Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL)

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Clinical risk classification is inaccurate in predicting relapse in adult patients with acute lymphoblastic leukemia, sometimes resulting in patients receiving inappropriate chemotherapy or stem cell transplantation (SCT). We studied minimal residual disease (MRD) as a predictive factor for recurrence and as a decisional tool for postconsolidation maintenance (in MRD^{neg}) or SCT (in MRD^{pos}). MRD was tested at weeks 10, 16, and 22 using real-time quantitative polymerase chain reaction with 1 or more sensitive probes. Only patients with t(9;22) or t(4;11) were immediately eligible for allogeneic SCT. Of 280 registered patients (236 in remission), 34 underwent an early SCT, 60 suffered from relapse or severe toxicity, and 142 were evaluable for MRD at the end of consolidation. Of these, 58 were MRD^{neg}, 54 MRD^{pos}, and 30 were not assessable. Five-year overall survival/disease-free survival rates were 0.75/0.72 in the MRD^{neg} group compared with 0.33/0.14 in MRD^{pos} ($P = .001$), regardless of the clinical risk class. MRD was the most significant risk factor for relapse (hazard ratio, 5.22). MRD results at weeks 16 to 22 correlated strongly with the earlier time point ($P = .001$) using a level of 10^{-4} or higher to define persistent disease. MRD analysis during early postremission therapy improves risk definitions and bolsters risk-oriented strategies. ClinicalTrials.gov identifier: NCT00358072. (Blood. 2009; 113:4153-4162)

Introduction

Although most adults with acute lymphoblastic leukemia (ALL) enter complete remission (CR), only 30% to 40% survive 5 or more years, at which time they are considered cured.[1-7] Survival depends on risk factors such as age, white cell count, time to CR, disease immunophenotype, cytogenetics, and molecular abnormalities. Traditionally, these features are used to identify risk groups with survival probabilities that range from less than 20% to greater than 50%.[2-4,8-13] However, risk models often lack prognostic precision at the level of individual patients. In fact, a considerable proportion of standard-risk (SR) patients treated with standard chemotherapy will eventually relapse—up to 40% to 50%, assuming a 5-year survival rate of 50% or higher for this group. The prognostic model is usually more accurate for high-risk (HR) patients, and there is a consolidated trend to perform hematopoietic stem cell transplantation (SCT) in these patients.[6,7,14-17] Paradoxically, approximately 20% to 25% of HR patients do not relapse. Therefore, a most important challenge is to establish a more precise prognostic definition to make better therapeutic decisions.

In recent years, several studies of childhood and adult ALL identified minimal residual disease (MRD) as an important independent prognostic factor for the duration of CR.[18-29] MRD can be evaluated at fixed time points during induction and consolidation therapy using cytofluorometry or patient-specific molecular probes. There is a close positive association between rapid MRD signal reduction (which is proof of chemosensitivity) and the duration of CR, independently of the applied treatments. In contrast, patients with persistent MRD almost always relapse. Thus, monitoring MRD may allow us to identify patients whose actual clinical course is unlikely to match the initial risk classification, and could help guide the decision to use SCT or postconsolidation maintenance. Such a strategy could spare some HR patients from the toxicity burden of SCT (and the attendant risk of remission death) as well as identify SR cases for whom standard chemotherapy is likely to fail.

Using MRD as the leading risk indicator, we designed an innovative prospective program in which the final treatment protocol was based on MRD study results. The program had 2 distinct phases. The first, phase A, was applicable to all patients, and had the dual aim of eradicating the disease in as many patients as possible while simultaneously allowing the MRD response to be defined. Only patients bearing t(9;22) or t(4;11) translocations could proceed straight to allogeneic SCT. The second,

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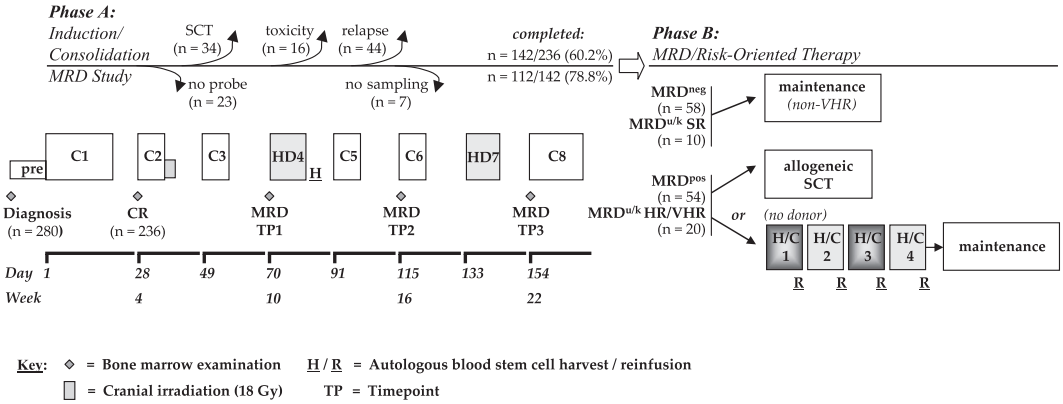

