Prognostic value of post-induction medullary myeloid recovery by flow cytometry in acute myeloid leukemia

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Abstract

Risk stratification and treatment response evaluation are key features in acute myeloid leukemia (AML) management. Immunophenotypic and molecular approaches all rely on the detection of persisting leukemic cells by measurable residual disease techniques.

A new approach is proposed here by assessing medullary myeloid maturation by flow cytometry through a myeloid progenitor ratio (MPR). The normal MPR range was defined using reference normal bone marrows (n= 48). MPR was considered balanced if between 1 and 4 and unbalanced if <1 or >4. MPR was retrospectively assessed at baseline and post-induction for 206 newly diagnosed AML patients eligible for intensive treatment from two different French centers.

All AML baseline MPR were unbalanced and thus significantly different from normal MPR (p<0.0001). Patients with an unbalanced MPR after induction had worse 3-year overall survival (OS) (44.4% *vs*80.2%, HR, 2.96; 95%CI, 1.81-4.84, p<0.0001) and 3-year relapse free survival (RFS) (38.7% *vs* 64.4%, HR, 2.11; 95%CI, 1.39-3.18, p<0.001). In multivariate analysis, post-induction unbalanced MPR was significantly associated with shorter OS and RFS regardless of the European LeukemiaNet 2010 risk stratification or *NPM1*/*FLT3*-ITD status. A balanced post-induction MPR conversely conferred favorable outcomes and reflects medullary myeloid recovery.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of diseases disrupting myeloid maturation by blocking hematopoietic cell maturation and resulting in myeloblast proliferation and accumulation in the bone marrow (BM) and/or peripheral blood (PB) (1, 2). Even for a single patient, the blastic population at diagnosis is heterogeneous, with co-existing clones expressing different genetic anomalies and immunophenotypic profiles. Minor clones hardly detectable at diagnosis can emerge after chemotherapy (3–5). The overall survival (OS) at 5 years, in a French epidemiologic cohort of AML patients, has been reported to be 21% for young patients and drastically dropped to 3% for patients older than 75-year-old (6, 7). Over the years, AML management has however improved through the identification of new prognostic factors allowing for better risk stratification and best treatment choice, which yielded increased survivals. The main pre-treatment prognostic factors can be patient-related or AML-related, with a major impact of genomic lesions. The European LeukemiaNet (ELN), considering cytogenetic and molecular anomalies, has proposed risk classifications applicable to classical chemotherapy regimen (8, 9).

After a first cycle of induction chemotherapy, 80% of the patients achieve complete remission (CR) but more than 50% relapse (10). AML is a dynamic myeloid neoplasm that evolves and shifts over time as co-existing and competitive subclones emerge either from natural disease progression or treatment selective pressure (11). Emerging clones may have lost initial baseline markers or acquired additional anomalies resulting in apparent immunophenotypic shifts. This clonal heterogeneity within a same patient complexifies AML monitoring. The CR status requiring BM free of AML blasts (8), based on less than 5%

blasts on microscopic BM smear examination, is not entirely satisfactory and other techniques are needed to detect smaller residual leukemic burden. Monitoring of measurable residual disease (MRD) is the biggest challenge and initial assessment of treatment response is crucial to guide therapeutic decisions as early as possible (12). MRD can be measured by multiparametric flow cytometry (MFC) or molecular techniques such as reverse transcriptase-quantitative PCR (RT-qPCR), next-generation sequencing or digital droplet PCR (ddPCR) (13, 14). However, a suitable molecular target for MRD monitoring is only present in about 60% of young AML patients (15).

Early MFC-MRD levels are known to be outcome predictors (16), complementary within genetic risk subgroups (17). The ELN consensus for AML MRD recommends a first assessment by MFC after two induction cycles with the combined strategies of “leukemia-associated immunophenotype (LAIP)” and “different from normal” (DfN) (8, 18, 19). The LAIP method identifies immunophenotypic aberrations at diagnosis in 90% of AML patients and tracks them during follow-up (20) but this approach requires the baseline sample and does not account for immunophenotypic shifts (21). The DfN strategy reveals immunophenotypic changes compared to the normal myeloid maturation processes in virtually all patients. This approach implies a deep knowledge of differentiation patterns and the availability of normal reference BM samples (18). Overall, MFC-MRD is not yet harmonized as antibody panels, instruments, protocols and expertise still differ between centers. The main challenge of this residual blasts hunting method is the detection of emerging subclones. Other approaches have been developed such as assessment of early peripheral blasts clearance (22), detection of leukemic stem cells (23, 24) or unsupervised clustering (25, 26) followed by supervised validation. The latter two, however, require a high level of expertise.

