Measurable residual disease by flow cytometry in acute myeloid leukemia is prognostic, independent of genomic profiling

Chezi Ganzel[a,*,1], **Zhuoxin Sun**[b,1], **Timour Baslan**[c], **Yanming Zhang**[d], **Mithat Gönen**[e], **Omar I. Abdel-Wahab**[e], **Janis Racevskis**[f], **Francine Garrett-Bakelman**[g,n,o], **Scott W. Lowe**[c,h], **Hugo F. Fernandez**[i], **Rhett Ketterling**[j], **Selina M. Luger**[k], **Mark Litzow**[l], **Hillard M. Lazarus**[m], **Jacob M. Rowe**[a], **Martin S. Tallman**[e], **Ross L. Levine**[e], **Elisabeth Paietta**[f]

[a]Hematology Department, Shaare Zedek Medical Center, and Faculty of Medicine, Hebrew University of Jerusalem, Israel

[b]Dana-Farber Cancer Institute, Boston, MA, USA

[c]Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

[d]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[e]Human Oncology and Pathogenesis Program and Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[f]Department of Oncology, Montefiore Medical Center, Bronx, NY, USA

[g]Department of Medicine, Weill Cornell Medicine, New York, NY, USA

[h]Howard Hughes Medical Institute, Chevy Chase, MD, USA

[i]Malignant Hematology and Cellular Therapy, Moffitt Cancer Center, Tampa, FL, USA

*Corresponding author. ganzelc@szmc.org.il (C. Ganzel).
[1]Contributed equally to this work

[j]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

[k]Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA

[l]Department of Medicine, Mayo Clinic, Rochester, MN, USA

[m]Case Western Reserve, Cleveland, OH, USA

[n]Departments of Medicine and Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, USA

[o]University of Virginia Cancer Center, Charlottesville, VA, USA

Abstract

Measurable residual disease (MRD) assessment provides a potent indicator of the efficacy of anti-leukemic therapy. It is unknown, however, whether integrating MRD with molecular profiling better identifies patients at risk of relapse. To investigate the clinical relevance of MRD in relation to a molecular-based prognostic schema, we measured MRD by flow cytometry in 189 AML patients enrolled in ECOG-ACRIN E1900 trial (NCT00049517) in morphologic complete remission (CR) (28.8 % of the original cohort) representing 44.4 % of CR patients. MRD positivity was defined as $\geq$ 0.1 % of leukemic bone marrow cells. Risk classification was based on standard cytogenetics, fluorescence-in-situ-hybridization, somatic gene analysis, and sparse whole genome sequencing for copy number ascertainment. At 84.6 months median follow-up of patients still alive at the time of analysis (range 47.0–120 months), multivariate analysis demonstrated that MRD status at CR (p = 0.001) and integrated molecular risk (p = 0.0004) independently predicted overall survival (OS). Among risk classes, MRD status significantly affected OS only in the favorable risk group (p = 0.002). Expression of CD25 (α-chain of the interleukin-2 receptor) by leukemic myeloblasts at diagnosis negatively affected OS independent of post-treatment MRD levels. These data suggest that integrating MRD with genetic profiling and pre-treatment CD25 expression may improve prognostication in AML.

Keywords

1. Introduction

Recurrent gene mutations in acute myeloid leukemia (AML) patients have biologic, prognostic and therapeutic relevance [1-4]. Mutational profiling can be integrated with cytogenetic analysis and clinical parameters to develop a more robust risk stratification in AML [2,4,5]. However, the risk of relapse and death from recurrent leukemia is not fully predicted by pre-therapy parameters, and there remains a pressing need to develop new biomarkers to guide therapeutic decisions in AML.

Measurable residual disease (MRD) assessment at the time of complete morphologic remission (CR) evaluates the quality of response to induction chemotherapy beyond the level of conventional histopathology. This notion has led to the definition of MRD-negative CR

as an important clinical endpoint [5]. Increasingly, studies are suggesting that MRD status serves as a prognostic tool in AML [6-9]. Nevertheless, MRD measurements are not yet routinely used to guide therapeutic decisions in AML, partly due to the lack of uniformity in methodologies used for MRD detection and interpretation [6-8,10-14]. In addition, the independent prognostic relevance of MRD relative to pre-therapeutic prognosticators, such as cytogenetic risk [10,15,16], expression of prognostic antigens, such as CD25 on myeloblasts [17], or molecular profiling [18-25] is not fully understood, particularly in the setting of large clinical trials with uniform treatment.

