Panel Based Error Corrected Next Generation Sequencing versus Flow Cytometry to Detect Measurable Residual Disease in Acute Myeloid Leukemia

Authors:
Nikhil Patkar1,2*, Chinmayee Kakirde1, Anam Fatima Shaikh1, Rakhi Salve1, Prasanna Bhanshe1, Gaurav Chatterjee1-2, Sweta Rajpal1,2, Swapnali Joshi1, Shruti Chaudhary1, Rohan Kodgule1, Sitaram Ghoghale3, Nilesh Deshpande3, Dhanalaxmi Shetty1, Syed Hasan Khizer2,4, Hasmukh Jain2,4, Bhausaheb Bagal2,4, Hari Menon5, Navin Khattry3,4, Manju Sengar3,4, Prashant Tembhare1,2, Papagudi Subramanian1,2, Sumeet Gujral1,2

*Corresponding Author

Affiliations:
1 Haematopathology Laboratory, ACTREC, Tata Memorial Centre, Navi Mumbai, India
2 Homi Bhabha National Institute (HBNI), Mumbai, India
3 Dept of Cytogenetics, ACTREC, Tata Memorial Centre, Navi Mumbai, India
4 Adult Haematolymphoid Disease Management Group, Tata Memorial Centre, Mumbai, India
5 Haemato-Oncology, CyteCare Cancer Hospital, Bangalore, India

Short Running Title: Error Corrected NGS based MRD in AML

Correspondence:
Dr Nikhil Patkar MD, DNB
Clinician Scientist & Associate Professor
Wellcome Trust – DBT Intermediate Fellow
Haematopathology Laboratory, CCE Building,
Advanced Centre for Treatment Research and Education in Cancer,
Tata Memorial Centre, Kharaghar, Maharashtra, India, Pin: 410210
Email: nvpatkar@gmail.com; npatkar@actrec.gov.in
Phone: +91-22-27405000, Ext: 5773

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Abstract:

A 35 gene error corrected next generation sequencing (NGS) panel was created using single molecule molecular inversion probes with applicability to 83% of acute myeloid leukemia (AML). We accrued 201 patients of adult AML treated with conventional therapy, in morphological remission and evaluated measurable residual disease using NGS (NGS-MRD) and multiparameter flow cytometry (FCM-MRD) at end of induction (PI) and consolidation (PC). A total of 344 mutations were detected (median VAF of 0.76%) during assessment of MRD. Nearly 71% of patients harbored PI NGS-MRD (and 40.9% harbored PC-MRD). Patients harboring NGS-MRD had a significantly higher cumulative incidence of relapse (CIR), inferior overall survival (OS) and relapse free survival (RFS) as compared to NGS-MRD negative patients at PI and PC time points. NGS-MRD was predictive of inferior outcome in intermediate cytogenetic risk and demonstrated potential in favorable cytogenetic risk AML. Patients who cleared PI NGS-MRD had a significantly improved survival as compared to patients who became negative subsequently indicating that kinetics of NGS-MRD clearance was of paramount importance. NGS-MRD identified over 80% of cases identified by flow cytometry at PI time point whereas FCM identified 49.3% identified by NGS. Only a fraction of cases were truly missed by NGS as compared to FCM-MRD. Both FCM and NGS MRD were important in predicting outcome however, PI NGS-MRD emerged as the most important independent prognostic factor predictive of inferior outcome. We demonstrate that panel based NGS-MRD is highly predictive of outcome and advantageous when compared to FCM-MRD in AML treated with conventional therapies.
Key Points:

- Panel based error corrected NGS-MRD assessment for acute myeloid leukemia is applicable to majority of AML
- NGS-MRD assessment in CR at early time-points in therapy is highly predictive for outcome
Introduction

Acute Myeloid Leukemia is a disease characterized by heterogeneous biology resulting in varying clinical outcomes including relapse.\(^1,^2\) There are limited novel treatment options such as targeted therapies using FLT3 or IDH2 inhibitors and most patients are treated based on the presumptive risk of relapse.\(^3,^4\) This risk adapted therapy typically considers standard cytogenetic and molecular variables as recommended by the European LeukemiaNet.\(^5\) Based on this risk, patients are recommended treatment with conventional (induction and consolidation) regimens or offered intensive therapy such as allogeneic bone marrow transplantation (BMT) after achievement of remission. Amongst these, the largest group remains as intermediate risk AML characterized by non-uniform clinical outcomes.

The monitoring of a patient’s response to chemotherapy, called, measurable residual disease (MRD) is one of the most important predictors of outcome in hematological malignancies. Several investigators have demonstrated the clinical utility of flow cytometry based MRD detection (FCM-MRD) in AML at early chemotherapy time points as well as in a pre-transplant setting.\(^6^-^14\) Although universally applicable, FCM-MRD suffers from suboptimal ability to predict relapse in AML compared to precursor B lineage acute lymphoblastic leukemia. A diverse array of sensitive molecular methods have been used to detect MRD in AML such as real time PCR\(^15\) and droplet digital PCR.\(^16\) These are useful for monitoring of individual gene mutations such as AML with mutated NPM1\(^17\) and chimeric gene fusions such as RUNX1-RUNX1T1.\(^18\) Next generation sequencing (NGS) is a promising tool for sensitive MRD monitoring and has been used successfully to monitor NPM1\(^19,^20\), RUNX1\(^21\) and FLT3\(^22\) mutations as well as chimeric gene fusions.\(^23,^24\) However, these methodologies can be utilized in only a subset of patients that harboured these mutations at diagnosis and this strategy discounts for clonal heterogeneity and evolution which are relevant to the pathogenesis of AML.

