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Minimal Residual Disease and Stem Cell Transplantation Outcomes

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Case 1:

A 52 year old female without comorbidity presented with AML. The karyotype was normal and molecular testing revealed mutations in *FLT3* (ITD, allelic ratio 1.0), *NPM1* and *IDH2*. She was enrolled into the NCRI AML19 study and received induction chemotherapy with daunorubicin, cytarabine and two doses of gemtuzumab. After second induction, *NPM1* mutant transcripts were detected (Figure 1) in both the blood and bone marrow aspirate and the patient was allocated to intensification with CPX-351 (Vxyeos). On regeneration, transcript levels had increased by ~1 log and the patient was withdrawn from the trial protocol and received salvage therapy with the FLAG-Ida regimen. On regeneration, an MRD assessment showed a further ~2 log increase in transcript levels and the patient developed painless enlarged lymph nodes in the left side of the neck. A fine needle aspirate confirmed extramedullary disease. Quizartinib was initiated at a dose of 20mg daily and was increased to 60mg daily over a period of three weeks. A PET-CT scan confirmed that the extramedullary disease was confined to the left side of the neck and the patient received 5 fractions of radiotherapy to sites of extramedullary disease and was scheduled for allograft from a matched

sibling. Pre-transplant MRD assessment showed a ~2 log reduction in disease-related transcripts and the patient proceeded to transplant. Conditioning was fludarabine (150mg/m^2 IV) and busulphan (12.8mg/kg IV) and GvHD prophylaxis was cyclosporin and mycophenolate. MRD assessment at D+30 was negative, weaning of immunosuppressive therapy was initiated and quizartinib was restarted. Further MRD assessments at D+60 and D+100 were negative. The patient developed chronic graft versus host disease affecting the skin and oral mucosa which was managed with extracorporeal photopheresis and topical steroids.

Case 2:

A 23 year-old male with severe obesity presented with AML. The karyotype was normal and molecular testing revealed a mutation in *NPM1* only. He did not enter a clinical trial and received induction therapy with daunorubicin and cytarabine and after second induction the bone marrow remained positive for disease related transcripts and the peripheral blood was not assessed. He received consolidation treatment with two cycles of high-dose cytarabine. MRD assessments performed on regeneration and 3 and 6 months after therapy showed persistence of *NPM1* mutant transcripts and at 9 months there was a >2 log rise in expression levels (Figure 2). This was confirmed on a second sample and molecular progression was diagnosed. Salvage therapy with FLAG-Ida was initiated and after the second cycle there had been a 2-3 log reduction in the level of MRD which remained positive. The patient proceeded with a haploidentical transplant with a parental donor, conditioning was fludarabine (160mg/m^2) and busulphan (12.8mg/kg) and GvHD prophylaxis was cyclophosphamide (50mg/kg on D+3 and +5), tacrolimus and mycophenolate. By D+100 all immunosuppression had been stopped and an MRD assessment showed persistent *NPM1* mutant transcripts. This was confirmed on a second sample and persistent molecular disease was diagnosed. Donor lymphocyte infusion at a dose of 10^5 cells / kg was administered. After four weeks, the level of *NPM1* mutated transcripts had decreased by 1 log and after eight weeks the patient tested MRD

negative. Molecular complete remission was maintained on several follow-up assessments up to 2 years post-transplant.

Introduction

With the improvement of therapeutic strategies in hematological malignancies, traditional morphological response definitions such as complete remission[1] are increasingly insufficient as they take no account of persistent malignant cells that are below the resolution of conventional techniques. Accurate measurement of this minimal or measurable residual disease (MRD) is crucial for more accurate prediction of relapse risk which in turn can be used to inform treatment intensity. Most of the methods for MRD detection are broadly similar across the hematological malignancies (Table 1). The role of MRD in acute lymphoblastic and chronic lymphocytic leukemia (ALL and CLL) and multiple myeloma (MM) has been summarized elsewhere[2–6]. The assessment of MRD in acute myeloid leukemia (AML) is in some ways more challenging due to molecular and phenotypic heterogeneity and MRD assessment is not yet completely standardized. This review focuses on the current status of MRD in AML and in particular the use of MRD for selecting patients for stem cell transplant, and for pre- and post-transplant interventions.

