Clearance of minimal residual disease after allogeneic stem cell transplantation and the prediction of the clinical outcome of adult patients with high-risk acute lymphoblastic leukemia

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ABSTRACT

Background and Objectives

The molecular analysis of minimal residual disease (MRD) may provide informaton on the risk of recurrence in patients with acute lymphoblastic leukemia (ALL). The aim of this study was to correlate the kinetics of MRD clearance after allogeneic transplantation with the clinical outcome of adults with ALL.

Design and Methods

MRD was evaluated by real-time quantitative polymerase chain reaction (RQ-PCR) using probes derived from fusion chimeric genes (*BCR/ABL* and *MLL/AF4*) (n=22) or rearrangements of the T-cell receptor or immunoglobulin genes (n =21). Forty-three adult patients with ALL were studied to correlate the kinetics of MRD clearance before and after allogeneic hematopoietic stem cell transplantation.

Results

At 36 months, the overall survival of patients who underwent transplantation in hematologic remission (n = 37) was 80% for those who were PCR-negative before transplantation (n = 12) compared to 49% for PCR-positive patients (n = 25)(p=0.17). For the same patients the cumulative incidence of relapse was 0% and 46%, respectively (p=0.027). Moreover, the relapse rate of patients who were PCR-negative at day +100 after transplantation was remarkably low (7%) compared to that among patients who were PCR-positive (80%, p=0.0006).

Interpretation and Conclusions

The kinetics of MRD clearance may help to identify patients at high risk of leukemia relapse after allogeneic stem cell transplantation. Patients not achieving an early molecular remission after transplantation require prompt and appropriate pre-emptive treatments such as infusions of donor lymphocytes or new experimental drugs.

Key words: MRD, adult ALL, Ph$^+$ ALL, allogeneic transplantation.

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Allogeneic stem cell transplantation represents a curative treatment option for patients with acute lymphoblastic leukemia (ALL) with high-risk features at diagnosis. However, the percentage of patients who relapse after transplantation is still high (30-40%) and transplant-related mortality further reduces the overall advantage provided by the procedure. Several studies have reported the importance of detecting and quantifying minimal residual disease (MRD) status before and after allogeneic hematopoietic stem cell transplantation (HSCT) in pediatric patients.[1-5] Children with molecularly detectable residual disease before allogeneic HSCT were predicted to have a higher risk of relapse and a poor outcome. Less information is available concerning the prognostic value of the molecular clearance of MRD after transplantation, particularly in adult patients with ALL.[6,7] Mortuza and co-workers, recently reported that MRD status *after* allogeneic bone marrow transplantation rather than *before* was an important predictor of outcome in a cohort of 13 adult ALL patients.[8] In the present study, in the context of a large multicenter treatment protocol, we investigated the clinical value of MRD analysis performed immediately before, then 30 and 100 days after allogeneic transplantation in a group of adult patients with high-risk ALL.

Design and Methods

Patients

Patients were diagnosed and treated in the context of multicenter Northern Italian Leukemia Group (NILG) protocols for adult ALL: NILG-ALL 08/96[9] (7 patients) and 09-00 (36 patients) (ClinicalTrials.gov Identifier: NCT00358072).[10] Patients were eligible for allogeneic transplantation in first complete remission only if they had adverse cytogenetic abnormalities including t(9;22) or t(4;11) or persistent MRD (>1 leukemic cell in 10^4 normal cells at week 16 or any level of detectable MRD at week 22) during consolidation chemotherapy. When imatinib became available, upon protocol amendment, it was included early during induction and consolidation. Eight t(9;22)-positive patients received imatinib. Other eligible patients were those with primary refractory or relapsed disease. To be included in this study, one or two sensitive molecular probes had to be available as well as serial bone marrow and peripheral blood samples obtained at diagnosis, during consolidation, before the conditioning regimen and at several times after transplantation. Table 1 shows the patients' characteristics: 37 patients had B-precursor ALL and six had T-ALL. Their median age was 30 years (range, 18-63). Twenty-nine patients were transplanted in first complete remission, eight in second complete remission and six with active disease. Twenty-four patients had a related donor and 19 patients had a matched unrelated donor (MUD). The conditioning regimen consisted of cyclophosphamide (120 mg/kg) in combination with total body irradiation (12 Gy) for 38 patients or busulfan (1 mg/kg/die × 4) for three patients. Two patients received a reduced intensity conditioning regimen because of advanced age and co-morbidity.