This work proposes an alternative to MFC-MRD with a simple and objective prognostic tool. It introduces the concept of evaluating myeloid maturation recovery by MFC in AML after induction chemotherapy. It was assumed that hematopoiesis respects a sequential myeloid maturation pattern with a pyramidal distribution where a common myeloid progenitor generates a balanced number of monocytic and granulocytic progenitors. Myeloid maturation assessed by MFC (referred to in this paper as “myeloid progenitor ratio” [MPR]) could be a biomarker reflecting this early recovery pathway which can be either balanced (regular BM maturation) or unbalanced (maturation blocking) regardless of the presence of leukemic cells. This new MFC tool, retrospectively assessed at post-induction for 206 newly diagnosed AML patients from two different centers appeared to carry a high prognostic value.

Methods

Reference bone marrow samples

A total of 48 reference bone marrow (RBM) samples was obtained from two French centers. One third came from healthy BM donors (n = 17), previously described for delineating normal BM subsets (ref. 27). The others came from patients with non-hematological malignancies, less than 75 years old (n = 31) (21

monoclonal gammopathy of undetermined significance, 5 immune thrombocytopenic purpura, 3 anemias from iron deficiency, 2 transient inflammatory syndromes).

Study cohort

A total of 206 AML patients newly diagnosed between 2015 and 2021 were retrospectively included in this study from two different French specialized centers for hematology, respectively 102 and 104 patients from the University Hospitals of Dijon and Bordeaux.

Patients ≥ 18 years old with a *de novo* AML were enrolled. The diagnosis had been performed on either PB or BM examination according to the 2016 WHO classification (28). AML subjects with acute promyelocytic leukemia, *BCR*:*ABL1*-positive AML or acute leukemia of ambiguous lineage were excluded. Post-induction response was evaluated according to ELN AML criteria as CR or CR with incomplete hematologic recovery (CRi) (8). All selected patients had achieved remission after 1 or 2 cycles of intensive induction chemotherapy (29) regardless of consolidation treatment. Patients who had received gemtuzumab ozogamycin at induction were excluded. Among these patients, only those with BM assessed by MFC after induction were retained. BM MFC data were retrospectively analyzed for each patient at baseline and post-induction. All patients provided informed consent in accordance with the Declaration of Helsinki.

Cytomorphology, cytogenetics and molecular data at diagnosis were also collected, and patients were classified in risk stratification subgroups according to ELN 2010 (ref. 30). The *NPM1*/*FLT3*-ITD status was also collected when available. Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} was considered as a favorable risk category (8).

Flow cytometry process

Total BM samples (RBM, baseline or post-induction), collected on EDTA, were processed in a “stain-lysis-no wash” protocol within 24 hours after collection. Each center applied their own panels with one mutual ten-color monoclonal antibody combination based on European recommendations (18, 31, 32), including CD34, CD13, CD33, CD117, CD15 or CD65, CD14, CD11b, CD16, CD7 and CD45. Antibody clones and fluorochromes could differ between centers (Supplemental Table 1). Sample acquisition was performed using Navios instruments (Beckman Coulter, Miami, FL). MFC data were analyzed using Kaluza® software, version 2.1 (Bekman Coulter). Instruments were not previously harmonized between centers since retrospective data were used from saved .fcs files.

Myeloid progenitor ratio MFC protocol

The MFC analysis protocol was designed on Kaluza® Software (Beckman Coulter) (Fig. 1). The aim of the MPR protocol was to focus on myeloid progenitors. First, debris were eliminated on an SSC/FSC histogram, then mononuclear cells were roughly selected on an SSC/CD45 histogram (Bermudes area) (32) (Fig. 1A). The second step consisted in a sequential clean-up gating strategy first excluding CD14$^+$ cells (mature monocytes) and CD16$^+$ cells (neutrophils and NK cells) on a CD14/CD16 histogram, then

excluding remaining $CD15^+$ immature ($CD16^-$) granulocytes (promyelocytes, myelocytes, promonocytes) and $CD11b^+$ basophils on a CD11b/CD15 histogram (Fig. 1B). The third step was to select all $CD33^+$ or $CD117^+$ myeloid progenitors on a CD33/CD117 histogram (Fig. 1C). The final step displayed the maturation pattern of the selected myeloid progenitors on a CD33/CD34 histogram where three populations could be identified (Fig. 1D), respectively (i) $CD34^+$ common myeloid progenitors (CMP), (ii) $CD34^-CD33^{++}$ monocyte progenitors (MP) and (iii) $CD34^-CD33^{low}$ granulocyte progenitors and contaminating erythroid progenitors. MPR was ultimately defined by dividing the number of CMP cells by that of MP cells in each sample.