DNA based focused target enrichment strategies (gene panels) are an attractive solution to detect MRD using NGS (NGS-MRD) in a mutation rich disease such as AML.\(^25^-^28\) However, short read sequencers are inherently prone to base calling errors limiting variant calling at 3-5% variant allele.
fraction (VAF).\textsuperscript{29} Although acceptable for diagnostic molecular pathology, this is undesirable assay performance for the detection of MRD. Error corrected sequencing involves the physical incorporation of random oligonucleotides or unique molecular identifiers (UMI) at the library preparation stage prior to amplification of DNA. This allows us to tag individual DNA molecules with an unique molecular fingerprint.\textsuperscript{30,31} Such approaches have been used for myelodysplastic syndromes\textsuperscript{32} and for pre transplant MRD monitoring of AML as demonstrated by Thol and colleagues.\textsuperscript{33} Thol utilized a sensitive patient specific mutation tracking approach using UMI based MRD detection. Although applicable to a broad spectrum of AML mutations, a tailored approach poses logistical and regulatory hurdles towards prospective MRD testing especially for early MRD timepoints.

In this study, we have evaluated the clinical utility of error corrected NGS to detect MRD in AML using single molecule molecular inversion probes (smMIPS).\textsuperscript{30,34} Each smMIP contains an 8 bp UMI and binds to a single molecule of DNA. Using consensus sequence-based variant calling we can detect somatic mutations including small indels in a sensitive manner. We demonstrate that error corrected NGS-MRD at early timepoints in therapy is significantly predictive of outcome in patients of AML treated with conventional therapies. Furthermore, we systematically compare multicolour FCM-MRD with error corrected NGS-MRD and assess the clinical utility of these two assays in a cohort of AML.
Methods

1. PATIENTS

a. Patient Accrual and Initial work up: The study was approved by the institutional ethics committee (IEC-III project 163) and participants were accrued after informed consent. A total of 393 adult patients of AML, diagnosed as per 2008 WHO criteria, were accrued in this study over a period of six years (Feb 2013 to May 2019). Cytogenetic (FISH and karyotyping) workup was as previously described.9,20 Somatic mutations at diagnosis were evaluated using a smMIPS based 50 gene myeloid panel as described previously by our group.35 Of these, the smMIPS MRD panel (see below) was applicable to 83.2% patients [327 out of 393 AMLs, median 2 mutations per case (range 1 – 6 trackable mutations); Supplementary Figure 1]. Of those (n=319) achieving morphological CR, the smMIPS MRD panel was applicable to 266 (83.4%). MRD assessment could be performed in 201 adult patients of AML (enrolment flow chart in supplementary figure 2). A summary of the clinical and laboratory characteristics of these 201 patients can be seen in Table 1.

b. Treatment of AML and MRD Sampling: All patients were treated with conventional “3+7” induction chemotherapy and further treated with high dose cytarabine (HiDAC) or allogeneic bone marrow transplantation (aBMT), if feasible.35 Only 15 patients received aBMT and their outcome was not different from the rest with respect to OS and RFS (p = not significant; supplementary figure 3) and are not considered separately. Sample for FCM-MRD was obtained from the bone marrow at end of induction (PI; n=200) and end of first consolidation cycle (PC, n=98). NGS MRD sample also obtained at the same time points (PI-196; PC-127) from the bone marrow (n=269; PI:181 and PC:88) or peripheral blood (n=51; PI:15 and PC:36).

c. Evaluation of Treatment Outcome: Overall Survival (OS) and Relapse Free Survival (RFS) were calculated as previously described.9,20,35 The prognostic impact of NGS and FCM-MRD assays on OS and RFS was computed using the Kaplan-Meier method and
compared using log-rank test. Multivariate analysis was performed using the Cox proportional-hazards regression analysis that considered FCM-MRD and NGS-MRD. Separate models were constructed for post induction and post consolidation MRD time points. Grey test was used to compare the cumulative incidences of relapse (CIR) and non-relapse mortality (NRM) using “cmprsk” module in R. The same module was used to generate representative graphs. Positive predictive value (PPV) and negative predictive value (NPV) were calculated as described.

