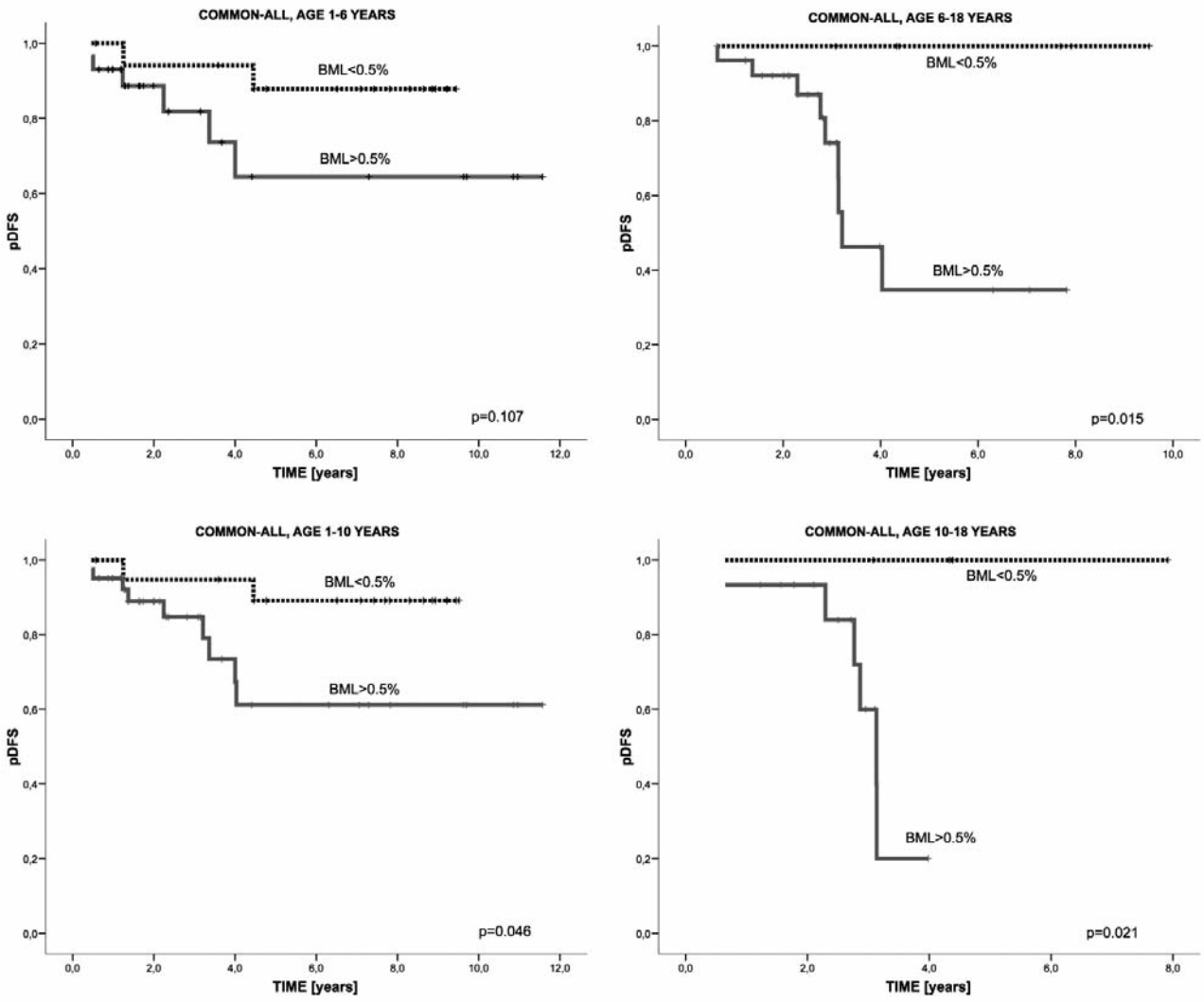


Figure 3. *pDFS for patients with common-ALL with respect to age and BML15 with cut-off value 0.5%.*

Interestingly, there are more data available that other simple tests based on morphology or FC analysis have strong prognostic value, better than previously accepted universal risk factors. Zweidler-McKay *et al*. showed that absolute lymphocyte count in PB higher than 1500/μl at day 29 of ALL therapy in children in protocol COG P9904/5/6 is a powerful prognostic factor with superior HR/*p*-value than MRD status at that day (20).

