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Minimal residual disease detection in childhood acute lymphoblastic leukaemia patients at multiple time-points reveals high levels of concordance between molecular and immunophenotypic approaches

Jacqueline Ryan,[1] Fiona Quinn,[1] Armelle Meunier,[1] Ludmila Boublikova,[1] Mireille Crampe,[1] Prerna Tewari,[1] Aengus O'Marcaigh,[3] Ray Stallings,[4] Michael Neat,[4] Ann O'Meara,[3] Fin Breatnach,[3] Shaun McCann,[2] Paul Browne,[2] Owen Smith[3] and Mark Lawler[1]

[1]*The John Durkan Laboratory for Leukaemia Research, Institute of Molecular Medicine, St. James's Hospital & Trinity College Dublin, University of Dublin, Dublin, Ireland,* [2]*Department of Haematology, St. James's Hospital, Dublin, Ireland,* [3]*National Paediatric Haematology & Oncology Centre, Our Lady's Children's Hospital, Dublin, Ireland, and* [4]*National Centre for Medical Genetics, Our Lady's Children's Hospital, Dublin, Ireland*

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Correspondence: Jacqueline Ryan, The John Durkan Laboratory for Leukaemia Research, Institute of Molecular Medicine, Trinity Health Science Centre, St. James's Hospital, Dublin 8, Ireland. E-mail: jmryan@tcd.ie

Summary

In this single centre study of childhood acute lymphoblastic leukaemia (ALL) patients treated on the Medical Research Council UKALL 97/99 protocols, it was determined that minimal residual disease (MRD) detected by real time quantitative polymerase chain reaction (RQ-PCR) and 3-colour flow cytometry (FC) displayed high levels of qualitative concordance when evaluated at multiple time-points during treatment (93·38%), and a combined use of both approaches allowed a multi time-point evaluation of MRD kinetics for 90% (53/59) of the initial cohort. At diagnosis, MRD markers with sensitivity of at least 0·01% were identified by RQ-PCR detection of fusion gene transcripts, *IGH*/*TRG* rearrangements, and FC. Using a combined RQ-PCR and FC approach, the evaluation of 367 follow-up BM samples revealed that the detection of MRD >1% at Day 15 ($P = 0·04$), >0·01% at the end of induction ($P = 0·02$), >0·01% at the end of consolidation ($P = 0·01$), >0·01% prior to the first delayed intensification ($P = 0·01$), and >0·1% prior to the second delayed intensification and continued maintenance ($P = 0·001$) were all associated with relapse and, based on early time-points (end of induction and consolidation) a significant log-rank trend ($P = 0·0091$) was noted between survival curves for patients stratified into high, intermediate and low-risk MRD groups.

Keywords: minimal residual disease, childhood Acute Lymphoblastic Leukaemia, real time quantitative PCR, flow cytometry, MRC UKALL 97/99.

In the treatment of childhood acute lymphoblastic leukaemia ALL, independent of all other risk factors, the detection of minimal residual disease (MRD) has emerged as a powerful prognostic tool and significant determinant of patient outcome (Brisco *et al*, 1994; Ciudad *et al*, 1998; van Dongen *et al*, 1998; Foroni *et al*, 1999; Biondi *et al*, 2000; Coustan-Smith *et al*, 2000; Eckert *et al*, 2001; van der Velden *et al*, 2001; Bjorklund *et al*, 2003; Goulden *et al*, 2003; Marshall *et al*, 2003). MRD levels detected at early stages of treatment reflect an individual patients' early response with a rapid clearance of residual disease predictive of a more favourable outcome (Jacquy *et al*, 1997; Cave *et al*, 1998; Panzer-Grumayer *et al*, 2000; Coustan-Smith *et al*, 2002; Dworzak *et al*, 2002; Nyvold *et al*, 2002; Zhou *et al*, 2007). Stratification based on early detection of MRD may improve survival rates by facilitating identification of patients with a high risk of relapse. These patients may benefit from further treatment intensification, or alternatively they may be candidates for early transplant or novel targeted drugs. Low-risk MRD patients may be candidates for a reduction in treatment intensification that would minimise toxicity.

