

# Monitoring of minimal residual disease in adult acute myeloid leukemia using peripheral blood as an alternative source to bone marrow

Luca Maurillo, Francesco Buccisano, Alessandra Spagnoli, Giovanni Del Poeta, Paola Panetta, Benedetta Neri, Maria Ilaria Del Principe, Carla Mazzone, Maria Irno Consalvo, Anna Tamburini, Licia Ottaviani, Daniela Fraboni, Chiara Sarlo, Paolo De Fabritiis, Sergio Amadori, Adriano Venditti

All authors from the Dept. of Hematology, Policlinico Tor Vergata and Ospedale S.Eugenio, Rome, Italy.

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Correspondence: Adriano Venditti, MD, Cattedra di Ematologia, Università Tor Vergata, viale Oxford 81, 001133 Rome, Italy. E-mail: [adriano.venditti@uniroma2.it](mailto:adriano.venditti@uniroma2.it)

## ABSTRACT

### Background and Objectives

To date, bone marrow (BM) is the most common source of cells to use in order to assess minimal residual disease (MRD) in acute myeloid leukemia (AML). In the present study, we investigated whether peripheral blood (PB) could be an alternative source of cells for monitoring MRD in AML.

### Design and Methods

Fifty patients with AML were monitored for MRD after the achievement of complete remission. Using multiparametric flow cytometry we compared the levels of MRD in 50 and 48 pairs of BM and PB after induction and consolidation, respectively.

### Results

After induction and consolidation therapy, the findings in BM and PB were significantly concordant ( $r=0.86$  and  $0.82$ , respectively,  $p<0.001$  for both comparisons). The cut-off value of residual leukemic cells in PB which correlated with outcome was  $1.5 \times 10^{-4}$ . Thirty-three of 43 (77%) patients with  $>1.5 \times 10^{-4}$  residual leukemic cells in PB after induction had a relapse, whereas the seven patients with lower levels did not ( $p=0.0002$ ). After consolidation, 38 patients had a level of MRD  $>1.5 \times 10^{-4}$  and 31 (82%) had a relapse; nine out of the remaining ten patients, whose levels of MRD were below  $1.5 \times 10^{-4}$ , are still relapse-free ( $p=0.00006$ ). In multivariate analysis, PB MRD status at the end of consolidation was found to have a significant effect on relapse-free survival ( $p=0.036$ ).

### Interpretation and Conclusions

These preliminary results indicate that: (i) PB evaluation can integrate BM assessment for MRD detection in patients with AML; (ii) PB MRD status at the end of consolidation therapy may provide useful prognostic information.

Key words: AML, MRD, multiparametric flow-cytometry, leukemia associated phenotype, peripheral blood.

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Current treatment strategies in adult patients with acute myeloid leukemia (AML) lead to complete remission (CR) in 50-80% of the patients.<sup>1-6</sup> However, most of these patients will eventually relapse due to the persistence of residual leukemic cells that escape the cytotoxic effect of the therapy and are undetectable by conventional light microscopy.<sup>7,8</sup> Recent studies have shown that assessment of minimal residual disease (MRD) may prove useful to modulate the intensity of post-remission therapy in AML.<sup>9-11</sup> Currently, the most widely used techniques to assess MRD are based on detection of either molecular or immunophenotypic markers expressed by the leukemic clone. Despite its high sensitivity (one target cell per  $10^3$  to  $10^6$  normal cells), the applicability of polymerase chain reaction (PCR) techniques is confined to those cases of AML (20-40%) characterized by the presence of fusion genes derived from chromosome translocations.<sup>8,9</sup> Multiparametric flow cytometry (MPFC) may allow a sensitivity of one leukemic cell per  $10^4$ - $10^5$  normal bone marrow cells and can be successfully applied in up to 80% of AML patients.<sup>8,12</sup> Our group and others have demonstrated that monitoring MRD by MPFC can provide useful prognostic information in adult AML, when bone marrow (BM) is used;<sup>13-16</sup> however, peripheral blood (PB) may represent an alternative source of cells for the purpose of these studies. This is based on the assumption that the presence of circulating blasts at the time of CR might be directly correlated to the persistence of malignant cells in the BM or might indicate the propensity of blast cells to exit prematurely from the BM, leading to a more aggressive course of disease. Initial studies to monitor MRD in PB used PCR and included patients with B-lineage acute lymphoid leukemia (ALL).<sup>17,18</sup> It was found that MRD is detectable and measurable in PB, with the levels usually being lower than those in BM. By using MPFC, Coustan-Smith *et al.* confirmed these findings in B-ALL, whereas in T-ALL similar proportions of MRD were observed in both BM and PB.<sup>19</sup> Although these results indicate that PB may be as useful a source of cells as BM for MRD studies, very few data have been reported in AML. A recent study<sup>20</sup> measuring the levels of MRD in PB and BM samples from AML patients with t(8;21), showed that quantitative PCR (RQ-PCR) is able to detect AML1-ETO fusion transcripts in both sources with a similar sensitivity. Based on these premises, we used MPFC to assess the levels of MRD in PB and BM samples from 50 adult patients with AML. The aim of the study was to verify the feasibility of MRD detection in PB and its prognostic relevance.

