A simplified minimal residual disease polymerase chain reaction method at early treatment points can stratify children with acute lymphoblastic leukemia into good and poor outcome groups

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

Minimal residual disease is an important independent prognostic factor in childhood acute lymphoblastic leukemia. The classical detection methods such as multiparameter flow cytometry and real-time quantitative polymerase chain reaction analysis are expensive, time-consuming and complex, and require considerable technical expertise.

Design and Methods

We analyzed 229 consecutive children with acute lymphoblastic leukemia treated according to the GBTLI-99 protocol at three different Brazilian centers. Minimal residual disease was analyzed in bone marrow samples at diagnosis and on days 14 and 28 by conventional homo/heteroduplex polymerase chain reaction using a simplified approach with consensus primers for *IG* and *TCR* gene rearrangements.

Results

At least one marker was detected by polymerase chain reaction in 96.4% of the patients. By combining the minimal residual disease results obtained on days 14 and 28, three different prognostic groups were identified: minimal residual disease negative on days 14 and 28, positive on day 14/negative on day 28, and positive on both. Five-year event-free survival rates were 85%, 75.6%, and 27.8%, respectively ($p<0.0001$). The same pattern of stratification held true for the group of intensively treated children. When analyzed in other subgroups of patients such as those at standard and high risk at diagnosis, those with positive B-derived CD10, patients positive for the *TEL/AML1* transcript, and patients in morphological remission on a day 28 marrow, the event-free survival rate was found to be significantly lower in patients with positive minimal residual disease on day 28. Multivariate analysis demonstrated that the detection of minimal residual disease on day 28 is the most significant prognostic factor.

Conclusions

This simplified strategy for detection of minimal residual disease was feasible, reproducible, cheaper and simpler when compared with other methods, and allowed powerful discrimination between children with acute lymphoblastic leukemia with a good and poor outcome.

Key words: minimal residual disease, acute lymphoblastic leukemia, childhood, *IG*, *TCR*.

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Introduction

Early response to treatment based on the rate of disappearance of leukemic cells in bone marrow has proven to be an independent prognostic factor in childhood acute lymphoid leukemias (ALL) and is being used by several groups as a criterion for the stratification of children for risk-adapted therapy.[1,2] Morphological analysis, although useful and applicable at any center, has proven to be subjective, of limited sensitivity and imprecise for the study of early response to treatment.[2-4] Sequential monitoring of minimal residual disease (MRD) using more sensitive and specific techniques, such as polymerase chain reaction (PCR) and flow cytometry analysis, with a detection power of one blast cell in 10^3 to 10^6 normal cells, has substantially refined the assessment of early response to treatment.[2,5-14] These methods, however, are expensive and complex and require considerable technological availability,[8,10,15,16] thus being inaccessible to most treatment centers, especially in developing countries.

Simplified methodologies for the assessment of the early response, including the evaluation of MRD, may provide good predictive criteria of an unfavorable course in children with ALL and could be used to identify patients at a high risk of relapse.[15,17]

The objective of the present study was to determine the prognostic impact of the presence of MRD on days 14 and 28 of induction therapy in children with ALL treated according to the protocol of the Brazilian Group of Treatment of Childhood Acute Leukemia (GBTLI-99) using a simplified PCR methodology with consensus primers for the detection of rearrangements in the immunoglobulin (*IG*) and T-cell receptor (*TCR*) genes.

Design and Methods

Characteristics of the patients

Samples were obtained from 229 patients with childhood ALL aged 1 to 18 years old, classified and treated according to the GBTLI-99 protocol at three different treatment centers and with frozen DNA samples of good quality for PCR. Of the 340 non-infant patients submitted to treatment at the participating institutions during the study period, 111 were excluded because of the lack of DNA at diagnosis or on days 14 and 28, three because of major violations of the protocol, and two because of loss to follow-up at the service of origin. Overall and event-free survival rates and the biological and clinical-laboratory characteristics were similar for the total group of patients and for the group of patients analyzed (*data not shown*). Twenty-six of the patients were also analyzed by flow cytometry for comparison of the MRD data obtained by PCR. The study was approved by the National Research Ethics Committee (CONEP, No. 1971/2005) and was based on the Helsinki convention criteria. The people responsible for the children gave written informed consent to participation in the study.

The diagnosis was made by standard morphological analysis and by flow cytometry immunophenotyping. Of the 229 patients studied, 192 (83.8%) had B-cell-derived ALL (pro-B ALL in 6 cases and common-ALL/pre-B ALL in 186), and 37 (16.2%) had T-cell-derived ALL; 139 were boys and 90 were girls (ratio 1.5:1) ranging in age from 13 months to 17 years (median 5 years). The presence of t(12;21), t(4;11), t(9;22) and t(1;19) was investigated by reverse transcription PCR (RT-PCR)[18] in 201 patients. The time of follow-up of patients in complete clinical remission ranged from 9 to 92 months, with a median observation of 50 months and a cut-off date in March 2008.