Table 1. Characteristics of the patients.

Number of patients	43
Male/female	27/16
Median age at transplantation (range)	30 (18-63)
Diagnosis	
T-ALL	6
B-precursor ALL	37
Status at transplant	
First complete remission	29
Second complete remission	8
Active disease	6
Cytogenetics	
Normal	12
t(9;22)	20
t(4;11)	2
Abnormal	6
Unknown	3
Donor	
Related	24
Unrelated	19
Conditioning	
*Myeloablative	41
Reduced intensity	2

**Myeloablative: cyclophosphamide 60 mg/kg/die × 2 + total body irradiation 12 Gy (n=38) or busulfan 1 mg/kg/die × 4 (n=3).*

Definitions of clinical and molecular remission

Patients with less than 5% blasts in the bone marrow were considered to be in complete clinical remission. Molecular analyses were performed preferentially on both bone marrow and peripheral blood samples. Molecular remission was defined by the absence of any positive molecular signal detectable by real-time quantitative (RQ)-polymerase chain reaction (PCR) in triplicate samples.

Clonal marker identification and MRD analysis

DNA and RNA were extracted using commercially available kits (Puregene, Gentra Systems, Minneapolis, MN, USA; RNeasy, Quiagen GmbH, Hilden, Germany) from mononuclear cells obtained from bone marrow or peripheral blood samples after centrifugation on a Ficoll-Hypaque gradient. Bone marrow samples were analyzed at diagnosis for the presence of the t(9;22), t(4;11), t(1;19) translocations for B-precursor ALL and for *TAL1* gene deletion for T-ALL.[11] For MRD detection in pre- and post-transplant samples chimeric *BCR/ABL* or *MLL/AF4* transcripts were amplified by RQ-PCR or in nested PCR (one patient with t(9;22)

Table 2. Sensitivity of the molecular probes.

Molecular Probes	*10^{-3}*	*10^{-4}*	*10^{-5}*	*Total*
Ig*	1	3	6	10
TcRγ	4	2	3	9
TcRδ	1	4	6	11
TcRβ	1	2	1	4
BCR-ABL	–	–	20	20
MLL-AF4	–	2	–	2
	7 (12%)	13 (23%)	36 (65%)	56**

**Ig probes encompass IgH and Igκ rearrangements; **Two molecular probes were available for 13 patients.*

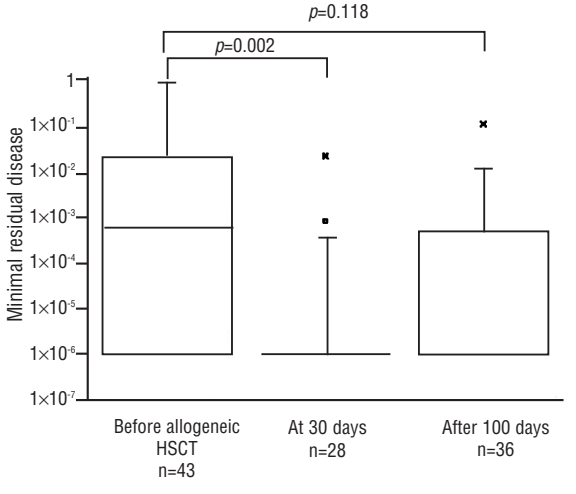

Figure 1. MRD level before and after allogeneic transplantation. The level of MRD is expressed as the logarithmic reduction of the disease detected in the bone marrow at diagnosis. The horizontal line indicates the median value observed. The box represents the range between the 25th and 75th percentiles. The bars extend to the 95th percentile. The minus symbol indicates the extremes. Small squares represent the mean value. Neoplastic cells were evaluated before as well as 30 days and 100 days after transplantation. Statistical analyses and *p* values were calculated using Wilcoxon's test for paired samples.

p190 e1a3 transcript) and quantified by parallel amplification of serial dilutions of *BCR/ABL* or *MLL/AF4*-containing plasmids.[11,12] When a chimeric fusion gene was not present, leukemia-specific probes were generated by genomic amplification and sequencing of VDJ regions of the immunoglobulin heavy chain (IgH) or κ light chain (Igκ), and T-cell receptor γ, δ and β (TcRγ, TcRδ, TcRβ) genes.[13-17] Clone-specific oligonucleotides were constructed 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.[18,19] The oligonucleotide sensitivities were tested on ten-fold serial dilutions of DNA obtained from leukemic cells isolated at diagnosis with DNA from a pool of eight healthy donors. MRD was quantified by amplification of 500 ng of sample DNA and the above-mentioned ten-fold serial dilutions of DNA. All samples were amplified in triplicate and the MRD level was expressed as the logarithmic reduction of the leukemic clone detected at diagnosis.[12,20]

