

MRD evaluation of AML in clinical practice

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1 **MRD EVALUATION OF AML IN CLINICAL PRACTICE: ARE WE THERE YET?**

2

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4

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1 **Abstract**

2 MRD technologies increase our ability to measure response in AML beyond the limitations of morphology.
3 When applied in clinical trials, molecular and immunophenotypic MRD assays have improved prognostic
4 precision, providing a strong rationale for their use to guide treatment as well as measure its effectiveness.
5 Initiatives such as from the European Leukemia Network (ELN) now provide a collaborative knowledge-based
6 framework for selection and implementation of MRD assays most appropriate for defined genetic subgroups.
7 For patients with mutated-*NPM1* AML, quantitative polymerase chain reaction (qPCR) monitoring of mutated-
8 *NPM1* transcripts post-induction and sequentially after treatment has emerged as a highly sensitive and
9 specific tool to predict relapse and potential benefit from allogeneic transplant. Flow cytometric MRD after
10 induction is prognostic across genetic risk groups and can identify those patients in the wild type *NPM1*
11 intermediate AML subgroup with a very high risk of relapse. In parallel to this data, advances in genetic
12 profiling have not only extended understanding of the etiology and the complex dynamic clonal nature of AML
13 but also created the opportunity for MRD monitoring using next generation sequencing (NGS). NGS AML MRD
14 detection can stratify outcomes and has potential utility in the peri-allogeneic transplant setting. There
15 however remain challenges inherent in the NGS approach of multiplex quantification of mutations to track
16 AML MRD. While further development of this methodology together with orthogonal testing will clarify its
17 relevance for routine clinical use, particularly for patients lacking a qPCR genetic target, established validated
18 MRD assays can already provide information to direct clinical practice.

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1 Case Presentation

2 *A 62yr old man with a normal WBC was diagnosed with cytogenetically normal AML and subsequent molecular*
3 *studies for ELN 2017 good and adverse risk mutations including mutated-NPM1 were negative. He was fit*
4 *enough to be treated with two courses of induction chemotherapy (standard UK NCRI AML treatment). MRD*
5 *response was assessed by flow cytometry. He achieved a CR but was MRD positive by flow cytometry (at 0.12%)*
6 *after the second cycle. Due to deterioration in performance status, his co-morbidities and preference, further*
7 *treatment options did not include transplant. He was known to have an IDH1 R132 and DNMT3A mutations*
8 *and was considered for novel regimens or azacytidine. Should he be monitored if he has further treatment*
9 *and how?*

10 Introduction

11 AML is a disease that consists of multiple genotypes with not only inter- but also intra-leukemic heterogeneity
12 that is influenced by treatment. It is therefore unsurprising that a range of MRD biomarkers and assay
13 platforms have been generated and are relevant to enable personalised AML MRD monitoring.¹ This however
14 contributes to the challenge that MRD testing in 2019 continues to present, i.e. deciphering how, when and
15 even whether it should apply to individual patients. From a survey in 2016-17, 69% of USA leukemia physicians
16 reported routine use of AML MRD, most commonly flow cytometry (MFC) followed by PCR for mutated-NPM1
17 but only 21% however had implemented serial PCR for longer term monitoring.² For those not incorporating
18 MRD testing into patient management, cited reasons were lack of resources and uncertainty regarding the use
19 of the results. Of note was the extent to which MRD directed decision-making varied in hypothetical clinical
20 scenarios. When asked about MRD test positivity post induction in a patient being considered for transplant,
21 responses were divided equally between recommending against a transplant vs additional chemotherapy vs
22 changing conditioning regimen. This variability reflects the paucity of high-quality evidence currently available
23 to inform such AML MRD based decisions. It also highlights the need for a more nuanced approach to MRD
24 test interpretation, taking into account that the available evidence from MRD testing differs between AML
25 subtypes. For example, in younger patients with mutated-NPM1 AML, post induction MRD in the blood by
26 qPCR predicts outcome independently of other mutations including FLT3³ and benefit from transplant (HR of
27 0.25 for overall survival).⁴ However in wild type NPM1 intermediate risk AML (as for patient in above clinical
28 case) or in older patients, although MRD positivity is also prognostic of poor outcomes,⁵⁻⁷ the effect of
29 additional intensified chemotherapy or transplant requires further evaluation.

30 It is perhaps easy to forget with the natural enthusiasm for the prospects of novel MRD technologies that the
31 well tested MRD platforms, namely qPCR and flow cytometry can provide sophisticated information for MRD
32 levels in most patients, identifying across all AML risk groups those most likely to relapse with current
33 treatment schedules. Clinical trials have assimilated these methods in real-time, testing the effect of

1 intensification, early intervention and novel approaches to improve prognosis, or evaluating MRD as an early
2 surrogate for therapeutic efficacy. Outcome data from these, together with amalgamated experience in
3 routine clinical practice step-wise informs how to deal with the results of MRD tests to best help AML patients.
4 In parallel, technical evolution and insights from leukemia biology together with collaborative efforts for
5 standardisation continuously progresses MRD detection. This review focuses on the more recent information
6 from MRD testing, including assay limitations and prospects, that with consensus recommendations can guide
7 current implementation.

8 **Recent Guidance from ELN and FDA**

9 Since 2018, guidance for the expanding application of MRD in AML has been proposed by two organisations.
10 The ELN through international collaboration published a consensus document from expert AML MRD
11 laboratories with recommendations for both flow cytometric and molecular MRD assays in clinical practice ⁷.
12 This addressed some of the key factors for MRD measurement including sampling, recommended approaches,
13 time-points and thresholds of positivity. Perhaps as importantly the document highlighted areas requiring
14 further work programs to harmonise and progress assays for which efforts by participating laboratories are
15 ongoing. The FDA after a series of workshops over several years, have invited comments on their draft
16 guidance for the use of MRD as a biomarker in regulatory submissions for haematological cancers.⁸ In this the
17 FDA list criteria for MRD assays, for example that reporting MRD negative results requires information on
18 detection limits and, perhaps more debatable for certain assays, sensitivity should be at least a log below the
19 cut-off for MRD positivity. Referring to AML, the guidance states that each selected MRD marker(s) should
20 reflect the leukemia and not underlying clonal hematopoiesis (false positives). Additionally, data should be
21 provided for the false negative rate that might result from relapse from a marker negative clone. The FDA
22 guidance comments that MRD could be used to stratify or enrich trial populations, a strategy applied for the
23 RELAZA2 trial ⁹ but when MRD is a trial endpoint any patient with missed MRD samples should be categorised
24 as unresponsive in the analysis.

25

26 **MRD Assay Considerations**

27 As highlighted by the ELN and FDA papers, there are several important factors to consider when selecting the
28 most appropriate of the current MRD assays and then interpreting results to guide clinical decisions for
29 individual patients. These comprise the 'S' factors; ¹⁰ specificity, sensitivity of the MRD marker(s) and its
30 stability during AML progression. Further considerations are the context of AML subtype and if known
31 associated relapse kinetics, sample, stage taken and extent of assay standardisation. Another 'S', price (\$), is
32 a practical inclusion to the list. Finally, the evidence for correlation with outcome in clinical studies is critical.

1 Preferably this clinical validation would include MRD testing in real time as in clinical practice and be
2 reproducible by other centers.

3 **Specificity: True vs False MRD Positives**

4 Perfect specificity for an assay has been defined as the ability of a method to assess unequivocally the analyte
5 in the presence of other components that are expected to be present. For AML MRD the analyte is a biomarker
6 of acute leukemia, however whether the presence of this analyte results in relapse will depend on the test
7 time point, subsequent interventions and the competing risk of death from other causes. The 'other
8 components' consist of any cells / genetic material that are not acute leukemia as well as artefactual assay
9 background (such as non-specific antibody binding and autofluorescence in flow cytometry or non-specific
10 primer binding in PCR). For example, MRD assays by flow cytometry and *WT1* RNA overexpression by qPCR
11 (both applicable in the majority of all AML patients) have intermediate specificity due mainly to analyte-type
12 signals (aberrant immunophenotypes or *WT1* transcription activity) from normal cells especially in
13 regenerating marrows.

14 Acute Promyelocytic Leukemia (APL) is the paradigm for MRD monitoring in AML. The APL genetic driver from
15 *PML-RARA* fusion (>95% of APLs) also provides the molecular MRD target of *PML-RARA* transcripts; ¹¹ this is
16 highly specific as only treatment resistant APL cells including those that may in time be relapse initiating with
17 have this MRD marker. Similarly, other AML subtypes including CBF (*RUNX1-RUNX1T1*, *CBFB-MYH11* fusions)
18 and mutated-*NPM1* AMLs have main genetic drivers that generate specific molecular MRD targets. These
19 analytes are typically representative of the residual AML independently of co-existing mutations but
20 additionally, are stable markers during AML progression and thus strong predictors of relapse by longer term
21 qPCR MRD monitoring.^{3,7,12,13} However even for these, genetic evolution is a consideration for interpretation.
22 ¹⁴ Mutated -*NPM1* AMLs may infrequently relapse as wild type *NPM1* albeit usually later (median of 43 months
23 in a recent report) with associated co-existing and persisting clonal hematopoietic mutations including
24 *DNMT3A*.¹⁵

25 It is now apparent that CHIP associated mutations including the *DNMT3A* point mutation in the above clinical
26 case, although frequent and stable in AML, can persist post treatment at high levels despite longer-term
27 disease free survival.¹⁶⁻¹⁹ These are therefore insufficiently specific MRD markers for AML relapse,
28 substantiating the FDA statement that marker selected to assess MRD should "not reflect underlying clonal
29 hematopoiesis". This does not preclude however the future utility of MRD assays tracking certain of these
30 mutations as has already been tested for *IDH* mutations,^{20,21} particularly for the efficacy evaluation of
31 appropriate targeted therapies such as *IDH1/2* inhibitors,^{22,23} a treatment option in our clinical case.
32 Moreover, clonal hematopoietic mutations that cooperate in the progression to acute leukemia could
33 conceivably be monitored in the future as specific bio-markers for pre-leukemic activity of novel agents. Of
34 particular interest in this regard are CHIP mutations in DNA damage response genes such as *TP53* and *PPM1D*

1 that are enriched after cytotoxic therapy and associated with an increased risk of developing leukemia.²⁴ Allelic
2 burdens of *DNMT3A*, *TET2* and *ASXL1* mutations, including as circulating tumour DNA, may also have potential
3 utility post allogenic stem cell transplantation to track ablation of patient clonal hematopoiesis.^{25,26}