According to the GBTLI-99 protocol, patients older than 9 years and/or with a white blood cell (WBC) count at diagnosis greater than 50×10^9/L were assigned to the group at high risk of relapse and the remaining ones were assigned to the low risk group, which received less intense treatment. Patients were characterized as poor responders, regardless of the initial risk group, if they met one or more of the following criteria during the phase of induction of remission: WBC count greater than 50×10^9/L on day 7, any blasts on the smear of peripheral blood on day 14, M3 bone marrow (>25% blasts) on day 14 or M2/M3 bone marrow (>5 and 25% blasts, respectively) on day 28. The choice of a WBC count of less than 50×10^9/L as a response criterion was due to its ability to discriminate children with good and poor outcomes following the GBTLI-93 protocol and to the fact that it is a parameter that can be easily measured at any treatment center. If patients belonged to the low risk group at the time of diagnosis (diagrisk), they were then reassigned to the treatment protocol of the high final risk group (finalrisk). All patients, regardless of risk group, received an induction regimen of 4 weeks including prednisone, vincristine, doxorubicin, L-asparaginase, and MADIT (intrathecal methotrexate, ara-C and dexamethasone). Low finalrisk patients received courses of consolidation [cyclophosphamide, ara-C, 6-mercaptopurine (6-MP)], intensification (methotrexate 2 g/m^2 x 4, 6-MP, MADIT), late consolidation [dexamethasone, vincristine, doxorubicin, L-asparaginase, cyclophosphamide, 6-thioguanine (6-TG), MADIT] and maintenance (6-MP, methotrexate, vincristine, dexamethasone, MADIT). Patients classified as high finalrisk of relapse received courses of: block A (methotrexate 2 g/m^2, 6-TG, ara-C 2 g/m^2 x 2, cyclophosphamide, MADIT), block B (methotrexate 2 g/m^2, 6-MP, ara-C 1 g/m^2 x 4, MADIT), intensification (dexamethasone, vincristine, daunomycin, cyclophosphamide, ara-C, 6-TG, MADIT), block C (methotrexate 2 g/m^2, 6-MP, ara-C 2 g/m^2 x 2, VP-16, MADIT), block D (ifosfamide, VP-16, MADIT), late consolidation (dexamethasone, vincristine, daunomycin, L-asparaginase, cyclophosphamide, ara-C, 6-TG, MADIT) and maintenance (6-MP, methotrexate, vincristine, dexamethasone, MADIT). Patients with CNS-3 (> 5% blast cells in cerebrospinal fluid) after day 14 of induction therapy received cranial radiotherapy (1200 Gy during the late consolidation phase) according to the GBTLI-99 protocol. Only four of the patients studied received cranial irradiation.

Cell samples and DNA isolation

Bone marrow samples were obtained from the patients at diagnosis and at the same two time points

used for morphological analysis according to the GBTLI-99 protocol: day 14 of induction and at the end of the induction therapy (day 28). Mononucleated cells were separated by a Ficoll-Paque centrifugation gradient and DNA was extracted with the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was quantified and evaluated by spectrophotometry (OD 260 nm/OD 280 nm) and tested for quality by agarose gel and standard PCR amplification for the beta-globin gene.

Minimal residual disease determined by polymerase chain reaction

DNA samples (50 ng) from the cells collected at diagnosis were amplified by PCR with consensus primers flanking the CDR-3 region of the *IGH* gene (*FR3A, LJH, VLJH*), *TCRG* (*Vγ I-IV* and *Jγ*1-3 families),[19,20] incomplete *TCRD* (*Vδ2Dδ3*)[21] and *IGK* rearrangements[22,23] (*Online Supplementary Appendix 1*). The PCR product was submitted to homo/heteroduplex analysis as previously described.[24] Clonality was characterized by the presence of a band of the expected size in the homo/heteroduplex analysis on 12% polyacrylamide gel. The expected fragment sizes are: 80 to 120 bp for CDR 3 (*IGH*), 80 to 100 bp for *TCRD*, 170 to 210 bp for *TCRG*,[19-21,25] 364 to 433 bp for *IGK* mix 1, and 175 to 443 bp for mix *IGK* mix 2.[23]

Multiplex PCR was initially applied for the *TCRG* gene with various primers of the V and J segments simultaneously (A and B mixes, *Online Supplementary Appendix 2*). To determine and verify the gene segments involved in a positive *TCRG* multiplex reaction, we performed split-out analysis using individual primer sets for segment V. Two negative controls were used in each PCR assay: one without DNA and the other containing polyclonal DNA obtained from peripheral blood mononucleated cells. The PCR protocols for the various primers are shown in *Online Supplementary Appendix 2*.