Statistical analyses

Clinical outcome data, collected up to April 2022 for patients enrolled in Bordeaux and November 2022 in Dijon, were analyzed with median follow-ups of 26 (range 4–82) and 22 months (range 4–69) respectively.

Data were tested using the Fisher's exact test for categorical variables and Mann-Whitney U or Anova tests for continuous variables.

The primary endpoints were OS and relapse-free (RFS) survivals as described by ELN 2017 recommendations.[8] They were evaluated using Kaplan-Meier graphical representation and log-rank test. For significant covariates in univariate analysis ($p < 0.20$, supplementary Table 2), a Cox proportional hazards model was used to identify independent predictive factors including center, sex, age at diagnosis, ELN 2010 risk stratification, mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}, number of intensive induction cycles needed to achieve CR/CRi, allogeneic hematopoietic stem cell transplantation (Allo-HSCT) and post-induction MPR (Table 2). Multivariate analysis was performed on 201 patients as *NPM1* status was not available for 5 patients. In all cases, estimates of hazard ratios (HR) are given with 95% confidence intervals (95%CI). P-values < 0.05 were considered statistically significant. Analyses were performed using MedCalc® Statistical Software version 20.006 (Ostend, Belgium) and all graphs were drawn using Graph Pad Prism® software version 9.5.0 (San Diego, CA).

Results

Reference bone marrows: outlining the MPR profile

The median age of RBM subjects was 53 years old (range 18 to 74), and 25 (52%) were female. MFC data analysis was performed in triplicates by three independent flow experts and no significant difference in MPR was found ($p = 0.41$). The mean MPR was 2.50 (± 2 standard deviations 1.25–3.75). The median MPR was 2.55 (range 1.27 to 3.80). MPR according to the origin of samples, *i.e.* healthy BM donors or patients with non-hematological malignancies, are shown in supplementary Fig. 1. For the rest of the study, a balanced MPR (bMPR) was set as ranging from 1 to 4. The MPR was considered unbalanced (ubMPR) if < 1 or > 4.

Patient characteristics

The whole cohort enrolled 206 patients with a median age of 62 years old (range 20–79), 42 of them (20.4%) being 70 or older. Ninety-six patients (46.6%) were female. Using the ELN 2010 risk stratification (30), 19 (9.2%) patients were classified as favorable risk, 153 (74.3%) as intermediate risk and 34 (16.5%) as adverse risk (8). The *NPM1*/*FLT3*-ITD status was obtained for 201 (97.6%) patients showing that 52 (25.9%) had a favorable prognosis (*i.e.* mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}).

Patients had required one (n = 182; 88.3%) or two cycles (n = 24; 11.7%) of intensive induction to reach CR/CRi. The median time between induction and evaluation was 35 days (range 21–92).

MPR at baseline

MFC data on BM samples were available for 195 (94.6%) patients at diagnosis. The 11 (5.4%) remaining had MFC data from PB. All MPR at baseline, evaluated on BM samples, were unbalanced ($p < 0.0001$) (Fig. 2). At baseline, ubMPR > 4 (n = 107 ; 54.9%) were more frequent in patients diagnosed as AML with minimal differentiation (AML0) (17/17, 100%) or AML with maturation (AML2) (31/54, 69%) whereas ubMPR < 1 (n = 88 ; 45.1%) were more recurrent in acute monoblastic and monocytic leukemia (AML5) (23/31; 74%) according to the morphological FAB classification (33) (Supplementary Fig. 2).

Post-induction MPR

Post-induction MPR were lower in patients in CR compared to CRi (median 2.90 *vs* 9.73, $p < 0.0001$) (Fig. 3A). For patients reaching CR, the proportion of post-induction bMPR was higher compared to those achieving CRi (61.9% *vs* 25.0%, $p < 0.0001$) (Fig. 3B). Patients with post-induction ubMPR needed more often 2 intensive induction cycles (n = 18) to reach CR/CRi than patients with bMPR (n = 6) (16.7% *vs* 6.1%, $p = 0.018$).

Post-induction MPR and baseline patient characteristics

Patients were divided in two groups according to their MPR status at the end of induction: balanced (n = 98) *vs* unbalanced (n = 108). Disease characteristics at baseline (Table 1), therapeutic lines and post-induction biology were then compared between these two groups.

There was no difference at baseline regarding ELN 2010 risk stratification (30) ($p = 0.08$), cytogenetic MRC 2010 stratification (34) ($p = 0.10$), ELN 2017 risk stratification (8) ($p = 0.21$) nor *NPM1*/*FLT3*-ITD status ($p = 0.88$) (Table 1).