2. DETECTION OF MRD USING NGS

a. smMIPS panel: We created a 35 gene panel comprising of a pool of 302 smMIPS (as seen in Supplementary Table 1). In brief, this panel covers regions of 35 commonly mutated genes in AML (ATM, BCOR, DNMT3A, EZH2, FLT3, GATA1, GATA2, IDH1, IDH2, JAK2, KDM6A, KIT, KMT2D, KRAS, NF1, NOTCH1, NOTCH2, NPM1, NRAS, PHF6, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SH2B3, SMC1A, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2). The panel was rebalanced (Supplementary Figure 4) to ensure uniform capture across regions. Approximately 600ng of genomic DNA was captured, treated with exonucleases and PCR amplified to create a sequencing ready library. Details pertaining to smMIPS design, assay standardization and sequencing are detailed in supplementary methods.

b. Bioinformatics: Reads were demultiplexed, trimmed, paired end assembled and mapped to the human genome (build hg19). Singleton reads (originating from one UMI) were discarded, and consensus family based variant calling performed using tools described in supplementary methods. We then created a site and mutation specific error model to ascertain the relevance of an observed variant at each site. Criteria for variant calling using the smMIPS MRD assay are described in supplementary methods.
c. **MRD for FLT3 internal tandem duplications (ITD):** FLT3-ITD were detected using a novel one-step PCR based NGS assay (see Supplementary Table 3). Variants were detected using a recently described algorithm for the accurate detection of FLT3-ITD.\(^{37}\)

d. **Orthogonal detection of MRD in NPM1 mutated AML:** NPM1 mutations were additionally tracked using an ultrasensitive orthogonal NPM1 MRD assay.\(^{20}\)

3. **DETECTION OF MRD IN USING MULTICOLOUR FLOW CYTOMETRY (FCM-MRD)**

FCM-MRD was detected using a previously described 10 colour two tube MRD assay.\(^{9,20,35,38}\) This approach uses a combination of leukemia associated immunophenotype and difference from normal approaches to detect MRD in AML.
Results

1. PATIENTS:

The median follow-up of the cohort was 42.3 months. The median OS was 35.9 months (95% CI: 27.2 to 42.8) for the entire cohort. The median RFS was 21.6 months (95% CI: 17.0 to 28.9) months. Additional patient characteristics can be seen in Table 1.

2. NGS BASED AML MRD:

a. Assay Characteristics:

We describe an NGS-MRD approach that is applicable to 83.2% of all AML. For patients in morphological CR (n=319) this approach was applicable to 83.4% (n=266). Of these, MRD could be assessed in 201 cases. A co-occurrence plot indicating interactions of mutations tracked by NGS-MRD, prior to therapy, can be seen in Figure 1A. The applicability of this MRD panel, when patients (n=201) are classified by cytogenetic risk is seen in supplementary table 2.

b. Limit of Detection:

In a limit of detection experiment (supplementary figure 5), we demonstrated that we could detect leukemic clones till a lower limit of 0.05% (0.03% for NPM1 mutation). Error modelling of normal samples indicated a higher prevalence of C>T and G>A changes consistent with oxidative DNA damage (supplementary figure 6). FLT3-ITD could be detected at a limit of 0.002% VAF (supplementary figure 7). For smMIPS based MRD, samples were sequenced at a median coverage of 14,728x (11,363x consensus coverage) whereas, for FLT3-MRD assay, the median coverage was 1,396,366x. A total of 344 mutations could be detected in 323 samples (Figure 1B,C) with a median VAF of 0.95% [0.76% after exclusion of mutations in DNMT3A, TET2, ASXL1 (DTA) genes]. Amongst positive patients, a median of 2 mutations could be detected per patient (range 1-4) at end of induction.

c. Clinical Relevance of NGS-MRD:
Nearly 71% (n=139; 70.9%) of patients harboured MRD at the end of induction and 40.9% (n=52) at the end of consolidation. Patients harbouring MRD had a significantly higher CIR as compared to MRD negative patients at PI time point as seen in Figure 2A, Table 2. For patients who were PC NGS-MRD positive a similar observation for CIR was made (Figure 2B, Table 2). The presence of NGS-MRD at the end of induction was associated with inferior OS and RFS (Figure 2C,E) as detailed in Table 3. Similarly, the presence of NGS-MRD (n=52; 40.9%) was predictive of inferior OS and RFS at the end of consolidation (Figure 2D,F; Table 3).

i. Cytogenetics and NGS-MRD: We did not observe a significant distribution of MRD results when cases were distributed by cytogenetic risk. However, the presence of NGS-MRD was predictive of inferior outcome in intermediate cytogenetic risk (and a tendency in favorable risk) as seen in supplementary figure 8.

ii. Change in mutations over MRD time points: Amongst paired samples (n=122), 83 samples were positive at PI time point, of which 37 became negative at end of consolidation. For 46 patients in whom MRD persisted a change in MRD profile occurred in 18 patients (39.13%). This included a loss of mutation in most cases (n=14) and gain in the rest (supplementary figure 9). There were 5 patients who were negative at PI time point and became positive at end of consolidation. Of these, relapse was seen in two patients.

iii. Clinical relevance of the kinetics of NGS-MRD clearance: Patients who cleared NGS-MRD at end of induction (and persisted) had a significantly improved OS [HR- 0.45; 95% CI- 0.22 to 0.9; (p=0.02)] and RFS [HR- 0.49; 95% CI- 0.27 to 0.89; (p=0.01)] as compared to patients who became negative at end of consolidation (supplementary figure 10). Similarly, patients who persistently harbour MRD had a significantly inferior outcome as compared to patients who were MRD negative at both time points.
(supplementary figure 11). There was no genetic difference observed between these two groups (supplementary figure 12).

d. Orthogonal detection of NGS-MRD in NPM1 mutated AML:

Orthogonal comparison of MRD detection in NPM1 mutated AML was performed in 75 patients (23.2% of all MRD samples; Supplementary Methods, supplementary figure 13). There was a good correlation observed with NPM1 NGS MRD assay (R²=0.71). The PPV of smMIPS MRD assay for NPM1 mutated AML was 58.5% (95%CI- 52.35 to 64.42%) as compared to a lower PPV of NPM1 NGS MRD assay [50% (95%CI- 45.34 to 54.66%)]. The NPM1 NGS MRD assay had a much lower specificity [11.11%(95%CI- 3.11 to 26.06%)] as compared to smMIPS MRD assay for MRD detection in NPM1 mutated AML [46.58%(95%CI- 34.80 to 58.63%)].