The samples analyzed on day 14 and day 28 of induction therapy were used at a DNA concentration of 500 ng/reaction and were considered to be positive when they presented the same migration pattern as the samples obtained at diagnosis and amplified in the same reaction for *IGH, IGK* and/or *TCR* rearrangements (see, for example, Figure 1). To obtain more objective data the samples were analyzed after scanning the gel and by two different observers. The MDR analyses were carried out in the laboratories of the three participating centers. To check the concordance and reproducibility of the assays, samples were reanalyzed at different time points in the same laboratory and also in different laboratories and the results were found to be closely concordant.

To validate the assay, samples of each amplicon were sequenced using the ABI-Prism Big-Dye Terminator Cycle Sequencing Read Reaction kit (Applied Biosystems, CA, USA) and the results were in agreement. PCR sensitivity ranged from 10^{-2} to 10^{-3} and was determined from serial dilutions of bone marrow DNA obtained at diagnosis and containing more than 90% blast cells in a pool of bone marrow DNA obtained from six patients with no hematologic disease.

The cost of the reagents and consumables used in this methodology was calculated to be about €10 for the samples obtained at diagnosis and €8 for those obtained at each time point analyzed.

Minimal residual disease determined by flow cytometry

Leukemic blasts were immunophenotyped using a two- or three-color combination of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCp) or CyChrome: IgG1-FITC/PE/CyChrome; CD1a-FITC; CD2-PE; CD3-PE/CyChrome; CD4-FITC; CD7-PE; CD8-PE; CD10-PE; CD15-FITC; CD19-FITC/CyChrome; CD20-FITC; CD33-FITC/PE; CD45-FITC; CD79a-PE (Becton Dickinson - Pharmingen, San Jose, CA, USA); CD10-FITC; CD22-PE; CD34-FITC/PE; CD45-PerCp; MPO-FITC; terminal deoxynucleotidyl transferase (TdT)-FITC (Becton Dickinson Biosciences, San Jose, CA, USA); CD13-PE; IgM-FITC (Dako, Carpinteria, CA, USA). The stain and lyse/wash technique was used as previously reported.[26] Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with the Cell Quest software program. At least one useful aberrant marker combination was identified at diagnosis and then used to monitor MRD in the follow-up samples.

Matched non-reactive fluorochrome-conjugated antibodies and the CD4-FITC/CD8-PE/CD3-CyChrome combination were used as negative and positive controls, respectively. A two-step acquisition procedure was applied. In the first step, a total of 15×10^3 non-gated events were acquired. In the second step, a live gate based on the lineage marker expression was set: CD19 for BCP-ALL and cytoplasmic (cy) CD3 for T-ALL, and low/ inter-

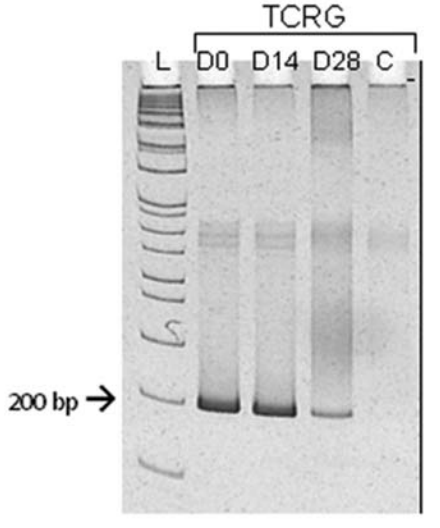

Figure 1. Example of homo/heteroduplex polymerase chain reaction for *TCRG* on samples taken from a single patient. (L) molecular weight marker; (D0), diagnostic sample; (D14), sample obtained on day 14 of induction; (D28), sample obtained on day 28 of induction therapy; (C-), negative control (polyclonal DNA from bone marrow mononuclear cells of patients without hematologic disease). Note that because of the similarity of the migration pattern and molecular weight of the three bands, this child was considered to be minimal residual disease positive on days 14 and 28 of induction.

mediate side scatter characteristics (SSC - lymphoid scatter). The total numbers of cells, usually 10^5-10^6 events passing though the flow cytometer, were recorded for each live gate acquisition.[26-28]

The expression of all markers was analyzed within the $CD19^+$ or $cyCD3^+$/ lymphocyte SSC gate to determine the presence of any cell subset with the aberrant marker.[26-28] In patients with no aberrant marker, MRD was monitored by the three triple-stainings CD45/CD10/CD19, CD34/CD22/CD19, and CD20/CD10/CD19.[26,27]

Data analysis was performed with Paint-a-Gate Pro software (Becton Dickinson, San Jose, CA, USA). A cluster of at least 10-20 events with expression of the patient's aberrant marker and adequate SSC was considered to demonstrate MRD. For comparison with the PCR results, *flow cyometry-detectable MRD* was defined as the expression of the leukemia-associated immunophenotype in 0.1% or more of the total events present in the sample.