Figure 1. Outline of protocol NILG-ALL 09/00, MRD study, and treatment realization. Induction/consolidation (C indicates cycle; HD, high-dose cycle): pre-phase (T-ALL only): cyclophosphamide (CY) 300 mg/m^2 intravenously and prednisolone (PDN) 20 mg/m^2 twice a day intravenously or by mouth on days − 3 to 0. C1: idarubicin (IDR) 10 mg/m^2 intravenously on days 1 and 2; vincristine (VCR) 2 mg intravenously on days 1, 8, and 15; L-asparaginase *(Erwinia)* 6000 IU/m^2 intravenously on days 8, 10, 12, 14, 16, and 18; PDN 30 mg/m^2 twice a day intravenously or by mouth on days 1 to 7 and 20 mg/m^2 twice a day on days 8 to 15 (then tapered); G-CSF 5 μg/kg subcutaneously from day 4 to resolution of neutropenia less than 0.5 × 10^9/L. C2, C3, C5, C6: IDR 12 mg/m^2 (10 mg/m^2 in C5-6) intravenously on days 1 and 2; VCR 2 mg intravenously on day 1; CY 750 mg/m^2 intravenously on day 2; dexamethasone (DXM) 4 mg twice a day intravenously or by mouth on days 1 to 4; G-CSF from day 4. HD4,7: Methotrexate (MTX) 1.5 g/m^2 intravenously on day 1 (20% in 1 hour, 80% over 23 hours); cytarabine (Ara-C) 2 g/m^2 twice a day intravenously on days 2 and 3 (1.2 g/m^2 if MTX plasma concentration > 25 mM); PDN 40 mg twice a day by mouth on days 1 to 3; folinic acid 15 mg/m^2 intravenously every 6 hours starting 24 hours from end of MTX to an MTX plasma concentration less than 0.1 mM; G-CSF from day 4. C8: IDR 6 mg/m^2 intravenously on days 1 and 8; VCR 1 mg/m^2 intravenously on days 1 and 8; PDN 20 mg/m^2 twice a day by mouth on days 1 to 15. Maintenance: CY 100 mg/m^2 by mouth on days 1 to 4 (months 1, 3, 5, 7, 9, 11); VCR 1 mg/m^2 intravenously on day 1; PDN 20 mg/m^2 twice a day by mouth on days 1 to 5 (months 2, 4, 6, 8, 10, 12); 6-mercaptopurine (6MP) 75 mg/m^2 by mouth on days 8 to 28 (months 1-12) and 1 to 28 (months 13-24); MTX 30 mg/weekly by mouth or intramuscularly (months 1-24). CNS prophylaxis: Intrathecal MTX 12.5 mg, Ara-C 50 mg, and PDN 40 mg on days 2 and 16 of C1; days 2 and 9 of C2; day 2 of C3, C5, C6, and C8; and day 1 of maintenance cycles 1, 3, 5, and 7, except if prior H/C(s). Hypercycles (H/C): H/C1, H/C3: etoposide (VP) 100 mg/m^2 twice a day intravenously and 6MP 225 mg/m^2 by mouth (in 3 divided doses) on days 1 to 4; melphalan (Mel) 100 mg/m^2 intravenously on day 5; autologous blood stem cell reinfusion on day 6 (1-2 × 10^6/kg CD34$^+$ cells); G-CSF from day 7. H/C2, H/C4: MTX 1.5 g/m^2 intravenously on day 1; Ara-C 3 g/m^2 twice a day intravenously on days 2 to 4 (2 g/m^2 if MTX plasma concentration > 25 mM); folinic acid rescue starting 24 hours from end of MTX, autologous blood stem cell rein fusion on day 6, and G-CSF from day 7. If CD20$^+$ ALL: rituximab 375 mg/m^2 intravenously on day 10 of each H/C. Dose reductions in patients older than 59 years: CY 75 mg/m^2 (pre-phase), 500 mg/m^2 (C2-3, C5-6), omitted (maintenance); IDR 8 mg/m^2 (C1-3) and 6 mg/m^2 (C5-6, C8); VCR 1 mg/m^2 (C1-3, C5-6, C8), omitted (maintenance); ASP 6000 IU total dose; PDN 20 mg/m^2 twice a day (C1, C8), omitted (maintenance); DXM omitted; VP 75 mg/m^2 twice a day (H/C1); 6MP 150 mg/m^2 (H/C1); ML 70 mg/m^2 (H/C1); MTX 1 g/m^2 (HD4,7, H/C2); Ara-C 1.2 g/m^2 twice a day (C4,7, H/C2); intrathecal MTX 10 mg, Ara-C 40 mg (CNS prophylaxis). Imatinib mesylate (600 mg/d orally) was added in January 2003 in patients with Ph$^+$ ALL, on days 15 to 21 of C1, days − 3 to 4 of C2 to C8, days 1 to 7 of each H/C, and long term during maintenance.

phase B, was experimental: treatment depended on MRD status, with maintenance therapy for MRD-negative (MRD^{neg}) patients, and high-dose treatments with SCT for MRD-positive (MRD^{pos}) patients. Five-year study results from an unselected patient cohort with a minimum follow-up of 1 year should allow us to assess whether it is realistic to use this MRD-based strategy in the management of adult ALL.

Methods

Diagnosis and clinical risk groups

Eligible patients had a diagnosis of untreated B- and T-precursor ALL according to the European Group for the Immunological Characterization of Leukemias (EGIL) criteria[30] and were between 16 and 65 years old. Three distinct clinical risk groups were identified based on known risk factors. The first group was a very high-risk (VHR) group, comprising patients with the Philadelphia (Ph) translocation (ie, t(9;22)), or t(4;11), or with corresponding gene rearrangements (*BCR-ABL*, *MLL-AF4*). The second was a high-risk (HR) group. The HR group included B- and T-lineage ALL cases with white blood cell (WBC) count higher than 30 × 10^9/L or higher than 100 × 10^9/L, respectively; patients achieving CR after cycle 2; and those with an adverse EGIL immunophenotype (pro-B or pre-/mature-T) or adverse cytogenetics. Adverse cytogenetics included monosomy 7, trisomy 8, del6q, t(8;14), low hypodiploidy with 30 to 39 chromosomes, near triploidy with 60 to 78 chromosomes, and complex karyotype (≥ 3 unrelated clonal abnormalities).[9,12,13,31] The third group was the standard-risk (SR) group in which patients had none of the aforementioned VHR/HR features.

Generation of patient-specific probes for MRD study

The molecular evaluation of MRD was performed centrally at the coordinating institution. DNA and RNA were extracted from mononuclear marrow cells using commercially available kits (Puregene [Gentra Systems, Minneapolis, MN]; RNeasy [QIAGEN, Hilden, Germany]). Samples were analyzed for *BCR-ABL*, *MLL-AF4*, *E2A-PBX1*, and *SIL-TAL1* chimeric genes.[32] Samples were amplified by real-time quantitative polymerase chain reaction (RQ-PCR) and quantified by parallel amplification of serial dilutions of transcript-containing plasmids according to BIOMED-1 and BIOMED-2 Concerted Action specifications.[32,33] Leukemia-specific probes were generated by genomic amplification and sequencing of the *VDJ/VJ* regions of immunoglobulin heavy chain (*IgH*) or the kappa light chain (*IgK*), and the T-cell receptor (*TCR*) gamma (*G*), delta (*D*), and beta (*B*) genes.[34-38] Clone-specific oligonucleotides were constructed based on the unique junctional region of each rearrangement and used in RQ-PCR experiments in combination with reverse primers and probes selected for the identified rearrangement.[39,40] Oligonucleotide sensitivities were tested on 10-fold serial dilutions of DNA from leukemic cells isolated at diagnosis and on DNA from a pool of 8 healthy donors. MRD quantification was performed by amplification of 500 ng sample DNA and the 10-fold DNA dilution series. All samples were amplified in triplicate, and the MRD level was expressed as the logarithmic reduction of the leukemic burden detected at diagnosis, after correction for DNA quality by amplification of a control gene.[26,33,41,42]

MRD study and risk model

For MRD assessment, 3 serial bone marrow (BM) samples were prospectively taken before cycles 4, 6, and 8, corresponding to the ends of treatment weeks 10, 16, and 22 (TP1-3, Figure 1). The critical point for assigning an MRD risk classification coincided with cycle no. 8, approximately 5 months after the presumed date of CR. All SR and HR patients were reclassified as MRD^{neg} or MRD^{pos}, respectively, according to the MRD results. Only probes with a sensitivity of 10^{-4} or higher were considered; however, in a minority of frail/elderly patients a sensitivity of 10^{-3} was accepted to support critical therapeutic decisions or to confirm persistent disease. When 2 MRD probes gave different results in the same patient, the higher MRD

level was considered valid for the purpose of the study. In the MRD risk model used here, MRD^{neg} patients had negative or low positive ($< 10^{-4}$) PCR signal(s) at week 16/TP2 and totally undetectable signal(s) at week 22/TP3. All other patients were classified as MRD^{pos}. This model reflected a priori decision, as per protocol, based on the need to have the early consolidation treatment completed as well as sufficient time to generate informative probes in most patients. MRD status at week 10/TP1 was initially not considered for the prospective study, based on the assumption that in adult ALL the maximum predictive power of MRD for relapse is manifested on or after the third month of chemotherapy.[25] As it became apparent that TP1 MRD status was also important, this result was included in the study analyses as retrospective prognostic variable. However, the original MRD risk definitions were not changed.