3. FCM BASED AML- MRD:

The presence of FCM-MRD was associated with inferior OS, RFS and CIR at the end of induction and consolidation as detailed in Tables 2, 3 and supplementary figure 14.

4. COMPARISON OF FCM AND NGS MRD:

On incorporating results combining both the MRD modalities, patients that were positive by both techniques (FCM+ NGS+) had a significantly inferior outcome with respect to OS, RFS and CIR at any MRD time point as compared to patients negative by both modalities as seen in Table 2,3 (Figure 3). A comparison of the baseline mutational profiles between dual PI MRD positive and negative groups revealed a significantly higher (p=0.04) prevalence of RUNX1 mutations in the dual MRD positive subset (supplementary figure 15).

a. Clinico-pathological correlates of FCM MRD Positive NGS MRD Negative AML:

A total of 20 patients were (FCM+NGS-) at assessed at PI and/or PC time points, of which 4 were detected at subsequent (or preceding) MRD time point. Of the remaining 16, in three patients a paired MRD sample was lacking. Amongst the rest, six patients (out of
13) had relapsed, three died due to non-relapse causes and four were alive at last follow-up (supplementary table 5).

b. **Accuracy of NGS-MRD in comparison with FCM-MRD:**

The PPV and NPV metrics of end of induction NGS-MRD to predict relapse in AML were 70.5% and 57.89% respectively with an accuracy of 66.84%. FCM MRD metrics at end of induction were comparable for PPV (75%), NPV (48.2%) and accuracy to predict relapse (60%). At the PI time point, NGS-MRD correctly identified 80% (68 out of 85) of cases classified as MRD positive by FCM, whereas FCM identified just 68 out of 138 cases (49.3%) identified by NGS. A detailed comparison of PPV, NPM and accuracy of combinations of patients detected between these two assays can be seen in supplementary table 6.

5. **MULTIVARIATE ANALYSIS:**

Both FCM and NGS MRD were important in predicting outcome as seen in Table 3. However, PI NGS-MRD emerged as the most important independent prognostic factor predictive of inferior outcome for OS [HR- 1.94; 95% CI- 1.15 to 3.27; (p<0.0001)] as well as RFS [HR- 2.05; 95% CI- 1.30 to 3.23; (p<0.0001)].
Discussion

Recently, Hourigan and colleagues performed ultradepth sequencing using a 13-gene panel to detect MRD in AML. In a pioneering effort, they demonstrated an advantage of myeloablative conditioning in preventing relapse in an AML cohort based on NGS MRD results. The authors, however, were unable to compare their results with other MRD assessment techniques. Here, we have assessed MRD at serial time points and have compared our results with 10 colour FCM-MRD, which is a widely used technique for the assessment of response to chemotherapy. We find that NGS-MRD is comparable in applicability and adds value, especially when a clear distinction of regenerating myeloid progenitors from leukemic blasts is absent. Advantages over FCM-MRD include potential for multi-institutional standardization, an ability to scale up operations and lack of requirement of expert operators. In our manuscript we demonstrate that NGS-MRD identified over 80% of cases identified by flow cytometry at PI time point. On evaluating 20 cases (from both PI and PC timepoints) missed by NGS-MRD (but detected by FCM), we observed that 20% of these were detected by NGS-MRD at a subsequent or preceding MRD time point. An additional 56.3% (n=9) are alive or have died due to causes other than relapse indicating that these could have been false positives. In that case, only a fraction of cases (n=7) are truly missed by NGS as compared to FCM-MRD. An additional novel insight gained is gained by studying the kinetics of MRD (supplementary figure 10). We demonstrate that patients who became negative at end of induction are likely to have a superior outcome as patients who turn negative at a subsequent time point.

Previously Jongen-Lavrencic have demonstrated clinical utility of NGS to detect MRD in AML by using computational error correction to mitigate sequencing errors. Such an approach although easy to implement discounts for batch effects and variability that occurs because of library clustering and batch dependent PCR artefacts. In that context, to the best of our knowledge, this is the first study to determine the clinical importance of (error corrected, panel based) NGS-MRD in AML treated with conventional therapies. Although our NGS-MRD strategy works in a majority of AML, we were curious about the genetic basis of cases (n=65 out of 393) in which this strategy did not work. The
cytogenetic and mutational landscape can be seen in supplementary figure 16. Nearly half of these patients did not harbour any mutation at diagnosis (n=29; 44.6%). Insight into rest of the cases revealed ASXL2 as a recurrently mutated gene (n=8, 12.3%). Incorporation of ASXL2 in future iterations of our panel will as well as other UMI based RNA sequencing approaches\textsuperscript{23,24} to monitor chimeric gene fusions will help in increasing the breadth of our approach.