Statistical analysis

Fisher's test was used to assess the association between the presence of MRD at the time points studied (day 14 and day 28) and the following clinical and biological variables: age, number of WBC at diagnosis, involvement of the central nervous system at diagnosis, immunophenotype, risk group, as well as the following response variables: number of WBC at day 7 and bone marrow status at day 14 and day 28 after the beginning of induction therapy. Event-free survival curves were constructed according to the Kaplan-Meier method. Primary resistance, relapse, or death due to any cause were considered unfavorable events. Curves for different groups were compared by the log-rank test. Multivariate analyses were done with the Cox model stratified by the type of assigned chemotherapeutic protocol which corresponds to the finalrisk group (see above). A step-down procedure (backward elimination) was adopted. The process was stopped when all the retained regression coefficients were significant at $p \leq 0.1$. All statistical analyses were performed using SPSS software version 15.0 (SPSS Inc., Chicago, USA), with the level of significance set at $p \leq 0.05$, except for multivariate analyses.

Results

Analysis of clonality in the samples obtained at diagnosis

At least one clonal rearrangement was identified in 221/229 (96.4%) of the patients studied: 185/192 (96.2%) for B lineage ALL and 36/37 (97.5%) for T-ALL. Two or more clonal markers were detected in 204/229 (89.1%) of cases: 30/37 (81.1%) for T-ALL cases and 163/192 (89.4%) for B-lineage ALL cases. For patients for whom no amplification was obtained for any of the screened markers, false negative results were ruled out by the amplification of the beta globin gene as a quality control test. The frequencies of detection of rearrangements were closely similar in the different participating laboratories (*data not shown*), suggesting the reproducibility of the assays.

For cases of B-lineage ALL, the most frequent rearrangement was *IGH* (148/198 patients, 77.4%), followed by *TCRG* (115/198 patients, 58.3%), incomplete *TCRD* (99/198 patients, 50%), and *IGK* (96/198 patients, 48.4%). For T lineage-derived ALL, the most frequent rearrangement was *TCRG* (36/37 patients, 97.5%), followed by incomplete *TCRD* (3/37 patients, 8.1%).

The frequencies of detection and the rearrangements obtained in both B-lineage and T-ALL were closely similar in the three participating laboratories (*data not shown*).

Minimal residual disease analysis

The clinical and biological data for the 229 patients evaluated in the present study are listed in Table 1. Unfavorable events (primary resistance, relapse or death) occurred in 43 patients (18.7%). Death during induction or during clinical remission occurred in 16 patients (6.9%) while 27 relapsed. Of the 121 patients initially classified as being at low risk, 12 were considered to be poor responders and were reclassified as having a high finalrisk according to GBTLI-99 criteria.

With the technique used, MRD was detected on day 14 and day 28 in 55/210 (26.2%) and 29/220 (13.2%) of cases, respectively. Only one patient with MRD detected on day 28 was negative on day 14. The clinical and biological variables that usually predict a poor response to treatment were significantly associated with the detection of MRD on days 14 and 28 of induction therapy (Table 2). Patient's age, presence of cerebrospinal fluid infiltrate at diagnosis, or *TEL/AML1* positivity were not associated with MRD data on day 14. Likewise age, risk group at diagnosis, immunophenotype, or *TEL/AML1* positivity were not associated with MRD on day 28. From 201 non-infant patients analyzed by RT-PCR, five had the translocation t(9;22) and only two the t(4;11). Although these patients had a higher incidence of MRD on day 14 (5/7 patients MRD positive, 71.4%) and on day 28 (4/7 patients MRD positive, 57.1%), the association of MRD and event-free survival remained statistically significant for all time points in the rest of the cohort after the Philadelphia chromosome-positive and *MLL*-positive patients were excluded (*data not shown*) due to the small number of patients.

As shown in Table 1, several prognostic factors for event-free survival were statistically significant. Figure 2A illustrates that, despite a more intensive therapeutic protocol, children in the high finalrisk group fared less well than those in the low risk group. The prognostic value of the presence of MRD on day 14 and day 28 is shown in Table 1 and Figures 2B-2C. The results led to patients being classified into three different prognostic groups according to MRD tests: (i) MRD-negative patients on days 14 and 28 (5-year event-free survival: 85%), (ii) MRD-positive patients on day 14 and negative on day 28 (5-year event-free survival: 76%), and (iii) MRD-positive patients on day 28 (5-year event-free survival: 28%) ($p<0.0001$, Figure 2D). When only the patients who were MRD-negative on days 14 and 28 were compared to those who were MRD-positive on day 14 and negative on day 28, although the 5-year event-free survival was higher in the former group, this difference was not statistically significant ($p=0.23$).

Table 1. Clinical and biological variables and 5-year event-free survival (±SD) according to the variables analyzed in a group of children with acute lymphoblastic leukemia.