Post-induction complete blood counts showed higher polymorphonuclear (mean 4.35x 10^9/L *vs* 2.8x10^9/L, $p = 0.0002$), monocyte (mean 0.90x10^9/L *vs* 0.64 x10^9/L, $p = 0.0019$) and platelet (mean 326 x10^9/L *vs* 175 x10^9/L, $p < 0.0001$) counts, together with less myelemia (mean 1.94% *vs* 5.41%, $p < 0.0001$) in patients with bMPR (Supplementary Fig. 3). Post-induction lymphocyte counts (mean 0.86x10^9/L *vs*

0.88x10^9/L, p = 0.74) and hemoglobin levels (mean 10.38 g/dL *vs* 10.14 g/dl, p = 0.10) were similar whatever the MPR group (Supplementary Fig. 3).

Post-induction MPR and clinical outcome

The median OS for patients with post-induction ubMPR was 36 months but was not reached for those with bMPR (HR, 2.96; 95%CI, 1.81–4.84; p < 0.0001, Fig. 4A). One year and 3-year OS were respectively 82.1% and 44.4% in patients with post-induction ubMPR *vs* 91.6% and 80.2% in patients with post-induction bMPR (p < 0.0001). In Cox model multivariate analysis, a status of ubMPR was significantly and independently associated with a worse OS (adjusted HR (aHR), 2.72; 95%CI, 1.42–5.20; p = 0.003, Table 2). Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (aHR, 0.36; 95%CI, 0.16–0.84; p = 0.017) and Allo-HSCT (aHR, 0.34; 95%IC, 0.18–0.65; p = 0.001) retained a significant positive impact on OS. ELN 2010 adverse risk held a statistically significant independent negative impact on OS (aHR, 2.99; 95%CI, 1.67–5.37; p = 0.0002).

The median RFS was 18 months and not reached for patients with post-induction ubMPR or bMPR (HR, 2.11; 95%CI, 1.39–3.18; p = 0.0004, Fig. 4B), respectively. One-year and 3-year RFS were respectively 57.7% and 38.7% in patients with post-induction ubMPR and 76.0% and 64.4% in those with bMPR (p = 0.0004). In multivariate analysis, post-induction ubMPR was significantly and independently associated with a shorter RFS (aHR, 2.27; 95%CI, 1.35–3.83; p = 0.002, Table 2). Both mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (aHR, 0.30; 95%CI, 0.15–0.60; p = 0.0007) and Allo-HSCT (aHR, 0.44; 95%IC, 0.26–0.75; p = 0.003) still retained a significant positive impact on RFS. Patients classified as ELN 2010 adverse risk had a significantly worse RFS (aHR, 1.95; 95%CI, 1.16–3.29; p = 0.012).

The prognostic value of post-induction MPR within ELN 2010 subgroups showed worse outcomes for patients with ubMPR classified as intermediate 1 or 2 (n = 153 patients), for both OS (HR, 2.69; 95%CI, 1.45–4.98; p = 0.0018) and RFS (HR, 2.22; 95%CI, 1.33–3.70; p = 0.0021) (supplementary Fig. 4). Conversely, no difference was found for patients stratified in ELN 2010 adverse (OS, p = 0.0642; RFS, p = 0.1369) or favorable (OS, p = 0.49; RFS, p = 0.74) subgroups.

Discussion

This work introduces a new, original and highly accessible MFC tool with a robust prognostic value, available for patients who reach CR or CRi, *i.e.* in the early stages of AML management. A simple myeloid maturation pathway can be identified through the strong and stable relationship between myeloid progenitors. The respective proportions of CD34$^+$/CD33^{low} CMP and CD34$^-$/CD33^{+-} MP, as established by the MPR, were demonstrated to lie in a tight range in normal BM. Conversely, AML BM cells at diagnosis were shown to be quite systematically outside of this normal range. In post-induction BM, however, CR translated in a return within the normal range in most cases, this medullary myeloid recovery being associated by a better prognosis.

Indeed, in this series of post-induction samples, all patients have been selected to obtain a CR or CRi. However, in the latter case, MPR was frequently more unbalanced possibly reflecting residual maturation blockade. Conversely, patients with post-induction bMPR, besides recovering MPR in the normal range, had higher levels of polymorphonuclears, monocytes and platelets suggesting strong myeloid regeneration, including the megakaryocytic component.

Post-induction ubMPR, associated with increased risks of relapse and death, was found to be independent from cytogenetic and molecular risk stratification. Within ELN 2010 intermediate (1 and 2) groups, a post-induction ubMPR was also associated with poor survivals.