4 **Sensitivity: True vs False MRD Negatives**

5 With the prerequisite of an adequate, representative sample, sensitivity for MRD assay targets ranges from
6 10^{-2} (current NGS mutation profiling) to 10^{-5} to 10^{-6} . The latter is achieved by the established qPCR assays for
7 which the target has high transcript expression (such as *NPM1* exon 12 insertion mutations). Current MRD
8 assays cannot test for AML eradication but reduction of MRD target below the lower limit of detection
9 /quantification at treatment time-points (CR_{MRD}- by ELN criteria²⁷) indicates AML clearance of up to 4 logs
10 greater depth than morphology. Not surprisingly, this significantly improves prognostic discrimination in
11 patient cohorts for survival as well as relapse. Younger adults in CR/CRi after their first course of induction in
12 the NCRI AML17 trial had a 5 year survival of 52%; if also categorised as CR_{MRD}- by flow cytometry (sensitivity
13 of 10^{-4}) survival increased to 63% overall and 70% when excluding poor risk patients.⁵ Observed clinical false
14 negatives (by relapse frequency) from single MRD assessment time points are 20-30% in good or intermediate
15 risk patients in multiple MRD studies, even for the most sensitive MRD assays.^{3,28} Monitoring at several time-
16 points, such as after each chemotherapy cycle and when applicable sequentially from end of treatment (such
17 as in Figure 1B/C schema), captures more information and consequently reduces false negatives.^{5,7,12,13}

18

19 **Sample Considerations for MRD Interpretation**

20 It is a sine qua non that a good quality bone marrow is most likely to be representative of residual AML for the
21 majority of patients without extramedullary disease. Hence, the present recommendation of a first pull bone
22 marrow for almost all AML MRD assays to reduce false negatives from suboptimal sensitivity. Leukocyte
23 numbers, cell viability (affected by transit time), hemodilution and hypoplasia all input into limiting the
24 sensitivity / lower level of quantification for any bone marrow sample, independently of the theoretical assay
25 sensitivity. Flow cytometric assays can assess and should incorporate information on all these factors in the
26 MRD report. MRD negativity from an antibody combination testing 250,000 leukocytes will only reach a
27 detection limit of 0.01% (with a 20% CV). Molecular assays include housekeeping gene (*ABL*) copies as control
28 for nucleated cell numbers but current assays cannot differentiate hemodilution and cell type unless
29 performed on pre-sorted cells. Single cell assays in the future may be able to combine phenotype as well as
30 RNA expression and mutation profile²⁹ but these currently are very expensive and can only evaluate low cell
31 numbers.

32 **Is Blood informative for AML MRD?**

33 Despite blood (PB) providing lower sensitivity than BM (1 log less for mutated-*NPM1*³), measuring mutated-
34 *NPM1* transcripts in PB post induction is highly prognostic for the mutated-*NPM1* subgroup.^{3,4} Interestingly

1 the 'false negative' relapse risk is not increased for MRD negativity in blood versus BM. ⁴ This implies that in
2 the ~25% of mutated-*NPM1* patients with only BM positivity post induction, either the MRD is at a level
3 concomitant with clearance by consolidation or is from non-leukemic initiating, more mature BM cells. Bone
4 marrow is recommended however for maximal sensitivity during later sequential monitoring since bone
5 marrow positivity by qPCR targets usually precedes that of blood providing an increased time window for any
6 interventions. ⁷
7 Reduced sensitivity may be compensated by a differential increase in specificity when testing blood for flow
8 cytometric MRD ³⁰ (due to fewer normal progenitors/precursors and therefore less 'noise'), as observed when
9 measuring *WT1* levels.³¹ Relapse free ³² and overall survival ^{30,33} appear significantly better for patients with
10 MRD negative blood samples post induction and consolidation in heterogeneous smaller cohorts. These
11 encouraging results merit further evaluation in older and intermediate / poor risk AML patients, most of whom
12 lack sensitive qPCR molecular markers. There is also preliminary evidence that PB clonal profiles may be
13 representative of BM during treatment³⁴ as well as at diagnosis, at least for higher frequency mutations. In a
14 small series of decitabine treated patients NGS measurements of VAF frequencies (at >5%) for mutation
15 profiles in PBs with <60% lymphocytes correlated well with paired BM results.³⁵

16

17 **How we would monitor: Core-binding-factor AMLs and mutated-*NPM1* AMLs**

18 Patients with CBF or mutated-*NPM1* AML, by the nature of their leukemic-specific fusion transcripts (*RUNX1-*
19 *RUNX1T1*, *CBFB-MYH11*) or insertion /deletion (InDel) mutations (*NPM1*), can be monitored by qPCR MRD
20 assays that combine high sensitivity with specificity and are extensively validated. Monitoring schedules (such
21 as in [Figure 1](#)) and reporting of results for comparability should be based on ELN guidelines ⁷ although these
22 are only evidence based for younger patients.³⁶ After treatment completion qPCR MRD assessments are
23 recommended for at least 2 years at 3 monthly intervals when prior result was negative or low copy numbers.
24 Average kinetics from molecular relapse to clinical relapse range from more than 3-4 months for *CBFB-MYH11*
25 to 2- 3 months for *RUNX1-RUNX1T1* or mutated-*NPM1* when *FLT3 ITD* positive.³⁷ There is no new data as yet
26 to support a survival benefit from pre-emptive intensification in CBF AMLs despite the clear association
27 between MRD status (post induction/consolidation or off-treatment) and clinical progression for younger
28 patients.^{7,38} Published studies indicate that allogeneic transplantation can be avoided in *FLT3 ITD* positive
29 mutated-*NPM1* younger adults when these patients remain in complete molecular first remission following
30 standard induction ⁷ but it is as yet uncertain whether concomitant treatment by *FLT3* inhibitors alters relapse
31 kinetics and therefore optimal off-treatment sampling intervals.

32 qPCR assays have been developed for other fusion transcripts such as from rearrangements involving *KMT2A*
33 (*MLL*), *NUP98* and *NUP214* rearrangements. Although these can track response ([Figure 1](#)), there is insufficient

1 data to provide guidance for their use hence it is recommended post induction to also assess response by flow
2 cytometric MRD.⁷

3 **What about patients with *FLT3* internal tandem duplications?**

4 Given the frequent prevalence in AML patients, the high probability and rapid kinetics of relapse, and the
5 continued development of effective targeted therapy there is great interest in monitoring of AML clones
6 containing *FLT3* internal tandem duplications (*FLT3*-ITD). *FLT3*-ITD mutations represent late events in
7 leukemic development and hence are not always detectable at relapse, particularly after targeted therapy,
8 often being “replaced” by another signaling variant (eg: *RAS*, *Kit* or a different *FLT3* variant).^{39,40} Despite the
9 limitation of potential false negative tests, *FLT3*-ITD MRD testing has utility as a positive result in an AML
10 patient otherwise thought to be in remission is highly suggestive of MRD and is associated with a high
11 likelihood of relapse often with a short lead-time.⁴¹⁻⁴³ *FLT3*-ITD mutations consists of nucleotide sequence
12 inserts of variable length and location between patients, making one universal approach to low-level
13 quantitative assessment by conventional PCR and bioinformatic mapping of data from NGS challenging. This
14 technical constraint has however been mitigated both by novel PCR methods⁴⁴ and sequencing
15 approaches.^{39,41,43,45} In approximately 50% of adult AML cases *FLT3*-ITD mutations will co-occur with a more
16 stable AML MRD marker such as mutated-*NPM1* or t15:17⁴⁶; it is therefore recommended to also track such
17 markers⁷ particularly for patients receiving *FLT3*-ITD directed therapy. For those without a second mutation
18 in addition to *FLT3*-ITD to track, the expression of *WT1* is known to be elevated in these patients^{31,47}
19 although the use of this AML MRD target for routine testing remains controversial. Flow cytometric MRD
20 detection is recommended for response evaluation and monitoring for those patients with AML that cannot
21 be tracked by a validated qPCR MRD assay.

22
23

24 **Newer Molecular Technologies in AML MRD Detection**

25 The past decade of focus on cancer genomics has elucidated the genetic basis of AML and provided a wide
26 range of molecular targets suitable not only for new drug development but also potentially for leukemic
27 disease burden tracking. While such approaches are highly publishable several limitations prevent direct
28 translation to the clinic at present. Digital PCR (dPCR), developed in the 1990s, allows absolute quantification
29 of abnormal DNA sequence by, unlike conventional quantitative PCR (qPCR, [Figure 2A](#)), partitioning each
30 template molecule for the PCR reaction into an individual compartment ([Figure 2B](#)). Advantages of this
31 technology include high sensitivity due to low background error rate, highly accurate quantification due to
32 elimination of template competition, limited bioinformatic requirements and a rapid “sample-to-result” turn-
33 around time.²⁸ Disadvantages include the need to develop and validate assays for each individual target
34 sequence, limited multiplexing capacity and the inability to perform discovery on serial samples (eg: to detect

1 selection of an independent *TP53* mutated clone during therapy) making it unsuitable to detect relapses
2 associated with clonal evolution. This technology is likely to be most useful for orthogonal validation of other
3 technology and for tracking of common, highly conserved, “hot-spot” variants such as those seen in *NPM1*
4 and *IDH1/IDH2* genes.^{20,48}