To investigate the clinical significance of MRD in relation to genetic profiling, we measured MRD by flow cytometry in AML patients age 60 and under enrolled in intergroup phase 3 trial, E1900 (NCT00049517) [2,26]. We previously reported that dose-intensification of daunorubicin during induction therapy improved CR rate and overall survival (OS) [26]. Moreover, expression of CD25, the alpha-chain of the interleukin-2 receptor, had prognostic relevance independent of mutational profiling [17]. We now assess whether MRD has prognostic value irrespective of induction enhancement, mutational profile, cytogenetic status, or CD25 expression.

2. Materials and methods

2.1. Patients

Eligibility criteria for E1900 have been published [26]. Of 657 accrued patients, 629 had baseline specimens submitted to the ECOG-ACRIN Leukemia Translational Research Laboratory (LTRL) for central immunophenotyping by multiparameter flow cytometry. Excluded from central MRD analysis in the LTRL were 83 patients from Rambam Medical Center, Haifa, Israel, for whom only frozen follow-up samples were received. Since submission of follow-up specimens was not mandatory, the LTRL received post-treatment samples for 388/546 remaining patients (71 %) at 1–15 time-points per patient. In 9 patients, only relapse material was submitted and, in 45 patients, baseline specimens, predominantly with monocytic phenotype, did not yield a Leukemia-Associated Immunophenotype (LAIP) suitable for MRD detection. For 189 of the 334 patients with suitable baseline LAIP, MRD data were available at CR, representing 28.8 % of the entire E1900 cohort and 44.4 % of patients who achieved a CR. No statistically significant differences were observed between these 189 patients and patients achieving CR without MRD data with regard to baseline characteristics and outcomes. Of the 189 patients, 87 had MRD samples available from CR as well as various time-points post-consolidation.

2.2. Flow cytometric analysis of baseline immunophenotypes and MRD

Heparinized samples were received and processed by the LRTL within 24 h of collection. MRD was assessed by 4-color flow cytometry in whole, unseparated samples and expressed as percent of nucleated white blood cells (WBC) based on staining with cell-permeant green fluorescent nucleic acid dye (Syto 16) (FACSCanto II flow cytometer, Beckton Dickinson, and FACSDiva analysis software). MRD detection was based on the diagnostic LAIP, using both asynchronous expression of antigens and aberrant antigen intensities in LAIP definition [10,27,28]. Any discrete cell population consistent of at least 10 events which

unequivocally expressed LAIP features was considered as MRD. If the quality of bone marrow aspirates precluded us from reliably determining the MRD status, we considered the MRD result as indeterminate. Indeterminate samples were not counted in our MRD analysis. Among Core-Binding-Factor (CBF) leukemias, LAIP features included expression of CD19 and/or CD56, absence of CD11a and CD7 and weak myeloid antigen expression for *RUNX1/RUNXT1* AML, and CD2 for *CBFβ/MYH11* AML [29]. If more than one leukemic immunophenotypic clone was detected at diagnosis, MRD assessment covered all of the antigen combinations of interest. Antibody combinations for MRD detection were designed individually for each patient based on the diagnostic LAIP. Whenever possible, a minimum of 100,000 events were acquired (median 176,000, range 30,000–400,000). If bone marrow was not submitted, peripheral blood was tested. However, due to decreased confidence in concordant negative MRD results in blood and bone marrow with our assay [30], only positive MRD results from blood specimens were included in the analysis.