Treatment protocol

Protocol NILG-ALL 09/00 (Figure 1) was approved by the Ethical Committee of the coordinating institution on December 5, 2000, and was later approved by the Ethical Committees of all participating centers. Written informed consent was obtained in accordance with the Declaration of Helsinki from patients or parents of patients who were minors. Induction-consolidation (phase A) was the same for all patients and consisted of 8 chemotherapy blocks administered over 25 weeks in association with central nervous system (CNS) prophylaxis. Collection of purified G-CSF–primed autologous blood stem cells was performed after cycle 4.[34]

For risk-oriented therapy (phase B), the consolidation program was considered concluded for MRD^{neg} cases. These patients began 2-year continuous maintenance therapy, reinforced by pairs of drugs alternated monthly. In contrast, it was intended that all MRD^{pos} patients with an available HLA-identical related or unrelated volunteer donor would undergo allogeneic SCT after high-dose cyclophosphamide plus total-body irradiation conditioning. For MRD^{pos} patients who were unable to undergo an allogeneic SCT, an intensification treatment supported by autologous blood stem cells was proposed, followed by the maintenance program described for MRD^{neg} cases. This regimen included 4 alternating "hyper-cycles" (H/C), 2 with methotrexate-cytarabine and 2 with melphalan-etoposide-mercaptopurine, each H/C supported by reinfusion of autologous stem cells; the cytotoxic humanized monoclonal antibody rituximab was given to patients with $CD20^+$ ALL. When the MRD risk class was unknown ($MRD^{u/k}$), phase B was maintenance therapy in clinical SR subsets and allograft or H/C maintenance in HR patients, respectively. VHR patients with Ph^+ and $t(4;11)^+$ ALL were always eligible for early allogeneic SCT, but were initially managed like other patients and were thus subject to MRD study. If an allogeneic SCT was not possible, the H/C phase plus maintenance option was indicated.

Definitions and statistics

Complete remission (CR) was defined by hemoglobin level higher than 100 g/L (10 g/dL), neutrophil count higher than 10^9/L, platelet count higher than 100×10^9/L, normocellular regenerating bone marrow without ALL cells (blast cells $< 5\%$), and clear cerebrospinal fluid (in patients with prior CNS involvement). Early death was defined as death before treatment response could be adequately assessed. Refractory ALL was defined by the persistence of leukemia after induction therapy, and a relapse was the reappearance of blast cells ($> 5\%$) in the bone marrow or extramedullary sites. Survival (overall survival, OS) was calculated from the date of diagnosis to the date of death by any cause. Disease-free survival (DFS) was the time from the date of CR to the date of relapse (in any site) or death in CR. The cumulative incidence of relapse (CIR) was calculated from the date of CR to recurrence in any site. Results were expressed by treatment intention.

Several prognostic factors were considered for comparative analyses: patient age and sex, immunophenotype subset and EGIL category, cytogenetics and molecular biology results, WBC count, clinical risk class, and MRD risk class. The probabilities of favorable outcome (CR, MRD negativity) were compared using the χ^2 test with Yates correction. DFS and survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Multivariate analyses were carried out by Cox linear regression model, including all variables expressing significant P values in univariate analysis. Statistical significance was associated with P values less than .05.

Table 1. Diagnostic characteristics of 280 adult patients with ALL

Parameter	No. of cases		
	Evaluable	Positive (%)	Median (range)
Age, y	280		38 (16-66)
Male sex, no.		154 (55)	
Hemoglobin, g/L			98 (24-165)
WBC, $\times 10^9$/L			16.5 (0.6-900)
Blast count, $\times 10^9$/L			7.4 (0-855)
Bone marrow blasts, %			90 (25-100)
Platelets, $\times 10^9$/L			45 (4-420)
Hepatomegaly, no.	277	80 (28.8)	
Splenomegaly, no.	275	115 (41.8)	
Lymphadenopathy, no.	277	81 (29.2)	
CNS involvement, no.	274	13 (4.7)	
FAB morphology, no.	270		
L1		76 (28.1)	
L2		194 (71.9)	
EGIL immunophenotype, no.	280		
B-I/pro-B		44 (16)	
B-II/common		129 (46)	
B-III/pre-B		43 (15.3)	
TII/pre-T		17 (6)	
T-III/cortical		31 (11)	
T-IV/mature-T		10 (3.6)	
T-undefined		6 (2.1)	
Cytogenetics/molecular genetics, no.*	276		
Adverse			
t(9;22) and/or *BCR-ABL*		68 (24.6)	
t(4;11) and/or *MLL-AF4*		20 (7.3)	
Other†		19 (6.9)	
Nonadverse			
t(1;19) and/or *E2A-PBX1*		5 (1.8)	
Hyperdiploid		12 (4.3)	
Other		20 (7.3)	
Normal		84 (30.4)	
Not known (cytogenetics)		48 (17.4)	
Clinical risk class, no.	280		
SR B-precursor		74 (26.4)	
T-precursor		22 (7.9)	
HR B-precursor		54 (19.3)	
T-precursor		37 (13.2)	
VHR t(9;22)/*BCR-ABL*		68 (24.3)	
t(4;11)/*MLL-AF4*		20 (7.1)	
Undefined (T cell)		5 (1.8)	

WBC indicates white blood cell.

*_BCR-ABL_, _MLL-AF4_, and _E2A-PBX1_ were tested in B-lineage only.

†Trisomy 8 (n = 6), near triploidy (n = 5), low hypodiploidy (n = 3), complex (n = 2), del6q (n = 2), and t(8;14) (n = 1).

Results

Patients and molecular probes

The study group consisted of 280 patients recruited starting in March 2000 (Table 1). Thirty-five patients (12.5%) were 60 years or older. Risk groups were evenly distributed, with approximately one-third of cases in each subset. Excluding VHR cases, the SR profile was more frequent in B-precursor (57.8%) than T-precursor (37.3%) ALL.