5
6 DNA sequencing, typically consisting of “panels” covering the small regions of the genome known to be often
7 somatically mutated in AML patients has increasingly become part of the standard initial diagnostic workup
8 given the importance of such mutations in both risk stratification and therapy selection²⁷ (Figure 2C). Given
9 detected mutations are thought to originate from the leukemic clone and may persist in remission at those at
10 increased risk of relapse¹⁷ it may be tempting to use the same NGS test for AML MRD detection.
11 Unfortunately, as suggested by the absence of recommendations on NGS in the current ELN AML MRD
12 guidelines, most NGS panels currently used at the time of AML diagnosis are not fit for the purpose of MRD
13 detection due to limited sample input, insufficient read-depth and lack of appropriate error correction (see
14 Table). NGS “AML panels” at diagnosis are used to determine likely somatic variants typically with a VAF
15 (variant allele frequency, also known as MAF for mutant allele frequency) of equal to or greater than 5% (ie: 5
16 variant reads per every 100 at that position). Accordingly, those tests use genomic DNA inputs as low as 10ng
17 which represents less than 8000 total cells (typically range of such tests: 10ng - 100ng). Given losses during
18 NGS library preparation, a requirement to detect 3-5 unique copies of each variant (each representing a
19 heterozygous mutation from a unique cell) to call MRD, and Poisson distribution sampling considerations at
20 the limit of detection (such that the FDA guidance recommended the MRD threshold be ten-fold higher than
21 the theoretical limit of detection) it is understandable that most high-quality AML MRD NGS research studies
22 have used DNA inputs in the 200-500ng range (ie: at least 1ml of marrow aspirate or blood). Similarly, most
23 AML MRD approaches using NGS, in recognition of massive number of false positive results particularly for
24 single nucleotide variants when considering potential variants less than 2% VAF, have used some form of error
25 correction in the form of laboratory techniques such as use of unique molecular identifiers (UMIs) followed by
26 consensus clustering and/or bioinformatic techniques including models incorporating nucleotide position
27 specific background error rates resulting from both PCR-based library preparation and sequencing itself^{17,49}
28 (Figure 2D). The sequencing depth requirement is often substantially higher for AML MRD than that routinely
29 done for diagnostic testing, as in addition to the increased sample input, typically each unique DNA molecule
30 will be sequenced 5 or 10 times in UMI-based error corrected sequencing. Finally, the prognostic significance
31 of AML “MRD” as detected by NGS compared with that detected by qPCR or flow cytometry may not be
32 equivalent, indeed there is already considerable evidence that detection of some somatic mutations may be
33 more prognostic than others.^{17,50,51}

1 In contrast, AML MRD targets for qPCR have already been extensively validated and have been approved by
2 the ELN consensus guidelines.⁷ This recently led to the development of a RNA-seq assay capable of
3 simultaneously detecting all of these molecular targets in a single standardized NGS workflow while
4 maintaining a limit of detection comparable with qPCR.⁵² Future NGS AML MRD assays may combine RNA and
5 DNA based approaches.

6

7 **Update for immunophenotypic MRD**

8 The ELN paper provides a framework for the harmonisation / standardisation of flow cytometric MRD
9 detection ⁷. Consensus guidance in this covers the most reliable of extensively tested markers for tracking
10 leukemic aberrant immunophenotypes, use of 8 colour panels (acceptable in clinical laboratories to increase
11 single-cell information), the importance of harmonising cytometer settings for comparable antibody stained
12 cell profiles and some technical recommendations for sample processing. Knowledge of the diagnostic LAIP
13 prevents reporting MRD negativity in the 5-10% of marker negative patients (no identifiable LAIP at diagnosis
14 as well as at follow-up) while a different-from-normal (DfN) analysis can detect phenotypic changes from
15 leukemia evolution.¹⁴ Consequently it is advised to combine information from both diagnostic LAIP and DfN
16 analysis in order to minimise false negatives. Defining MRD positivity by $\geq 0.1\%$ of leukocytes (1 log greater
17 than limit of detection) is proposed as this threshold is relevant in most studies. The proviso for this however
18 is that some patients with quantifiable MRD at $< 0.1\%$ may have residual AML that is prognostic, for example
19 due to MRD under-representation by hemodilution, or potentially due to smaller but more chemo-resistant
20 amounts after consolidation ^{53,54} or upfront higher intensity treatment ⁵⁵ or in older patients. ⁶ Risk
21 discrimination from low level MRD may also depend on genetic subgroup; detectable but $< 0.1\%$ flow
22 cytometric MRD after a first induction course was associated with a significantly increased relapse risk in
23 mutated-*NPM1* and CBF AMLs but not in wild type *NPM1* intermediate risk patients.⁵

24 **Feasibility of Harmonisation / Standardisation**

25 Experience from acute lymphoblastic leukemia (ALL) flow cytometric MRD work has shown that multicenter
26 standardisation⁵⁶ can be achieved with interpretive discordance reduced amongst experienced laboratories
27 by feedback schemes.⁵⁷ Recent efforts by some multicenter flow cytometry laboratory networks such as in
28 Germany and France are showing that this is also feasible for AML MRD despite differences in cytometers etc.
29 [Figure 3](#) shows an example of an implemented harmonisation strategy (Personal Communication, Plesa A.
30 and Roumier C., on behalf of Acute Leukaemia French Association Intergroup). The ELN are testing a consensus
31 standardised tube as a template to simplify inter-laboratory set-up and comparability for multicenter
32 interpretation.⁵⁸

33 **Immunophenotypic Leukemic Stem Cell Assays**

1 Weak/negative CD38 expression on CD34+ cells (CD34+CD38-) identifies progenitors that are enriched for
2 functional HSC activity in normal bone marrow. High frequencies of CD34+CD38- cells in AML at diagnosis
3 (variability shown in Figure 4), have an independent adverse prognostic impact⁵⁹⁻⁶¹ consistent with this
4 immunophenotypic subpopulation as a biomarker for LSC-like chemoresistance in some AMLs. Leukemic
5 CD34+CD38- cells can express aberrant markers⁶² that provide flow cytometric 'LSC' targets with higher assay
6 specificity due to less background from normal counterparts than with bulk progenitors. The approach has
7 been refined and standardised for a 'different from normal' strategy by construction of a single 'LSC' tube that
8 combines multiple CD34+CD38- aberrant 'LSC' markers.⁶³ Immunophenotypic quantitation of an
9 immunophenotypic LSC- population prior to allogeneic transplant was prognostic for relapse free survival.⁶⁴
10 Moreover immunophenotypic LSC frequency in CR had significant additive prognostic value to standard MRD
11 by LAIP or mutated-*NPM1* qPCR in a large cohort, mainly due to increased specificity for very poor outcomes
12 in the ~10% of patients with positivity for both LSC and standard MRD.⁶⁵ Although increasing assay sensitivity
13 up to 1 in a million by testing more cells could reduce the false negative relapse frequency observed in this
14 study (3yr CIR of 35% for double LSCneg / MRDneg), weak/negative CD38 may not be an appropriate or stable
15 marker for relapse-initiating LSCs in a subset of patients.^{66,67}

16 **Future Perspectives in Immunophenotypic MRD**

17 Ongoing discovery of leukemic aberrant phenotypes expands and rationalises the repertoire of informative
18 markers that can inform future routine AML MRD antibody panels⁶⁸ as well as for immunotherapy targets.
19 For example IL1RAP has been identified by both proteomic⁶⁶ and single cell RNA sequencing²⁹ strategies as a
20 discriminatory marker particularly in *FLT3-ITD* AML.

21 Hi-dimensional immunophenotyping using Cytometry by Time of flight (CyTOF) when available allows deeper
22 and/or broader population coverage due to increased parameters (>40) than that possible with flow
23 cytometry. When combined with novel data analysis algorithms,⁶⁹ CyTOF data can reveal biologically
24 important leukemic and immune cell subpopulations that are missed by traditional approaches. While CyTOF
25 has proven to be a powerful discovery tool, its slow acquisition rates (500 events/sec) preclude applying CyTOF
26 technology to clinical laboratory assays that require high cell numbers, MRD assays included. However, as
27 individual samples can be metal barcoded there may be potential for multiplexing of diagnostic samples,
28 achieving higher throughput and reducing data collection variation. This approach could screen for the most
29 informative markers to guide construction of improved flow cytometric MRD panels or to enable personalised
30 profiling for immunotherapeutic targets. The analysis algorithms developed for high dimensional
31 immunophenotyping have also been applied to flow cytometric data⁷⁰ and may with further development
32 provide tools for unsupervised or partially unsupervised AML MRD analysis in the future⁷¹.

33 **Immunophenotyping versus Molecular MRD detection: *Friend or Foe***

1 Initial evidence points to molecular and flow cytometric MRD providing complimentary independent
2 information at the same time-points during the active treatment, particularly when the molecular assays have
3 lower sensitivity and specificity.^{17,18,21,72,73} About 30% of patients in two studies^{17,18} had discordant results after
4 induction between flow cytometric and standard NGS MRD (excluding the main CHIP mutations) and these
5 had an intermediate risk of relapse (~50% at 4yr) in the larger cohort.¹⁷ In order to understand how best to
6 improve and deploy these assays it will be helpful in the research setting to define if their respective false
7 positive / negative results cluster in certain AML subtypes and any overlap. Of particular interest in this regard
8 are AMLs without a sensitive qPCR target including those with greater clonal complexity such as the chromatin-
9 spliceosome group.

10 **Flow cytometry versus Morphology to assess remission**

11 Flow cytometric assays continue to provide the fastest turn-around time for evaluating blast percentages and
12 MRD so that routine results may be available concurrently if not before morphology reporting. Inter-observer
13 variability for morphological BM blast counts has been recently reported as greatest for blast percentages
14 from 2-10% (and only moderate agreement for <2% and >10%) while reference percentages calculated from
15 digital trephine images, correlated well with flow cytometric blast percentages of first pull BM.⁷⁴ Evaluation
16 of leukemic aberrant immunophenotypes by MRD antibody panels adds specificity as well as sensitivity for
17 discriminating leukemic from normal blasts. Adults^{75,76} and children^{77,78} who are refractory by morphology
18 but MRD negative by flow cytometry after first induction have a good prognosis (60% 3-year survival in 2 adult
19 trial cohorts⁷⁵), equivalent to those in CRMRD-.⁷⁵ Also of note is that in a series of 87 patients morphologically
20 categorised as relapsed, none were MRD negative by flow.⁷⁹ Together these results support refining the
21 present criteria of refractory and relapsed AML to incorporate flow cytometric MRD negative results when a
22 validated assay is available.

23 Since MRD positivity in CR and morphologic refractory disease appear equivalent for outcomes pre allogeneic
24 transplant^{53,80} (with the caveat of unavoidable selection bias), does blast enumeration for the criteria of
25 resistant disease and partial remission add prognostic information? In younger adults treated in the NCRI
26 AML17 trial, patients in partial remission IWG criteria) or MRD positive CR after a first course of induction had
27 an equivalent intermediate prognosis (~40% 5yr survivals) with the exception of MRD positive patients with
28 incomplete count recovery who had a much poorer prognosis (19% 5yr survival). Patients with resistant
29 disease had a similar outcome to the latter.⁵ Thus when flow cytometric MRD is incorporated to response
30 assessment post first induction, the prognostic effect from blast percentages of ≥5% by morphology appears
31 restricted to the subgroup with resistant disease.