Consistent with previous reports, we find that in some patients, mutations in DTA genes are present at high VAF at MRD time points (Figure 1) indicating an origin from an ancestral clone possibly originating from clonal haematopoesis.\textsuperscript{26,33,39} We are in agreement with Thol and colleagues who indicated that mutations in TET2 could reliably be used as MRD markers, at least in a fraction of cases.\textsuperscript{33} Unlike amplicon based\textsuperscript{27,33} approaches, the advantage of a smMIPS based capture includes a stable panel which can be used across a spectrum of cases and no susceptibility to allelic skew or PCR induced errors prior to incorporation of the UMI barcode. Disadvantages of smMIPs include poor performance for GC rich genes such as CEBPA gene and inability to capture low yield or poor quality of DNA (a problem not infrequently seen with MRD samples). The library preparation process, is relatively low cost in nature and the overall process has a realistic, turnaround time of five to seven days. Our observation is that sensitivity in the clinic for most mutations is close to 0.1% VAF. A lower can be obtained for complex indels such as NPM1 and FLT3-ITD. Improvements with sensitivity may be possible through duplex sequencing based methods albeit at a much higher cost of sequencing.\textsuperscript{29}

Lastly, based on this data we find that mutations in NPM1, FLT3, NRAS, KIT, IDH1, IDH2, WT1, RUNX1, GATA2, U2AF1 and PHF6 were most helpful in making an positive NGS-MRD call. (supplementary figure 17).

To conclude, we demonstrate that panel based error corrected NGS-MRD is clinically relevant and possibly advantageous when compared to FCM-MRD based AML MRD.
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Authorship and conflict-of-interest statements:

NP designed the study, conducted research, analyzed, interpreted the data, and wrote the manuscript.

AFS, CK, RS, PB, SR, SJ, SC, RK conducted research and analyzed data. DS, SG, GC, ND, PT, PGS, SG conducted research and analyzed data. BB, HM, NK, MS recruited patients and analyzed data.

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Agreement to Share Publication-Related Data and Data Sharing Statement:

Dr Nikhil Patkar, Haematopathology Laboratory, CCE Building, Advanced Centre for Treatment Research and Education in Cancer, Tata Memorial Centre, Kharghar, Maharashtra, India, Pin: 410210. Email: nvpatkar@gmail.com; npatkar@actrec.gov.in. Phone: +91-22-27405000, Ext: 5773
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Figure legends

Figure 1: Somatic mutations in AML detected at diagnosis and during therapy. Figure 1A: The interaction of mutations at baseline is demonstrated here using Fisher’s Exact test. Co-occurrence is indicated in grey colour and mutual exclusivity is indicated in red. Figure 1B: The total number of mutations detected per patient and the number of such patients in the cohort is displayed. The total number of mutations in DNMT3A-TET2-ASXL1 genes is indicated here as a fraction. Figure 1C: Variant allele frequencies of mutations detected at MRD time points for patients of AML in morphological remission. The bars indicate median values with interquartile ranges.

Figure 2: Clinical relevance of error corrected NGS-MRD. Presence of NGS MRD at post induction (A) and post consolidation time points (B) is associated with a higher cumulative incidence of relapse (CIR). Kaplan Meyer plots indicate the clinical relevance of NGS-MRD with respect to OS and RFS at post induction (C,E) and post consolidation time points (D,F).

Figure 3: Comparison between FCM and NGS-MRD. The clinical relevance of positive or negative results for detection of MRD during complete remission when measured by FCM or error corrected NGS at post induction (A,B) and post consolidation time points (C,D).
| Parameter                                                                 | Observation (%) |
|--------------------------------------------------------------------------|-----------------|
| Demographics:                                                             |                 |
| Age                                                                      | Range: 18-63 years  |
|                                                                          | Median: 36 years |
| Sex                                                                      | Male : Female : 1.48 : 1 |
| Clinical Characteristics:                                                |                 |
| Total Number of Patients Accrued                                         | 201             |
| Remission Characteristics:                                              |                 |
| Complete remission (CR)                                                  | 31              |
| CR with incomplete hematologic recovery (CRi)                            | 170             |
| Bone Marrow Transplantation:                                            |                 |
| Patients who underwent BMT                                               | 15              |
| Laboratory Characteristics:                                             |                 |
| Blood Counts at Presentation                                            |                 |
| 1. More than 50,000/mm$^3$                                              | 49              |
| 2. Less than 50,000/mm$^3$                                              | 152             |
| Classification according to Cytogenetic Risk:                           |                 |
| Favorable Risk                                                           | 48 (23.9%)      |
| Intermediate Risk                                                        | 136 (67.7%)     |
| Poor Risk                                                                | 17 (8.5%)       |
| Post Induction Flow MRD (n=200):                                        |                 |
| MRD Positive                                                             | 88 (44.0%)      |
| MRD Negative                                                             | 112 (56.0%)     |
| Post Consolidation Flow MRD (n=98):                                     |                 |
| MRD Positive                                                             | 21 (21.4%)      |
| MRD Negative                                                             | 77 (78.6%)      |
| Post Induction NGS MRD (n=196):                                         |                 |
| MRD Positive                                                             | 139 (70.9%)     |
| MRD Negative                                                             | 57 (29.1%)      |
| Post Consolidation NGS MRD (n=127):                                     |                 |
| MRD Positive                                                             | 52 (40.9%)      |
| MRD Negative                                                             | 75 (59.1%)      |
| Comparative Analysis of FCM MRD and NGS MRD (Post Induction; n=195)      |                 |
| NGS MRD+ FCM MRD+                                                       | 68 (34.9%)      |
| NGS MRD+ FCM MRD-                                                       | 70 (35.9%)      |
| NGS MRD- FCM MRD+                                                       | 17 (8.7%)       |
| NGS MRD- FCM MRD-                                                       | 40 (20.5%)      |
| Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation; n=87)   |                 |
| NGS MRD+ FCM MRD+                                                       | 09 (10.3%)      |
| NGS MRD+ FCM MRD-                                                       | 28 (32.2%)      |
| NGS MRD- FCM MRD+                                                       | 08 (9.2%)       |
| NGS MRD- FCM MRD-                                                       | 42 (48.3%)      |