In the monitoring of MRD, it is important to employ methods of sufficient specificity and sensitivity. Two techniques that fulfil these requirements, with broad applicability

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and sensitivities between 10^{-3} and 10^{-6}, are real-time quantitative polymerase chain reaction (RQ-PCR) and flow cytometry (FC). While PCR methods are considered to be highly sensitive, FC has the advantage of being fast and relatively inexpensive. Currently, most centres evaluate MRD by a single method, and to date, only a limited number of studies have reported concurrent detection of MRD by both RQ-PCR and FC with varying qualitative concordance levels of between 72% and 97% (Veltroni *et al*, 2003; Malec *et al*, 2004; Neale *et al*, 2004; Kerst *et al*, 2005; Coustan-Smith *et al*, 2006).

On previous Medical Research Council (MRC) protocols (UKALL X and XI), it was found that 55% (33/59) of childhood ALL samples at the end of induction had levels of MRD >0·01% and this was associated with a 70% risk of relapse, while >0·1% MRD prior to the second delayed intensification conferred an 89% risk of relapse (Evans *et al*, 1998; Goulden *et al*, 1998). However, while the overall significance of MRD kinetics has been established by early PCR MRD studies for MRC protocols and other large prospective studies (Cave *et al*, 1998; van Dongen *et al*, 1998; Coustan-Smith *et al*, 2000; Dworzak *et al*, 2002; Nyvold *et al*, 2002), consensus as to what represents significant levels of MRD and at which early time-points still requires clarification and is highly dependent on the protocols for treatment, methods of MRD detection, sensitivity of detection methods, and interlaboratory differences in the interpretation of results (zur Stadt *et al*, 2001). Therefore, it is recognised that it may not be feasible to directly extrapolate the results from one study for the analysis of MRD to another study in a different centre using different protocols and approach to MRD detection. Thus MRD kinetics for a given protocol should be established on an individual study basis.

In this prospective blinded study, we analysed MRD levels in 53 patients with 367 follow-up bone marrow (BM) samples from childhood ALL patients enrolled in Ireland in the modified MRC UKALL97/99 trial. At a single centre, samples were independently assessed for MRD detection by RQ-PCR and 3-colour FC. Following completion, the concordance between the two methods of detection of MRD was evaluated, and MRD results were correlated with clinical data and patient outcome to evaluate this combined approach.

Patients, materials and methods

Patients and samples

Diagnostic bone marrow samples from 59 newly diagnosed childhood ALL patients, were sequentially collected over a 3-year period. Six patients were excluded from the study due to insufficient sample volume, poor quality nucleic acid in the diagnostic samples, or failure to identify a suitable target for MRD detection by any method. The MRD cohort (n = 53) comprised of 48 B-precursor cell ALL and 5 T-cell ALL patients. The patients were represented by 26 males and 27 females with a mean age of 7 years at diagnosis (range 1–15 years). All patients were enrolled in Our Lady's Children's Hospital, Dublin, Ireland and treated on modified MRC UK ALL97/99 protocols (Appendix SI). Informed consent was obtained from parents/guardians and study protocols were approved by hospital ethics committee. Patients were treated on regimen A, B, or C. Poor prognosis was associated with Risk Group C (Presence of t(9;22), t(4;11) if aged between 1 and 2 years, or hypodiploidy) (n = 2). An intermediate prognostic risk was given to Risk Group B (age >10 years or white cell count (WCC) >50 × 10^9/l at diagnosis) (n = 22). Risk Group A represented the standard risk patients with no risk factors (n = 29). Six Risk Group B Patients with >25% blasts at Day 8 were switched to the high risk Regimen C.

Follow-up bone marrow samples (n = 367) were collected at multiple time-points during treatment encompassing all potentially relevant treatment stages [day 8 (n = 31) and day 15 (n = 34)], at the end of induction [day 28 (n = 51)], during consolidation treatment [∼week 7–14 (n = 42)], prior to first delayed intensification [∼week 17–22 (n = 47)], end of first intensification [∼week 23–30 (n = 23)], prior to second delayed intensification [∼week 31–39 (n = 46)], end of second intensification [∼week 37–45 (n = 7)], at the start of continued maintenance [∼week 39–52 (n = 46)], and during and at the end of continued maintenance (n = 40). Bone marrow samples were collected in preservative-free heparin and processed within 2–6 h. Mononuclear cells (MNCs) were isolated by centrifugation on density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway), washed twice in phosphate-buffered saline (PBS) and resuspended in PBS with 2% bovine serum albumin and 0·1% sodium azide at a concentration of 10^7 cells/ml for immediate flow cytometric analysis. In parallel, RNA and DNA were extracted from MNCs using Qiagen Kits (Qiagen, Valencia, CA, USA) for molecular analysis.