2.3. Cytogenetic and mutational analyses

Karyotypes from all cases were centrally reviewed. The definition of favorable, intermediate, indeterminate and unfavorable cytogenetic data followed that published by Slovak et al [31]. Fluorescence-in-situ hybridization (FISH) for t(8;21), inv(16), t(9;22), t(15;17), + 8, – 7/del(7q), – 5/del(5q), rearranged *KMT2A* and *EVI1* genes was performed centrally at the Mayo Cytogenetics Laboratory, Rochester, MN, as described [32]. PCR analysis for *RUNX1/RUNX1T1*, *CBFβ/MYH11*, *PML/RARα*, *BCR/ABL* transcripts and partial tandem duplication of the *KMT2A*-gene (*KMT2A*-PTD) was done centrally at the LTRL [26]. Somatic mutational analysis, including for *FLT3* and *NPM1* gene aberrations, and integrated mutational/cytogenetic risk stratification were as reported previously [2]. This risk classification was chosen since patients with *FLT3-ITD* mutations could not be grouped according to the ratio of the mutant to wild-type alleles, as suggested by the 2017 ELN recommendation [5].

2.4. Sparse whole genome sequencing (sWGS) for copy number ascertainment

sWGS was performed using 50 ng of bulk DNA purified from bone marrow or peripheral blood mononuclear cells with a blast count of at least 25 %. TruSeq indexed libraries were generated, sequenced in multiplex fashion targeting a coverage of 2 million sequencing reads per sample, and data subsequently processed for copy number analysis as described [33]. In brief, sequencing reads were mapped to human reference genome hg19 with uniquely mapped reads sorted and indexed. Uniquely mapped reads were then counted in genomic bins while partitioning the genome in 20,000 bins using a described algorithm (Varbin) [34]. Read bin counts were normalized genome wide with subsequent processing using Circular Binary Segmentation to retrieve chromosomal segments that exhibited copy number states deviating from a euploid, 2N state.

2.5. Statistical analysis

Baseline characteristics were compared using Fisher's exact test if they were categories and Wilcoxon rank sum tests if they were continuous. OS was defined as time from date of CR to death from any cause. Disease-free survival (DFS) was defined as the time from documented CR to relapse or death from any cause. Kaplan-Meier estimates were

used to estimate the event-time distributions. Univariate and multivariate Cox models were performed on OS and DFS. Multivariate models were adjusted for induction treatments, age (continuous), gender (binary), cytogenetic risk (categorical), baseline WBC count (continuous), platelet count (continuous), hemoglobin levels (continuous), and allogeneic transplant (time-varying covariate).

To examine whether MRD status after consolidation had prognostic relevance, a time-varying stratum Cox analysis was performed on patients who entered consolidation and had MRD data available at time points after consolidation. As MRD status may change over time, it was included into the Cox models as a time-varying covariate where patients enter the MRD-positive stratum at any time after consolidation when an MRD test is positive and enter back to MRD-negative stratum when an MRD test is negative. All P values were based on 2-sided tests.

3. Results

3.1. Determining the cut-off point for MRD positivity

When determining the clinically most informative cut-off point for defining MRD positivity with our assay sensitivity threshold and patient population, hazard ratios (HR) (MRD negative versus MRD positive) in univariate Cox analysis at CR concerning OS were 0.32 (95 % CI, 0.19–0.56; $p < 0.001$) when 0.01 % of blasts defined positive MRD status, 0.35 (95 % CI, 0.21–0.59; $p < 0.001$) when 0.05 % of blasts were used, and 0.41 (95 % CI, 0.26–0.65; $p < 0.001$) when 0.1 % of blasts defined positive MRD status, indicating a consistently similar worse prognosis with MRD positivity at each of these MRD levels. There was a significant difference in OS between patients with CR MRD levels between undetectable (0 %) and < 0.1 % and those with MRD levels 0.1 % -< 1 % (HR 0.43, 95 % CI, 0.27–0.69, $p = 0.002$). However, there was no difference in OS between patients with MRD levels of 0.1 %– 1 % and those with MRD levels > =1 % (HR 0.74, 95 % CI, 0.42–1.28, $p = 0.28$). We, therefore, chose an MRD level of 0.1 % to define MRD positivity.