Variable	Strata	Percentage (%)	5y-EFS±SD[1] (%)	*p*
Age (n=229)	1-9 years (n=168)	73.4	80.2±3.6	0.015
	>9 years (n=61)	26.6	70.5±6.1	
Diagnostic WBC (n=229)	<50×10⁹/L (n=158)	69	77.9±3.7	0.76
	>50×10⁹/L (n=71)	31	77.2±5.5	
Diagnostic CNS (n=220)	CNS 3 (n=9)	4.1	79.4±3.1	0.0001
	CNS 1 or 2 (n=211)	95.9	40±17.4	
WBC on day 7 (n=225)	<50×10⁹/L (n= 208)	92.4	80.3±3.0	0.001
	≥50×10⁹/L (n=17)	7.6	34.9±17.4	
BM on day 14 (n=199)	M1/M2 (n=190)	95.5	81.8±3.1	<0.0001
	M3 (n=9)	4.5	19.8±17.3	
BM on day 28 (n=226)	M1 (n=220)	97.3	79.0±3.1	0.004
	M2/M3 (n=6)	2.7	44.4±22.2	
Response (n=205)	Good responder (n=177)	86.3	83.8±3.1	<0.0001
	Poor responder (n=28)[2]	13.7	30.7±12.2	
Risk group at diagnosis[3] (n=229)	Low risk (n=121)	52.8	79.8±4.2	0.14
	High risk (n=108)	47.2	75.3±4.5	
Final risk group[4] (n=229)	Low risk (n=109)	47.6	84.1±4.0	0.016
	High risk (n=120)	52.4	71.7±4.7	
Immunophenotype (n=229)	Pre-B/common (n=186)	81.2	77.6±3.5	0.64
	T (n=37)	16.2	78.3±7.3	
	Pro-B (n=6)	2.6	66.7±19.3	
TEL/AML1 (n=201)	Positive (n=51)	25.4	78.8±6.5	0.89
	Negative (n=150)	74.6	78.0±3.8	
MRD on day 14 (n=210)	Negative (n=155)	78.3	84.6±3.2	0.0001
	Positive (n=55)	26.2	57.4%±7.5	
MRD on day 28 (n=220)	Negative (n=191)	86.8	82.9±3.0	<0.0001
	Positive (n=29)	13.2	27.8±12.0	
MRD on days 14/28 (n=211)	Negative day 14 (n=151)	71.6	85.0±3.2	<0.0001
	Positive day 14/negative day 28 (n=31)	14.7	76.0±8.0	
	Positive day 28 (n=29)	13.7	27.8±12.0	

[1]Kaplan-Meier method and log rank test. [2]Poor responders: patients who fulfilled one or more of the following criteria during the phase of induction of remission: WBC on day 7 >5×10⁹/L, any blasts on the smear of peripheral blood on day 14, bone marrow M3 (>25% blasts) on day 14 or bone marrow M2 or M3 (>5 and 25% blasts, respectively) on day 28. [3]Low risk at diagnosis: age between 1-9 years AND diagnostic WBC<50×10⁹/L; High risk: all others. [4]Low final risk group: Low risk at diagnosis AND good responders; High final risk: high risk at diagnosis AND poor responders who were initially assigned to low risk at diagnosis.

In a multivariate analysis, stratified by the type of chemotherapeutic protocol (i.e. final risk group), and initially containing age, WBC at diagnosis, morphological status of bone marrow on day 28, and MRD on day 28, only age and MRD were statistically significant. The risk of an event in children with positive MRD on day 28 was 4.9 times higher (95% confidence interval 2.4-9.7; $p<0.00001$) than that in children with negative MRD. The risk in children older than 9 years was 2.2 higher (95% confidence interval 1.05-4.8; $p=0.04$) than that in children between 1 and 9 years old.

It is interesting to note that the 5-year probability of event-free survival for children intensively treated (high finalrisk group) was significantly dependent on the MRD on day 28. The event-free survival for those with negative MRD (n=92) was 80.1±4.4% and for those with positive MRD (n=24), 23.7±12.6% ($p=0.00001$; Figure 3A). Analysis of the low finalrisk group was hampered by the small number of children with positive MRD on day 28 (n=5). When the high finalrisk patients were stratified into three groups according to (i) absence of MRD on days 14 and 28, (ii) presence of MRD on day 14 and absence of MDR on day 28, and (iii) presence of MRD on day 28, again a progressively lower 5-year event-free survival was observed, with the separation into distinct groups with good prognosis, intermediate prognosis and poor prognosis (87.3±4.3%, 60.8±11.9%, and 23.7±12.6%, respectively, $p=0.00001$; Figure 3B). When

Table 2. Association between the clinical and biological variables of the group of patients analyzed as a whole and the presence of minimal residual disease on days 14 and 28 determined by Fisher's exact test.