Flow cytometric and molecular assessment of MRD

Both PCR and FC studies were performed independently, without exchange of results for the duration of the study, at a single laboratory. In the identification of molecular markers at diagnosis, rearrangements in immunoglobulin heavy chain (*IGH*), kappa chain (*IGK*), and T-cell receptor gamma (*TRG*) genes were identified using standardized clonality kits from Invivoscribe (Invivoscribe Technologies, San Diego, CA, USA), developed according to BIOMED-2 protocols and a fluorescent PCR approach incorporating Genemapper (Applied Biosystems, Foster City, CA, USA) analysis (Tables SI, SII and SIII) (van Dongen *et al*, 2003). Allele specific oligonucleotides (ASOs), complementary to unique junctional regions of V-J (*IGK* and *TRG*) or V-D-J (*IGH*) rearrangements, were designed with a reproducible sensitivity of ≥0·01% and used in combination with consensus primers and probes complementary for joining (J) gene segments to quantify MRD by RQ-PCR (Verhagen *et al*, 2000; van der Velden *et al*, 2002a,b). In addition, a standardized RT-PCR approach for the detection of five chromosomal translocations commonly associated with

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childhood ALL [t(9;22) *BCR-ABL1* p210 (n = 0); t(9;22) *BCR-ABL1* p190 (n = 1); t(1;19) *TCF3-PBX* (n = 1); t(12;21) *ETV6-RUNX1* (n = 9); and t(4;11) *MLL-AFF1* (n = 3)] was employed according to the European BIOMED-1 protocols (van Dongen *et al*, 1999). RQ-PCR primers and probes used for the detection of fusion gene transcripts were designed and approved by the Europe Against Cancer Programme (Gabert *et al*, 2003). In all RQ-PCR assays, patient diagnostic DNA/RNA was serially diluted with non-leukaemic polyclonal DNA/RNA (from ten donor MNCs) and standard curves generated for the quantitation of MRD in follow up samples. In all samples, fluorescence data was collected during the annealing/extension phase of each PCR cycle using the ABI Prism 7700 Sequence Detection System incorporating a 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA).

Flow cytometry analysis of MRD, adapted from Campana and Behm (2000), was performed using 3-colour analysis of a panel of multiple antibody combinations (Table SIV) (Campana & Behm, 2000). Leukaemia-associated immunophenotypes (LAIPs), which enabled the detection of one leukaemic cell in a background of 10^4 or more normal bone marrow cells, were identified at diagnosis. Samples were acquired using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the data was analysed using CellQuest software (Becton Dickinson). For each follow up sample, MRD was determined as the percentage of LAIP positive cells in the total number of gated cells for that sample.

Detection of MRD

Minimal residual disease detection analysis was successfully performed, with sensitivity of at least 0·01%, in 53 diagnostic samples and 367 follow up bone marrow samples (Table SV). Concurrent MRD analysis was successfully performed by both RQ-PCR and 3-colour FC in 29 diagnostic and 151 follow-up bone marrow samples. Where MRD was detected by both methods for individual patients (29 patients), the assay with the greatest sensitivity was used in the final risk analysis. Where both assays had the same sensitivity (e.g. 10^{-4}) then the assay with the highest % values at early time-points (day 15, 28, consolidation, prior to first DI) was used. The limits of sensitivity of MRD detection for individual patient assays ranged from 0·01% to 0·001% for *IGH*/*TRG* RQ-PCR assays (n = 36), 0·01–0·001% for fusion gene transcript assays (n = 12), and 0·01% for FC (n = 41).

Statistical analysis

The Kaplan–Meier method was used to estimate the distribution of relapse-free survival (RFS). RFS was the time between day 1 of treatment and date of relapse, censored at date of last time of contact. Two remission deaths (unique patient number [UPN] 025 and 035) were not included in analysis. Univariate associations between MRD groups were tested using log-rank tests. P-values <0·05 were considered significant.