This new MFC approach does not require baseline diagnostic samples nor the use of RBM. The latter were only used here to establish the normal range of MPR, shown to be robust on different instruments and different panels, only relying on the proper biparametric gating strategy.

Post-induction MPR stands out as a novel prognostic factor based on the dynamic properties of BM to recover an adequate differentiation ability after chemotherapy in AML patients (35). This assay is simple to use, robust and universal. MFC results are available on the same day as post-induction BM sampling. MPR assessment thus meets standards for delivering a quick and reliable answer on treatment effectiveness that could guide therapeutic decision making (36).

Further studies are needed to understand how the persistence of clonal leukemic cell affects medullary myeloid maturation processes. MRD and MPR should first be evaluated separately in order to assess their respective impact on survival outcome. Both could then be used in a combined strategy to improve prognostic risk stratification. Hematopoiesis recovery signature through the MPR could be a new marker differing from MRD approaches.

Declarations

AUTHORSHIP CONTRIBUTIONS

C.R. and J.G. designed the study and analyzed the data. C.R., J.G., N.L., J-P.V., A.M. and M-C.B. provided and analyzed flow cytometry data. J.-N.B., D.C., E.S., A.P., T.L., M.C., M.M., and PY.D. provided clinical and biological data; and all authors wrote the manuscript and approved its final version.

COMPETING INTERESTS DISCLOSURES

All authors declare no conflict of interest and no competing financial interests for this study.

DATA AVAILIBILITY STATEMENT

For original data, detailed flow cytometry method and myeloid progenitor ratio protocol (.protocol) please contact the corresponding author.