Risk classification at diagnosis

Detailed characterization of patients using a range of diagnostic techniques is essential for optimal treatment of acute myeloid leukemia (AML)[7]. Morphology remains the cornerstone of diagnosis and can distinguish different subtypes based on cellular and differentiation features[8], most obviously the M3 FAB subtype, which allows initiation of urgent therapy including ATRA[9]. Flow cytometry (FCM) is mandatory to confirm the diagnosis of AML and can be used to assign subtype based on specific cell surface markers that are expressed in particular phases during differentiation of hematopoietic cells

(i.e. cluster of differentiation or CD markers)[8]. Although there is no clear relationship between immunophenotype and outcome, multi-color flow cytometry (MFC) allows rapid determination of cell surface antigen expression status which is critical given the increasing availability of immunotherapies (e.g. gemtuzumab ozogamycin). MFC can also provide early clues to the underlying cytogenetic and molecular lesion, for example both APL and *NPM1* mutated AML have characteristic immunophenotypes[10,11]. Cytogenetic analysis provides the most powerful prognostic information in AML and a full karyotype , complemented by fluorescence in-situ hybridization (FISH), is needed to identify recurrent chromosome abnormalities which are strongly predictive of outcome and inform the ELN guidelines for risk classification[7]. Molecular genetic analysis provides further essential prognostic information[12], which is particularly informative in patients with a normal karyotype, for example in the patients described here. As well as risk group assignment, molecular analysis is increasingly important for selection of patients for targeted therapies including small molecule inhibitors of *FLT3*, *IDH1* and *IDH2*, which are now becoming widely available.

In most AML clinical studies, the majority of patients are classified as intermediate risk and despite detailed molecular analysis at diagnosis, outcome prediction remains imperfect. Therefore, particularly in this group, treatment emergent factors such as MRD may be particularly informative with respect to selection of appropriate consolidation therapy[13].

Measurable residual disease (MRD) assessment during treatment

The majority of AML patients treated with induction chemotherapy achieve a morphological complete remission (i.e. <5% blasts by morphology[1]). However, this is not a very sensitive method to accurately determine the residual load of leukemia cells[13,14]. More sophisticated methods for detection of residual disease can provide sensitivity orders of magnitude higher than that achieved by morphology. MRD status during treatment effectively provides a read out of multiple patient and leukemia specific characteristics, not all of which are well understood. There are several methods to

detect MRD in blood and bone marrow of AML patients during therapy which include MFC, amplification of leukemia specific transcripts by reverse-transcription quantitative PCR (RT-qPCR)[15–20] and more recently, detection of leukemia-specific mutations using next-generation sequencing[21–23]. Details of the applicability of the different methods are summarized in Table 1.

MRD status has been robustly correlated with risk of relapse in many independent large clinical trials. The prognostic value has been shown as early as after the first cycle of treatment. Many studies use MRD status after the second cycle of treatment to further refine risk classification since this time point has significant prognostic value and still allows enough time to initiate logistics for stem cell transplantation (SCT) when necessary.