32

33

34 **Treatment Changes based on MRD Detection**

1 **MRD Status after Induction** In the case of mutated-*NPM1* AML⁴ and potentially for wild type *NPM1*
2 intermediate risk AML, ⁵ exploratory analyses in published studies support directing allogeneic transplant in
3 first remission only to those patients testing positive for MRD post induction chemotherapy (by qPCR for
4 mutated-*NPM1* or flow cytometry for wild type *NPM1*). However when ELN2017 adverse risk patients are
5 included, the benefit from allogeneic transplant as consolidation appears at least equivalent between MRD
6 negative and MRD positive patients.^{81 82} This supports allogeneic transplant in first remission as the best
7 approach for adverse risk patients even when achieving MRD negativity early in treatment.

8 The extent to which outcome is altered by intensifying consolidation in those who are MRD positive at the
9 post-induction time-point will be further informed by forthcoming data in trials implementing this strategy in
10 younger adults (such as NCRI AML17 /19 and HOVON 132 for ELN intermediate risk) and older adults (NCRI
11 AML18).

12

13 **MRD Status after Consolidation**

14 The CETLAM AML12 and the GIMEMA AML1310 phase 2 trials have investigated adjusting allogeneic
15 transplant allocation by post-consolidation MRD levels in addition to genetic risk. Initial reports from these^{83,84}
16 show the feasibility of real-time MRD treatment stratification and in the GIMEMA study, disease-free-survival
17 was similar between MRD positive and MRD negative intermediate genetic risk patients. The low patient
18 numbers in the analyses of the effect of transplant directed by post-consolidation MRD reinforces the difficulty
19 of testing non-biased transplant interventions.

20 **Monitoring off-treatment**

21 A suggested MRD directed treatment algorithm for AML patients with stable molecular qPCR MRD targets is
22 shown in Figure 1. When patients remain low risk by MRD levels after induction and consolidation, sequential
23 3 monthly qPCR MRD monitoring is recommended to allow a greater time-window for treatment decisions as
24 an increase in target transcript levels (as defined by the ELN⁷) precedes clinical relapse.

25 For AML patients without a qPCR MRD target, the role of off-treatment sequential monitoring requires further
26 evaluation. However as well as MRD status after induction and consolidation, pre- and potentially post-
27 transplant MRD testing can inform risk of poorer outcomes.

28

29 **MRD Status pre Transplant**

30 While it is clear that persistent MRD positivity in AML patients in morphological remission prior to allogeneic
31 transplant ^{49,53,80,85} is associated with increased relapse and decreased survival after transplant, due to the
32 lack of randomized clinical trials it is unproven if additional intervention can improve clinical outcomes rather
33 than simply increase treatment toxicity. Results from ultra-deep NGS on pre-transplant blood of AML patients
34 on the BMT CTN phase III randomized trial 0901 were recently presented demonstrating, in those with an AML

1 associated variant detected, increased post-transplant relapse risk and inferior overall survival in those
2 randomized to reduced intensity conditioning compared with those randomized to myeloablative
3 conditioning.⁸⁶ This study provides the best current evidence so far that intervention on the MRD state in AML
4 may improve clinical outcomes.

5 **MRD post Transplant**

6 For those patients who develop MRD positivity off treatment, pre-emptive treatment may be feasible even
7 after allogeneic transplant. In the RELAZA2 phase 2 trial, azacytidine converted 36% of 53 patients back to
8 CR_{MRD-} with 20% maintaining this during the 2yr follow-up. While such non-intensive interventions directed
9 to younger patients progressing to MRD positivity post-transplant are of interest for further evaluation,
10 optimising delivery will be challenging due to the variables of relapse kinetics and tolerability.

11

12 **Comment on Case Presentation**

13 *A 62yr old man with a normal WBC was diagnosed with cytogenetically normal AML and subsequent molecular*
14 *studies for ELN 2017 good and adverse risk mutations including mutated-NPM1 were negative. He was fit*
15 *enough to be treated with two courses of induction chemotherapy (standard UK NCRI AML treatment). MRD*
16 *response was assessed by flow cytometry. He achieved a CR but was MRD positive by flow cytometry (at 0.12%*
17 *after the second cycle. Due to deterioration in performance status, his co-morbidities and preference, further*
18 *treatment options did not include transplant. He was known to have an IDH1 R132 and DNMT3A mutations*
19 *and was considered for novel regimens or azacytidine. Should he be monitored if he has further treatment*
20 *and how?*

21 This patient even if he were younger has a very high risk of relapse by his post induction MRD status even
22 when treated with 3-4 courses of standard induction / consolidation chemotherapy (3yr CIR 89%, data from
23 NCRI AML17 trial for younger adults⁵). Since he could not proceed to allogeneic transplant other treatment
24 options include azacytidine maintenance (although it is uncertain if MRD positive patients derive benefit) or,
25 when available, novel regimens incorporating Bcl2⁸⁷ or IDH1 inhibition (venetoclax and ivosidenib
26 respectively).²² There are no recommendations available for MRD monitoring off-trial in the setting of less
27 intensive treatment. As he is MRD positive by flow cytometry, conversion to MRD negativity by this assay
28 (observed in 32% of older AML patients attaining CR/CRi with venetoclax and low-dose cytarabine⁸⁷ and in
29 40% treated by hypomethylating agents⁸⁸) may be encouraging for a more durable response although not as
30 yet a surrogate for survival. Parallel monitoring of IDH1 R132 mutation²⁰⁻²² may be particularly informative
31 for tracking on-target effect of an IDH1 inhibitor. Clearance of IDH1 mutations by digital PCR (limit of detection
32 2–4×10⁻⁴) in this context appears promising as a surrogate for outcome.²²

1

2 **Concluding remarks**

3 AML MRD evaluation in clinical practice is happening and will continue to increase. Upfront intensification
4 that includes allogeneic transplantation plus incorporation of available novel agents and maintenance
5 schedules expand current possibilities to improve outcomes but require evidence from randomized clinical
6 trials to establish benefit. MRD-guided therapy added to diagnostic genetic profiling has the potential to
7 target these therapeutic options appropriately and thus improve the ratio of benefit to toxicity and costs.
8 While MRD at single time-points has strong prognostic (and for some treatments predictive) value at a cohort
9 level, consecutive measurements of currently recommended MRD targets during and after treatment are
10 more likely to provide accurate information for individual patients by tracking any increase in their MRD levels.
11 This also reduces the potential impact from false negative and false positive MRD results inherent in any single
12 MRD test.

13 As in the case of ALL, CML, CLL and myeloma, trial participation is likely to continue as a key factor for enabling
14 familiarity with the clinical use of MRD as well as advancing methodology. Clinical trial networks provide
15 resources and direction in addition to investigating unresolved questions. This has been observed for example
16 in the UK, where MRD-informed treatment for non-APMLs has been integrated into UK NCRI trials since 2012
17 with availability of coordinated advice for interpretation of results and management decisions. An important
18 task for the next few years will be the evaluation of error-corrected NGS MRD assays, particularly in those AML
19 subgroups without available sensitive PCR assays. As there is no uniform marker of either leukemia or clonality
20 in AML unlike CML and lymphoid malignancies, the most appropriate, prognostic MRD platform and whether
21 orthogonal testing best measures leukemic reservoirs of relapse will depend on AML subtype. In this regard,
22 AMLs with greater intra-tumoral genetic heterogeneity (and consequently also increased mechanisms for
23 treatment escape) are particularly challenging for tracking relevant MRD by mutations.

24

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1 References

- 2 1. Hourigan CS, Gale RP, Gormley NJ, et al: Measurable residual disease testing in acute
3 myeloid leukaemia. *Leukemia* 31:1482-1490, 2017
- 4 2. Epstein-Peterson ZD, Devlin SM, Stein EM, et al: Widespread use of measurable residual
5 disease in acute myeloid leukemia practice. *Leuk Res* 67:92-98, 2018
- 6 3. Ivey A, Hills RK, Simpson MA, et al: Assessment of Minimal Residual Disease in Standard-Risk
7 AML. *N Engl J Med* 374:422-33, 2016
- 8 4. Balsat M, Renneville A, Thomas X, et al: Postinduction Minimal Residual Disease Predicts
9 Outcome and Benefit From Allogeneic Stem Cell Transplantation in Acute Myeloid Leukemia With NPM1
10 Mutation: A Study by the Acute Leukemia French Association Group. *J Clin Oncol* 35:185-193, 2017
- 11 5. Freeman SD, Hills RK, Virgo P, et al: Measurable Residual Disease at Induction Redefines
12 Partial Response in Acute Myeloid Leukemia and Stratifies Outcomes in Patients at Standard Risk Without
13 NPM1 Mutations. *J Clin Oncol* 36:1486-1497, 2018
- 14 6. Freeman SD, Virgo P, Couzens S, et al: Prognostic relevance of treatment response measured
15 by flow cytometric residual disease detection in older patients with acute myeloid leukemia. *J Clin Oncol*
16 31:4123-31, 2013
- 17 7. Schuurhuis GJ, Heuser M, Freeman S, et al: Minimal/measurable residual disease in AML: a
18 consensus document from the European LeukemiaNet MRD Working Party. *Blood* 131:1275-1291, 2018
- 19 8. Food and Drug Administration H: Hematologic Malignancies: Regulatory Considerations for
20 Use of Minimal Residual Disease in Development of Drug and Biological Products for Treatment; Draft
21 Guidance for Industry; Availability in Services USDoHaH, Administration FaD, Research CfDEaRCfBEa (eds).
22 Federal Register, 2018, pp 52225
- 23 9. Platzbecker U, Middeke JM, Sockel K, et al: Measurable residual disease-guided treatment
24 with azacitidine to prevent haematological relapse in patients with myelodysplastic syndrome and acute
25 myeloid leukaemia (RELAZA2): an open-label, multicentre, phase 2 trial. *Lancet Oncol* 19:1668-1679, 2018
- 26 10. Selim AG, Moore AS: Molecular Minimal Residual Disease Monitoring in Acute Myeloid
27 Leukemia: Challenges and Future Directions. *J Mol Diagn* 20:389-397, 2018
- 28 11. Grimwade D, Lo Coco F: Acute promyelocytic leukemia: a model for the role of molecular
29 diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia.
30 *Leukemia* 16:1959-73, 2002
- 31 12. Hoffmann H, Thiede C, Glauche I, et al: The prognostic potential of monitoring disease
32 dynamics in NPM1-positive acute myeloid leukemia. *Leukemia*, 2019
- 33 13. Hollein A, Jeromin S, Meggendorfer M, et al: Minimal residual disease (MRD) monitoring and
34 mutational landscape in AML with RUNX1-RUNX1T1: a study on 134 patients. *Leukemia* 32:2270-2274, 2018
- 35 14. Martinez-Losada C, Serrano-Lopez J, Serrano-Lopez J, et al: Clonal genetic evolution at
36 relapse of favorable-risk acute myeloid leukemia with NPM1 mutation is associated with phenotypic changes
37 and worse outcomes. *Haematologica* 103:e400-e403, 2018
- 38 15. Hollein A, Meggendorfer M, Dicker F, et al: NPM1 mutated AML can relapse with wild-type
39 NPM1: persistent clonal hematopoiesis can drive relapse. *Blood Adv* 2:3118-3125, 2018
- 40 16. Gaidzik VI, Weber D, Paschka P, et al: DNMT3A mutant transcript levels persist in remission
41 and do not predict outcome in patients with acute myeloid leukemia. *Leukemia* 32:30-37, 2018
- 42 17. Jongen-Lavrencic M, Grob T, Hanekamp D, et al: Molecular Minimal Residual Disease in
43 Acute Myeloid Leukemia. *N Engl J Med* 378:1189-1199, 2018
- 44 18. Morita K, Kantarjian HM, Wang F, et al: Clearance of Somatic Mutations at Remission and
45 the Risk of Relapse in Acute Myeloid Leukemia. *J Clin Oncol* 36:1788-1797, 2018
- 46 19. Ottone T, Alfonso V, Iaccarino L, et al: Longitudinal detection of DNMT3A(R882H) transcripts
47 in patients with acute myeloid leukemia. *Am J Hematol* 93:E120-E123, 2018
- 48 20. Ferret Y, Boissel N, Helevaut N, et al: Clinical relevance of IDH1/2 mutant allele burden
49 during follow-up in acute myeloid leukemia. A study by the French ALFA group. *Haematologica* 103:822-829,
50 2018