	MRD day 14	MRD day 28
Age	0.47	0.26
Diagnostic WBC	0.02	0.05
CNS	0.21	0.02
WBC on D7	0.001	0.002
MO on D14	<0.001	<0.001
MO on D28	0.001	0.003
Response[1]	<0.001	<0.001
Risk group at diagnosis[2]	0.04	0.23
Final risk group[3]	<0.001	0.001
Immunophenotype[4]	0.04	0.31
TEL/AML1	0.27	1.00

[1]Response: good responders versus poor responders (see Table 1); [2]See Table 1 [3]See Table 1. [4]Immunophenotype: pre-B/common ALL versus T and pro-B ALL.

comparing only patients who were MRD-negative on days 14 and 28 to those who were MRD-positive on day 14 and negative on day 28, a significantly higher 5-year event-free survival was observed in the former group (p=0.02). MRD on day 28 was also a significant prognostic factors when other subgroups of patients were considered: those at standard and high risk at diagnosis (p<0.00001 and p=0.001, respectively), and those who were B-lineage CD10 positive (p<0.00001), B-lineage CD10-negative (p=0.02), *TEL/AML*-positive (p<0.00001), and *TEL/AML*-negative (p=0.0006). Analysis for T-cell patients was hampered by the small number of children who were positive for MRD on day 28 (n=6).

Considering the morphological status of the bone marrow on day 28 (<5% blasts versus ≥5% blasts), a traditional prognostic factor, those with a marrow *in remission* but with positive MRD in the same marrow fared worse than those without MRD in the marrow (5-year event-free survival 31.0±13.2% and 83.0±3.0%, respectively; p=0.00001; Figure 3C). Six out of 226 patients (3 died before day 28) were not in morphological remission on day 28; four had concomitant positive MRD: three relapsed and one was in remission 11 months after diagnosis. Two children although not in morphological remission on day 28 did not have detectable MRD at this point; both are in long-term remission 5 and 7 years since diagnosis. It is possible that in these cases blast cells in the marrow may have been misinterpreted as leukemic blasts.

When the 26 cases studied by PCR and flow cytometry were analyzed using the same 0.1% cut-off point to define the presence or absence of MRD, agreement between methods was demonstrated in 24 of them (92%) on day 14. In two cases, MRD was found to be positive when determined by flow cytometry and negative when determined by PCR. On day 28 the agreement was 100%. The frequencies of MRD detection at each time point, as well as the MRD-based survival curves determined by PCR at the three different centers were closely similar (*data not shown*).

Discussion

MRD monitoring by flow cytometry and by real time quantitative PCR (RQ-PCR) has been significantly correlated with clinical prognosis, being particularly useful for the evaluation of an early response and thus permitting a refined stratification of treatment for both children[2,7,10-15,29-32] and adults.[33-35] Stratification into risk groups according to criteria not based on MRD has proven to be less accurate compared to stratification according to criteria based on MRD, especially for low risk patients.[36]

With the technique used in the present study, the presence of at least one clonal rearrangement for the study of MRD was identified in 96.4% of the patients studied. The presence of two or more markers was detected in 88.4% of the cases investigated, showing that the simpli-