Flow cytometric MRD can assess the efficacy of induction / consolidation or salvage on the dominant diagnostic (or relapse) leukemic blast populations by identifying leukemia associated immune phenotypes (LAIPs) on at least 10-20% of leukemic blasts in the diagnostic (or relapse) sample and monitoring these specific LAIPs during therapy[24] . Since the LAIP is based on aberrant expression of CD markers on the cell surface that are not present in healthy bone marrow, residual leukemia cells can also potentially be detected during therapy without knowledge of the diagnostic (or relapse) LAIPs. This is referred to as the "different from normal approach" (DfN) and can be applied when no diagnostic (or relapse) samples are available, for example when patients are referred to a transplant center without having previously been monitored there. Additionally the DfN approach can detect any phenotypically aberrant leukemia subpopulations that may have been minor or undetectable at diagnosis but have expanded during therapy, potentially due to clonal evolution such as that observed following transplants[25,26]. Therefore the recommendation of the ELN is to combine both flow cytometric-MRD methods instead of limiting CD marker panels to the LAIPs found at diagnosis[27]. In the recently completed HOVON 132 trial, all markers were measured so when clinical data become available, potential relevance of novel upcoming clones can be established. The DfN approach however can lead to false positive results and therefore reduced specificity particularly when there is

insufficient knowledge of progenitor phenotypes resulting from regeneration of the bone marrow after chemotherapy or transplant. Tracking the diagnostic or relapse LAIPs as biomarkers of the major pre-treatment leukemic populations may also be more appropriate when assessing the efficacy of novel treatment strategies to reduce the dominant leukemic clones. At present however, flow cytometric MRD assays are not yet standardized and validated enough to be used as a surrogate endpoint. Joint efforts of current MRD assessments implemented in the majority of clinical trials should facilitate this. Several groups are currently collaborating to standardize (where needed) and harmonize (where possible) the flow cytometric MRD methods[28]. Additionally, several studies are currently directed towards computational approaches to objectify, simplify and speed up MRD assessments[29].

Molecular MRD analysis provides a highly sensitive alternative to MFC in patients with a validated target for RT-qPCR i.e. patients with recurrent in-frame gene fusions or *NPM1* mutations (together accounting for ~60% of younger adults)[30]. For patients with *NPM1* mutated AML, molecular MRD analysis has demonstrated remarkable discrimination in a number of large prospective clinical trials and is the most powerful prognostic factor for these patients[31–33], identifying those who will benefit from upfront transplantation. Apart from *NPM1*, prognostic impact of *PML-RARA*[34], *RUNX1-RUNX1T1* and *CBFB-MYH11*[15,19] fusion transcripts during treatment and follow-up is well established and the relevance of transcript status for other rarer fusion genes is currently being investigated in large prospective trials such as NCRI AML19 and MyeChild01. Levels of expression of these leukemia-specific transcripts at diagnosis varies markedly and this impacts on assay sensitivity[35] for example, *NPM1* mutant transcripts are highly expressed at diagnosis, resulting in sensitivity of up to $1:10^7$ whereas assays to monitor *KMT2A* fusions may only afford sensitivity orders of magnitude lower[36]. Molecular markers considered unsuitable on their own to monitor MRD include *FLT3*, due to its relative instability at relapse[37].

Assays for *WT1* mutation and expression are generally no longer considered satisfactory for MRD measurement; while often upregulated or mutated at relapse, expression is insufficiently specific whereas mutation status is insufficiently sensitive to reliably detect relapse[38].

The most valuable addition to the current battery of assays[35] would be the ability to measure all AML-associated mutations with the sensitivity required for MRD analysis using NGS. Therefore, many initiatives are currently ongoing to improve sensitivity and reproducibility of NGS-MRD, these will need to be completed before NGS can be uniformly introduced for MRD directed treatment allocation for clinical trials and in routine practice[39]. In addition to harmonization of the MRD assays, the combination of MFC and molecular analyses needs further evaluation, since it has recently been shown that these methods are complementary[23].

All current AML MRD platforms would benefit from further standardization and from unified criteria for MRD-positivity. Considering the progress in this[40], it is anticipated that MRD might be accepted by the FDA as surrogate endpoint for treatment response in the near future.

Besides improvements to the methods for MRD detection, uncertainties remain regarding thresholds for MRD positivity across AML subtypes, treatment strategies and informative time-points as well as the utility of MRD by monitoring in peripheral blood samples.[41–43] This is currently still under investigation and should become apparent within the next few years.