3.2. Association of MRD with presenting biologic characteristics

Table 1 summarizes the distribution of MRD-negative and MRD-positive patients by gender, age, presenting WBC count, hemoglobin, platelet count and cytogenetic risk group. For only 6 patients, neither standard cytogenetics nor FISH nor sWGS data were available. There was no significant difference between MRD-positive and -negative patients with respect to clinical or demographic factors. However, MRD-negative patients had a higher likelihood of being in the favorable cytogenetic risk group (32.7 %) and less likely to have unfavorable cytogenetics (7.3 %) than MRD-positive patients (14.9 % and 16.4 %, respectively) ($p = 0.03$). In agreement with this finding, among all MRD-negative patients, 20 % had favorable *RUNX1/RUNX1T1* CBF-leukemia compared with 4 % among MRD-positive patients ($p = 0.004$). However, no such difference was seen for patients with *CBFβ/MYH11* CBF-AML. None of the other genetic abnormalities detected by FISH or sWGS was associated with MRD status. Regarding specific somatic mutations, among 18 genes tested (Supplementary

Table S1), $DNMT3A^{R882}$ mutation was the only mutation significantly associated with the presence of MRD at CR, consistent with what we have previously reported [35].

3.3. Association of MRD with overall and disease-free survival

At the time of CR, 55 (29.1 %) patients were MRD-negative and 134 (70.1 %) patients were MRD-positive. At 84.6 months median follow-up of patients still alive at the time of analysis (range 47.0–120 months), the median OS in MRD-negative patients had not yet been reached versus 25.2 months for MRD-positive patients (HR 0.41, 95 % CI: 0.26, 0.65, p < 0.001, Fig. 1). In multivariate analysis, the impact of MRD positivity on OS remained significant (HR 0.45, 95 % CI: 0.28, 0.72, p = 0.0009, Supplementary Table S2). Similarly, the median DFS in MRD-negative patients had not yet been reached versus 12.0 months for MRD-positive patients (univariate analysis HR 0.39, 95 % CI: 0.25, 0.61, p < 0.001, Supplementary Fig. S2; and multivariate analysis HR 0.42, 95 % CI: 0.26, 0.66, p = 0.0002).

We also assessed the effect of MRD on outcome when measured at any time post-consolidation using Cox models as a time-varying covariate where patients can enter the MRD-positive and MRD-negative strata at any time post-consolidation. MRD data for both CR and post-consolidation therapy were available for 87 patients. Of those, 6 had converted from MRD-negative at CR to positive at the first post-consolidation evaluation and 7 patients who were MRD-positive at CR subsequently converted to MRD-negative status. In both univariate (for OS, HR: 0.24, 95% CI: 0.12, 0.47, p < 0.001; for DFS, HR: 0.21, 95% CI: 0.11, 0.41, p < 0.001) and multivariate analysis (for OS, HR: 0.26, 95% CI: 0.13, 0.52, p < 0.001; for DFS, HR: 0.22, 95% CI: 0.11, 0.44, p < 0.001), data again showed worse outcome for patients who were MRD-positive at any time-point post-consolidation.

3.4. Association of MRD at complete remission with daunorubicin dose

Of patients with MRD data at CR, 88 had been randomized to receive standard-dose daunorubicin (45 mg/m^2) and 101 patients to receive high-dose daunorubicin (90 mg/m^2) during induction. There was no significant association of MRD status at CR and daunorubicin dose (p = 1.0). However, high-dose, but not standard-dose, daunorubicin was associated with improved OS in MRD-negative (univariate analysis HR: 0.42, 95 % CI: 0.18, 0.99, p = 0.05; multivariate analysis HR: 0.40, 95 % CI: 0.16, 1.06, p = 0.06), but not in MRD-positive patients (univariate analysis HR: 0.87, 95 % CI: 0.58, 1.30; p = 0.49; multivariate analysis HR: 0.93, 95 % CI: 0.61, 1.42, p = 0.75) (Fig. 2), suggesting that dose-intensified induction therapy increased the depth of CR in chemotherapy-sensitive patients. Similar results were observed for DFS (Supplementary Fig. S1).