- 1 21. Ok CY, Loghavi S, Sui D, et al: Persistent IDH1/2 mutations in remission can predict relapse in
2 patients with acute myeloid leukemia. *Haematologica* 104:305-311, 2019
- 3 22. DiNardo CD, Stein EM, de Botton S, et al: Durable Remissions with Ivosidenib in IDH1-
4 Mutated Relapsed or Refractory AML. *N Engl J Med* 378:2386-2398, 2018
- 5 23. Stein EM, DiNardo CD, Fathi AT, et al: Molecular remission and response patterns in patients
6 with mutant-IDH2 acute myeloid leukemia treated with enasidenib. *Blood* 133:676-687, 2019
- 7 24. Desai P, Hassane D, Roboz GJ: Clonal Hematopoiesis and risk of Acute Myeloid Leukemia.
8 *Best Pract Res Clin Haematol* 32:177-185, 2019
- 9 25. Kim T, Moon JH, Ahn JS, et al: Next-generation sequencing-based posttransplant monitoring
10 of acute myeloid leukemia identifies patients at high risk of relapse. *Blood* 132:1604-1613, 2018
- 11 26. Nakamura S, Yokoyama K, Shimizu E, et al: Prognostic impact of circulating tumor DNA status
12 post-allogeneic hematopoietic stem cell transplantation in AML and MDS. *Blood*, 2019
- 13 27. Dohner H, Estey E, Grimwade D, et al: Diagnosis and management of AML in adults: 2017
14 ELN recommendations from an international expert panel. *Blood* 129:424-447, 2017
- 15 28. Parkin B, Londono-Joshi A, Kang Q, et al: Ultrasensitive mutation detection identifies rare
16 residual cells causing acute myelogenous leukemia relapse. *J Clin Invest* 127:3484-3495, 2017
- 17 29. van Galen P, Hovestadt V, Wadsworth li MH, et al: Single-Cell RNA-Seq Reveals AML
18 Hierarchies Relevant to Disease Progression and Immunity. *Cell* 176:1265-1281 e24, 2019
- 19 30. Zeijlemaker W, Kelder A, Oussoren-Brockhoff YJ, et al: Peripheral blood minimal residual
20 disease may replace bone marrow minimal residual disease as an immunophenotypic biomarker for
21 impending relapse in acute myeloid leukemia. *Leukemia* 30:708-15, 2016
- 22 31. Cilloni D, Renneville A, Hermitte F, et al: Real-time quantitative polymerase chain reaction
23 detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute
24 myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol* 27:5195-201, 2009
- 25 32. Maurillo L, Buccisano F, Spagnoli A, et al: Monitoring of minimal residual disease in adult
26 acute myeloid leukemia using peripheral blood as an alternative source to bone marrow. *Haematologica*
27 92:605-11, 2007
- 28 33. Guenot C, Lacombe F, Allou K, et al: Peripheral blood minimal/measurable residual disease
29 assessed in flow cytometry in acute myeloblastic leukemia. *Leukemia*, 2019
- 30 34. Wong HY, Sung AD, Lindblad KE, et al: Molecular Measurable Residual Disease Testing of
31 Blood During AML Cytotoxic Therapy for Early Prediction of Clinical Response. *Front Oncol* 8:669, 2018
- 32 35. Duncavage EJ, Uy GL, Petti AA, et al: Mutational landscape and response are conserved in
33 peripheral blood of AML and MDS patients during decitabine therapy. *Blood* 129:1397-1401, 2017
- 34 36. Buccisano F, Dillon R, Freeman SD, et al: Role of Minimal (Measurable) Residual Disease
35 Assessment in Older Patients with Acute Myeloid Leukemia. *Cancers (Basel)* 10, 2018
- 36 37. Ommen HB: Monitoring minimal residual disease in acute myeloid leukaemia: a review of
37 the current evolving strategies. *Ther Adv Hematol* 7:3-16, 2016
- 38 38. Boddu P, Gurguis C, Sanford D, et al: Response kinetics and factors predicting survival in
39 core-binding factor leukemia. *Leukemia* 32:2698-2701, 2018
- 40 39. Bibault JE, Figeac M, Helevaut N, et al: Next-generation sequencing of FLT3 internal tandem
41 duplications for minimal residual disease monitoring in acute myeloid leukemia. *Oncotarget* 6:22812-21,
42 2015
- 43 40. McMahon CM, Ferng T, Canaani J, et al: Clonal selection with Ras pathway activation
44 mediates secondary clinical resistance to selective FLT3 inhibition in acute myeloid leukemia. *Cancer Discov*,
45 2019
- 46 41. Levis MJ, Perl AE, Altman JK, et al: A next-generation sequencing-based assay for minimal
47 residual disease assessment in AML patients with FLT3-ITD mutations. *Blood Adv* 2:825-831, 2018
- 48 42. Schnittger S, Schoch C, Dugas M, et al: Analysis of FLT3 length mutations in 1003 patients
49 with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study
50 and usefulness as a marker for the detection of minimal residual disease. *Blood* 100:59-66, 2002

- 1 43. Zuffa E, Franchini E, Papayannidis C, et al: Revealing very small FLT3 ITD mutated clones by
2 ultra-deep sequencing analysis has important clinical implications in AML patients. *Oncotarget* 6:31284-94,
3 2015
- 4 44. Lin MT, Tseng LH, Dudley JC, et al: A Novel Tandem Duplication Assay to Detect Minimal
5 Residual Disease in FLT3/ITD AML. *Mol Diagn Ther* 19:409-17, 2015
- 6 45. Blatte TJ, Schmalbrock LK, Skambraks S, et al: getITD for FLT3-ITD-based MRD monitoring in
7 AML. *Leukemia*, 2019
- 8 46. Papaemmanuil E, Gerstung M, Bullinger L, et al: Genomic Classification and Prognosis in
9 Acute Myeloid Leukemia. *N Engl J Med* 374:2209-2221, 2016
- 10 47. Candoni A, De Marchi F, Zanini F, et al: Predictive value of pretransplantation molecular
11 minimal residual disease assessment by WT1 gene expression in FLT3-positive acute myeloid leukemia. *Exp*
12 *Hematol* 49:25-33, 2017
- 13 48. Mencia-Trinchant N, Hu Y, Alas MA, et al: Minimal Residual Disease Monitoring of Acute
14 Myeloid Leukemia by Massively Multiplex Digital PCR in Patients with NPM1 Mutations. *J Mol Diagn* 19:537-
15 548, 2017
- 16 49. Thol F, Gabdoulline R, Liebich A, et al: Measurable residual disease monitoring by NGS
17 before allogeneic hematopoietic cell transplantation in AML. *Blood* 132:1703-1713, 2018
- 18 50. Bhatnagar B, Eisfeld AK, Nicolet D, et al: Persistence of DNMT3A R882 mutations during
19 remission does not adversely affect outcomes of patients with acute myeloid leukaemia. *Br J Haematol*
20 175:226-236, 2016
- 21 51. Debarri H, Lebon D, Roumier C, et al: IDH1/2 but not DNMT3A mutations are suitable targets
22 for minimal residual disease monitoring in acute myeloid leukemia patients: a study by the Acute Leukemia
23 French Association. *Oncotarget* 6:42345-53, 2015
- 24 52. Dillon LW, Hayati S, Roloff GW, et al: Targeted RNA-sequencing for the quantification of
25 measurable residual disease in acute myeloid leukemia. *Haematologica* 104:297-304, 2019
- 26 53. Araki D, Wood BL, Othus M, et al: Allogeneic Hematopoietic Cell Transplantation for Acute
27 Myeloid Leukemia: Time to Move Toward a Minimal Residual Disease-Based Definition of Complete
28 Remission? *J Clin Oncol* 34:329-36, 2016
- 29 54. Buccisano F, Maurillo L, Spagnoli A, et al: Cytogenetic and molecular diagnostic
30 characterization combined to postconsolidation minimal residual disease assessment by flow cytometry
31 improves risk stratification in adult acute myeloid leukemia. *Blood* 116:2295-303, 2010
- 32 55. Ravandi F, Jorgensen J, Borthakur G, et al: Persistence of minimal residual disease assessed
33 by multiparameter flow cytometry is highly prognostic in younger patients with acute myeloid leukemia.
34 *Cancer* 123:426-435, 2017
- 35 56. Bruggemann M, Schrauder A, Raff T, et al: Standardized MRD quantification in European ALL
36 trials: proceedings of the Second International Symposium on MRD assessment in Kiel, Germany, 18-20
37 September 2008. *Leukemia* 24:521-35, 2010
- 38 57. Keeney M, Wood BL, Hedley BD, et al: A QA Program for MRD Testing Demonstrates That
39 Systematic Education Can Reduce Discordance Among Experienced Interpreters. *Cytometry B Clin Cytom*
40 94:239-249, 2018
- 41 58. Brooimans RA, van der Velden VHJ, Boeckx N, et al: Immunophenotypic measurable residual
42 disease (MRD) in acute myeloid leukemia: Is multicentric MRD assessment feasible? *Leuk Res* 76:39-47, 2019
- 43 59. Jentzsch M, Bill M, Nicolet D, et al: Prognostic impact of the CD34+/CD38- cell burden in
44 patients with acute myeloid leukemia receiving allogeneic stem cell transplantation. *Am J Hematol* 92:388-
45 396, 2017
- 46 60. Khan N, Freeman SD, Virgo P, et al: An immunophenotypic pre-treatment predictor for poor
47 response to induction chemotherapy in older acute myeloid leukaemia patients: blood frequency of CD34+
48 CD38 low blasts. *Br J Haematol* 170:80-4, 2015
- 49 61. Terwijn M, Zeijlemaker W, Kelder A, et al: Leukemic stem cell frequency: a strong biomarker
50 for clinical outcome in acute myeloid leukemia. *PLoS One* 9:e107587, 2014