Use of MRD to inform pre-transplant management

Using a combination of comprehensive diagnostic profiling and MRD status, patients who are likely to be cured with chemotherapy alone and who can be safely monitored in CR1 can be discriminated from those who will benefit from upfront transplantation[31,32]. Although there are currently no

randomized data to support this approach, based on currently available non-randomized data this is approach has generally been adopted and is implemented in a number of large clinical trials (see for examples Table 2). Ongoing prospective studies will provide further information to guide decision making and in particular the UK NCRI "monitor versus no monitor" randomization will report next year and will be informative regarding the benefit of MRD-directed consolidation therapy.

MRD positivity is thus a marker for selection of patients for allo-SCT. However, it has been shown that the risk of post-transplant relapse is lowest in MRD negative patients[21,44–48] the question is therefore whether MRD-positive patients would benefit from intensification of treatment before SCT in order to convert to MRD negativity. Although superficially attractive, the potentially drug resistant MRD positive bone marrow may not easily become MRD negative, and this could lead to additional toxicity without benefit, which could potentially compromise long-term survival. There are currently no prospective data to inform such decisions, however ongoing studies such as UK NCRI COSI may be informative in this regard. In the absence of such data, individual patient management can be informed by sequential MRD measurement, and this is potentially an opportunity for use of novel relatively non-toxic targeted agents for the elimination of drug-resistant cell reservoirs prior to transplant.

It also remains unclear whether augmented conditioning can improve the outcome for patients who are MRD positive before transplant; retrospective studies comparing myeloablative and reduced intensity conditioning for these patients have yielded conflicting results[48,49]. A retrospective study has suggested an advantage for MRD positive patients who receive umbilical cord blood transplants[50] although this remains controversial[51] and importantly this approach removes the option of using donor lymphocyte infusion after transplant. Prospective studies to define the optimal management for these patients are urgently required.

After stem cell transplantation

For patients who remain persistently MRD positive after transplant, relapse is inevitable without further intervention. Treatment options depend on the clinical situation and time from transplant. Data to inform management of MRD positivity in the post-transplant setting are fairly sparse and indeed there is only 1 published prospective study to date. The RELAZA2 study demonstrated that azacitidine can prevent or delay hematological relapse in a proportion of patients with ongoing MRD positivity after treatment and this may be more effective in patients who have been transplanted, potentially indicating an immunological effect of this treatment[52]. Post-transplant MRD status may also be useful to plan withdrawal of immunosuppression; after this has been tapered persistent MRD positivity should prompt consideration of donor lymphocyte infusion (DLI). A number of studies have reported very good success rates in this setting[53,54]. It appears that DLI can convert patients to MRD negativity and long-term disease-free survival rates of 80-100% have been reported in these small retrospective studies. Certainly, the success rates for the use of DLI in the MRD setting appear significantly higher than when DLI is used at hematological relapse[55,56]. Azacitidine in combination with DLI has also been reported to be effective in this situation[56] and could be considered if DLI alone fails or the level of MRD is very high. DLI is also reported to have activity in post-transplant MRD positivity in other hematological malignancies such as ALL[57] and CML[58]. Based on these effective options for persistent or re-emergent MRD positivity, it is therefore recommended to perform serial MRD monitoring after transplant[59] particularly for patients with a sensitive molecular marker where impending relapse can be predicted months in advance, providing a time window for intervention[36]. We suggest that post-transplant surveillance is continued for at least 1-2 years after transplant as this period is associated with the highest risk of relapse. A proportion of patients will be unable to receive DLI due either to the donor status or the presence of graft-versus-host disease (GVHD) and some of these patients will either not respond to, or be unable to tolerate azacitidine. For these patients, management is more challenging. In the absence of evidence, novel agents could be considered for example, FLT3 or BCL2 inhibitors. Further prospective clinical trials that utilize MRD directed post-transplant interventions are now required. Some studies that are in progress should

give further insights in the near future; in this respect HOVON recently initiated a prospective phase III trial to determine the efficacy of panobinostat maintenance therapy versus standard of care after allo-SCT, which includes MRD assessment before and at several time points after SCT.