3.5. Prognostic effect of MRD in cytogenetic risk groups

Among patients with favorable cytogenetics (n = 38), MRD status had a significant impact on OS (univariate HR: 0.23, 95 % CI: 0.08, 0.71, p = 0.005, Supplementary Fig. S3; multivariate HR 0.20, 95 % CI, 0.05, 076, p = 0.02). The OS for MRD-negative patients with favorable cytogenetics (47 %) has not yet been reached compared with 31.6 months for MRD-positive patients. In patients with intermediate-risk cytogenetics (n = 79), MRD status had a marginally significant impact on OS in univariate (HR 0.51; 95 % CI: 0.25, 1.02, p = 0.051, Supplementary Fig. S4) but not multivariate analysis. Median OS for

MRD-negative intermediate-risk patients has not yet been reached compared with 27.5 months in MRD-positive patients. In the unfavorable cytogenetic risk groups (n = 26), OS did not differ by MRD status (HR: 1.05, 95 % CI: 0.31, 3.62, p = 0.94, Supplementary Fig. S5). MRD negativity was achieved in only 4/26 patients with unfavorable cytogenetics.

3.6. MRD status and integrated molecular profiling

Integrated molecular profile combined molecular and cytogenetic risk classes into a single risk stratification schema. The incidence of MRD-positivity at CR was: 56 % in patients with a favorable-risk, 76 % of patients with intermediate-risk and 78 % in patients with unfavorable-risk profile. The frequency of MRD-positivity increased with increasing risk of relapse as stratified by integrated molecular profiling (p = 0.015). Multivariate analysis demonstrated that post-induction MRD status (p = 0.0004) and pre-therapy integrated molecular risk (p < 0.001) independently impacted OS. The effect of MRD status on outcome was significant in all risk classes, though most pronounced in favorable-risk patients (univariate HR: 0.28, 95 % CI: 0.11, 0.69, p = 0.006; multivariate HR: 0.22, 95 % CI: 0.08, 0.61, p = 0.004). Intermediate-risk (univariate HR: 0.34, 95 % CI: 0.12, 0.97, p = 0.04; multivariate HR: 0.30, 95 % CI: 0.09, 1.00, p = 0.05) and integrated adverse-risk MRD-negative patients also had better OS compared to MRD-positive patients (univariate HR: 0.63, 95 % CI: 0.29, 1.37, p = 0.24; multivariate HR: 0.36, 95 % CI: 0.14, 0.92, p = 0.03).

3.7. MRD at CR and CD25 status at baseline

E1900 patients whose myeloblasts at diagnosis expressed CD25, the α-chain of the interleukin 2 receptor, had a significantly poorer outcome than patients without CD25 expression independent of molecular profiling [17]. The presence of a distinct CD25 expressing myeloblast population (range, 19 %–99 %) defined CD25 positivity. CD25 data were available for 185 patients with MRD data at CR. *NPM1* (p = 0.014), *FLT3-ITD* (p < 0.001), and *DNMT3A^{R882}* (p = 0.02) were the only mutations tested significantly associated with CD25 positivity (Supplementary Table S3). Of the 185 patients, 26 (14 %) had been CD25-positive at diagnosis, comparable to the rate of CD25-positivity in the entire E1900 cohort (13 %) [17]. Of CD25-positive patients, 92 % were MRD-positive at CR. Among 131 MRD-positive patients at CR, 24 (18.3 %) had been CD25-positive at diagnosis, while only 2 of 54 MRD-negative patients had been CD25-positive (3.7 %) (p = 0.009). Median expression levels of CD25 were 64 % in the two CD25-positive/MRD-negative and 55 % in the 131 CD25-positive/MRD-positive patients. MRD-negative/CD25-negative patients had better OS than MRD-negative/CD25-positive patients (HR: 0.16, 95 % CI: 0.03, 0.72, p = 0.02). MRD-positive/CD25-negative patients also had significantly better OS compared with MRD-positive/CD25-positive patients (HR: 0.55, 95 % CI: 0.34, 0.91, p = 0.02) (Fig. 3). Similar results were observed for DFS (Supplementary Fig. S6). A Cox model confirmed that MRD status at CR (HR: 0.54, 95 % CI: 0.33, 0.87, p = 0.01) and CD25 status at presentation (HR: 0.46, 95 % CI:0.27, 0.77, p = 0.004) were significantly and independently associated with OS (Supplementary Table S4).