- 1 62. Pollyea DA, Jordan CT: Therapeutic targeting of acute myeloid leukemia stem cells. *Blood*
2 129:1627-1635, 2017
- 3 63. Zeijlemaker W, Kelder A, Oussoren-Brockhoff YJ, et al: A simple one-tube assay for
4 immunophenotypical quantification of leukemic stem cells in acute myeloid leukemia. *Leukemia* 30:439-46,
5 2016
- 6 64. Bradbury C, Houlton AE, Akiki S, et al: Prognostic value of monitoring a candidate
7 immunophenotypic leukaemic stem/progenitor cell population in patients allografted for acute myeloid
8 leukaemia. *Leukemia* 29:988-91, 2015
- 9 65. Zeijlemaker W, Grob T, Meijer R, et al: CD34(+)CD38(-) leukemic stem cell frequency to
10 predict outcome in acute myeloid leukemia. *Leukemia*, 2018
- 11 66. Boyd AL, Aslostovar L, Reid J, et al: Identification of Chemotherapy-Induced Leukemic-
12 Regenerating Cells Reveals a Transient Vulnerability of Human AML Recurrence. *Cancer Cell* 34:483-498 e5,
13 2018
- 14 67. Ho TC, LaMere M, Stevens BM, et al: Evolution of acute myelogenous leukemia stem cell
15 properties after treatment and progression. *Blood* 128:1671-8, 2016
- 16 68. Coustan-Smith E, Song G, Shurtleff S, et al: Universal monitoring of minimal residual disease
17 in acute myeloid leukemia. *JCI Insight* 3, 2018
- 18 69. Kimball AK, Oko LM, Bullock BL, et al: A Beginner's Guide to Analyzing and Visualizing Mass
19 Cytometry Data. *J Immunol* 200:3-22, 2018
- 20 70. Hu Z, Jujjavarapu C, Hughey JJ, et al: MetaCyto: A Tool for Automated Meta-analysis of Mass
21 and Flow Cytometry Data. *Cell Rep* 24:1377-1388, 2018
- 22 71. Lacombe F, Dupont B, Lechevalier N, et al: New Concepts of Flow Cytometry Analysis in
23 Oncohematology: Application to Diagnosis and Follow up (Minimal Residual Disease) in AML, ALL and MDS,
24 *Am Soc Hematology*, 2017
- 25 72. Marani C, Clavio M, Grasso R, et al: Integrating post induction WT1 quantification and flow-
26 cytometry results improves minimal residual disease stratification in acute myeloid leukemia. *Leuk Res*
27 37:1606-11, 2013
- 28 73. Getta BM, Devlin SM, Levine RL, et al: Multicolor Flow Cytometry and Multigene Next-
29 Generation Sequencing Are Complementary and Highly Predictive for Relapse in Acute Myeloid Leukemia
30 after Allogeneic Transplantation. *Biol Blood Marrow Transplant* 23:1064-1071, 2017
- 31 74. Hodes A, Calvo KR, Dulau A, et al: The challenging task of enumerating blasts in the bone
32 marrow. *Semin Hematol* 56:58-64, 2019
- 33 75. Freeman SD, Hills RK, Russell NH, et al: Induction response criteria in acute myeloid
34 leukaemia: implications of a flow cytometric measurable residual disease negative test in refractory adults.
35 *Br J Haematol*, 2018
- 36 76. Ouyang J, Goswami M, Tang G, et al: The clinical significance of negative flow cytometry
37 immunophenotypic results in a morphologically scored positive bone marrow in patients following
38 treatment for acute myeloid leukemia. *Am J Hematol* 90:504-10, 2015
- 39 77. Inaba H, Coustan-Smith E, Cao X, et al: Comparative analysis of different approaches to
40 measure treatment response in acute myeloid leukemia. *J Clin Oncol* 30:3625-32, 2012
- 41 78. Loken MR, Alonzo TA, Pardo L, et al: Residual disease detected by multidimensional flow
42 cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: a report from
43 Children's Oncology Group. *Blood* 120:1581-8, 2012
- 44 79. Zhou Y, Wood BL, Walter RB, et al: Is there a need for morphologic exam to detect relapse in
45 AML if multi-parameter flow cytometry is employed? *Leukemia* 31:2536-2537, 2017
- 46 80. Hourigan CS, Goswami M, Battiwalla M, et al: When the Minimal Becomes Measurable. *J Clin*
47 *Oncol* 34:2557-8, 2016
- 48 81. Versluis J, Kalin B, Zeijlemaker W, et al: Graft-versus-leukemia effect of allogeneic stem-cell
49 transplantation and minimal residual disease in patients with acute myeloid leukemia in first complete
50 remission. *JCO Precision Oncology* 1:1-13, 2017

- 1 82. Lambert J, Lambert J, Thomas X, et al: Early Detection of WT1 minimal Residual Disease
2 Predicts Outcome in Acute Myeloid Leukemia and Identify Patients with High Risk of Relapse Independently
3 of Allogeneic Stem Cell Transplantation, Am Soc Hematology, 2017
- 4 83. Sierra J, Garrido A, Vives S, et al: Therapy for Acute Myeloid Leukemia (AML) Adjusted to
5 Genetic Data and Minimal Residual Disease: Results of the AML12 Trial of the Spanish Cetlam Group in
6 Adults up to the Age of 70 Years. Blood 130:567-567, 2017
- 7 84. Venditti A, Piciocchi, A., Candoni, A., Melillo, L., Calafiore, V., Cairoli, R., De Fabritiis, P.,
8 Storti, G., Salutari, P., Lanza, F, Martinelli, G.: Risk-adapted, MRD-directed therapy for young adults with
9 newly diagnosed acute myeloid leukemia: results of the AML1310 trial of the GIMEMA group. . Presented at
10 the EHA, 2017
- 11 85. Buckley SA, Wood BL, Othus M, et al: Minimal residual disease prior to allogeneic
12 hematopoietic cell transplantation in acute myeloid leukemia: a meta-analysis. Haematologica 102:865-873,
13 2017
- 14 86. Hourigan CS DL, Logan B, Scott B, Ghannam J, Gui G, Li Y, Licon A, Fei M, Alyea E, Bashey,
15 AH. Deeg J, Devine S, Fernandez H, Giralt S, Hamadani M, Horowitz M, Howard A, Maziarz R, Porter D,
16 Warlick E, Pasquini M, Horwitz M: Impact of Conditioning Intensity of Allogeneic Transplantation for Acute
17 Myeloid Leukemia with Genomic Evidence of Residual Disease Presented at the EHA, 2019
- 18 87. Wei A, Strickland SA, Hou J-Z, et al: Venetoclax with Low-Dose Cytarabine Induces Rapid,
19 Deep, and Durable Responses in Previously Untreated Older Adults with AML Ineligible for Intensive
20 Chemotherapy. Blood 132:284-284, 2018
- 21 88. Boddu P, Jorgensen J, Kantarjian H, et al: Achievement of a negative minimal residual
22 disease state after hypomethylating agent therapy in older patients with AML reduces the risk of relapse.
23 Leukemia 32:241-244, 2018
- 24 89. Dillon R, Hills RK, Freeman SD, et al: Pre-Transplant *NPM1* Mutant Transcript Level Is Highly
25 Predictive of Outcome after Allograft and Thresholds Are Dependent on *FLT3* ITD Status. Blood 132:2739-
26 2739, 2018

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28

1 **Figure Legends**

2 **Figure 1 legend**

3 **A.** Suggested management algorithm for patients with AML with a molecular MRD target.

4 [1] ELN favourable risk patients with <4 log reduction in *NPM1* mutant transcripts after first induction shown
5 to benefit from a CR1 allograft⁴ and any positivity in the PB after second induction is associated with a very
6 high risk of relapse.³ [2] MRD positivity >200 copies / 10^5 *ABL* (i.e. molecular persistence) and serially rising
7 transcript levels after treatment (i.e molecular progression) reliably predicts relapse⁷.

8 [3] At the end of treatment, patients with core binding factor AML with high or serially rising transcript levels
9 are destined to relapse (relevant thresholds are >500 copies (per 10^5 *ABL*) of *RUNX1/RUNX1T1* in the BM or
10 >100 copies in the PB, and >50 copies of *CBFB/MYH11* in the BM or >10 copies in the PB)⁷. Salvage according
11 to panel C should be considered for these patients although there is currently no evidence that this improves
12 outcome. Conversely patients with low copy numbers below these thresholds can be safely monitored
13 according to panel B. [4] Although CBF patients with an early unfavourable MRD response have a higher risk
14 of relapse there is insufficient evidence to warrant treatment change⁷ however this may prompt initiation of
15 an early donor search; salvage may be considered in cases with extremely poor early response or if there is
16 rise while on treatment (i.e. molecular progression).

17 [5] Although there is currently no evidence that standard risk patients who remain MRD positive benefit from
18 transplant, this is a reasonable approach and adopted in the current NCRI AML19 and MyeChild01 protocols.