Table 1: Summary of clinical, laboratory and MRD characteristics of patients accrued in this study.
### Cumulative Incidence of Relapse

|                      | Cumulative Incidence of Relapse (CIR) | Non-Relapse Mortality (NRM) |
|----------------------|--------------------------------------|----------------------------|
|                      | HR 3-year CIR (95% CI)               | P-value                     |
| Post Induction NGS MRD |                                      |                            |
| MRD Negative         | 1 25.7 % (14.9 to 37.9)              | 0.003                       |
| MRD Positive         | 8.81 47.5 % (38.7 to 55.8)           | 0.03                        |
| Post Consolidation NGS MRD |                                  |                            |
| MRD Negative         | 1 36.2 % (25.1 to 47.4)              | 0.03                        |
| MRD Positive         | 4.79 52.8 % (37.8 to 65.7)           | 0.03                        |
| Post Induction FCM MRD |                                  |                            |
| MRD Negative         | 1 33.6 % (24.7 to 42.7)              | 0.003                       |
| MRD Positive         | 6.97 49.8 % (38.6 to 59.9)           | 0.003                       |
| Post Consolidation FCM MRD |                                |                            |
| MRD Negative         | 1 35.9 % (25.1 to 46.9)              | 0.003                       |
| MRD Positive         | 6.03 61.9 % (36.3 to 79.7)           | 0.03                        |

### Comparative Analysis of FCM MRD and NGS MRD (Post Induction)

|                      | Cumulative Incidence of Relapse (CIR) | Non-Relapse Mortality (NRM) |
|----------------------|--------------------------------------|----------------------------|
|                      | HR 3-year CIR (95% CI)               | P-value                     |
| NGS MRD- FCM MRD-    | 1 18.7 % (8.0 to 32.7)               | 0.003                       |
| NGS MRD+ FCM MRD-    | - 41.9 % (29.6 to 53.7)              | 0.003                       |
| NGS MRD- FCM MRD+    | - 41.2 % (17.5 to 63.6)              | 0.003                       |
| NGS MRD+ FCM MRD+    | 14.01 52.5 % (39.6 to 63.9)          | 0.003                       |

### Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation)

|                      | Cumulative Incidence of Relapse (CIR) | Non-Relapse Mortality (NRM) |
|----------------------|--------------------------------------|----------------------------|
|                      | HR 3-year CIR (95% CI)               | P-value                     |
| NGS MRD- FCM MRD-    | 1 24.8 % (12.6 to 39.0)              | 0.003                       |
| NGS MRD+ FCM MRD-    | - 50.0 % (29.9 to 67.1)              | 0.003                       |
| NGS MRD- FCM MRD+    | - 87.5 % (17.2 to 98.9)              | 0.003                       |
| NGS MRD+ FCM MRD+    | 18.81 55.5 % (14.7 to 83.5)          | 0.003                       |

### Dual Timepoint

|                      | Cumulative Incidence of Relapse (CIR) | Non-Relapse Mortality (NRM) |
|----------------------|--------------------------------------|----------------------------|
|                      | HR 3-year CIR (95% CI)               | P-value                     |
| MRD Negative         | 1 27.8 % (13.5 to 44.0)              | 0.04                        |
| MRD Positive         | 6.46 52.9 % (37.0 to 66.6)           | 0.04                        |