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

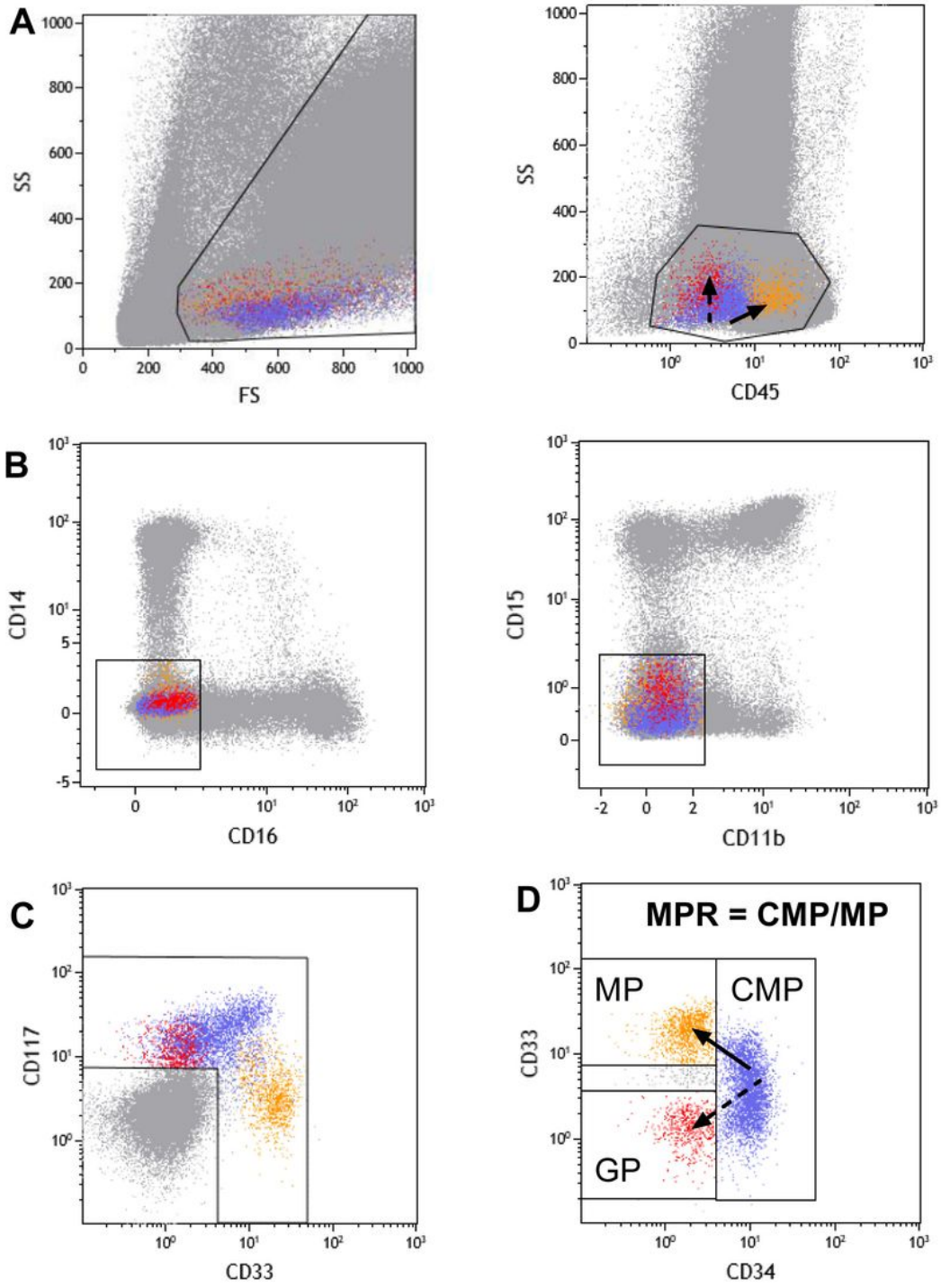

Figure 1

Myeloid progenitor ratio protocol for multiparametric flow cytometry

Using ten-color monoclonal antibody combination CD34, CD13, CD33, CD117, CD15 or CD65, CD14, CD11b, CD16, CD7 and CD45, the MFC analysis protocol was designed on Kaluza® Software (Beckman Coulter) and was built on a sequential 4 steps approach:

(A) Debris eviction (SS/FSC) and Bermudes area (SS/CD45)

(B) Cleaning up cells (CD14/CD16 and CD11b/CD15)

(C) Selecting myeloid progenitors (CD33/CD117)

(D) Populations of interest (CD33/CD34): common myeloid progenitor (CMP), monocytes progenitor (MP) and granulocytes progenitor (GP)