Case discussions

Case 1. This patient was ELN intermediate risk based on molecular and cytogenetic features at diagnosis however failure to achieve MRD negativity in the peripheral blood after second induction is associated with a very high risk of relapse[20]. For patients with *FLT3* ITD, pre-transplant MRD positivity is associated with a high risk of post transplant relapse (Dillon EHA abstract 2019) therefore intensification was attempted but was unsuccessful and resulted in molecular and extramedullary progression indicating chemorefractory disease. In this situation, novel targeted agents may be useful to reduce disease burden prior to, and to sustain remission after transplantation, as illustrated by the effect of quizartinib in this case.

Case 2. This patient was ELN favorable risk based on presentation at presentation, however at the end of treatment he remained persistently MRD positive indicating impending relapse. Due to a high risk of treatment related mortality, intervention was only undertaken after a significant rise in MRD levels. Although molecular complete remission was not regained after salvage therapy, patients who are *FLT3* ITD negative with low levels of *NPM1* mutant transcripts have a generally good outcome after transplant (Dillon EHA abstract 2019). Despite this, he remained MRD positive after withdrawal of immunosuppression. Donor lymphocyte infusion resulted in a rapid clearance of residual disease providing an example of the graft-versus-leukemia effect for eliminating MRD after transplant.

Future perspectives

Risk classification based on diagnostic cytogenetic and molecular characterization and refined by MRD assessment at early time points is critical for proper clinical decision making in AML. MRD provides the most powerful predictor of outcome in intermediate risk AML and its measurement both in clinical trials and everyday practice is strongly recommened[60]. MRD data can be exploited to tailor treatment intensity according to response and as shown here can serve as a trigger for application of novel therapies (such as FLT3 inhibitors, IDH1/2 inhibitors, splicing modulators or epigenetic modifiers)[40,61].

Although current MRD platforms provide very powerful prognostic information, further improvement is possible through standardization of assays and accumulation of larger data sets. These collaborative efforts will lead to a clearer definition of the optimum time points, sample sources and thresholds for clinical decision making. With regard to MFC MRD, a deeper understanding of the characteristics of relapse initiating cells may further improve prognostic value [62,63]. A recently designed one tube assay to assess leukemia stem cell (LSC) load is used in HOVON studies, which takes clonal evolution into account and which is associated with clinical outcome both at diagnosis and during therapy[64]. In addition, it has been shown that combining molecular and MFC data also aids in distinguishing a very poor risk group who may benefit from intensified treatment strategies[63].

It is currently being investigated whether MRD can be used as surrogate endpoint in clinical trials to assess effectivity of treatment. This might considerably improve the development of new treatment options for the patient subgroups most likely to benefit from the intervention[65].

Finally, although few studies have yet been published regarding the use of MRD results to optimize peri-transplant management, this now appears to be an extremely promising field of study with the potential to test MRD-triggered interventions which could have a major impact on rate of post-

transplant relapse by focusing interventions on those patients at highest risk. Results of these emerging studies are eagerly anticipated.

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Legends to figures

Figure 1. Examples of molecular MRD monitoring of *NPM1* mutations before and after therapy.

A) Example of MRD guided pre-transplant management. See text for full case history. DA: daunorubicin and cytarabine. GO: gemtuzumab ozogamycin. FLAG-Ida: fludarabine, cytarabine, granulocyte colony stimulating factor and idarubicin. DXT: radiotherapy. FB4 Sib Allo: fludarabine and busulphan conditioned sibling allograft.

B) Example of MRD guided therapy and peri-transplant management. See text for full case history. DA: daunorubicin and cytarabine. HDAC: high dose cytarabine. FLAG-Ida: fludarabine, cytarabine, granulocyte colony stimulating factor and idarubicin. FBC Haplo: fludarabine and busulphan conditioned haplo-identical allograft with and post-transplant cyclophosphamide. DLI: donor lymphocyte infusion.