Statistical analyses

Actuarial probabilities of overall survival (OS) and cumulative incidences of relapse were calculated at 36 months, using the Kaplan-Meier method. Wilcoxon's test was used to assess the statistical significance of the MRD reduction at different time points.

Results

Identification of molecular probes and MRD analysis

Diagnostic samples from 43 ALL patients were analyzed to identify a molecular probe suitable for MRD evaluation. Twenty-two patients with B-precursor ALL were positive for the presence of either *BCR/ABL* chimeric transcripts (e1a2 in 13 patients, e1a3 in one patient, b2a2 in two patients and b3a2 in four patients) or the *MLL/AF4* gene (two patients) (Table 2). The remaining patients (six with T-ALL and 15 with B-precursor ALL) were studied for the presence of clonal Ig or TCR gene rearrangements (Table 2). A total of 56 molecular probes were generated with a median sensitivity ranging from 10^{-3} (12%) to 10^{-5} (65%). Probes derived from chimeric genes reached a homogeneous sensitivity of 10^{-5} for *BCR-ABL* and 10^{-4} for *MLL/AF4*.[11] For 13 patients two informative Ig or TCR-derived probes were available which gave concordant results in 11 cases and discordant in the other two. The sensitivity of the probes by which patients were actually monitored during the follow-up was low (10^{-3}) in three cases (7%), intermediate (10^{-4}) in ten (23%) and high (10^{-5}) in 30 (70%).

Clearance of MRD after allogeneic HSCT

As shown in Figure 1, a median value of one leukemic cell in 10^3 normal cells (ranging from 0 up to 1 in 10 normal cells) was documented in the bone marrow before starting the conditioning regimen. According to the study design, five patients were selected for allogeneic HSCT because they were MRD-positive at week 16-22 during consolidation, although they proved negative immediately before conditioning. Patients with t(9;22) or t(4;11) positive ALL underwent allogeneic HSCT regardless of the course of MRD during consolidation and with detectable (15 cases) or undetectable (7 cases) MRD at the time of conditioning. The early and repeated evaluation of MRD in these 43 ALL patients allowed us to quantify the degree of leukemia debulking obtained by the conditioning regimen. At 30 days after transplantation, a median 3-log reduction of leukemic cells was documented so that 20 of the 28 evaluable