In conclusion, evidence is gathering that FC analysis of BM at day 15 of induction therapy in childhood ALL has strong prognostic value, comparable to that obtained by PCR MRD studies at later time points. In general, the presence of blasts by day 15 of therapy in childhood ALL is a poor prognosis factor. In this study, we determined an adverse prognostic factor based on the presence of >0.5% lymphoblasts in BM detected by FC. The unfavorable impact of BM blast count >0.5% by day 15 was especially prominent in common/pre-B patients aged above 6 years.

## Acknowledgements

The study was supported by grants: MNiSW N407 541339; Ph.D. stipend 2008/2009 ZPORR, UMK 10/2009 and Ph.D. stipend EFS 2010.

## References

1 Basso G, Veltroni M, Valsecchi MG, Dworzak MN, Ratei R, Silvestri D, Benetello A, Buldini B, Maglia O, Masera G, Conter V, Arico M, Biondi A and Gaipa G: Risk of relapse of childhood acute lymphoblastic leukemia is predicted by flow cytometric measurement of residual disease on day 15 bone marrow. J Clin Oncol *27*: 5168-5174, 2009.

2 Mejstrikova E, Fronkova E, Kalina T, Omelka M, Batinic D, Dubravcic K, Pospisilova K, Vaskova M, Luria D, Cheng SH, Ng M, Leung Y, Kappelmayer J, Kiss F, Izraeli S, Stark B, Schrappe M, Trka J, Stary J and Hrusak O: Detection of residual B precursor lymphoblastic leukemia by uniform gating flow cytometry. Pediatr Blood Cancer *54*: 62-70, 2010.
3 Szczepanski T, Orfao A, van der Velden VH, San Miguel JF and van Dongen JJ: Minimal residual disease in leukaemia patients. Lancet Oncol *2*: 409-417, 2001.
4 Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, Ludwig R, Ritter J, Stollmann B and Henze G: Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83). Klin Padiatr *199*: 151-160, 1987.
5 Gaynon PS, Desai AA, Bostrom BC, Hutchinson RJ, Lange BJ, Nachman JB, Reaman GH, Sather HN, Steinherz PG, Trigg ME, Tubergen DG and Uckun FM: Early response to therapy and outcome in childhood acute lymphoblastic leukemia: A review. Cancer *80*: 1717-1726, 1997.
6 Moricke A, Reiter A, Zimmermann M, Gadner H, Stanulla M, Dordelmann M, Loning L, Beier R, Ludwig WD, Ratei R, Harbott J, Boos J, Mann G, Niggli F, Feldges A, Henze G, Welte K, Beck JD, Klingebiel T, Niemeyer C, Zintl F, Bode U, Urban C, Wehinger H, Niethammer D, Riehm H and Schrappe M: Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: Treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95. Blood *111*: 4477-4489, 2008.
7 Morimoto A, Kuriyama K, Hibi S, Todo S, Yoshihara T, Kuroda H and Mashuku S: Prognostic value of early response to treatment combined with conventional risk factors in pediatric acute lymphoblastic leukemia. Int J Hematol *81*: 228-234, 2005.
8 Ratei R, Basso G, Dworzak M, Gaipa G, Veltroni M, Rhein P, Biondi A, Schrappe M, Ludwig WD and Karawajew L: Monitoring treatment response of childhood precursor B-cell acute lymphoblastic leukemia in the AIEOP-BFM-ALL 2000 protocol with multiparameter flow cytometry: Predictive impact of early blast reduction on the remission status after induction. Leukemia *23*: 528-534, 2009.
9 van der Velden VH, Panzer-Grumayer ER, Cazzaniga G, Flohr T, Sutton R, Schrauder A, Basso G, Schrappe M, Wijkhuijs JM, Konrad M, Bartram CR, Masera G, Biondi A and van Dongen JJ: Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. Leukemia *21*: 706-713, 2007.