4. Discussion

Although molecular, cytogenetic, and clinical features have an important impact on survival in AML, there remains a critical need to identify new predictors of outcome. In adult ALL, MRD status has emerged as a robust measure of therapeutic sensitivity and depth of response with substantive prognostic value [36-38]. In contrast, despite several reports suggesting the prognostic importance of MRD in AML [8, 39,41], it is not considered routine practice in adult AML [40]. To investigate the clinical relevance of MRD in relation to a molecular-based prognostic schema, we measured MRD by flow cytometry in 189 AML patients enrolled in the ECOG-ACRIN E1900 clinical trial (NCT00049517) in morphologic complete remission (CR) (28.8 % of the original cohort) representing 44.4 % of CR patients. We provide evidence that MRD, as assessed by central flow cytometry, has prognostic value in a uniformly treated AML cohort independent of integrated cytogenetic/molecular profiling. This result is particularly noteworthy as our 4-color LAIP-based flow cytometric MRD assay, available during the duration of the E1900 trial (December 2002-November 2008), would nowadays be considered sub-standard [5], confirming the robust nature of MRD as a prognostic parameter. The maturity of the data, on the other hand, provided us with a follow-up of up to 10 years.

To answer the question of optimal timing of MRD measurement, we assessed MRD at the time of hematologic CR and any-time post-consolidation. MRD status at either time-point was strongly predictive of outcome. The other parameter of interest was the cut-off for MRD positivity. Though the standard MRD positivity threshold is 0.1 % [10], we found no difference between several cut-offs (0.1 %, 0.05 % or 0.01 %) with respect to MRD positivity and outcome. These data suggest that it is best to consider MRD as a continuous, quantitative variable, and that whatever cutoff can be enumerated by the flow cytometric method used and retrospectively found to be associated with outcome has prognostic significance in individual AML trials using equivalent treatment strategies. For the majority of patients, the 4-color LAIP-based MRD assessment was not sufficiently sensitive to detect levels below 0.01%. Data with improved methodology suggest that any level of MRD may be prognostically important [41,42].

One important question in the field is how best to incorporate MRD in current risk stratification schema in AML. Previous studies have performed multivariate analyses including both MRD and cytogenetics[43], or MRD and specific mutations, such as *FLT3* [44,45] and *NPM1* [19,20,44], to show that MRD has independent prognostic value. We show that in the context of comprehensive cytogenetic and molecular risk stratification, MRD has an important prognostic impact. By incorporating sWGS with FISH and standard cytogenetics, we both confirmed recurrent translocations and copy-number alterations which had been identified by cytogenetic analysis and increased the portion of patients with genomic information to 97 %. Because the impact of MRD status on prognosis was significant in all AML patients, whether classified as favorable, intermediate or adverse integrated risk, we conclude that MRD-negativity selects the subset of patients within these risk profiles who have chemo-sensitive disease and thus the most favorable outcome. There is clearly a need of more extensive mutational profiling [46], preferably at the single-cell level [24,47,48], to further underscore this notion.

Surface expression of the CD25 antigen at diagnosis was a powerful adverse prognostic factor in E1900 patients [17]. We now show a significantly higher rate of MRD-positivity at CR in CD25-positive compared to CD25-negative patients. Importantly, CD25 and MRD status were significantly and independently associated with OS, irrespective of age, WBC count and cytogenetic risk. Our data expand on an earlier report by Terwijn et al [49]. which showed a correlation of expression of CD25 on > 10 % myeloblasts at diagnosis with significantly higher MRD frequency after the first cycle of chemotherapy. Based on previous gene expression profiling studies in the E1900 cohort [17] and studies by others on the biology of CD25 in AML [50,51], we hypothesize that CD25 tracks with leukemic stem cell burden/identity, and that MRD-positivity and/or CD25-expression mark AML stem cells which are less sensitive to cytotoxic chemotherapy.