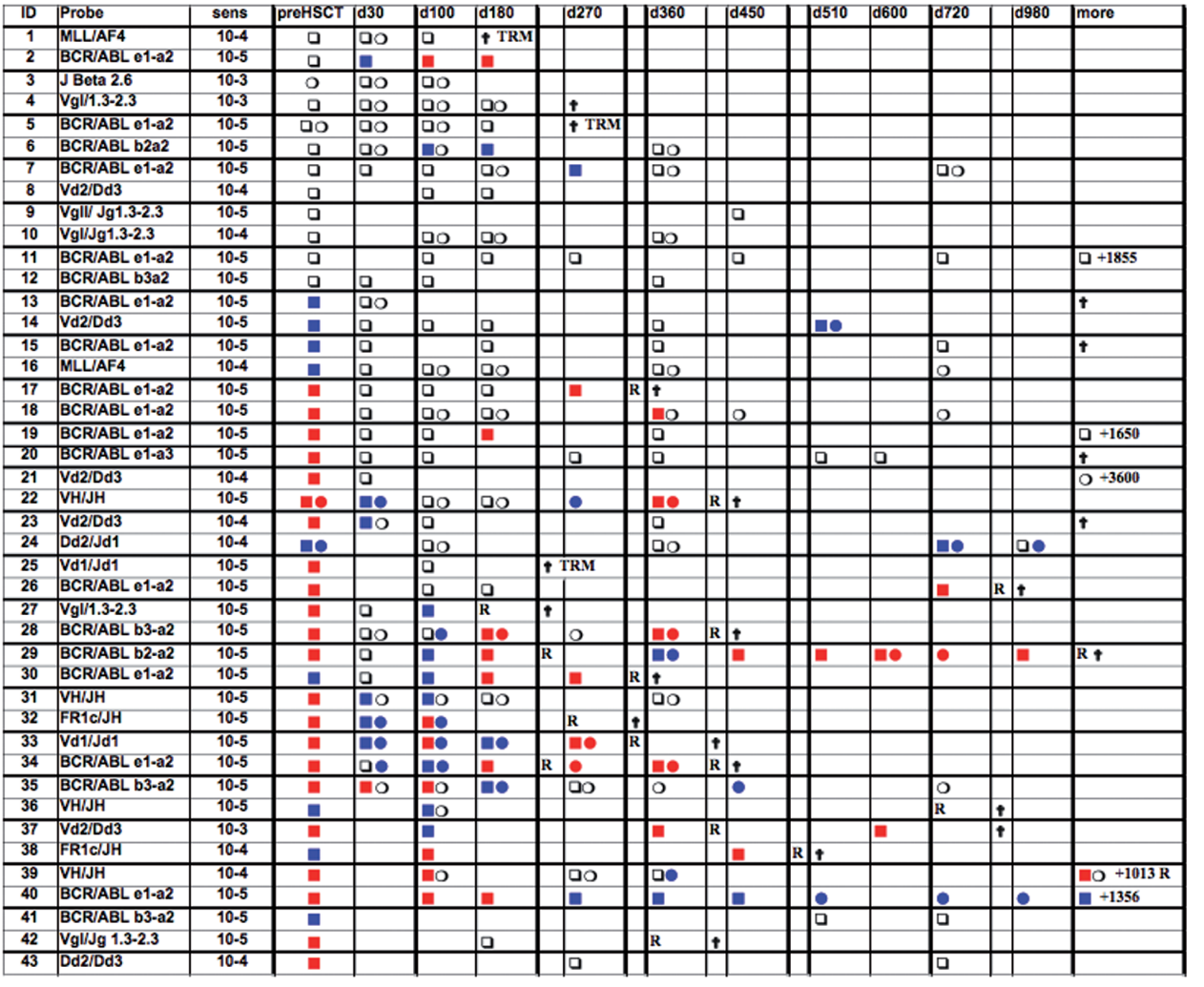

Figure 2. Molecular follow-up of the 43 adult patients with ALL. Squares and circles represent bone marrow and peripheral blood samples, respectively. Open symbols represent MRD negative values, blue symbols represent low positive values (1 positive cell in 1,000-100,000 negative cells); red symbols represent high positive values (1 positive cell in 10-1,000 negative cells).

patients (71%) had converted to a molecularly negative status (Figures 1 and 2). At day +100 a significant 3 log mean reduction of the leukemia burden was confirmed in the bone marrow of the 36 patients analyzed but evidence of leukemia persistence/progression was documented in 16 of the 36 samples (44%) available for evaluation (Figures 1 and 2). As expected, the median MRD value at conditioning was ten times higher in the bone marrow of most patients who failed to achieve molecular remission by day +30 (Figure 2, patients 22, 23, 31-35). Similarly, 11 out of 16 patients who did not achieve or rapidly lost molecular remission by day +100 had a high leukemia burden before transplantation (Figure 2).

Prognostic value of MRD monitoring after allogeneic HSCT

With a median follow-up of 23 months (range 4 - 138), the overall survival at 36 months of the 43 ALL patients studied is 48% (95% CI 31-63) (Figure 3, Panel A). The degree of heterogeneity observed in the number of leukemic cells detectable in the bone marrow before the conditioning regimen prompted us to verify whether this biological information could be related to long-term clinical outcomes. At 36 months, the overall survival of patients who underwent transplantation in hematologic remission (n=37) was 80% for those who were PCR-negative before transplantation as compared to 49% for PCR-positive patients (95% CI, 20-67), (p=0.17) (Figure 3, Panel B). For the same patients, the cumulative incidence of relapse was 0% for PCR-negative patients and 46% for PCR-positive patients (p=0.027) (Figure 3, Panel C). Moreover, the relapse rate calculated according to the molecular results at day +100 confirmed that the achievement or maintenance of a PCR-negative status within 3 months following transplantation was significantly associated with a lower incidence of relapse at 36 months (7% vs. 80%, p=0.0006) (Figure 3, panel D). Looking for