## Design and Methods

### Patients

A total of 50 consecutive adult patients with *de novo* AML diagnosed at the Department of Hematology, S.

Eugenio Hospital, University Tor Vergata, were analyzed. All patients underwent intensive chemotherapy according to the EORTC/GIMEMA protocols AML-10, AML-12 and AML-13. The expression of a *leukemia-associated* phenotype at the time of diagnosis and the achievement of morphologic CR after induction therapy were criteria for inclusion in the study. We studied 50 pairs of BM and PB samples at the end of induction therapy. Two patients had an early relapse before consolidation; therefore, 48 pairs of BM and SP samples were evaluated at the end of consolidation treatment. Approval for this study was obtained from the institutional review board. The patients involved provided informed consent according to the declaration of Helsinki.

### Treatment protocols

The EORTC/GIMEMA AML-10 randomized trial included patients aged 18-60 years.<sup>21</sup> Induction treatment combined cytarabine ( $100 \text{ mg/m}^2$  days 1-10), etoposide ( $50 \text{ mg/m}^2$  days 1-5), and on days 1, 3 and 5, either daunorubicin ( $50 \text{ mg/m}^2$ ), mitoxantrone ( $12 \text{ mg/m}^2$ ) or idarubicin ( $10 \text{ mg/m}^2$ ) according to randomization. As consolidation, patients received cytarabine ( $500 \text{ mg/m}^2/\text{q12 hours}$  days 1-6) and the same anthracycline as in induction. Patients with an HLA-compatible sibling were allografted, whereas the others were randomly assigned to PB or BM autologous stem cell transplantation. In the AML-12 EORTC/GIMEMA trial, patients received the daunorubicin arm of AML-10 as standard remission induction and cytarabine ( $500 \text{ mg/m}^2/\text{q12 hours}$  days 1-6) plus daunorubicin ( $50 \text{ mg/m}^2$  on days 4-6) as consolidation. Patients with an HLA-compatible sibling were allografted, whereas the others underwent PB autologous stem cell transplantation, followed by no further therapy or subcutaneous maintenance therapy with interleukin-2, according to a second randomization. Patients older than 60 years of age were entered into the EORTC/GIMEMA AML-13 randomized trial.<sup>22</sup> In this protocol, patients received mitoxantrone ( $7 \text{ mg/m}^2$  days 1, 3 and 5), cytarabine ( $100 \text{ mg/m}^2$  days 1-7) and etoposide ( $100 \text{ mg/m}^2$  days 1-3), as induction therapy. Upon achievement of CR, patients were randomly assigned to receive either an intravenous or an oral consolidation program (two cycles). Intravenous consolidation consisted of idarubicin ( $8 \text{ mg/m}^2$  days 1, 3 and 5), cytarabine ( $100 \text{ mg/m}^2$  days 1-5) and etoposide ( $100 \text{ mg/m}^2$  days 1-3). Oral consolidation consisted of idarubicin ( $20 \text{ mg/m}^2$  days 1, 3 and 5), etoposide ( $50 \text{ mg/m}^2$  twice a day, days 1-3), and subcutaneous cytarabine ( $50 \text{ mg/m}^2$  twice a day, days 1-5).