Results

Comparison of MRD results determined by RQ-PCR and 3-colour flow cytometry

Minimal residual disease analysis was concurrently performed by both RQ-PCR and FC in 29 diagnostic patient samples and 151 follow-up bone marrow samples. To establish the qualitative concordance between FC and RQ-PCR results, a 0·01% positive cut-off was chosen, in line with the assay with the least level of sensitivity in all cases. Using this cut-off, comparison between RQ-PCR and FC MRD results revealed an overall qualitative concordance in 151 early and late time-points samples of 93·38% (Table I). Time-points at which concordance was noted at less than 90% in the first 3 months of treatment included the end of induction (day 28) and consolidation (~week 7–12). At all remaining early time-points in treatment, a qualitative concordance of >90% was observed.

Qualitative discordance was noted in 10/151 samples (6·62%) assessed by RQ-PCR and FC (6 RQ-PCR+/FC− and 4 FC+/RQ-PCR−) (Fig 1 and Table II). In six samples (from five patients) MRD was detected by RQ-PCR but not by FC. The detected MRD levels by RQ-PCR ranged from 0·011–0·141%. The failure of detection by FC in a number of cases may have been due to the closeness of measured MRD to the 0·01% cut-off. Alternatively, it may be plausible that false negatives in FC are attributed to manually 'gated out' leukaemic cells. However in other cases, such as UPN 005, where MRD was detected at 0·141% by PCR but not by FC at day 28, a possible explanation is the detection of non-viable cells in the PCR assay at the end of induction while apoptotic cells were gated out in the FC assay. This may be of particular relevance when stratifying patients into a higher risk group based on MRD levels at the end of induction.

In four samples, from four patients, MRD was detected by FC but not by RQ-PCR. The detected MRD levels by FC ranged from 0·01% to 0·091%. Discordance may be attributed to the detection of regenerating bone marrow by FC at individual time-points prior to the first and second delayed intensification and continued maintenance.

Quantitative concordance analysis was performed on the 53 samples with detected levels of MRD >0·01% by both RQ-PCR and FC (Fig 1). In 40/53 samples, higher levels of MRD were detected by RQ-PCR compared with FC. Twenty-eight samples were up to 1log higher and 12 samples were 1–2 log higher by RQ-PCR analysis (Fig S1). Good quantitative concordance was determined as 75·47% (40/53) using sample values that differed by less than 1 log. Quantitative discordance was due to the high sensitivity of RQ-PCR assays in the majority of cases.

A combined RQ-PCR and FC approach to the assessment of MRD kinetics

Minimal residual disease detection analysis was successfully performed by RQ-PCR and/or FC for 53 patients with a total

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Table I. Qualitative concordance between RQ-PCR and FC MRD results with a 0·01% positive cut-off level.

Time-points during treatment	Samples	PCR+/FC+*	PCR−/FC−	PCR+/FC−	PCR−/FC+	Concordance
During induction						
Day 8	n = 13	13	0	0	0	100%
Day 15	n = 13	13	0	0	0	100%
End of induction						
Day 28	n = 25	11	10	3	1	84%
Consolidation treatment						
~Week 7–12	n = 17	6	9	2	0	88·23%
Pre first delayed intensification (DI)						
~Week 13–20	n = 17	3	13	0	1	94·18%
End first DI/interim maintenance						
~Week 21–30	n = 16	1	13	1	1	87·5%
Pre second DI						
~Week 31–40	n = 21	2	18	0	1	95·23%
Start continued maintenance						
~Week 41–52	n = 16	2	14	0	0	100%
Continued maintenance						
~Week 52–165	n = 13	2	11	0	0	100%
Early samples (<3 months)	n = 68	43	19	5	1	91·17%
Late samples (>3 months)	n = 83	10	69	1	3	95·18%
Total	n = 151	53	88	6	4	93·38%

*PCR, real time quantitative polymerase chain reaction (RQ-PCR), FC, flow cytometry.