**Table 2:** Prognostic influence of NGS-MRD, FCM-MRD and a combination of these modalities on the cumulative incidence of relapse (CIR).
## Univariate Cox Analysis

|                          | Overall Survival (OS) | Relapse Free Survival (RFS) |
|--------------------------|-----------------------|----------------------------|
| **Post Induction FCM MRD** |                       |                            |
| MRD Negative             | 1                     | 0.0002                     |
| MRD Positive             | 2.1 (1.40 to 3.13)    | 1.8 (1.26 to 2.60)         |
| **Post Induction FCM MRD** |                       |                            |
| MRD Negative             | Mean OS: 58.0 months; 95% CI (51.2 to 64.8 months), Median OS: Not Reached | Mean OS: 63.2 months; 95% CI (54.4 to 72.0 months), Median OS: Not Reached |
| MRD Positive             | Mean OS: 36.3 months; 95% CI (29.6 to 43.0 months), Median OS: 18.4 months; 95% CI (15.1 to 33.9 months) | Mean OS: 32.9 months; 95% CI (28.9 to 36.9 months), Median OS: 27.0 months; 95% CI (17.9 to 42.8 months) |
| **Post Consolidation FCM MRD** |                       |                            |
| MRD Negative             | Mean OS: 58.0 months; 95% CI (51.2 to 64.8 months), Median OS: Not Reached | Mean OS: 63.2 months; 95% CI (54.4 to 72.0 months), Median OS: Not Reached |
| MRD Positive             | Mean OS: 36.3 months; 95% CI (29.6 to 43.0 months), Median OS: 18.4 months; 95% CI (15.1 to 33.9 months) | Mean OS: 32.9 months; 95% CI (28.9 to 36.9 months), Median OS: 27.0 months; 95% CI (17.9 to 42.8 months) |
| **Post Induction NGS MRD** |                       |                            |
| MRD Negative             | 1                     | 0.001                      |
| MRD Positive             | 2.2 (1.47 to 3.42)    | 2.3 (1.58 to 3.31)         |
| **Post Induction NGS MRD** |                       |                            |
| MRD Negative             | Mean OS: 63.2 months; 95% CI (54.4 to 72.0 months), Median OS: Not Reached | Mean OS: 54.9 months; 95% CI (45.7 to 64.2 months), Median OS: Not Reached |
| MRD Positive             | Mean OS: 32.9 months; 95% CI (28.9 to 36.9 months), Median OS: 27.0 months; 95% CI (17.9 to 42.8 months) | Mean OS: 26.1 months; 95% CI (22.4 to 29.6 months), Median OS: 16.7 months; 95% CI (14.5 to 22.4 months) |
| **Post Consolidation NGS MRD** |                       |                            |
| MRD Negative             | 1                     | 0.008                      |
| MRD Positive             | 0.001                 |                            |

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| MRD Positive | 1.9 (1.14 to 3.22) | 1.9 (1.18 to 3.06) |
|-------------|-------------------|-------------------|
| **Post Consolidation** | **Overall Survival (OS)** | **Relapse Free Survival (RFS)** |
| **NGS MRD** | **HR (95% CI)** | **P** | **HR (95% CI)** | **P** |
| MRD Negative | Mean OS: 55.5 months; 95% CI (47.2 to 63.7 months), Median OS: Not Reached | **0.008** | Mean OS: 46.9 months; 95% CI (38.8 to 54.9 months), Median OS: 33.0 months; 95% CI (21.4 to 42.0 months) | **0.004** |
| MRD Positive | Mean OS: 30.1 months; 95% CI (23.7 to 36.5 months), Median OS: 18.1 months; 95% CI (16.1 to 36.5 months) | | Mean OS: 24.6 months; 95% CI (18.7 to 30.6 months), Median OS: 15.4 months; 95% CI (11.2 to 17.5 months) | |
| **Dual Timepoint** | **Overall Survival (OS)** | **Relapse Free Survival (RFS)** |
| **NGS MRD** | **HR (95% CI)** | **P** | **HR (95% CI)** | **P** |
| MRD Negative | 1 | **0.03** | 1 | **0.01** |
| Either MRD Positive | 1.2 (0.66 to 2.28) | | 1.2 (0.71 to 2.21) | |
| MRD Positive | 2.5 (1.33 to 4.61) | | 2.5 (1.45 to 4.48) | |
| **Comparative Analysis of FCM MRD and NGS MRD (Post Induction)** | **Overall Survival (OS)** | **Relapse Free Survival (RFS)** |
| **NGS MRD** | **HR (95% CI)** | **P** | **HR (95% CI)** | **P** |
| NGS MRD- FCM MRD- | 1 | **0.0002** | 1 | **0.0001** |
| NGS MRD+ FCM MRD- | 1.6 (0.96 to 2.61) | | 1.4 (0.87 to 2.12) | |
| NGS MRD- FCM MRD+ | 1.3 (0.58 to 2.74) | | 1.3 (0.63 to 2.52) | |
| NGS MRD+ FCM MRD+ | 4.7 (2.71 to 8.00) | | 4.0 (2.51 to 6.47) | |

Comparative Analysis of FCM MRD and NGS MRD (Post Induction)

| NGS MRD- FCM MRD- | Mean OS: 71.5 months; 95% CI (62.4 to 80.7 months), Median OS: Not Reached | **0.0002** | Mean OS: 63.7 months; 95% CI (53.5 to 73.8 months), Median OS: Not Reached | **0.0001** |
| NGS MRD+ FCM MRD- | Mean OS: 37.7 months; 95% CI (31.8 to 43.5 months), Median OS: 42.8 months; 95% CI (19.8 to 42.8 months) | | Mean OS: 29.2 months; 95% CI (23.8 to 34.5 months), Median OS: 19.1 months; 95% CI (14.9 to 33.1 months) | |
Table 3: Difference in OS and RFS between FCM-MRD, NGS-MRD and comparative analysis between the two modalities at post induction and post consolidation timepoints. OS: Overall Survival, RFS: Relapse Free Survival, CI: confidence interval.