Table 2. Details of 308 molecular probes obtained in 223 patients

Probe type and sensitivity	First probe, 223 patients, no.				Second probe, 85 patients, no.				Total probes, no. (%)
	10^{-5}	10^{-4}	10^{-3}	Total	10^{-5}	10^{-4}	10^{-3}	Total	
***IgH/TCR* rearrangements**									
IgH	25	14	1	40	6	7	3	16	56 (18.2)
Ig—kappa	2	5	1	8	3	5	—	8	16 (5.2)
TCRD	14	15	4	33	5	14	4	23	56 (18.2)
TCRB	5	6	—	11	6	7	—	13	24 (7.8)
TCRG	22	15	7	44	2	10	13	25	69 (22.4)
Fusion genes									
BCR-ABL	61	—	—	61	—	—	—	—	61 (19.8)
MLL-AF4	—	20	—	20	—	—	—	—	20 (6.5)
E2A-PBX1	5	—	—	5	—	—	—	—	5 (1.6)
SIL-TAL1	—	1	—	1	—	—	—	—	1 (0.3)
Total (%)	134 (60)	76 (34)	13 (6)	223 (100)	22 (25.9)	43 (50.6)	20 (23.5)	85 (100)	308 (100)

The search for patient-specific probes was performed in 253 (90.3%) of 280 cases. Eight cases were excluded because of early death, and the marrow harvest was insufficient in 19 cases. A total of 308 probes were obtained from 223 patients (88.1%; Table 2); in 30 patients, no clonality marker could be identified. A single probe was available in 138 cases (61.8%), and 2 probes were available in 85 (38.2%). Apart from fusion genes, the most frequent case-specific markers involved *IgH*, *TCRD*, and *TCRG* rearrangements (58.8%), followed by *IgK* and *TCRB* subtypes (13% cumulative incidence). Most *IgH/TCR* probes expressed a high sensitivity between 10^{-4} and 10^{-5} (89%). The less sensitive markers almost exclusively involved the *IgH*, *TCRD*, and *TCRG* subclasses (sensitivity 10^{-3} in 7.1%, 14.2%, and 29%, respectively). In summary, a clonal marker was available for nearly 90% of the patients studied, with a sensitivity level of 10^{-4} or higher in 94.2%. When considered separately for cell lineage, study failures were less frequent in B-precursor ALL: 17 (8.8%) of 192 versus 13 (21.3%) of 61 in T-lineage ($P = .009$).

Overall treatment results

CR was achieved in 236 patients (84.3%), 23 died during induction, and 21 proved to have refractory ALL. The median time to CR was 29 days, with only 7 late responders. Only age older than 35 years ($P = .001$) and adverse pre-/mature-T phenotype ($P = .023$) were associated with lower CR probability. As of September 1, 2008, 109 patients were alive (38.9%), 91 in first CR and 18 beyond first CR. Postinduction failures were due to relapse (n = 117, 49.6%) or treatment-related mortality after allogeneic SCT (13/59, 22%), H/C therapy (4/32, 12.5%), or chemotherapy (11/177, 6.2%; $P = .002$). The 5-year OS probability was 0.34 (95% CI: 0.28-0.40); it was 0.49 for the SR group, and 0.27 and 0.24 in the HR and VHR groups, respectively (Figure 2). DFS probability was 0.33 (95% CI, 0.26-0.40), ranging from 0.42 in SR to 0.28 in HR and 0.23 in VHR. The OS of remitters was 0.39 (95% CI, 0.32-0.46), varying from 0.54 in SR to 0.32 and 0.27 in HR and VHR, respectively (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Induction-consolidation and MRD study (phase A)

The study flowchart is included in Figure 1. An MRD-based risk definition was obtainable in 112 of 142 patients who completed treatment phase A. Of these, 58 were MRD^{neg} (51.8%) and 54 MRD^{pos} (48.2%), yielding a success rate for the MRD study of 78.9%. Fifty-eight of 112 MRD evaluable patients were studied at TPs 2 and 3 with 2 distinct probes, and 10 (17%) proved MRD^{pos} with only 1 marker. In 8 of them the positivity was close to the sensitivity limit of the probe ($\geq 10^{-4}$). Of 30 $MRD^{u/k}$ patients, 23 lacked a suitable marker and 7 were not adequately sampled at TP2 or TP3. The other reasons for the loss of 94 patients from the MRD evaluation were as follows. SCT was provided to one group of 34 subjects with VHR ALL who were automatically removed from the study. In 16 patients, treatment-related toxicity caused patient death or permanent discontinuation of therapy. However, the major obstacle to completion of the MRD analysis was early relapse, which occurred in 44 subjects (18.6%). This observation prompted a search for correlations between early relapse rate and the MRD response in different risk groups (Table 3), which demonstrated a lower incidence of relapse in B-type SR ALL, with a progressive rise in HR and T-ALL, and especially in $t(4;11)^+$ ALL and in cases with leukocyte count higher than 100×10^9/L. Nevertheless, once phase A therapy was concluded, the MRD^{neg} rates were comparable among different prognostic groups, even if there were relatively few patients in some of the subsets examined.

Risk-oriented therapy (phase B)

The key objective of the study was outcome analysis of the 2 MRD risk groups. This is reflected in the prospective therapeutic shift prompted by the new risk definition from standard maintenance in MRD^{neg} cases to high-dose therapies with SCT rescue in MRD^{pos} patients (and the VHR subgroup), whereas $MRD^{u/k}$ patients were allocated by clinical risk class (Figure 1; Table 4). Adherence to the study protocol was substantial, though not absolute: of 58 MRD^{neg} patients, 47 had maintenance therapy (81%), but there were 6 violations and 5 VHR patients who were allocated to H/C therapy. Conversely, 36 (66.7%) of 54 MRD^{pos} cases proceeded to SCT or to the H/C phase, whereas others had maintenance therapy or suffered from pretransplantation relapse. In terms of therapeutic response,

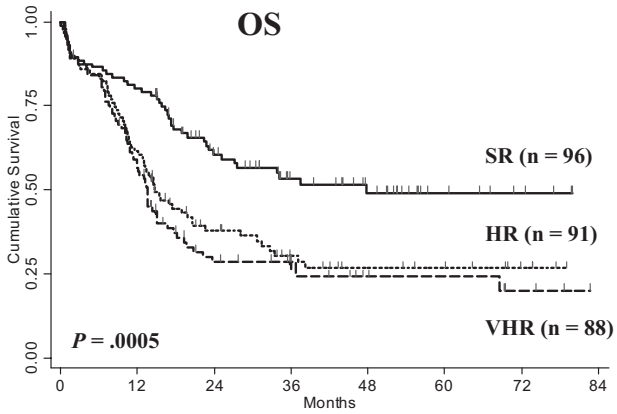

Figure 2. Overall survival by risk class.