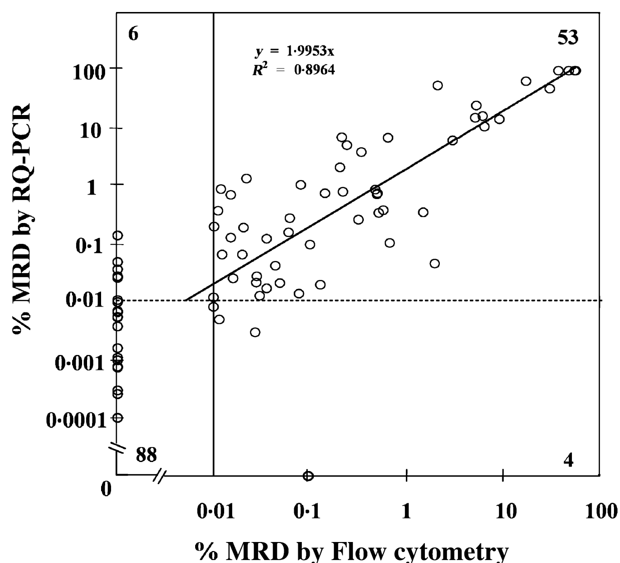

Fig 1. MRD results detected by both real time quantitative polymerase chain reaction (RQ-PCR) and flow cytometry (FC) for 151 follow-up bone marrow samples. Circles represent detected levels of MRD. Limits of sensitivity for RQ-PCR assays ranged from 0·01% to 0·001% and 0·01% for FC. A positive cut-off point of 0·01% is indicated by the dashed lines.

of 367 follow up bone marrow samples. Up to and including 1 February 2008, eleven patients had relapsed (relapses occurring between 17 and 61 months from diagnosis). Two further patients died early in treatment and were not included in further MRD analysis; UPN 035 died from liver failure post remission induction, UPN 025 died from pseudomonas sepsis (Week 37). The mean follow up time for the remaining 40 patients was 276 weeks (69 months) with a range of 220–336 weeks (55–84 months). The 5-year RFS on 1 February 2008 was 78% (40/51).

We assessed the significance of different MRD levels detected at several time-points during Year 1 of treatment (Table III). In line with the assay for MRD detection with the least level of sensitivity in all cases, we used 0·01% as the cut-off point and detected values below this threshold were not assessed. At early time-points during treatment, we found levels of MRD >0·01% at the end of induction (P = 0·0207), consolidation (P = 0·0139), and prior to the first delayed intensification treatment (P = 0·0104) to be significantly associated with relapse in our cohort. In addition, we also determined that levels of MRD >1% at day 15 during the induction therapy were significantly associated with relapse (P = 0·0416) (Fig S2). At later time-points in the first year of treatment, we found patients with >0·1% MRD before the second delayed intensification (P = 0·0014) and patients with >0·1% MRD at the start of continued maintenance (P = 0·001) were associated with relapse.

Based on our findings, we retrospectively stratified 51 patients into MRD risk groups based on MRD levels > or <0·01% at the end of induction (EOI) and consolidation (Fig 2). In the cases where a week 7–12 samples was unavailable, patients were classified as high-risk MRD when they had ≥0·01% MRD at Day 28 and >0·01% MRD between weeks 13 and 20 and/or ≥1% MRD at day 28. Intermediate risk (>0·01% at EOI but <0·01% at consolidation), low risk (<0·01% at EOI).

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Table II. Discordant MRD results in RQ-PCR and flow cytometry.

UPN	Time-point	RQ-PCR %MRD	Flow cytometry %MRD
005	End of induction	0·141	UN
	Consolidation	0·0263	UN
019	End of induction	0·037	UN
028	Consolidation	0·028	UN
039	Prior to second DI	0·011	UN
041	End of induction	0·05	UN
002	End of induction	0·003	0·027261
005	Prior to second DI	UN	0·091
009	Prior to first DI	0·0083	0·010358
033	Prior to CM	0·005	0·011416

A positive cut-off of 0·01% was applied.
UPN, unique patient number; UN, MRD not detected; DI, delayed intensification; CM, continued maintenance; RQ-PCR, real time quantitative polymerase chain reaction.

A significant log-rank trend ($P = 0{\cdot}0091$) was noted between survival curves for each risk group.

Discussion

Our single centre prospective assessment of MRD detection in MRC UK ALL97/99 protocols identified a high complementarity between RQ-PCR and FC detection at multiple time-points. This combined approach enabled informative retrospective stratification of the majority of our childhood ALL patients.