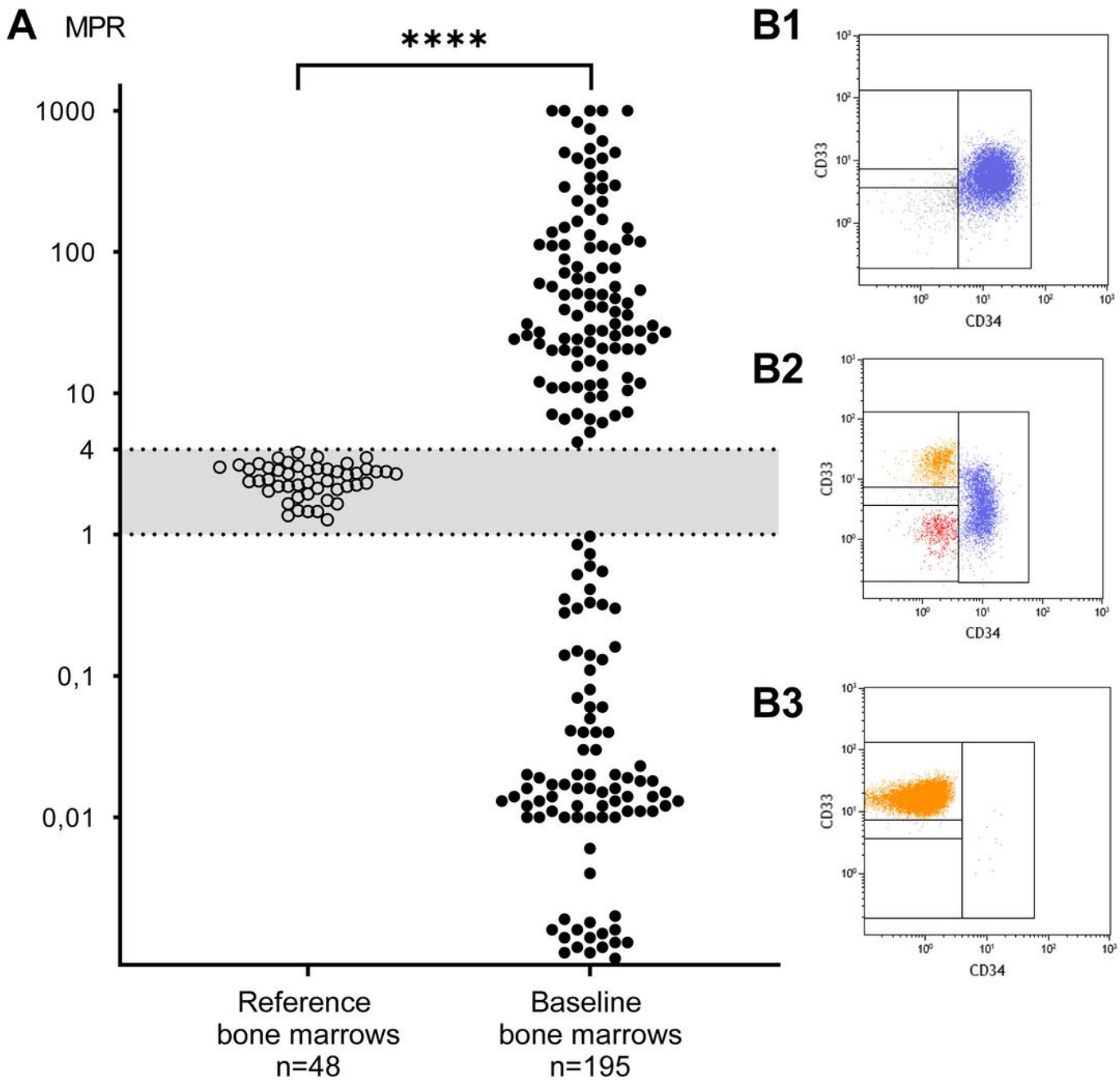

Figure 2

Myeloid progenitor ratio in reference bone marrow and baseline bone marrows

(A) MPR value

(B) MPR flow profile: (B1) unbalanced MPR >4 ; (B2) balanced MPR ; (B3) unbalanced MPR <1

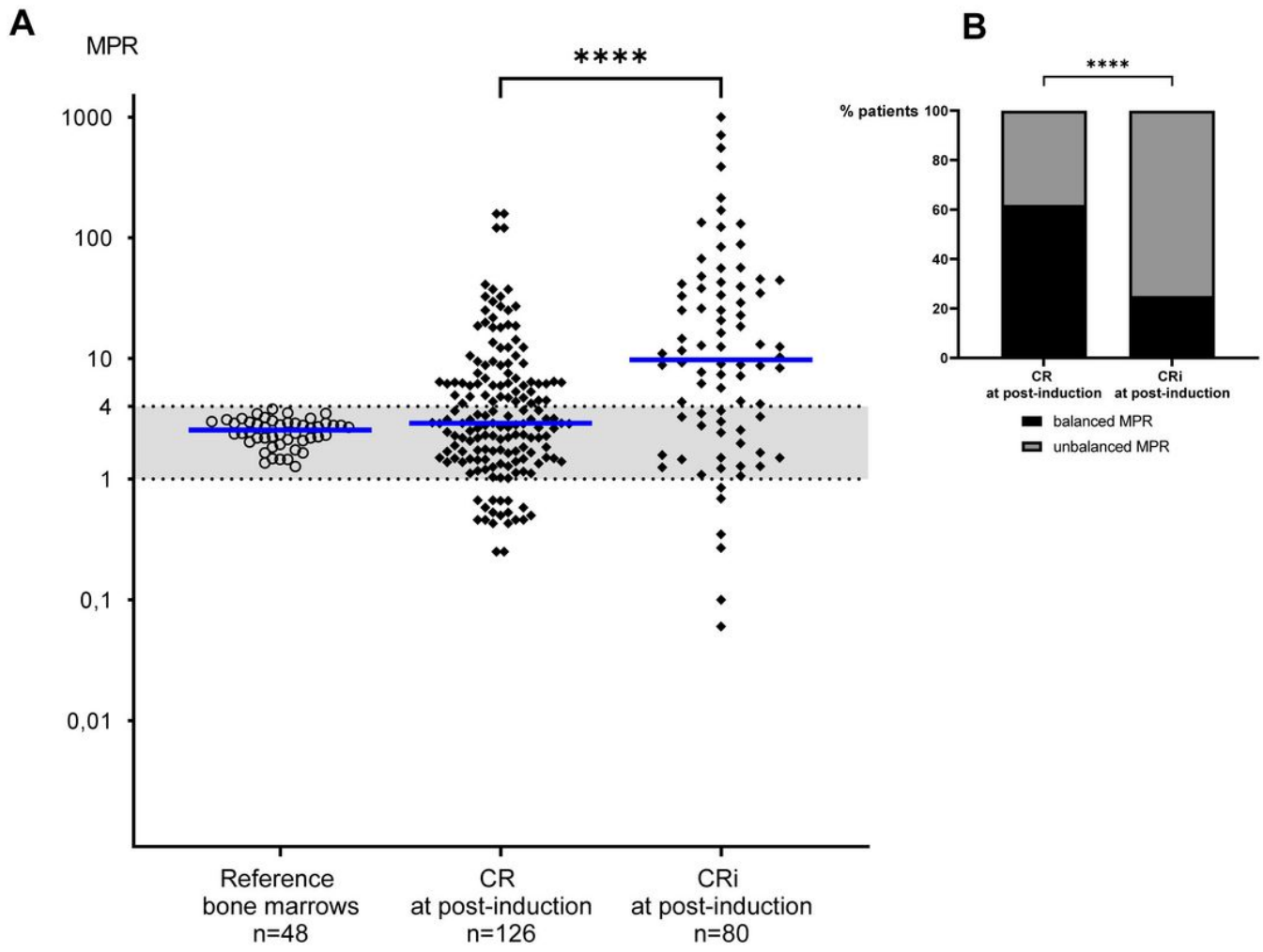

Figure 3

Myeloid progenitor ratio in reference bone marrows and at AML post-induction bone marrow evaluation