Table 1. Characteristics of the different currently used MRD methods

MRD Method	Technique	Markers	Patient material	Leukemia type	Sensitivity	Advantage	Disadvantage	Relevant references
Molecular								
Rearrangements	RT-Q-PCR ddPCR	IGH TCR	DNA	ALL Myeloma CLL	10^{-4} - 10^{-5}	Patient specific Standardized method	Requires diagnosis sample Clonal evolution missed	4,5,66,67
	NGS Including ClonoSEQ		DNA	ALL Myeloma CLL	10^{-3} - 10^{-6}	Does not require construction of patient-specific reagents, May detect clone shifts	Expensive	68
Fusion genes	RT-Q-PCR	BCR/ABL AML/ETO CBFB-MYH11 PML/RARa RUNX1/RUNX1T1 BCL3 translocations Less common fusions	RNA	ALL, CML AML AML APL ALL, AML CLL	10^{-4} - 10^{-5}	Standardized primers	Applicable in a limited number of patients	34,35,58,69
Mutations	RT-Q-PCR	NPM1	RNA	AML	10^{-4} - 10^{-7}	Extremely sensitive	Applicable in a limited number of patients (~30%) Clonal evolution missed	31,70
	NGS	Mutation panels	DNA	AML	10^{-3} - 10^{-5}	Applicable in many patients May detects clone shift	Requires further development for assay harmonization /standardization Insensitive without Error correction	39
Over expression	RT-Q-PCR	WT1	RNA	AML	10^{-5}	Sensitive	Applicable in a limited number of patients	38,53,71,72
Immunophenotypic								

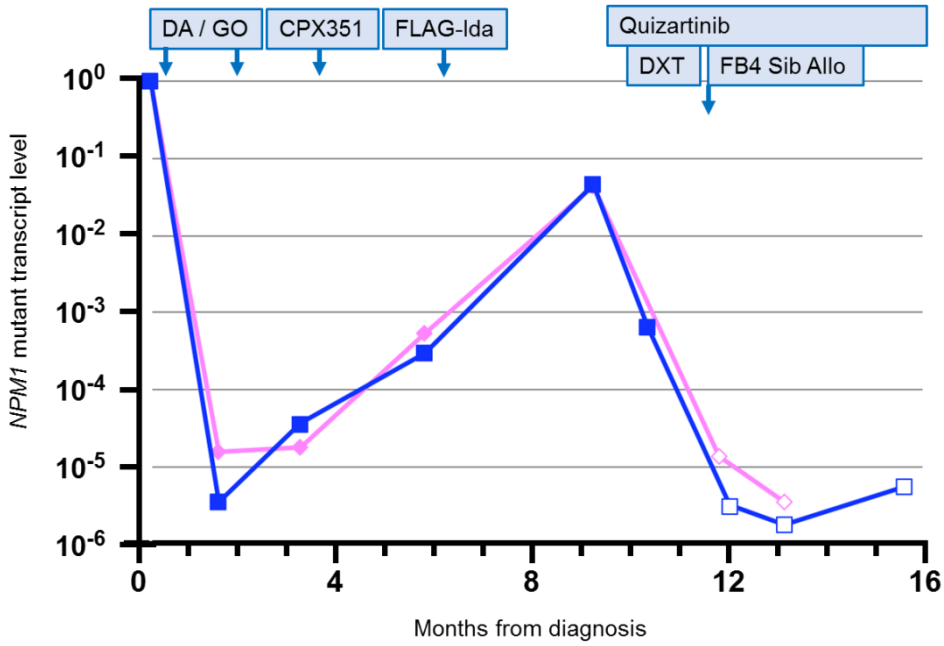
Leukemia/myeloma Associated Immune Phenotype	Distinctive antibody panels	Diagnosis and follow-up cells	AML ALL CLL Myeloma	10^{-4}	More informative with diagnosis sample Almost all patients Relatively fast	Clonal evolution missed Immunophenotypic profiles required	[73–75]
Different from Normal	Distinctive antibody panels	Follow-up cells only	AML	10^{-4}	Almost all patients Relatively fast	Extensive knowledge of normal and regenerating bone marrow required	[76]
Leukemia stem cells	CD34+/CD38- Plus distinctive antibody panels	Diagnosis and follow-up cells	AML	10^{-6}	High prognostic relevance to identify a poor risk group	Only CD34+ leukemia Large number of cells required	[63]