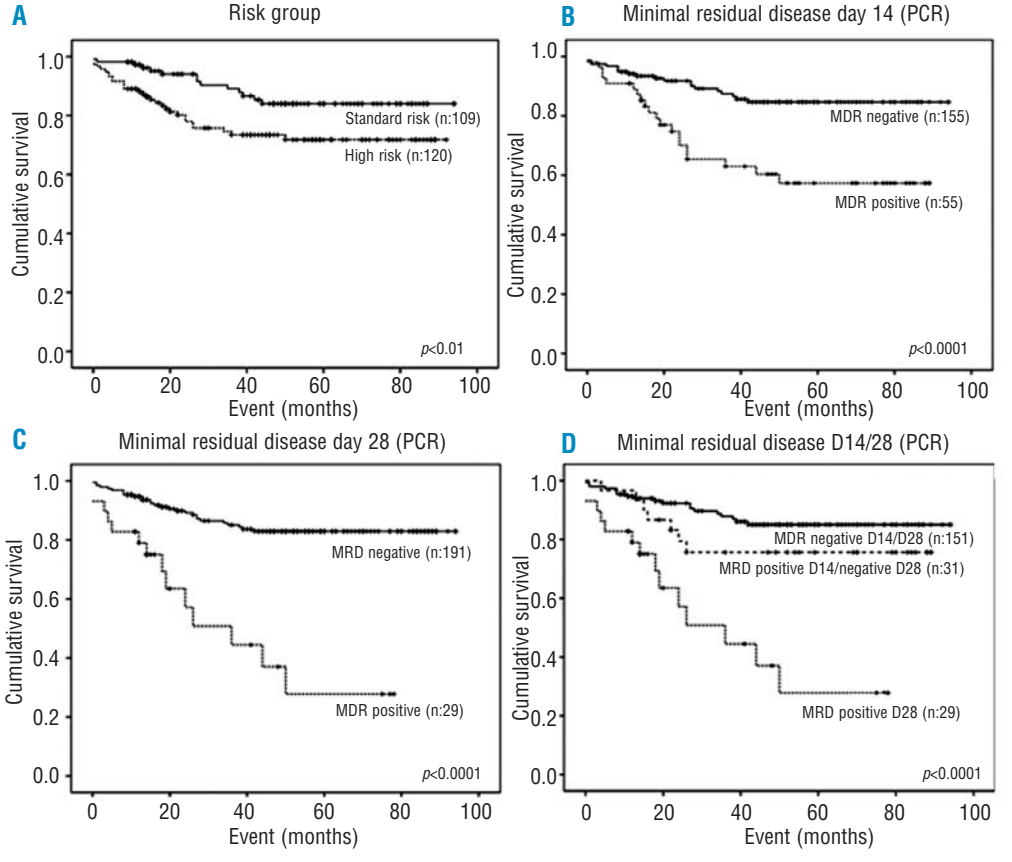

Figure 2. Event-free survival according to (A) risk group on day 28 of induction (final risk group), (B) minimal residual disease on day 14 by polymerase chain reaction, (C) minimal residual disease on day 28 by polymerase chain reaction and (D) minimal residual disease on days 14 and 28 by polymerase chain reaction.

fied technique can be used for the study of MRD in most children with ALL and confirming the preliminary data obtained with a similar methodology.[17] Although the present method is less sensitive, the results obtained were similar to those reported with the use of more complex techniques such as RQ-PCR and flow cytometry, mainly in the higher risk group.[2,8-15]

In addition, the frequency of detection of the rearrangements at diagnosis and the frequency of positivity for MRD during the phases analyzed were closely similar at the three treatment centers, showing good reproducibility of the technique used. Comparison of the two methods in the 26 patients for whom both methods were used showed 92% agreement of the results obtained on day 14 and 100% agreement of the results obtained on day 28, suggesting a good correlation between them despite the small number of cases investigated. Similar results from comparisons of RQ-PCR and flow cytometry have been reported previously.[8,37,38] The frequency of MRD cases that were positive on both day 14 and day 28 was significantly higher among patients classified as being at high final risk and in patients with T-ALL, in agreement with data reported by others.[30,31,36,39] MRD positivity on days 14 and 28 was also found to be associated with the initial response to treatment. The group of patients considered to be good responders had a lower frequency of positive MRD results compared to the group of poor responders (p<0.0001).

In this study the presence of MRD on day 28 was associated with relapse and/or death rates in all groups studied and was an independent adverse prognostic factor compared to the classical stratification factors used by the GBTLI-99. The present study demonstrates that the combination of MRD data obtained on day 14 and day 28 was able to define three groups with distinct prognoses: (i) patients without MRD on days 14 and 28 had event-free survival rates higher than 85%, (ii) patients who were positive for MRD on day 14 and negative on day 28 had an intermediate prognosis, and (iii) patients who were positive for MRD on day 28 had a high relapse rate. This pattern of stratification of patients was also observed for the group of intensively treated children (Figure 3B). These data support previously published preliminary results concerning a small number of patients submitted to two different treatment protocols[17] and suggest that positive MRD detection by a simplified technique at the end of induction therapy may be the most important prognostic factor in children with ALL. As also observed in the present study, the detection of high MRD rates (10^{-2} to 10^{-3}) on day 15[30,31] or day 19[15] and between weeks 4 and 12[5-14] of induction therapy has been associated with an unfavorable prognosis. It should be pointed out that in the aforementioned studies the patients were submitted to different induction schemes with or without a pre-phase with corticosteroids, a fact that may impair comparison of the results.

Simplified techniques using standard PCR,[40,41] RQ-PCR[42] or GeneScan[43,44] have proven to be valid for the study of MRD in ALL. Recently, a simplified and relatively inexpensive technique of MRD detection on day 19 of induction therapy by flow cytometry using a panel of only three monoclonal antibodies was able to identify children with B-lineage ALL with a very good response to treatment.[15] The choice of the method for MRD detection on which patient stratification could be based is highly relevant in terms of adhesion to the study by the various institutions. In addition to simplicity and accuracy, the choice of the laboratory method should also consider the cost, especially when financial resources are limited. The