### Immunophenotypic studies and MRD detection

At diagnosis, immunophenotypic, chromosomal and genetic studies were performed as detailed elsewhere.<sup>13,23,24</sup> *Leukemia-associated* phenotypes were detected by staining leukemic cells with several combinations of monoclonal antibodies conjugated to fluorescein

**Table 1.** Clinical characteristics of the patients (n=50).

Age (yrs)	
Median (range)	49 (21-73)
< 60 yrs	34 patients
> 60 yrs	16 patients
Sex	
Male	33
Female	17
FAB subtype	
M0/M1/M2	8/13/9
M4/M5/M6	8/11/1
Cytogenetic risk group*	
Favorable	4
Intermediate	35
Unfavorable	5

\*Data available for 44 out of 50 patients. Patients were stratified, according to the Medical Research Council (MRC) classification of cytogenetic risk, into "favorable" [cases with t(8;21), t(15;17) or inv(16)/del(16q), irrespective of the presence of additional cytogenetic abnormalities], "adverse" [cases lacking these favorable risk aberrations with complex cytogenetic changes (five or more unrelated abnormalities), -5, del(5q), -7, or 3q abnormalities] and "intermediate" [cases with normal karyotype, 11q23 abnormalities, trisomy 8 (+8), del(7q), del(9q), complex karyotypes ( $\geq$  three abnormalities but  $<$  five abnormalities) or all chromosomal changes of unknown prognostic significance] risk groups.

isothiocyanate, phycoerythrin, peridinin chlorophyll protein, and allophycocyanin. A given combination of markers was regarded as relevant if expressed in  $\geq 50\%$  of the blasts. This step served to define a *leukemia immunophenotypic fingerprint* which in turn was used to track possible residual leukemic cells during follow-up at specific time points. At least two antibody combinations for each case were selected to minimize pitfalls due to *phenotypic switches* that have been described to be occasionally associated with relapses.<sup>25-27</sup> The study of a series of normal BM and PB samples from healthy donors or regenerating samples from patients with lymphomas created an internal standard reference to distinguish normal from leukemic patterns.<sup>13,23,24</sup> CellQuest (Becton Dickinson) software was used for acquisition of the flow cytometric data, applying *live gates* on the forward-light/orthogonal light scatter (blast region) and fluorescence plots. Samples were then analyzed using PAINT-A-GATE<sup>PRO</sup> software program (Becton Dickinson), as previously described.<sup>13,23,24</sup> MRD studies during remission were performed on erythrocyte-lysed whole BM and PB samples using the same antibody combination defining the specific *leukemia immunologic fingerprint*. During data acquisition a live-gate including the lymphomonocytic/granuloblastic region and excluding debris and platelet aggregates was used with  $10^6$  total events being acquired in all samples. The acquired events were analyzed with the PAINT-A-GATE software, also applying the MouseTRAX Control option, as described elsewhere.<sup>13,23,24</sup>

### Statistical analyses

Spearman's rank correlation ( $r$ ) was used to assess the correlation between PB and BM MRD levels after induc-

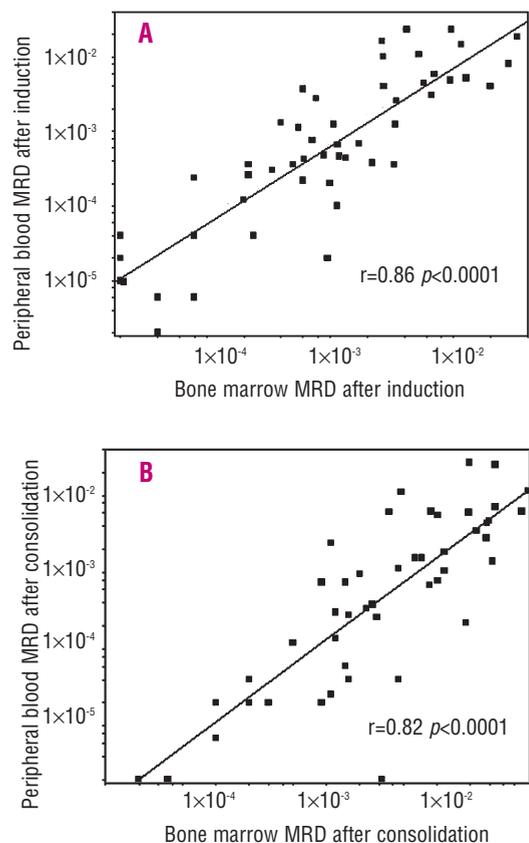
tion and consolidation. Values of MRD levels, evaluated after induction and consolidation therapies, were tested for possible cut-offs by means of maximally selected log-rank statistics.<sup>28</sup> The relationship of PB MRD levels with patients' characteristics and response to treatment was estimated by a two-sided  $\chi^2$  test (or Fisher's exact test when either group included fewer than 20 cases). A  $p$  value of 0.05 or less was considered to be statistically significant.