| NGS MRD - FCM MRD+ | Overall Survival (OS) | Relapse Free Survival (RFS) |
|---------------------|-----------------------|-----------------------------|
| Mean OS: 32.7 months; 95% CI (22.2 to 43.0 months), Median OS: 21.0 months; 95% CI (14.1 to 33.8 months) | Mean OS: 27.0 months; 95% CI (17.5 to 36.5 months), Median OS: 19.6 months; 95% CI (11.8 to 33.0 months) |

| Comparative Analysis of FCM MRD and NGS MRD (Post Induction) | Overall Survival (OS) | Relapse Free Survival (RFS) |
|---------------------------------------------------------------|-----------------------|-----------------------------|
| HR (95% CI) | P  | HR (95% CI) | P  |
| NGS MRD - FCM MRD+ | 1  | 0.02 | 1 | 0.001 |
| NGS MRD+ FCM MRD- | 2.2 (0.60 to 7.83) | 2.2 (0.64 to 7.65) |
| NGS MRD- FCM MRD+ | 2.0 (0.43 to 9.34) | 1.6 (0.35 to 7.02) |
| NGS MRD+ FCM MRD+ | 3.7 (1.07 to 13.0) | 4.1 (1.27 to 13.70) |

| Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation) | Overall Survival (OS) | Relapse Free Survival (RFS) |
|---------------------------------------------------------------|-----------------------|-----------------------------|
| HR (95% CI) | P  | HR (95% CI) | P  |
| NGS MRD - FCM MRD+ | Mean OS: 33.5 months; 95% CI (29.2 to 37.8 months), Median OS: Not Reached | Mean OS: 33.5 months; 95% CI (29.2 to 37.8 months), Median OS: Not Reached | 0.02 | 0.001 |
| NGS MRD+ FCM MRD- | Mean OS: 34.6 months; 95% CI (26.1 to 43.2 months), Median OS: 20.5 months; 95% CI (17.3 to 36.5 months) | Mean OS: 28.3 months; 95% CI (20.1 to 36.4 months), Median OS: 16.7 months; 95% CI (15.1 to 35.5 months) | |
| NGS MRD- FCM MRD+ | Mean OS: 43.9 months; 95% CI (20.7 to 367.1 months), Median OS: 21.0 months; 95% CI (11.7 to 33.9 months) | Mean OS: 24.9 months; 95% CI (8.3 to 41.5 months), Median OS: 14.1 months; 95% CI (10.1 to 32.6 months) | |
| NGS MRD+ FCM MRD+ | Mean OS: 18.2 months; 95% CI (8.04 to 28.4 months), Median OS: 12.6 months; 95% CI (5.60 to 27.0 months) | Mean OS: 13.4 months; 95% CI (5.1 to 21.8 months), Median OS: 9.6 months; 95% CI (4.7 to 15.4 months) | |

| Multivariate Cox Analysis |
|---------------------------|
| **Post Induction Timepoint** | **Overall Survival (OS)** | **Relapse Free Survival (RFS)** |
| HR (95% CI) | P  | HR (95% CI) | P  |
| NGS MRD Positive | 1.94 (1.15 to 3.27) | <0.000 | 2.05 (1.30 to 3.23) | <0.000 |
| FCM MRD Positive | 1.88 (1.21 to 2.84) | 1.61 (1.12 to 2.31) |
| **Post Consolidation Timepoint** | **Overall Survival (OS)** | **Relapse Free Survival (RFS)** |
| HR (95% CI) | P  | HR (95% CI) | P  |
| NGS MRD Positive | 1.82 (1.09 to 3.29) | 0.02 | 1.82 (1.06 to 3.11) | 0.002 |
| FCM MRD Positive | 2.03 (1.05 to 3.94) | 2.46 (1.34 to 4.47) |

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A. Post Induction NGS MRD - Cumulative Incidence of Relapse

Relapse $p=0.003$
NRM $p=0.33$

Number at risk
Group: NGS MRD Positive 139
Group: NGS MRD Negative 57

B. Post Consolidation NGS MRD - Cumulative Incidence of Relapse

Relapse $p=0.028$
NRM $p=0.532$

Number at risk
Group: NGS MRD Positive 52
Group: NGS MRD Negative 75

C. Post Induction NGS MRD - Overall Survival

Survival probability (%)

Number at risk
Group: NGS MRD Positive 139
Group: NGS MRD Negative 57

D. Post Consolidation NGS MRD - Overall Survival

Survival probability (%)

Number at risk
Group: NGS MRD Positive 52
Group: NGS MRD Negative 75

E. Post Induction NGS MRD - Relapse Free Survival

Survival probability (%)

Number at risk
Group: NGS MRD Positive 139
Group: NGS MRD Negative 57

F. Post Consolidation NGS MRD - Relapse Free Survival

Survival probability (%)

Number at risk
Group: NGS MRD Positive 52
Group: NGS MRD Negative 75