Table 3. Early relapse rate and MRD study results in different risk subsets

	CR patients			MRD study, no. (%)		MRD study results, no. (%)		
	No.	Phase A relapse (%)	*P*	End phase A	MRD evaluable	MRD^{pos}	MRD^{neg}	*P*
Risk class								
SR	85	9 (10.6)	.060	71	61 (85.9)	26 (42.6)	35 (57.4)	.308
HR	73	17 (23.3)		52	37 (71.1)	19 (51.4)	18 (48.6)	
VHR	74	17 (23)		17	14 (82.3)	9 (64.3)	5 (35.7)	
Age, y								
55 or younger	197	37 (18.8)	.903	122	100 (81.9)	51 (51)	49 (49)	.631
Older than 55	39	7 (17.9)		20	12 (60)	7 (58.3)	5 (41.7)	
WBC, $\times 10^9$/L								
0-30	150	9 (6)	.001	108	84 (77.8)	39 (46.4)	45 (53.6)	.348
More than 30 to 100	49	18 (36.7)		22	18 (81.8)	8 (44.4)	10 (55.6)	
More than 100	37	17 (46)		12	10 (83.3)	7 (70)	3 (30)	
Immunophenotype								
B	183	27 (14.7)	.004	111	90 (81)	45 (50)	45 (50)	.44
T	53	17 (32)		31	22 (71)	9 (41)	13 (59)	
B by subtype								
Common/pre-B	148	18 (12.2)	.042	92	77 (83.7)	39 (50.7)	38 (49.3)	.764
Pro-B	35	9 (25.7)		19	13 (68.4)	6 (46.2)	7 (53.8)	
T by subtype								
Cortical-T	29	10 (34.5)	.551	17	14 (82.3)	6 (42.9)	8 (57.1)	.806
Pre-/mature-T	19	5 (26.3)		12	8 (66.7)	3 (37.5)	5 (62.5)	
Cytogenetics/molecular genetics								
Nonadverse/normal	107	20 (18.7)	.566	81	70 (86.4)	30 (42.9)	40 (57.1)	.133
Adverse	91	20 (22)		31	23 (74.2)	14 (60.9)	9 (39.1)	
Adverse by subtype								
t(4;11)	16	9 (56.2)	.001	2	2 (100)	1 (50)	1 (50)	.829
Ph^+	58	8 (13.8)		15	12 (80)	8 (66.7)	4 (33.3)	
Other	17	3 (17.6)		14	9 (64.3)	5 (55.6)	4 (44.4)	

WBC indicates white blood cell.

the results were strikingly improved in MRD^{neg} patients compared with the MRD^{pos} cohort (DFS: 0.72 vs 0.14 at 5 years, median not reached vs 1.16 years, $P = .001$ [Figure 3]; OS: 0.75 vs 0.33, median not reached vs 1.98 years, $P = .001$). This was clearly related to a reduced CIR rate in the MRD^{neg} group (24.1% vs 68.5% in MRD^{pos}, $P = .001$; Figure S2). The details of the 14 relapsing MRD^{neg} patients are reported as Table S1. There were 2 extramedullary relapses and one BM recurrence associated with disappearance of the IgH rearrangement observed at diagnosis (7.1%). Two of these patients had a single marker with 10^{-3} sensitivity. These subjects were part of a group of 9 patients, 5 MRD^{pos} and 4 MRD^{neg}, with suboptimal marker sensitivity who were retained in the study because of medical considerations. When only BM relapse in patients with sensitive probes ($\geq 10^{-4}$) was considered, the recurrence rate was only 18.5% (10/54) with a 5-year DFS probability of 0.78 (Figure 4A). Similar results were obtained for 45 SR and HR patients who had maintenance therapy as planned (DFS 0.76), excluding 6 SCT violations. No remission deaths were registered among these patients. Moreover, the clinical risk class no longer predicted relapse (Figure 4B), and the use of 1 or 2 molecular probes was just as informative (Figure 4C). For the MRD^{pos} cases, there was an advantage for 36 patients who had an allogeneic SCT or the H/C plus maintenance sequence (median H/C number, 2; 5 patients received all 4 planned H/C treatments; Figure 5A). Among 18 patients unable to undergo SCT or H/C, 4 had a suitable SCT donor and 11 sufficient autologous blood stem cells for H/C therapy. Fourteen relapsed quickly after a median of 1.6 months from TP3 (range, 0.8-4.9 months), partly in relation to refusal to continue (Table 4) or treatment delay. In addition, there was little or no difference between the procedures (Figure 5B), with further benefit when MRD negativity was attained afterward (Figure 5C). Transplantation-related mortality was registered as

Table 4. Phase B therapy by risk class (MRD and clinical)

Risk class		Phase B therapy, no.			
MRD	Clinical	Maintenance	SCT	H/C	None (reason)
MRD^{neg} (n = 58)	SR (n = 35)	32	1	2	—
	HR (n = 18)	15	2	1	—
	VHR (n = 5)	0	0	4	1 (relapse)
MRD^{pos} (n = 54)	SR (n = 26)	3	10	7	6 (relapse 5, refusal 1)
	HR (n = 19)	1	8	4	6 (relapse 5, refusal 1)
	VHR (n = 9)	0	4	3	2 (relapse 1, toxicity 1)
$MRD^{u/k}$ (n = 30)	SR (n = 10)	8	0	1	1 (refusal)
	HR (n = 15)	4	1	8	2 (relapse 1, off study 1)
	VHR (n = 3)	0	0	2	1 (relapse)
	Undefined (T-cell) (n = 2)	1	0	0	1 (relapse)

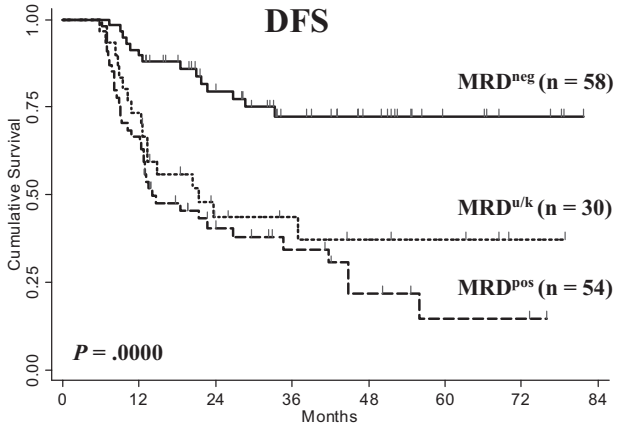

Figure 3. DFS according to MRD study results.

expected (allogeneic SCT: 5/22 or 22.7%; H/C phase: 3/14 or 21.4%).

Prognostic significance of MRD risk class versus clinical risk factors

A multivariable Cox regression analysis was conducted for 93 patients with a complete data set for major risk features including MRD risk model, WBC count (examined both above 30×10^9/L and above 100×10^9/L), immunophenotype, cytogenetics/genetics, and age. Before that, an univariate analysis was conducted on continuous risk variables such as WBC count and patient age (Figures S3, S4). Persistence of MRD was the most significant independent risk factor for both DFS and BM relapse (Table 5), followed by a cell count higher than 100×10^9/L and, to a lesser degree, higher than 30×10^9/L.