CR and relapse were defined by standardized criteria.<sup>30</sup> Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Relapse-free survival (RFS) was measured from achievement of CR until relapse. The Kaplan-Meier method<sup>29</sup> was used to estimate OS and RFS and the log-rank test was applied to compare the OS and RFS of the two groups. To assess the independent effect of different variables on duration of RFS, a multivariate analysis was performed using a Cox proportional hazard model including predictive variables which were significant in univariate analysis. A  $p$  value of 0.05 or less was considered to be statistically significant in all cases.

## Results

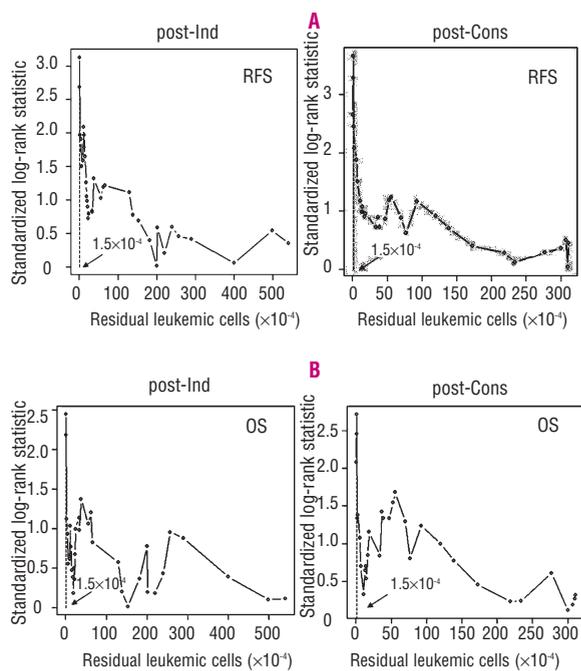
The clinical characteristics of the 50 patients included in the study are shown in Table 1. Thirty-three (66%) patients relapsed after a median time of 10 months (range, 2-24); the median follow up was 18 months (range, 3-85).

Firstly, we compared the levels of MRD in 50 paired BM and PB samples collected simultaneously after induction therapy. MRD was consistently detectable in both BM and PB. The median value of the residual leukemic cells (RLC) in BM and PB was  $5.2 \times 10^{-3}$  (range  $1 \times 10^{-4}$ - $1.64 \times 10^{-1}$ ) and  $2.85 \times 10^{-3}$  (range  $1 \times 10^{-5}$ - $1.25 \times 10^{-1}$ ), respectively; a significant correlation was found between the two sources ( $r=0.86$ ,  $p<0.0001$ ) (Figure 1A). In 12 (24%) of the 50 paired samples, the level of RLC was more than 10-fold higher in the BM than in the PB; conversely, in three cases (6%), the level of RLC was more than 10-fold higher in the PB than in BM samples. As two patients relapsed early after induction, 48 paired samples were available for the analysis after consolidation therapy; at this stage, the median value of BM RLC and PB RLC was  $4.1 \times 10^{-3}$  (range  $2 \times 10^{-5}$ - $6.3 \times 10^{-2}$ ) and  $3.7 \times 10^{-3}$  (range  $1 \times 10^{-5}$ - $1.34 \times 10^{-1}$ ), respectively, and again, a significant correlation between the two sources of cells was found ( $r=0.82$ ,  $p<0.0001$ ) (Figure 1B). In 12 pairs (25%), MRD in BM was 10-fold higher than in PB, whereas in three pairs (6%) the opposite was true. Therefore, in our AML cases the proportion of MRD in PB significantly reflected that in BM. The three cases in which PB MRD levels were 10-fold higher than in BM samples (both after induction and consolidation), were all monoblastic leukemias