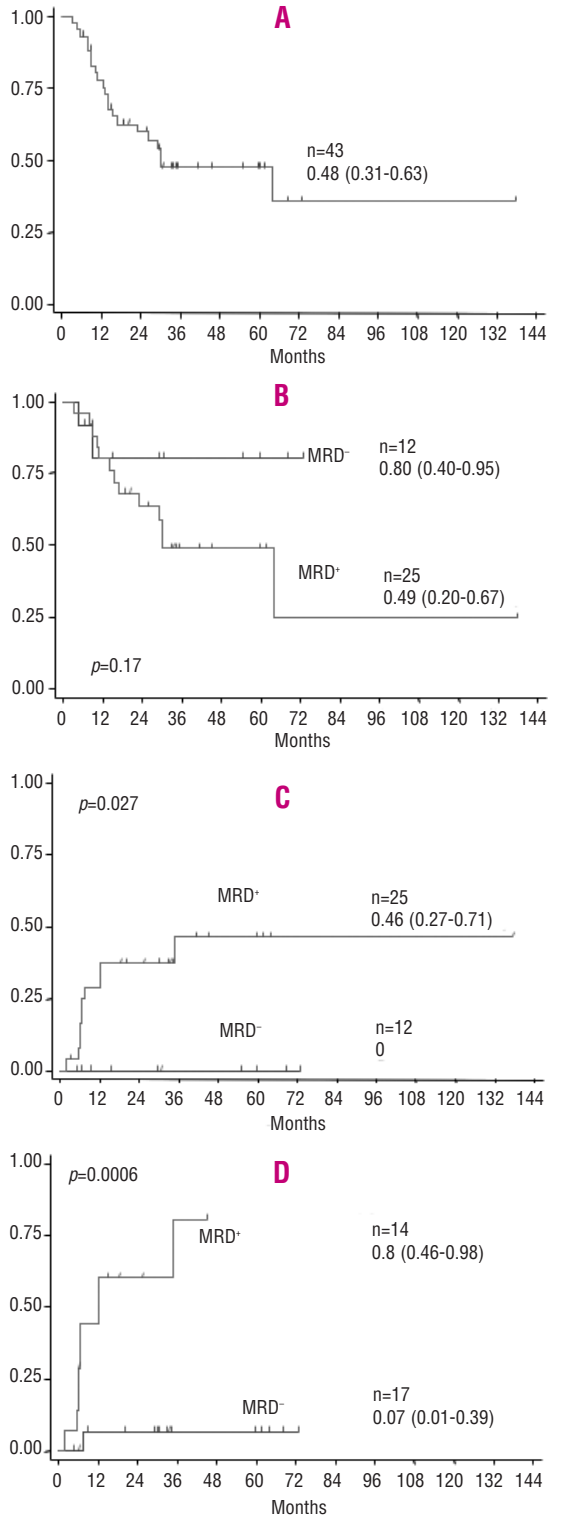

Figure 3. Overall survival and cumulative incidence of relapse. Overall survival of the 43 transplanted patients (Panel A). Overall survival according to MRD status before transplantation (Panel B), cumulative incidence of relapse by MRD status at transplanta tion (Panel C) and by MRD status at day +100 (Panel D) of patients undergoing transplantation in complete hematologic remission.

Table 3. Factors predicting molecular complete remission (CR) at 100 days after allogeneic transplantation.

		Univariate		*Multivariate (Logistic analysis)*	
Variable		*CR (%)*	*p value(χ^2)*	*OR (95% CI)*	*p value*
Age	>30 ≤30	10/19 (53) 10/17 (59)	0.70	0.29 (0.04 - 1.83)	0.19
Immuno-phenotype	T B	2/4 (50) 18/32 (56)	0.80	0.33 (0.01-9.98)	0.53
Adverse cytogenetics*	Yes No	11/19 (58) 9/17 (53)	0.80	1.23 (0.21 - 7.07)	0.81
Blasts at diagnosis >10,000**	Yes No	6/13 (46) 14/23 (61)	0.40	0.34 (0.04 -2.35)	0.27
Hematologic CR at conditioning	No Yes	3/5 (60) 17/31 (55)	0.80	4.85 (0.22 - 102)	0.31
Molecular CR at conditioning***	No Yes	11/25 (44) 9/11 (82)	0.035	0.09 (0.012-0.73)	0.024

** t(9;22) or t(4;11); ** counted in the peripheral blood; *** evaluated by quantitative PCR on bone marrow samples.*

informative predictors of the achievement of a molecular complete remission at day +100 we used univariate and multivariate analyses to investigate the role of conventional clinical findings (age, immunophenotype, adverse cytogenetics and the number of leukemic blasts at diagnosis) as well as the clinical status (first complete remission vs second complete remission vs active disease) and molecular complete remission evaluated immediately before the beginning of the conditioning regimen. By multivariate analysis, only molecular complete remission before conditioning proved to be a significant predictor of the achievement of molecular negativity at day 100 after transplantation (Table 3).