Prognostic value of early MRD data and MRD/white cell count interaction

Last, we examined the relationship between MRD risk class (focusing on TP2 and TP3 data) and the earliest MRD results from TP1/week 10. Ten patients without TP1 determinations were included only in the final MRD risk model. There was excellent correlation between TP1 MRD and the final risk model (Table 6), with MRD limits set at 10^{-4} or higher for MRD^{pos}, less than 10^{-4} for MRD$^{low\text{-}pos}$ (low positive), and totally undetectable for MRD^{neg}. DFS estimates were then recalculated using the TP1 MRD to assess the sheer prognostic influx of an earlier molecular response. An absence of TP1 MRD conferred a clear prognostic advantage (Figure 6A) that was only slightly inferior to that observed at the end of the study. Interestingly, the 10 patients who were MRD^{neg} by TP2/3 model but MRD$^{pos/low\text{-}pos}$ by TP1 model accounted for only 2 of 14 relapses in the MRD^{neg} cohort (cases no. 4 and 9 in Table S1). Because hyperleukocytosis had been used to define HR risk subsets and was an independent risk factor for relapse (Table 5), a joint analysis was performed to determine the cumulative prognostic effect of TP1 MRD and cell count in B- and T- precursor ALL, respectively (Figure 6B,C). TP1 MRD seemed to have greater predictive power in B- than T-lineage disease, since the latter had a higher BM relapse rate (42% vs 13.9% in the B subset; $P = .019$).

Discussion

The Northern Italy Leukemia Group (NILG) study 09/00 for adult ALL had 2 primary objectives. First, it was designed to confirm

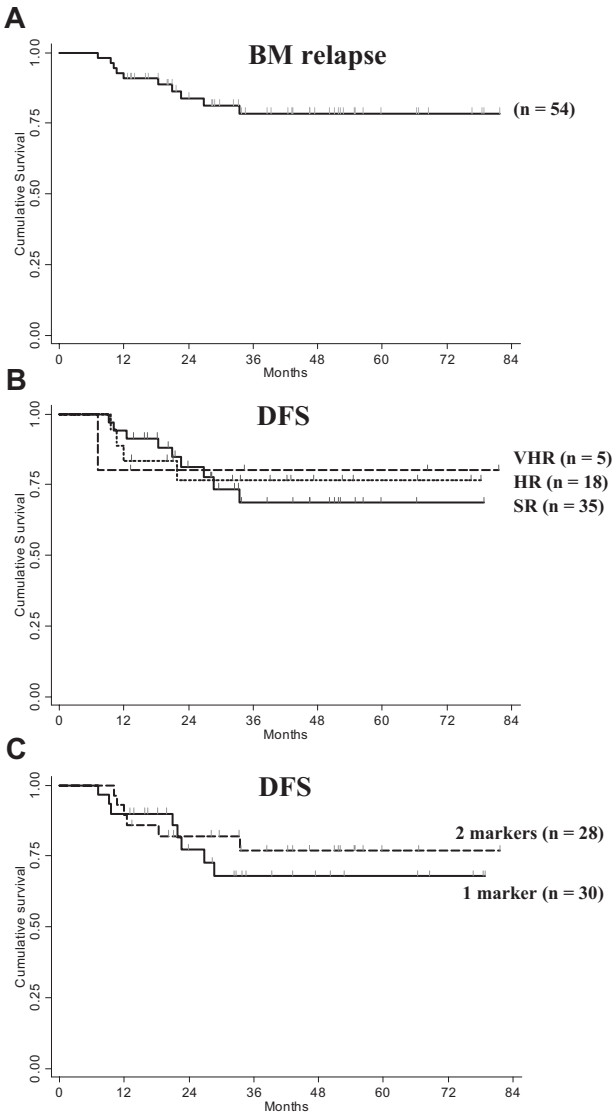

Figure 4. Treatment outcome of MRD^{neg} group. (A) Duration of CR (vs BM relapse) in cases with probe(s) sensitivity of 10^{-4} or higher; (B) DFS according to clinical risk class and (C) number of molecular markers used for MRD analysis.

prospectively the role of MRD as a main predictor for relapse, superseding the clinical risk class definition in unselected patients in first CR. A second objective was to determine whether risk classification based on MRD results could be used to guide the final treatment strategy (which currently ranges from maintenance to allogeneic SCT) with tangible results, especially when the clinical risk profile alone would have suggested a different treatment choice. With a median follow-up that was more than 3 years (with a minimum follow-up of 1 year from diagnosis), 280 patients entered the trial and overall DFS and survival rates by risk subset were comparable with other major clinical series[2-4,6,7] despite relatively advanced patient age (median age, 38 years) and the prevalence of unfavorable prognostic subsets (65% of the total). Beyond that, the results clearly showed that MRD is the best prognostic indicator to date for the majority of SR and HR patients, and that using MRD results allows design of individualized treatment programs that have an unprecedented degree of accuracy. We also performed concurrent analyses to assess (a) how and when to collect the MRD information, with regard to the number of molecular probes and the optimal time points; (b) among the subsets of patients identified

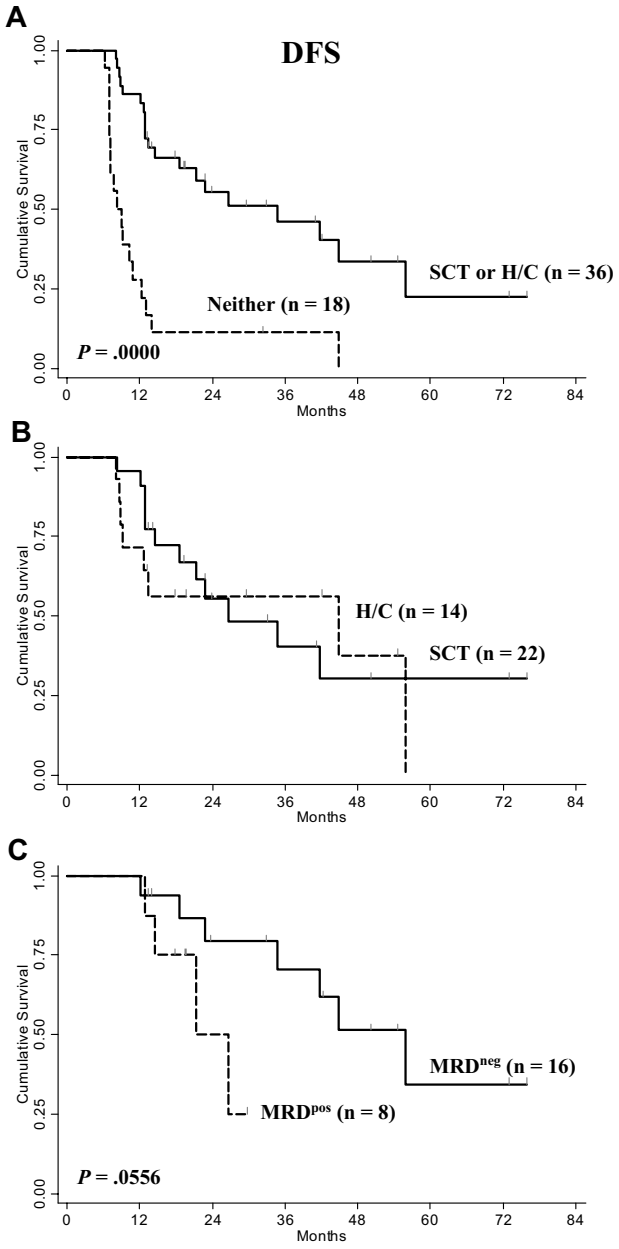

Figure 5. DFS of MRD^{pos} group. DFS in patients undergoing (A) SCT-based treatment, (B) allogeneic SCT or H/C therapy; (C) DFS in patients who converted or not to MRD^{neg} status after H/C or SCT. DFS probability at 4 years: SCT or H/C 0.33 versus 0 with neither (A); H/C 0.37 versus SCT 0.30 (B); MRD^{neg} 0.51 versus MRD^{pos} 0 (C).

using clinical prognostic factors, which are likely to benefit most from this strategy; and (c) considering currently available treatment options, what can be achieved therapeutically in the 2 major risk categories redefined by MRD analysis.