**Figure 1.** Comparison of MRD levels in PB and BM samples showing a significant correlation between MRD values from the two cell sources. **1A.** Regression curve of the 50 paired PB and BM samples collected simultaneously after induction therapy. **1B.** Regression curve of the 48 paired PB and BM samples collected simultaneously after consolidation therapy.

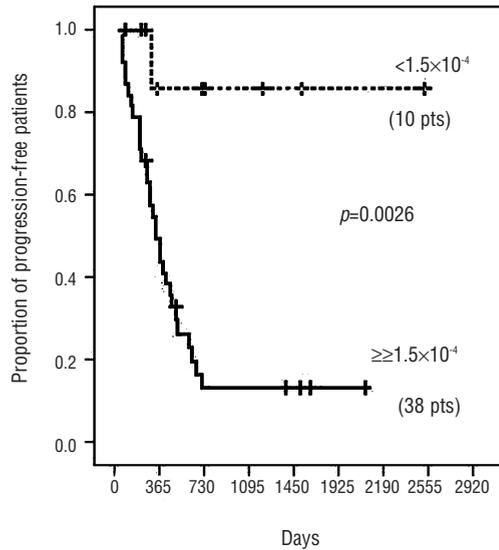
with extramedullary localization at presentation (one gingival hypertrophy, one gingival hypertrophy associated with localization in Waldeyer's ring, one with marked splenomegaly). The levels of PB RLC were tested to identify the optimal cut-offs yielding the best separation of AML patients into two groups with different probabilities of RFS and/or OS. To do this, we evaluated the trend of standardized log-rank statistics using RFS (Figure 2A) and OS (Figure 2B) as dependent variables, and the value of PB RLC, determined at post-induction (post-Ind) and post-consolidation (post-Cons) checkpoints, as independent variables. The experimental cut-off point identified as the absolute peak in standardized log-rank statistics plots (vertical dotted line in Figures 2A and 2B) was  $1.5 \times 10^{-4}$  RLC for both post-induction and post-consolidation. According to these data we decided to utilize the value of  $1.5 \times 10^{-4}$  PB RLC to discriminate MRD<sup>-</sup> from MRD<sup>+</sup> cases, after both induction and consolidation. Therefore, patients with PB RLC values below the cut-off of  $1.5 \times 10^{-4}$  are referred to as PB MRD<sup>-</sup>, whereas those with PB RLC equal or exceeding the  $1.5 \times 10^{-4}$  level are classified as PB MRD<sup>+</sup>.



**Figure 2.** Experimentally determined cut-off values splitting patients into groups with different RFS (A) and OS (B) probabilities. Maximally selected log-rank statistics were applied to values of RLC, evaluated at both post-Ind and post-Cons checkpoints, to estimate the most appropriate cut-off values capable of splitting patients into groups with different RFS (A) or OS (B) probabilities. For each plot, the dotted line and the arrow indicate the experimentally determined cut-off point.

This cut-off was significantly correlated with outcome; in fact, 77% (33/43) of the patients who were PB MRD<sup>+</sup> after induction (PBMRDInd<sup>+</sup>) had a relapse, whereas the seven PB MRD<sup>-</sup> (PBMRDInd<sup>-</sup>) patients did not ( $p=0.0002$ ). No significant difference in OS was observed between the PBMRDInd<sup>+</sup> and PBMRDInd<sup>-</sup> groups, however PBMRDInd<sup>+</sup> patients had a significantly shorter RFS ( $p=0.001$ ). After consolidation, 38 patients were PB MRD<sup>+</sup> (PBMRDCons<sup>+</sup>), and 31 (82%) of them experienced a relapse; nine out of the remaining ten patients, who were PB MRD<sup>-</sup> (PBMRDCons<sup>-</sup>), are in continuous complete remission ( $p=0.00006$ ). No significant difference in OS was observed between the PBMRDCons<sup>+</sup> and PBMRDCons<sup>-</sup> groups; the median duration of RFS was not reached among the patients with a PBMRDCons<sup>-</sup> status, whereas it was 11 months among those in the PBMRDCons<sup>+</sup> group ( $p=0.0026$ ; Figure 3).