In the detection of MRD, a limited number of studies have reported varying qualitative (69–97%) and quantitative (71–100%) concordance for PCR and FC MRD assays (Neale *et al*, 1999, 2004; Munoz *et al*, 2000; Chen *et al*, 2001; Malec *et al*, 2001, 2004; Veltroni *et al*, 2003; Kerst *et al*, 2005; Robillard *et al*, 2005; Coustan-Smith *et al*, 2006) (Table SVI). Overall, qualitative concordance has been high but the exact techniques applied and the application of a positive cut-off point for results (>0·1%, >0·01%, or >0·001%) has varied. In all of the studies described, comparison of methods was performed at a single time-point (usually end of induction) or at a limited number of follow up time-points. In our cohort, the concordance of 3-colour FC and RQ-PCR MRD results were evaluated at eight different time-points during the first year of treatment and during maintenance therapy. To our knowledge, this is the most comprehensive evaluation of MRD concordance between RQ-PCR and FC, encompassing all potentially relevant time-points for MRD analysis. In each case, sensitivity of ≥0·01% was achieved. Consistency was provided by the fact that it was also a single centre study and enriched MNCs were used for both FC and RQ-PCR analysis. Overall, qualitative concordance between the two methods was good (93·38%) and in agreement with the largest study to date,

Table III. Relapse-free survival in patients grouped by levels of MRD detected at multiple time-points during treatment.

Time-point	Total samples (*n*)	>MRD cut-off	*n*	Relapse *n*	< MRD cut-off	*n*	Relapse *n*	Logrank Test	HR**
Day 8 (induction)	30	>1%	19	3	<1%	11	2	$P = 0{\cdot}7863$	0·7813
		>0·1%	29	5	<0·1%	1	0	N/A	N/A
		>0·01%	30	5	<0·01%	0	0	N/A	N/A
Day 15 (induction)	32	>1%	12	5	<1%	20	2	$P = 0{\cdot}0416$*	4·684
		>0·1%	20	6	<0·1%	12	1	$P = 0{\cdot}1866$	3·766
		>0·01%	27	7	<0·01%	5	0	$P = 0{\cdot}2256$	N/A
Day 28 (end of induction)	49	>1%	5	3	<1%	44	8	$P = 0{\cdot}0159$*	4·424
		>0·1%	14	6	<0·1%	35	5	$P = 0{\cdot}0336$*	3·353
		>0·01%	24	9	<0·01%	25	2	$P = 0{\cdot}0207$*	5·062
~Week 7–12 (consolidation†)	41	>1%	2	2	<1%	39	8	$P < 0{\cdot}0001$*	11·91
		>0·1%	6	3	<0·1%	35	7	$P = 0{\cdot}0553$	3·451
		>0·01%	15	7	<0·01%	26	3	$P = 0{\cdot}0139$*	4·652
Pre first (delayed intensification)	47	>0·01%	12	6	<0·01%	35	5	$P = 0{\cdot}0104$*	4·152
Pre second (delayed intensification)	46	>0·1%	4	3	<0·1%	34	8	$P = 0{\cdot}0014$*	6·478
		>0·01%	11	4	<0·01%	35	7	$P = 0{\cdot}2790$	1·946
Start of continued maintenance	46	>0·1%	4	3	<0·1%	34	8	$P = 0{\cdot}001$*	6·755
		>0·01%	10	4	<0·01%	36	7	$P = 0{\cdot}1609$	2·344

†According to MRC modified UK ALL97/99 protocols, the end of consolidation sampling ranges from week 7 to 14 and pre first delayed intensification samples ranges from week 16 to 22 for Regimens A, B and C. Certain variation in treatment timing is necessary for individual patients. Therefore following analysis of patient sample timing, for Regimen C patients, up to week 16 are included here for end of consolidation and up to week 26 for pre first delayed intensification. Regimen A and B patient samples fell within the protocol ranges week 7–12 for consolidation and week 17–22 for pre first delayed intensification.
HR**, hazard ratio, N/A, not applicable.
*Significant ($P < 0{\cdot}05$).