Table 6. Correlation between TP1 MRD results and final MRD risk model

		TP2- and TP3-based risk model, no. (%)		
TP1 MRD	No.	MRD^{neg}	MRD^{pos}	*P*
Negative	47	41 (87.2)	6 (12.8)	.001
Low positive ($< 10^{-4}$)	16	7 (43.8)	9 (56.2)	
Positive ($\geq 10^{-4}$)	39	3 (7.7)	36 (92.3)	

It is useful to discuss the core study results first. We found an outstanding correlation between MRD status as defined by the study model (integrating TP2 and TP3), related therapeutic choices, and 5-year DFS rate (Figure 3). In fact, among the risk factors examined, MRD risk analysis was the best for predicting long-term outcome. Its relevance is illustrated by the impact of MRD status on therapeutic choices: maintenance therapy should be used in MRD^{neg} cases, regardless of whether the patient is SR or HR, whereas in the study, transplantation procedures were reserved for MRD^{pos} cases (and by design to VHR cases). Notably, there were 2 reasons that we did not halt chemotherapy in the MRD^{neg} subgroup, as in the German study in SR patients.[20] First, we treated unselected patients, including HR patients, so that a higher cumulative risk of relapse was anticipated in our patient population. Second, based on the sensitivity threshold of MRD analysis, it was reasonable to assume that residual ALL cells (below the detectable level of 10^{-4} to 10^{-5}) could still cause disease recurrence, and this guided our decision to continue with therapy and monitoring. With this strategy, in unselected SR/HR patients who turned MRD^{neg} and were studied using at least one sensitive probe ($\geq 10^{-4}$), the BM relapse rate was less than 20% and the 5-year DFS nearly 80%, without remission mortality and with excellent quality of life. This result is probably beyond the reach of any SCT-based strategy considering transplantation-related mortality, which is seldom less than 20% (as in the present study) and has been reported to be as high as 36% in HR patients.[7]

In totally unselected MRD^{neg} patients (clinically SR, HR, and VHR, the latter eligible for SCT treatment), long-term DFS was still as good as 72%. This group included a few patients with suboptimal probes (sensitivity 10^{-3}) or who suffered from extramedullary relapse. Taken together, these findings cast doubt on the indication for SCT as the preferred therapy in unselected patients with ALL in first CR,[6,7,15-17] and call for an alternative MRD-assisted decisional approach. Conversely, outcome was definitely poorer in the MRD^{pos} subset, again with no difference related to the original clinical risk class. Interestingly, a proportion of these patients was effectively rescued by SCT. Although some retrospective reports have already suggested this possibility,[25,43,44] the present study may provide the first prospective evidence of the actual salvage rate of MRD^{pos} patients submitted to allogeneic (or autologous) SCT. Given that treatment-related mortality is around 20%, DFS estimates indicate that approximately one half of

Table 5. Multivariable prognostic analysis for DFS and risk of BM relapse (on 93 patients evaluable for all risk variables)

		DFS				BM relapse			
Risk factor	No. (%)	Hazard ratio	SE	95% CI	*P*	Hazard ratio	SE	95% CI	*P*
MRD^{pos}	44 (47.3)	5.88	2.16	2.86-12.08	.001	5.33	2.20	2.38-11.96	.001
WBC more than 100	9 (9.7)	5.13	2.38	2.06-12.75	.001	4.32	2.25	1.56-11.99	.005
WBC more than 30	24 (25.8)	2.57	0.88	1.32-5.02	.006	2.27	0.90	1.04-4.96	.040
HR phenotype	16 (17.2)	1.68	0.64	0.79-3.55	.176	2.04	0.85	0.90-4.63	.085
HR cytogenetics	23 (24.7)	1.04	0.38	0.52-2.12	.901	0.96	0.41	0.42-2.21	.934
Age older than 55 y	15 (16.1)	1.36	0.67	0.52-3.59	.530	1.44	0.80	0.49-4.29	.508

WBC indicates white blood cell ($\times 10^9$/L).

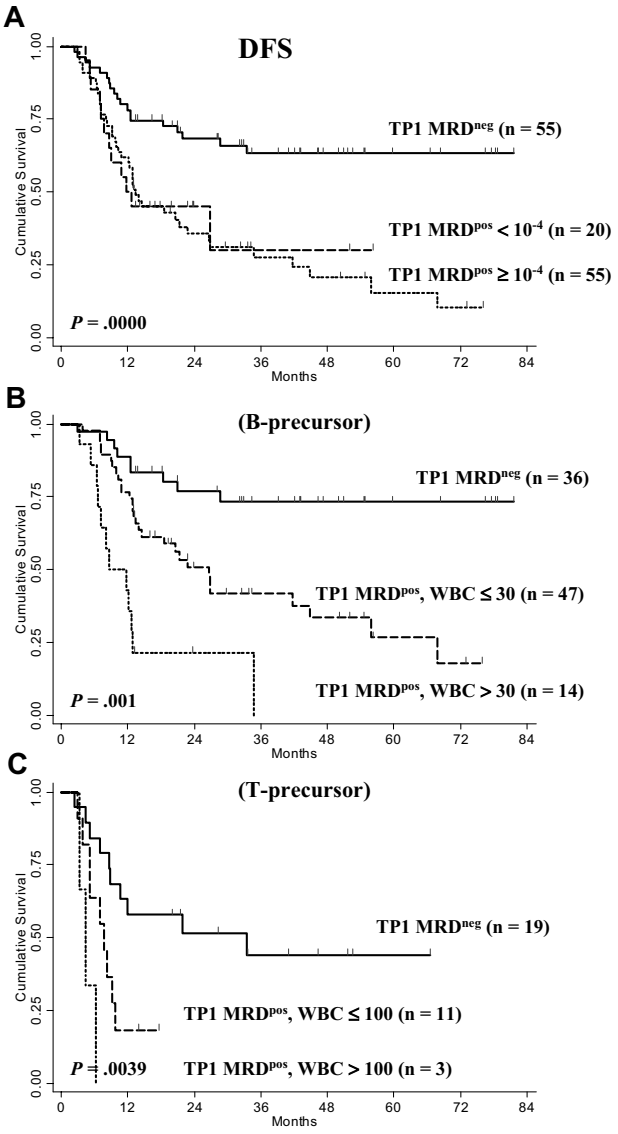

Figure 6. DFS by TP1 MRD and MRD/white cell count interaction. (A) DFS according to TP1 MRD status in unselected patients; DFS according to TP1 MRD status and white blood cell (WBC) count ($\times 10^9$/L) in (B) B-precursor ALL and (C) T-precursor ALL. DFS probability at 4 to 5 years: MRD^{neg} 0.63 versus $MRD^{low\text{-}pos}$ (< -4) 0.30 versus MRD^{pos} (≥ -4) 0.15; B-precursor: MRD^{neg} 0.73 versus MRD^{pos} (any level) with WBC of 30 $\times$ 10^9/L or less, 0.18 and WBC more than 30 $\times$ 10^9/L, 0; T-precursor: MRD^{neg} 0.44 versus MRD^{pos} (any level) with WBC lower than or equal to or more than 100 $\times$ 10^9/L, 0 (C).