In univariate analysis, white blood cell count (WBC), P-glycoprotein 170 (P-gP), PB MRD status after consolidation, and BM MRD status after consolidation were significantly associated with RFS (Table 2A). To explore whether PB MRD and BM MRD status after consolidation were independent prognostic factors affecting RFS estimates, the relevant variables that were prognostic in univariate analysis were pooled into a multivariate model. PB MRD and BM



**Figure 3.** Relapse-free survival (RFS) according to PB MRD status after consolidation treatment. Patients who were PB MRDCons<sup>+</sup> (PB RLC  $\geq 1.5 \times 10^{-4}$ ) had a median RFS time of 11 months, a point not reached by those who were PB MRDCons<sup>-</sup> (PB RLC  $< 1.5 \times 10^{-4}$ ).

MRD status after consolidation were analyzed separately because of their co-linearity, resulting in no significance when entered together in the multivariate model. MRD<sup>+</sup> status in either PB or BM was found to be an independent variable significantly associated with a shorter duration of RFS (Table 2B and 2C,  $p=0.036$  and  $p=0.04$ , respectively).

Although PB MRDInd<sup>+</sup> status was not an independent prognostic factor affecting RFS, we observed that the number of PB RLC at the end of induction significantly affected the degree of cytoreduction achieved with consolidation. In fact, the patients who achieved a PB MRDCons<sup>-</sup> status had a median number of  $7.5 \times 10^{-5}$  PB RLC after induction; in contrast, patients who were PB MRDCons<sup>+</sup> had a median level of  $5.9 \times 10^{-3}$  malignant residual cells after induction therapy ( $p=0.0004$ ) (Figure 4).

## Discussion

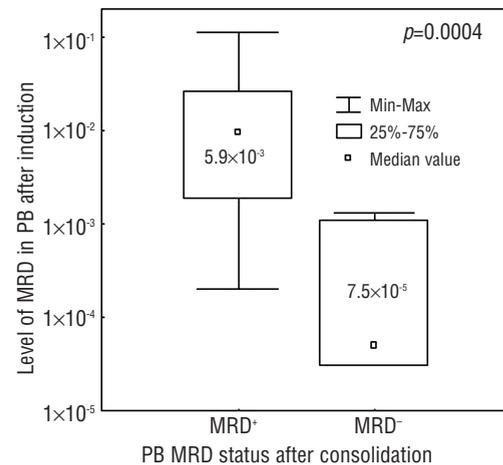
Over the years, MRD assays based on MPFC have been improved by advances in the quality and variety of antibodies used and by the refinement of flow cytometers. MPFC holds great promise for clinical application because of its simplicity and wide availability. Previous studies performed on BM samples demonstrated strong correlations between MRD levels and treatment outcome in AML patients,<sup>13-15,31</sup> lending support to the reliability of this technique.

More recently, it has been proposed that PB may represent an alternative source of cells for monitoring MRD in patients with acute leukemia.<sup>19,20,32</sup> In fact, the

**Table 2. A.** Variables significantly associated with RFS in univariate analysis. **B.** In multivariate analysis, post-Cons PB MRD status was independently associated with RFS. **C.** In multivariate analysis, post-Cons BM MRD status was independently associated with RFS.

Variable	p value
<b>A</b>	
MDR1 phenotype	0.02
White blood cell count	0.05
Post-cons PB MRD status	0.002
Post-cons BM MRD status	0.02
<b>B</b>	
MDR1 phenotype	NS
White blood cell count	NS
Post-cons PB MRD status	0.036
<b>C</b>	
MDR1 phenotype	NS
White blood cell count	NS
Post-cons BM MRD status	0.04

MRD: minimal residual disease; Post-Cons: post consolidation, PB: peripheral blood; BM: bone marrow; MDR1: multidrug resistance; NS: not significant.