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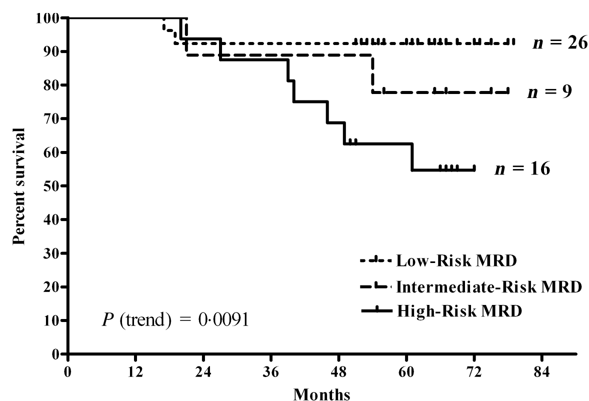

Fig 2. Kaplan–Meier curves for the three minimal residual disease (MRD) risk groups show significant differences with a log rank trend value <0·01 ($P = 0·0091$). High-risk MRD was defined as ≥0·01% at the end of induction (day 28) and consolidation (week 7–12), intermediate-risk MRD was defined as ≥0·01% at the end of induction but <0·01% at end of consolidation, and low-risk MRD was defined as <0·01% at the end of induction.

performed at St. Jude Children's Hospital, reporting high concordance (96%) between either limiting dilution PCR or RQ-PCR and 4-colour flow in 1375 follow up samples (736 BM and 639 PB) (Neale *et al*, 2004). In our multiple time-point analysis the qualitative concordance ranged from 84–100%. At the end of induction (day 28) and post-induction at consolidation (week 7–12), concordance levels were <90%. The discordance noted may reflect the inability of PCR to distinguish between viable and apoptotic cells. In addition, false positive results by FC may be associated with normal bone marrow regeneration during interim maintenance phases of treatment. Modulation of antigen expression or immunophenotypic shift during treatment can also result in the loss of an LAIP marker, resulting in a false negative result (van Wering *et al*, 1995; van Lochem *et al*, 2000; van der Sluijs-Gelling *et al*, 2005). Emergence of a subclone at relapse can also cause a false negative result (Gaipa *et al*, 2005), although we did not observe this.

When evaluating quantitative concordance between the two methods (75%), we noted a more pronounced difference. Quantitative concordance for other studies ranged between 71% and 100%, although the majority had less than 20 samples with positive MRD results (>0·01%) by both methods (Table SVII), with the exception of a St Jude Children's Hospital study that determined 90% quantitative concordance in 94 samples assessed by both PCR and FC (Neale *et al*, 2004). We attribute lower quantitative concordance to increased sensitivity of RQ-PCR over 3-colour FC. Lower quantitative concordance may also be attributed to our analysis of multiple time-points. In addition, both approaches measure very different targets and therefore complete quantitative concordance is unlikely.

A major drawback in choosing one method for MRD detection is that, at present, neither PCR nor 3-colour FC alone can be applied to all patients. On the current UKALL 2003 study, MRD stratification is according to PCR assessment only. In our study, 70% (41/59) of patients could be monitored with sufficient sensitivity (0·01%) using 3-colour FC and 70% (41/59) could be monitored using an RQ-PCR approach. While this independent applicability is lower than reported in other studies, we suggest that this can be attributed to the use of a 3 rather than 4-colour FC approach and the strict criteria that was applied to the design of RQ-PCR assays (targeting monoclonal rearrangements with sensitivity of at least 0·01%). However, our results highlight the difficulty of providing sensitive MRD results for all patients using a single approach. In view of our observation that a combined RQ-PCR and FC approach enabled monitoring of MRD levels in 90% of our cohort, we conclude that, ideally, both methods should be used in tandem to enable MRD to be monitored in the majority of patients. An important consideration is the cost of applying both methods, which may be prohibitive in certain centres. The employment of the new ≥6–colour FC (currently being developed by a number of groups including the EuroFlow Consortium) should undoubtedly allow for further increased sensitivity and applicability of this approach and is relatively inexpensive (Orfao *et al*, 2006). This may prove more practical in a clinical setting for the assessment of a majority of patients whereas a PCR approach may be employed in patients without sufficiently specific immunophenotypic markers. However, this would need to be assessed in prospective studies.

In our combined PCR/FC approach to the assessment of MRD kinetics for UKALL97/99 protocols, we determined that levels of MRD >0·01% at the end of induction, end of consolidation, and prior to the first delayed intensification treatment were all significantly associated with relapse, and a significant log trend ($P = 0·0091$) for patients stratified according to MRD risk group was noted.

Although our cohort involved <100 patients, these results are in agreement with earlier larger studies by either FC or PCR alone, emphasising the importance of MRD detection at early time-points.