Table 2: Clinical validation of MRD before and after transplant

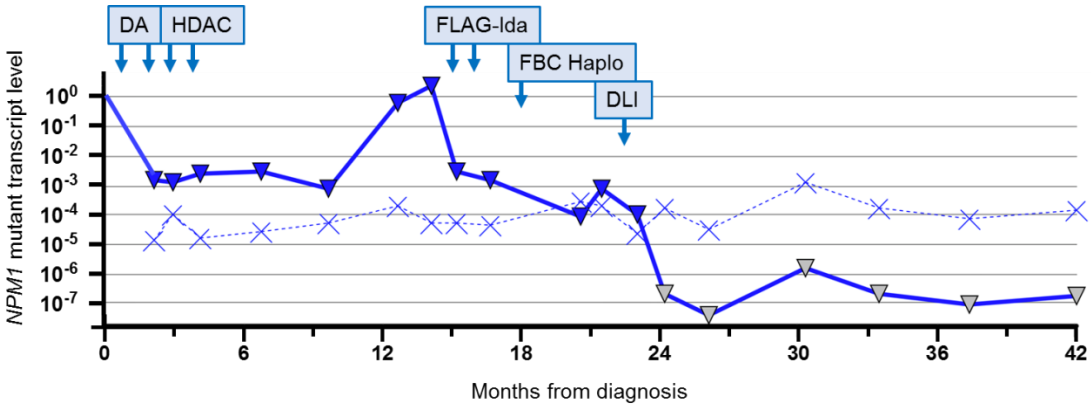
Study	Patients (N)	Patient group	MRD method	Time point	Threshold	Outcome	Ref.
Meta-analysis	1,431	Adult Non-APL	MFC and molecular	Pre-transplant	0.1 % for FCM	Prognostic for relapse and survival	[77]
EORTC/GIMEMA	81	MRD+ Eligible for SCT	MFC	After consolidation	0.035%	No contra-indication for allo-SCT in MRD+ patients	[78]
IRCCS Genoa	224	Transplanted in CR1/CR2	MFC and *WT1*	Pre-transplant	2.5×10^{-4} 250 copies/Abl × 10^4	Prognostic value of MRD for pre- and post-transplant interventions	[71]
Toronto/Korea	104	Adult AML eligible for transplant	NGS	Diagnosis Pre- and post-transplant (day 21)	$VAF^{0.2\%}$-post-HCT^{D21}	Good prediction of relapse	[79]
Seattle	279	Adult	MFC	Pre- and post-transplant	0.1 %	MRD+ poor prognosis irrespective of myelo-ablative conditioning	[80]
Hannover	116	> 18 years	NGS	In CR before transplant	Error corrected	Predictive for relapse and survival Refine SCT	[21]
RELAZA2	60	MRD+ > 18 years	*NPM1* Fusion gene MFC	After induction/consolidation Pre-emptive	Depending on test used	Predictive for relapse	[52]

Figure 1.

A)


DA / GO
CPX351
FLAG-Ida
Quizartinib
DXT
FB4 Sib Allo
NPM1 mutant transcript level
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
0
4
8
12
16
Months from diagnosis

B)


DA
HDAC
FLAG-Ida
FBC Haplo
DLI
NPM1 mutant transcript level
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
0
6
12
18
24
30
36
42
Months from diagnosis