(A) MPR value. Error bar at median.

(B) Percentages of patients with balanced/unbalanced MPR in CR *vs* CRi at post-induction evaluation

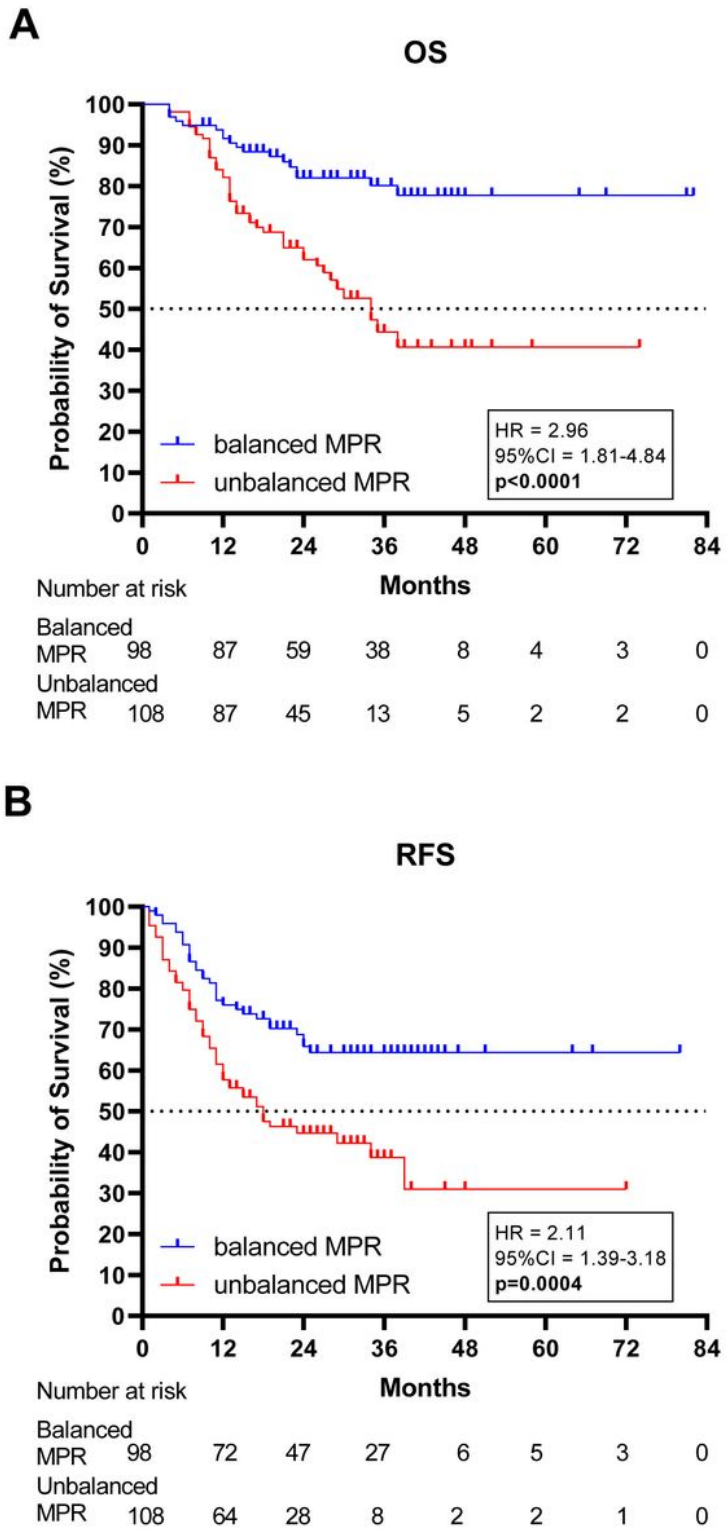

Figure 4

Myeloid progenitor ratio at post-induction and clinical outcome

(A) Overall Survival ; (B) Relapse Free Survival

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Table2.xlsx
- MPRSupplementary.pdf