Taken together, we show that the presence of flow cytometric MRD is an independent prognostic factor in newly diagnosed AML patients 60 years old, both at time of CR and after consolidation. This effect was independent of pre-therapeutic molecular risk and level of CD25 expression, both biomarkers with significant prognostic implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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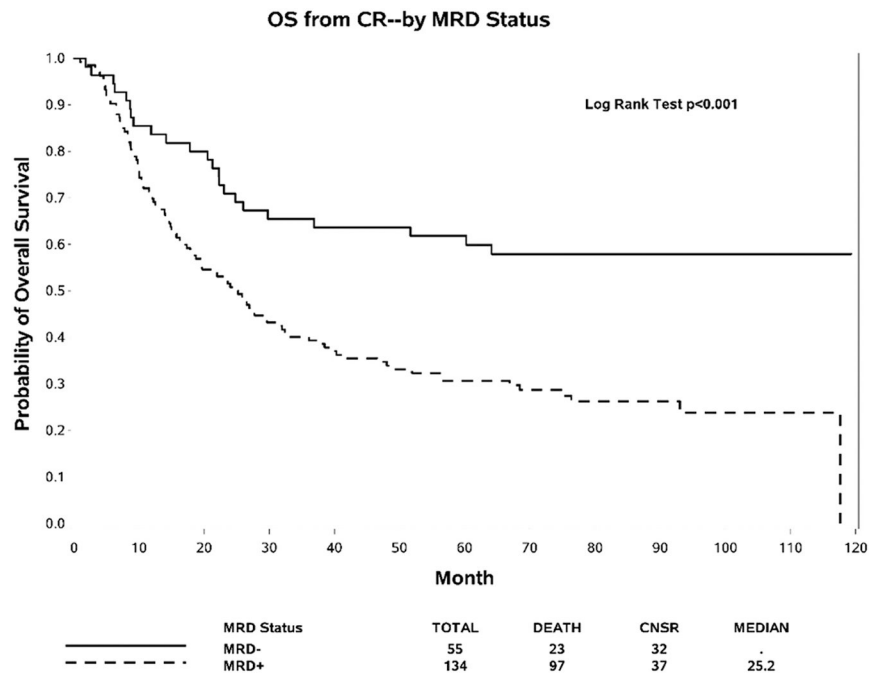
OS from CR--by MRD Status

Fig. 1.
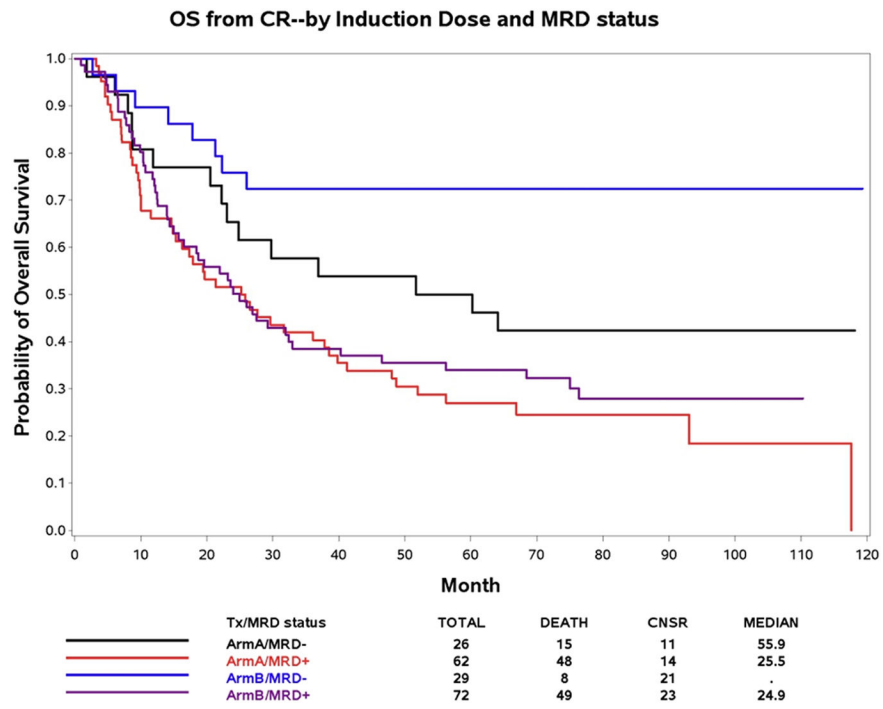Overall Survival (OS) according to MRD status at time of complete hematologic remission (CR).