Discussion

In this study we investigated the prognostic value of MRD in ALL patients undergoing allogeneic HSCT. The sensitivity of the molecular probes we used for MRD detection was high in the great majority of patients and this ensured a low incidence of possible false negative results. The molecular monitoring of MRD that we performed in this cohort of ALL patients undergoing stem cell transplantation provides two major insights.

In keeping with previous observations in pediatric patients[1,3,21,22] we showed that the incidence of leukemia relapse after transplantation was significantly higher in all patients with molecularly

detectable residual disease before the conditioning regimen. Indeed, the relapse rate was negligible in patients who were in molecular remission before transplantation while 46% of patients relapsed if any level of MRD, no matter the amount, was detectable at this time. In fact, by multivariate analysis, the PCR negativity before the conditioning regimen proved to be the only significant predictor of clinical and molecular complete remission at day 100 after transplantation. This observation highlights the need to deliver therapeutic programs that can achieve molecular remission before transplantation. This is particularly important for patients bearing the t(9;22) chromosome translocation who may benefit from the early addition of imatinib [23,24] or the newer tyrosine kinase inhibitors to the chemotherapeutic programs offered at diagnosis.[25,26]

The second insight comes from the kinetics of MRD clearance. The relapse rate among patients who achieved PCR negativity by day +100 post-transplantation was significantly lower than that among patients who never achieved or rapidly lost molecular remission. Although the probability of converting to a stable PCR negative remission was higher among patients with a lower MRD level at conditioning, almost half the patients with high levels of MRD before transplantation achieved a molecular remission by day +100. This result clearly reflects the biological heterogeneity of ALL cells in terms of sensitivity to both the conditioning regimens (chemo-radiotherapy in most of our cases) and the immune-mediated graft-versus-leukemia effect. This latter mechanism probably also explains some of the cases of late and durable clearance of MRD, which albeit rarely, were occasionally seen in our patients. At variance to what has previously been reported,[2] our results indicate that the check point at day +100, rather than earlier time points, should be considered crucial for subsequent therapeutic decisions. In fact, patients not achieving a molecular remission at this time point should be selected for early discontinuation of immune suppression and possibly given infusions of donor lymphocytes or leukemia-specific cytotoxic T-lymphocytes,[27] natural killer cells[28] or molecularly targeted drugs, when indicated. Unlike other analyses, our results pertain only to adult patients at high risk of leukemia relapse either because of the presence of adverse cytogenetic abnormalities (i.e., t(9;22) or t(4;11)) or because of the persistence of molecularly detectable MRD after consolidation chemotherapy. It should be said that the adverse clinical and biological features of our patients may represent a negative selection bias, leading to an underestimation of the therapeutic potential of allogeneic HSCT. In addition, a major limitation to the molecular evaluation of MRD is the enormous amount of laboratory work which requires highly specialized and fully committed staff.[29]

In conclusion, sequential and prospective MRD evaluation may be of crucial importance for accurate clinical monitoring after allogeneic HSCT since it may not only reveal a pending leukemia relapse in advance but also, more importantly, it may provide a guide to early post-transplantation treatment. Nonetheless, the number of patients analyzed in this study is limited and so our results must be considered preliminary and not definitive. A prospective trial is currently underway to confirm our findings.

Authors' Contributions

OS, BP, MT, VG performed the molecular analyses; AS and MCZ produced the cell preparations and performed immunophenotyping; EO performed the statistical analysis, AG, TI, CM followed the patients and provided blood and marrow samples; GR, PF, GL-D, EA designed the study and revised the manuscript; TB and RB applied for funding and critically revised the manuscript; OS and AR planned the project, supervised the work and wrote the manuscript.

Conflict of Interest

The authors reported no potenatial conflicts of interest.

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