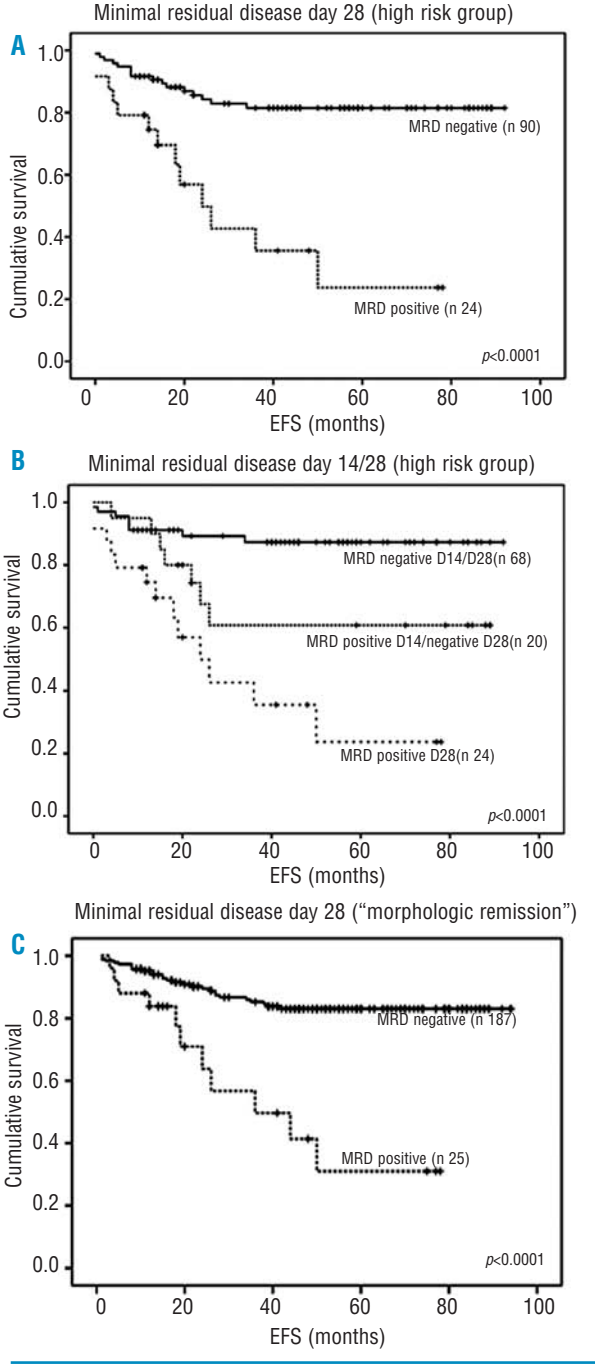

Figure 3. Event-free survival for the high final risk group, i.e, for those intensively treated, according to (A) minimal residual disease on day 28 by polymerase chain reaction; (B) minimal residual disease on days 14 and 28 by polymerase chain reaction; (C) Event-free survival for children who had a bone marrow smear on day 28 read as "in morphological remission" (M1 marrow), according to minimal residual disease on day 28 by polymerase chain reaction.

cost of the reagents and consumables needed to establish MRD markers at diagnosis in Europe has been estimated to be €190 for flow cytometry and €250 for real time quantitative PCR (RT-PCR) and €55 for each sample analyzed during follow-up with both methods.[8] These costs may be even higher in developing countries such as Brazil since some of the reagents used must be imported. In addition to being very expensive, these methods are quite labor intensive and require more sophisticated technological equipment. Other simplified methods such as GeneScan,[43,44] although cheaper than RQ-PCR and flow cytometry and perhaps having a slightly better sensitivity than our simplified strategy, require the use of DNA sequencing apparatus, fluorescent probes and specific software for analysis, thus being beyond the reach of most services of pediatric oncology and hematology, especially in developing countries. The cost of the present simplified technique was 10 to 15 times lower than that of flow cytometry or RT-PCR. The method proved to be highly reproducible and relatively simple, requiring only a standard thermocycler and a system for polyacrylamide gel runs.

It should be pointed out that the present methodology may have deficiencies that should be considered. Due to its low sensitivity, the assay mainly recognized children at higher risk of relapse (MRD positive group). Our MRD negative group comprised a larger proportion than that observed in studies using more sensitive methods, suggesting that highly sensitive methods are required for the recognition of truly low risk patients. In order to obtain better sensitivity we used a large amount of DNA (500 ng) in the tests carried out on days 14 and 28, when the number of blasts in bone marrow was reduced. In addition, in order to prevent the occurrence of non-specific bands, which may lead to false-positive results, it is important to use simultaneous amplification of a normal control containing bone marrow and/or peripheral blood cells without hematologic disease and a run on homo/heteroduplex gel for the unequivocal identification of clonal amplification.[24] To decrease subjectivity, the analysis of the pattern of migration at diagnosis and on samples taken on days 14 and 28 should be performed after gel scanning and by more than one observer.

In summary, the detection of MRD is already part of modern care for patients with leukemia. The challenge is how to incorporate the information obtained in studies on MRD into the therapeutic scheme and the design of new treatment protocols. It is hoped that a more sensitive and specific assessment of remission and of the initial response to treatment will result in clinical approaches that will increase the cure rate for children with ALL. The refinement of methods for MRD detection and the use of simpler techniques of lower cost such as the one developed in the present study may enable the benefit of MRD monitoring to be extended to all children with leukemia.

Authorship and Disclosures

CAS, JGA, MAG, JAY, SRT, AB, MBV, ASP, SRB and LGT: conception and design of the research. CAS, JGA, MAG, MA, SRT, MLML, ED, RPQ, MBV, and JAY: acquisition of data and analysis of results. CAS, JGA, MLML, ED, MBV, JAY, SRB, and LGT: wrote the paper. All authors revised the manuscript and approved the final version.

The authors reported no potentia conflicts of interest.

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