In addition, we also determined that levels of MRD >1% at day 15 were significantly associated with relapse ($P = 0·04$) in a subset of 12 patients with slow clearance of disease (all 12 patients had ≤5% blasts detected in the bone marrow at day 15). This is in agreement with other studies that found MRD detection at day 15 during induction to be associated with outcome (Panzer-Grumayer *et al*, 2000; Nyvold *et al*, 2002). Patients with ≥1% MRD at day 15 may be stratified earlier into a high risk MRD group but, as our cohort was small, a larger cohort treated on our protocols would be required to verify these findings.

At the end of induction, 51% of samples had levels of MRD ≥0·01%. In other large prospective studies, this value ranged from 24% to 60%, reflecting method sensitivity and differences in treatment protocols (Cave *et al*, 1998; van Dongen *et al*, 1998; Coustan-Smith *et al*, 2000; Dworzak *et al*, 2002; Nyvold *et al*, 2002; Zhou *et al*, 2007) (Table SVII). In addition, levels of MRD detected at <0·01% by other studies varied greatly

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from 5% to 20% (with the exception of Nyvold *et al*, 2002; who reported 40% with <0·01% including undetected levels). We identified 20% of samples that had detectable levels of MRD by RQ-PCR that fell below the 0·01% cut-off, which, while not always accurately quantifiable, cannot be described as a ''true'' negative result, as outlined by recent European Study Group-MRD-ALL guidelines (van der Velden *et al*, 2007). This is important when considering therapy reduction based on MRD kinetics. In addition, 29% of samples in our cohort had undetected MRD at the end of induction and these patients may represent potential candidates for treatment reduction. This value of 29% is less than the 40% expected in the current Berlin-Fankfürt- Münster (BFM) ALL2000 trial or the 62% reported by the recent Dana-Farber Cancer Institute (DFCI) ALL Consortium, but is more in line with an unpublished pilot study report of UKALL97/99 protocols from the UK MRD Laboratory Network (contained in the current MRC UKALL2003 protocols), detailing that 20% of patients (16/80) had undetected levels of MRD, 30% had detected levels <0·01%, and 50% had levels >0·01% MRD by PCR at the end of induction. Observed differences are either treatment-related (the UKALL97/99 Regimen A protocol has a 3 drug induction and no prednisone pre-phase) and/or due to increased sensitivity of MRD assays.

Minimal residual disease assessment is now regarded as a valid indicator of relapse and an independent prognostic factor in childhood ALL. However, incorporating MRD results into the clinical management of all patients remains a challenge. We eagerly await the results and observations of large randomized studies incorporating MRD stratification, such as the ongoing Associazione Italiana Ematologia Oncologia Pediatrica -BFM ALL2000, MRC UKALL 2003, and current DFCI clinical trials.

In summary, this study shows that MRD detection by both FC and RQ-PCR on UKALL protocols is highly concordant and a combined approach may be used to ensure that a majority of patients can benefit from MRD stratification.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Quantitative concordance in 53 samples with levels of MRD ≥0·01% detected by both RQ-PCR and FC. Circles indicate higher levels of MRD detected by RQ-PCR than FC in each of 40 samples. Squares indicate higher levels of MRD detected by FC than RQ-PCR in each of 13.

Figure S2. Kaplan–Meier curves showing distribution of relapse free survival (RFS) (A) During induction (B) at the end of induction (C) Consolidation treatment (D) Pre first delayed intensification treatment. RFS was the time between day 1 of treatment and date of relapse, censored at date of last time of contact. Univariate associations between MRD groups (>/<0·01% or >/<1% for day 15) were tested using log-rank tests.

Table SI. Frequency and distribution of rearrangements in IgH, IgK,and TCRγ.

Table SII. Frequency of monoclonal rearrangements identified in IgH, IgK, or TCRγ.

Table SIII. Detection of monoclonal rearrangements at diagnosis in 52 childhood ALL patients.

Table SIV. Panel of monoclonal antibodies for the detection of MRD.

Table SV. Childhood ALL cohort for MRD detection.

Table SVI. Studies of tandem application of FC and PCR in the detection of MRD in childhood ALL.

Table SVII. Prospective minimal residual disease studies in childhood acute lymphoblastic leukaemia.

Appendix SI. Medical Research Council Modified UK ALL 97/99 Protocols.

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