10 Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grumayer R, Moricke A, Arico M, Zimmermann M, Mann G, De Rossi G, Stanulla M, Locatelli F, Basso G, Niggli F, Barisone E, Henze G, Ludwig WD, Haas OA, Cazzaniga G, Koehler R, Silvestri D, Bradtke J, Parasole R, Beier R, van Dongen JJ, Biondi A and Schrappe M: Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: Results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood *115*: 3206-3214, 2010.
11 Coustan-Smith E, Ribeiro RC, Stow P, Zhou Y, Pui CH, Rivera GK, Pedrosa F and Campana D: A simplified flow cytometric assay identifies children with acute lymphoblastic leukemia who have a superior clinical outcome. Blood *108*: 97-102, 2006.
12 Coustan-Smith E, Sancho J, Behm FG, Hancock ML, Razzouk BI, Ribeiro RC, Rivera GK, Rubnitz JE, Sandlund JT, Pui CH and Campana D: Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia. Blood *100*: 52-58, 2002.
13 Schrappe M, Reiter A, Ludwig WD, Harbott J, Zimmermann M, Hiddemann W, Niemeyer C, Henze G, Feldges A, Zintl F, Kornhuber B, Ritter J, Welte K, Gadner H and Riehm H: Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: Results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM study group. Blood *95*: 3310-3322, 2000.
14 Pui CH, Pei D, Sandlund JT, Ribeiro RC, Rubnitz JE, Raimondi SC, Onciu M, Campana D, Kun LE, Jeha S, Cheng C, Howard SC, Metzger ML, Bhojwani D, Downing JR, Evans WE and Relling MV: Long-term results of St. Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. Leukemia *24*: 371-382, 2010.
15 Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, Ludwig WD, Ritter J, Harbott J, Mann G, Klingebiel T, Zintl F, Niemeyer C, Kremens B, Niggli F, Niethammer D, Welte K, Stanulla M, Odenwald E, Riehm H and Schrappe M: Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia *24*: 265-284, 2010.
16 Conter V, Arico M, Basso G, Biondi A, Barisone E, Messina C, Parasole R, De Rossi G, Locatelli F, Pession A, Santoro N, Micalizzi C, Citterio M, Rizzari C, Silvestri D, Rondelli R, Lo Nigro L, Ziino O, Testi AM, Masera G and Valsecchi MG: Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. Leukemia *24*: 255-264, 2010.
17 Dworzak MN, Gaipa G, Ratei R, Veltroni M, Schumich A, Maglia O, Karawajew L, Benetello A, Potschger U, Husak Z, Gadner H, Biondi A, Ludwig WD and Basso G: Standardization of flow cytometric minimal residual disease evaluation in acute lymphoblastic leukemia: Multicentric assessment is feasible. Cytometry B Clin Cytom *74*: 331-340, 2008.
18 Lorenzana R, Coustan-Smith E, Antillon F, Ribeiro RC and Campana D: Simple methods for the rapid exchange of flow cytometric data between remote centers. Leukemia *14*: 336-337, 2000.
19 Pui CH: Toward a total cure for acute lymphoblastic leukemia. J Clin Oncol *27*: 5121-5123, 2009.
20 Zweidler-McKay P, Gramatges MM, Borowitz M, Palla S, Shi X, Margolin J and Rabin K: Refining MRD-based risk stratification in pediatric ALL using absolute lymphocyte counts (abstract 301b). Pediatr Blood Cancer *54*: 788, 2010.

*Received February 10, 2011*
*Revised March 6, 2011*
*Accepted March 8, 2011*