19 **B.** Suggested algorithm for sequential monitoring after treatment. Patients conversion to MRD positivity,
20 confirmed on a second sample with >1 log rise should be diagnosed with molecular relapse and treated
21 according to panel B⁷.

22 **C.** Possible peri-transplant management strategy. [1] Patients with an *NPM1* mutation without *FLT3* ITD who
23 have transcript levels below 1000 copies in the BM or 200 copies in the PB have a very good outcome after
24 allograft, it is uncertain whether these patients benefit from salvage chemotherapy.⁸⁹ [2] Patients with high
25 levels of MRD after salvage, without an adequate response to DLI and those to whom these standard therapies
26 cannot be given should be considered for investigational approaches.

27 Figure by Richard Dillon, NCRI Group.

28

29 **Figure 2 Legend**

30 A) Quantitative polymerase chain reaction (qPCR) is a common method for quantification of nucleic acid with
31 real-time monitoring of the amplification of target of interest (eg: variant sequence shown with red cross).

1 Advantages include ubiquitous in most clinical laboratories, fast turn-around time, high sample throughput
2 and broad dynamic range. Disadvantages include limited number of suitable targets/assays available, relative
3 lack of multiplexing ability, need to validate each target/assay individually, potential for false negative results
4 due to sample impurity, limited ability to accurately discriminate between very low levels of target as seen in
5 MRD.

6 B) Rather than performing the PCR reaction in “bulk”, digital PCR partitions template of interest into individual
7 compartments (top) improving performance compared with qPCR due to lower background error rate (lower
8 right), elimination of template competition and digital result output allowing absolute quantification (lower
9 left). Lack of deep multiplexing ability and the need to validate each target/assay individually remain
10 limitations.

11 C) Next generation sequencing (NGS) has revolutionized initial clinical diagnostic evaluation of AML by
12 allowing for simultaneous evaluation of multiple target regions typically selected from those known to be
13 often mutated in AML. NGS is useful for discovery of mutations present in the range from 5% to 100% of a
14 sample (Variant Allele Frequency, VAF). Not all variants detected will be pathogenic somatic mutations
15 however, and care should be taken to consider the possibility of identification of homozygous or
16 heterozygous germline variants as well as loss of heterozygosity (LOH) events. Variant discovery below a
17 VAF of 5% using panels designed for profiling variants at diagnosis is challenging due to lack of sensitivity and
18 high false positive rates.

19 D) NGS for AML MRD performed in recent high-quality research studies has typically included error
20 correction, by (upper) incorporation of unique molecular identifiers followed by consensus determination of
21 true (red) variants versus false positives introduced by the technique (green) and/or (lower) bioinformatic
22 approaches to model background error rates at each nucleotide position in those not having a variant and
23 determine probability that observed variant is a true positive (red).

24 Figure by Erina He, NIH Medical Arts.

25

26 **Figure 3 legend**

27 **A.** Strategy applied for Flow Cytometric AML MRD multi-center harmonisation by Acute Leukaemia French
28 Association (ALFA) Intergroup.

29 [1] Rationale of AML MRD Flow panel design was based on simplicity, reproducibility and cost. Tube 1 was a
30 core combination for Leukemic Associated ImmunoPhenotypes (LAIP) detected at diagnosis and /or by

1 Different-from-Normal analysis. Tube 2 was targeted to aberrancies of CD34+CD38- cells (immunophenotypic
2 LSCs) and Tube 3 was an optional development tube for monocytic aberrancies.

3 [2] Flow cytometer fluorescent settings were harmonised ('mirrored') between Canto vs Navios cytometer
4 platforms. Voltages were set to reach target MFI values by acquisition of rainbow calibration beads without
5 compensation for fluorescent channels FL1 to FL8 on the Canto cytometers; these rainbow bead settings were
6 transposed to Navios cytometers by applying $MFI_{target} = Canto_{target} / 256$. Mirrored (superimposable)
7 target peaks for both cytometers shown for FL1 and FL8 fluorescent channels with an example of resulting
8 comparable antibody profiles between cytometer platforms.

9 [3] Quality controls for reproducibility of staining profiles from harmonised cytometer settings /sample
10 processing between cytometers/laboratories. Examples shown are for CD117 and CD38 expression intensity
11 on CD34+ gated mononuclear cells of Tube 1 from 10 shared BM samples stained and then acquired on Canto
12 vs Navios . Intensity profiles are similar between cytometer platforms for each sample.

13 [4] External quality assessment for all harmonised steps from pre-analytical to final gating analyses by
14 distribution of a normal bone marrow to 22 participating laboratories (cytometer platforms : 12 Cantos, 10
15 Navios). Example shows that strong reproducibility can be achieved in the detection of rare events (shown
16 for CD34+CD38-) among 22 participating laboratories.

17 Figure by Christophe Roumier and Adriana Plesa.

18

19 **Figure 4 legend**

20 CD34/CD38 expression pattern of blasts from 5 diagnostic AML samples showing the variability in the
21 frequency of the most immature leukemia cells (CD34+CD38-, in orange) compared to normal BM. (CN,
22 normal cytogenetics).

23 Figure by Adriana Plesa and Christophe Roumier.

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Table 1: Limitations of use of next generation sequencing to detect residual disease in AML

Problem	Significance	Solution
NGS AML panels used at diagnosis are unfit for purpose of AML MRD	Insufficient sample input/sequencing depth limits assay sensitivity. Insufficient error correction increases false positive rate at low VAF.	NGS for research not clinical use in AML MRD currently. ELN guidelines on use of NGS in AML MRD coming.
Association between variant tracked and residual AML clone(s)	Variants closely linked to AML, eg: FLT3-ITD, often subclonal/unstable. "Stable" variants, such as those in DMNT3A, also seen in ARCH/CHIP.	Tracking a panel of gene regions in remission will likely improve predictive power, at increased cost. Larger datasets will provide more information on which variants detected at MRD stage are the most associated with subsequent relapse.
Genetic clonal heterogeneity of AML = risk of false negative tests.	Variants detected at diagnosis are not necessarily present in the AML clone remaining after unsuccessful treatment responsible for relapse.	Tracking a panel of gene regions in remission will likely improve predictive power, at increased cost. Deep profiling of diagnostic sample to screen for minor subclones (eg: <i>TP53</i>) may have utility.
Error rates intrinsic to NGS = risk of false positive tests.	Traditional NGS approaches cannot reliably identify novel variants present at low VAF (less than 2-5%) with sufficient specificity (ie: many false positive variant calls mask rare true positive variant).	ECS using UMI consensus clustering and/or bioinformatic approaches such as background error models (Figure D) helpful. Low VAF variants seen in diagnostic sample or multiple surveillance samples more likely true variants (but see above)
Correlation of NGS results with other measures of AML MRD	Tests designed to detect residual disease in AML may classify patient sample differently based on modality used (qPCR vs. flow cytometry vs. NGS). No single test represents "gold standard" for detecting, in patients in remission, those cells that will subsequently lead to AML relapse.	Studies designed to integrate information from different AML MRD tests performed on the same sample cohorts are underway.
Lack of uniform reporting standards	How many UMI reads needed to call a variant (3, 5)? How many distinct UMI read families per variant needed to call MRD? How many genomic equivalents as input? Standardized filtering, consensus clustering and variant calling needed? What about controls, duplicates, platforms?	NGS suited for research not clinical use in AML MRD currently. FDA guidance for MRD in Hematological Malignancies published in draft form. ELN guidelines on use of NGS in AML MRD coming.