Fig. 2.
Overall survival (OS) after achievement of complete remission (CR) by MRD status and induction treatment. Arm A– Daunorubicin 45 mg/M^2, Arm B– Daunorubicin 90 mg/M^2.

OS from CR--by CD25 and MRD Status

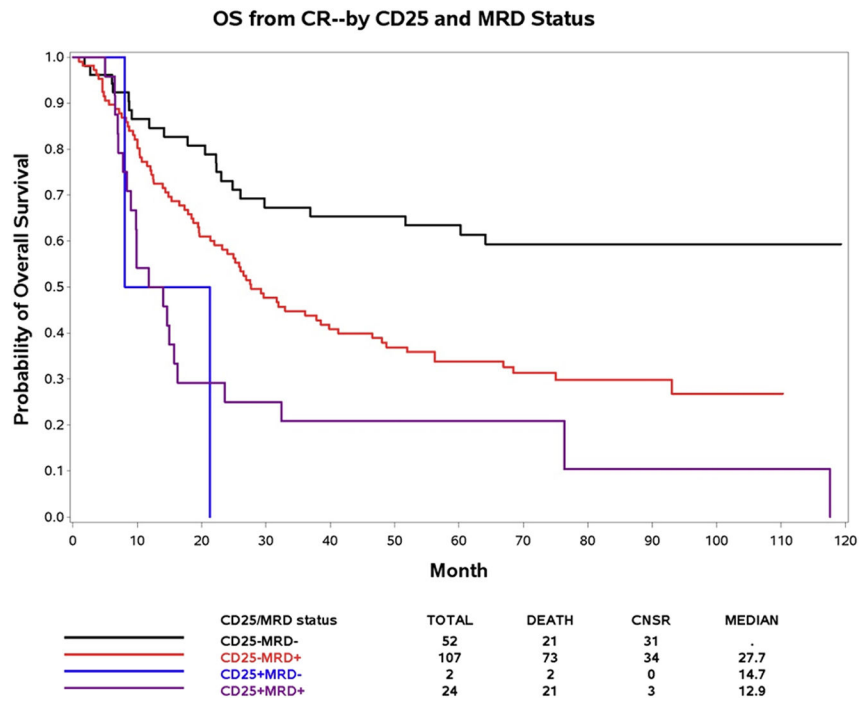| CD25/MRD status	TOTAL	DEATH	CNSR	MEDIAN
CD25-MRD-	52	21	31	.
CD25-MRD+	107	73	34	27.7
CD25+MRD-	2	2	0	14.7
CD25+MRD+	24	21	3	12.9

Fig. 3.

Overall Survival (OS) according to MRD status and CD25 at time of complete hematologic remission (CR).

Table 1

a Demographics and disease characteristics of the CR patients with MRD status data (continues variables).

	MRD−			MRD+			Total		
	Median	Min	Max	Median	Min	Max	Median	Min	Max
Age (years)	45.0	19	60	50.0	18	60	48.0	18	60
WBC count (/mm3)(x1000)	9.7	1	107	14.6	1	176	11.2	1	176
Hemoglobin (g/dl)	9.2	6	16	9.2	5	30	9.2	5	30
Platelet count (/mm3)(x1000)	46.0	6	246	56.0	1	479	54.3	1	479

b Demographics and Disease Characteristics of the CR patients with MRD status data (categorical variables).

	MRD−		MRD+		Total	
	N	%	N	%	N	%
Gender						
Female	25	45.5	66	49.3	91	48.1
Male	30	54.5	68	50.7	98	51.9
Cytogenetics						
Favorable	18	32.7	20	14.9	38	20.1
Indeterminate	11	20.0	35	26.1	46	24.3
Intermediate	22	40.0	57	42.5	79	41.8
Unfavorable	4	7.3	22	16.4	26	13.8