**Figure 4.** Correlation between the level of PB MRD after induction therapy and the type of PB MRD status after consolidation. Patients with the highest probability of achieving a MRDCons<sup>-</sup> status had two log fewer RLC after induction therapy than those who were MRDCons<sup>+</sup>.

presence of circulating morphologically undetectable blasts at the time of CR might be directly correlated to the persistence of malignant cells in BM or might indicate the propensity of blast cells to exit from the BM prematurely and therefore a more aggressive disease. In the present analysis, we were able to detect MRD in

PB of all patients enrolled in our clinical trials; the level of residual leukemic cells in PB significantly reflected that observed in BM at each time-point. In fact, a significant correlation between RLC in the two cell sources was found after induction and consolidation. To our knowledge this is the first report showing such a correlation in AML using MPFC, and our results are in keeping with those of recent studies using PCR-based assays to monitor MRD in PB and BM of AML patients carrying core binding factor fusion genes.<sup>20,32</sup> We, therefore, investigated whether PB MRD status had a prognostic role and which time-point was the most informative to predict disease outcome. We found that a level of RLC  $>1.5 \times 10^{-4}$  in PB after consolidation therapy was associated with a significant likelihood of subsequent relapse and a shorter duration of RFS. By contrast, nine out of the ten patients with  $\leq 1.5 \times 10^{-4}$  RLC in PB after consolidation therapy are still in CR with a median follow-up of 18 months. In previous studies, performed on BM samples, we found that post-consolidation levels of MRD were highly predictive of disease outcome;<sup>13,23-24</sup> in the present study, we demonstrated that PB MRD assessment may be equally informative. In addition, we observed that the prognostic role of MRD status after consolidation is not affected by the post-induction level of MRD. In fact, three out of ten patients who were MRD<sup>-</sup> after consolidation were positive after induction; nevertheless they had the same favorable outcome as the six who became MRD<sup>-</sup> soon after induction. However, the magnitude of debulking obtained with consolidation therapy seems to be affected by the level of MRD after induction. In fact, patients who entered a MRDCons<sup>-</sup> status had, after induction, 2 log fewer PB RLC than those who were MRDCons<sup>+</sup>. These findings suggest that the absence of MRD post-induction is a factor predisposing to a favorable prognosis, but only the further debulking achieved after consolidation therapy results in an improvement of outcome. This assumption is confirmed by the statistical observation that PB MRD status after consolidation

retained statistical significance in both univariate and multivariate analyses.

In the present study, no pre-treatment characteristics were predictive of the outcome; cytogenetics in particular, did not affect the duration of either OS or RFS.<sup>13,22</sup> This unusual result may be explained by the over-representation of intermediate karyotypes (35 out of 44 cases cytogenetically evaluable) in our series. Finally, seven patients are in continuous CR in spite of detectable disease after consolidation; two underwent allogeneic stem cell transplantation and we hypothesize that the residual leukemia is being kept under control by a graft-versus-leukemia effect. In the other five patients, we believe that AML is still present, but the limited follow-up time may explain why we have not yet observed a relapse.

In conclusion, our findings demonstrate that: (i) MRD is detectable and measurable in PB of AML patients using MPFC; (ii) MRD levels in PB are correlated to those measured in BM and, therefore, PB may be a complementary source of cells for MRD studies in patients with AML; (iii) PB MRD determination after consolidation therapy has a prognostic role; (iv) combined assessment of MRD in BM and PB might increase the value of sub-stratification of risk categories and thus improve MRD monitoring in AML patients.

Studies including larger series of patients are warranted in order to further standardize MRD monitoring procedures and confirm these preliminary results.

#### Authors' Contributions

LM, FB, GDP and MID: conception and design of the study and interpreting data; AS performed statistical analysis; PP, MIC and DF: immunophenotyping, karyotypic and FISH analyses; BN, CM, LO, CS and MA: conducting the work and analyzing the results; PDF, SA and AV: supervised the project. All authors contributed to the design of the study and revision of the manuscript. AV: primary responsibility for the publication.

#### Conflict of Interest

The authors reported no potential conflicts of interest.

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