MRD^{pos} patients surviving the toxicities of the SCT procedure could have a real therapeutic benefit (Figure 5). This reflects residual sensitivity to high-dose chemotherapy or radiotherapy or the graft-versus-leukemia effect, as confirmed by MRD conversion rates after both H/C-type autologous therapy or allografting. What remains to be determined is the clinicobiologic profile of MRD^{pos} cases likely to respond to transplantation-based therapy. We were unable to associate response with any known pretreatment diagnostic variable or with MRD status itself. Studies of ALL cell genomics, proteomics, and pharmacogenetics in SCT-resistant MRD^{pos} cases may eventually identify disease clusters for which treatment with investigational new agents and biomodifiers is indicated.[45,46]

In regard to technical issues, the MRD molecular study was carried out using 1 or 2 case-specific markers with sensitivity of 10^{-4} or higher, and only occasionally with less sensitive probes with a detection power of 10^{-3}; the latter were useful only for confirming the presence of residual disease, not its absence ($< 10\%$ of the cases). Accordingly, the MRD protocol was similar to some[25,29] but not all of the reported studies and expert recommendations.[20,27] Naturally, this hampers interstudy comparisons. For instance, in the German study, 2 sensitive probes were available in 59% of the patients studied, and all other cases were excluded from MRD analysis. In our study, less than 40% of the cases had 2 probes, but none of those with only a single molecular marker was excluded and the study was successful in almost 90% of the eligible subjects. These variations are tied to technical issues at the laboratories performing the molecular analysis, and MRD procedures must be standardized. However, it is notable that our data did not show any prognostic difference by probe number in the MRD^{neg} group (Figure 5C), validating for the time being the use of a single clonal marker as a reliable prognostic indicator, in agreement with recent conclusions from a large pediatric study.[47] Nonetheless, we recognize as possible limitation the lack of a simultaneous immunophenotypic confirmation of CR, and the absence of the correlation between molecular and immunologic MRD. This approach may provide comparable results with higher success rates, at least in younger patients.[22,28,45,48]

Concerning study time points, the current risk model was derived from TP2 (week 16) and TP3 (week 22) MRD results in light of a published study in which the greatest prognostic power related to MRD was manifest after 3 to 6 months of therapy.[25] Although childhood protocols usually require earlier MRD assessment (from as early as day 8 and up to week 12),[27,48] therapeutic results in adult ALL are typically inferior and a more prolonged evaluation of the course of residual disease seemed more appropriate for describing the potential for failure or cure of different MRD groups. There was a small group of patients ($< 10\%$) identified by the GMALL study[20] who had very early molecular clearance of disease at days +14 and +24 of therapy and an excellent outcome (DFS 100% at 3 years). However, the molecular risk for relapse was defined in most cases using MRD results at week 16, which is not very different from our study. Moreover, analyzing MRD TP1 results at week 10, we found an excellent correlation index of approximately 90% for both MRD^{pos} and MRD^{neg} groups using totally negative RQ-PCR results to define MRD negativity and a level of 10^{-4} or higher for positivity. All the data reviewed so far indicate that weeks 4 to 16 of therapy comprise the most critical interval for MRD analysis in adult ALL to obtain patient-specific information that supersedes the prognostic capability of clinical risk classifications. Although we cannot predict yet which patients will be positive for MRD in CR, an earlier MRD evaluation might identify very good responders[20] (none of whom would escape subsequent identification), whereas the early identification of patients expected to remain MRD^{pos} would allow to anticipate an intensified SCT-based treatment. Later MRD analysis is more commonly confirmatory as part of long-term MRD monitoring.

The early failure analysis by risk category (Table 3) aimed to identify patients for whom delaying the therapeutic decision until after MRD TP2 and TP3 might result in an unacceptably high risk of relapse, which could be countered (in theory) only by an earlier choice to perform allogeneic SCT. These patients had higher white cell counts, particularly higher than 100 $\times$ 10^9/L, a diagnosis of T-ALL, and adverse cytogenetics such as t(4;11), all features that are associated with an early relapse risk of 30% to 50% or greater with the stated chemotherapy program. Although the early consolidation schedule can be significantly improved in T-ALL, as reported by others,[3,4,49] and Ph^+ or $t(4;11)^+$ ALL patients are always eligible for early allogeneic SCT, we strongly discourage applying an MRD-guided treatment policy based on evaluation

time points longer than 3 months in subsets at very high risk of early relapse. These include cases with hyperleukocytosis more than 100×10^9/L, HR T-ALL with pro/pre/mature T phenotype (noncortical), and rarer poor-risk cytogenetic subtypes whose numeric paucity prevents drawing conclusions about early MRD response and associated clinical course. In our opinion, in these subsets, which represent 20% or less of all SR/HR subjects, even when MRD^{neg} status could be achieved at end of consolidation therapy, there remains a clear indication to proceed rapidly to an allogeneic SCT and the clinical definition of VHR category can be applied.

In summary, molecular analysis of MRD performed with at least one sensitive probe during the first months of induction/consolidation therapy is an unrivalled early prognostic indicator in unselected adult patients with SR and HR ALL. It is applicable to 80% or more of cases and greatly improves clinical risk classification. In the large fraction of patients who can be safely observed during the time needed to complete the MRD study, this information can be used to individually optimize therapeutic strategy, which currently ranges from standard maintenance to allogeneic SCT. The expected benefits are reduced overall toxicity and mortality when allotransplantation can be avoided initially, a more rational use of therapeutic resources, and the identification of VHR subjects for whom novel experimental treatments are indicated. Like another published study,[20] the study described here belongs to the first generation of MRD-based trials. Further progress is to be expected in the coming years as we gain more knowledge about the molecular analysis of MRD and as new treatment modalities drive the developing field of risk-oriented therapy for adult ALL patients.

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Authorship

Contribution: R.B., T.B., and A.R. designed the study; R.B., O.S., and A.R. wrote the paper; E.O. and T.I. analyzed the data and designed the figures and tables; O.S., M.T., and B.P. performed the molecular study; R.B., T.B., A.R., T.I., G.R., E.B., E.M.P., E.T., P.F., V.C., G.L.-D., A.C., A.B., G.G., F.C., M.B., A.G., D.M., E.D.B., C.R., and A.M.S. were involved in the conduct of the study at treatment centers.

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