Box: Eight “S’s of AML MRD

Specificity of test

Sensitivity of test

Stability of MRD marker

Subtype of AML

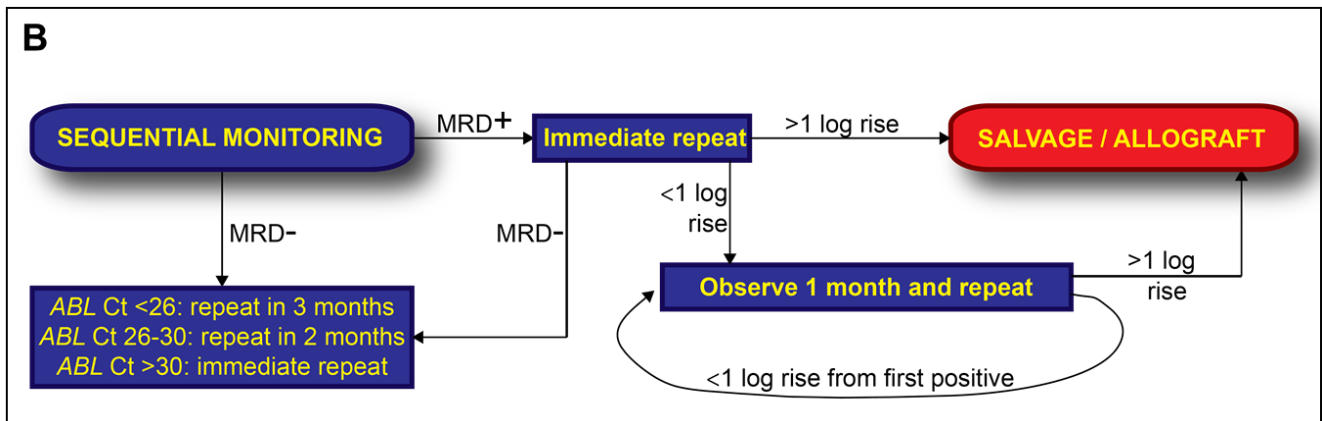
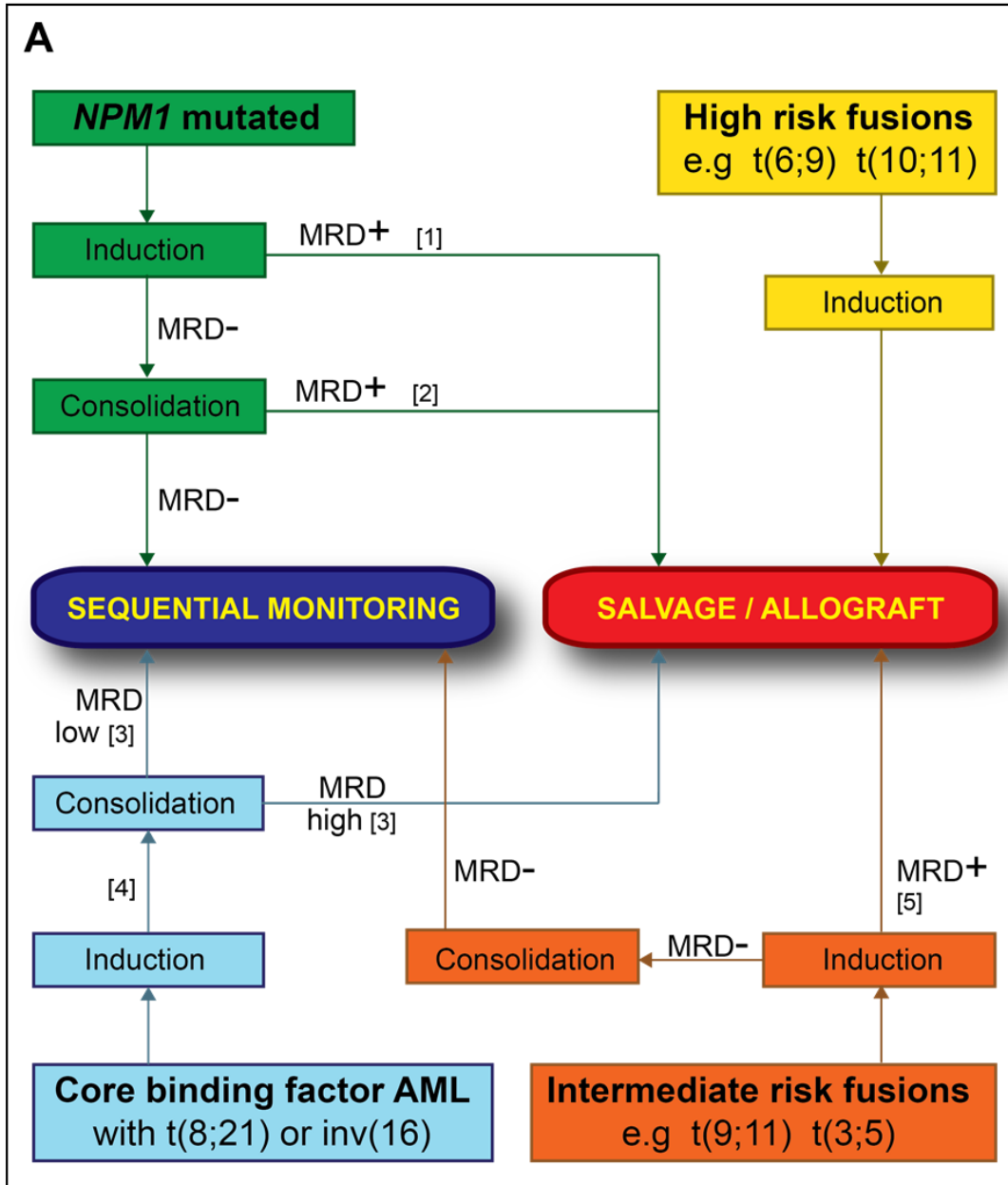
Sample type

Stage during treatment sample taken

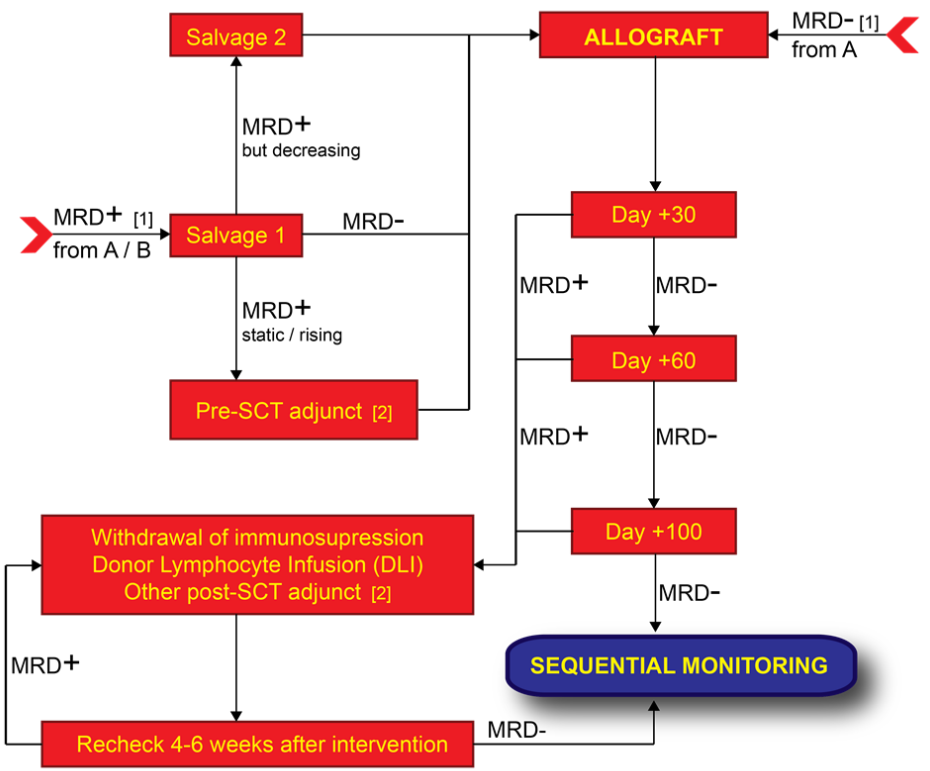
Standardisation of Assay

Sprice (\$)

Figure 1



C



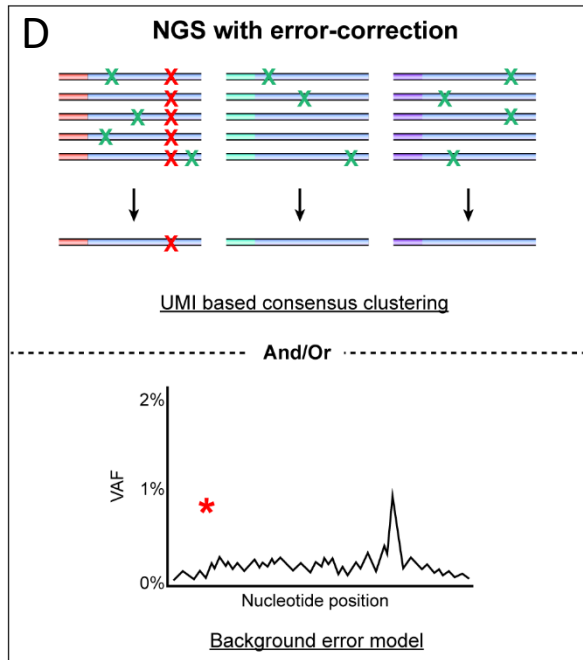
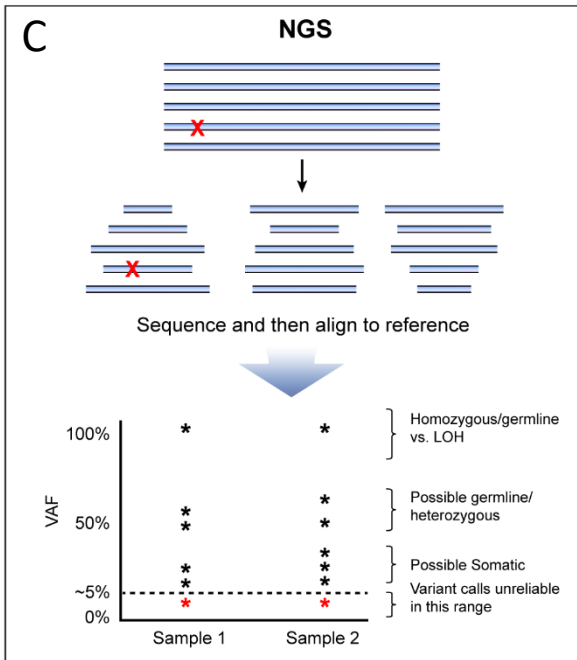
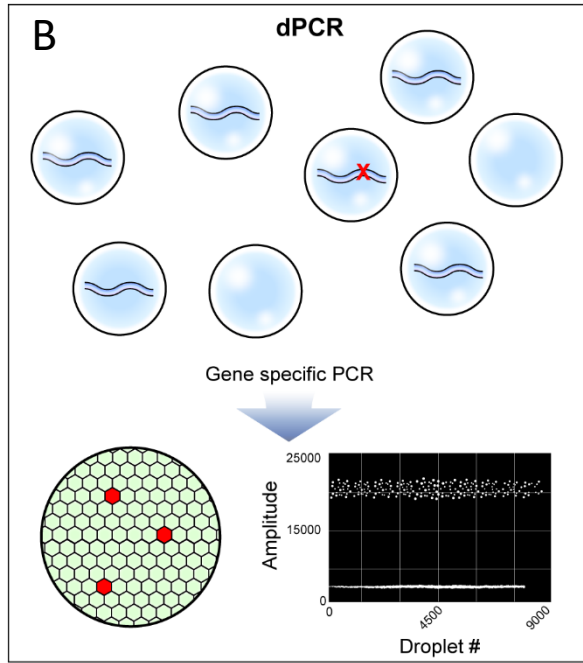
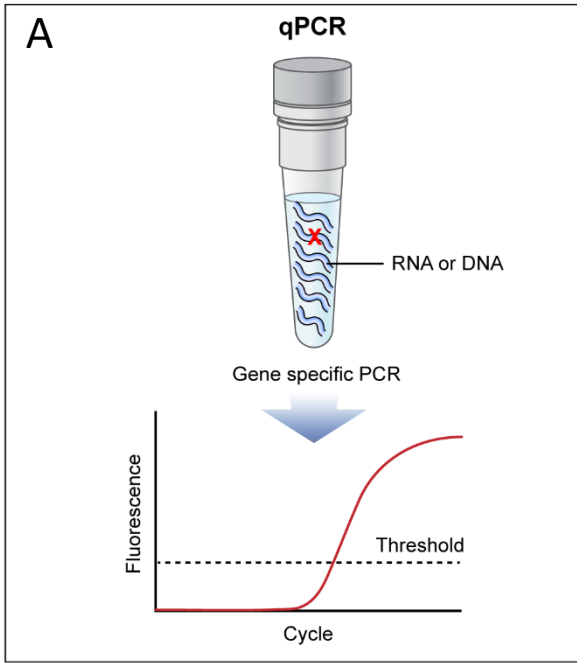
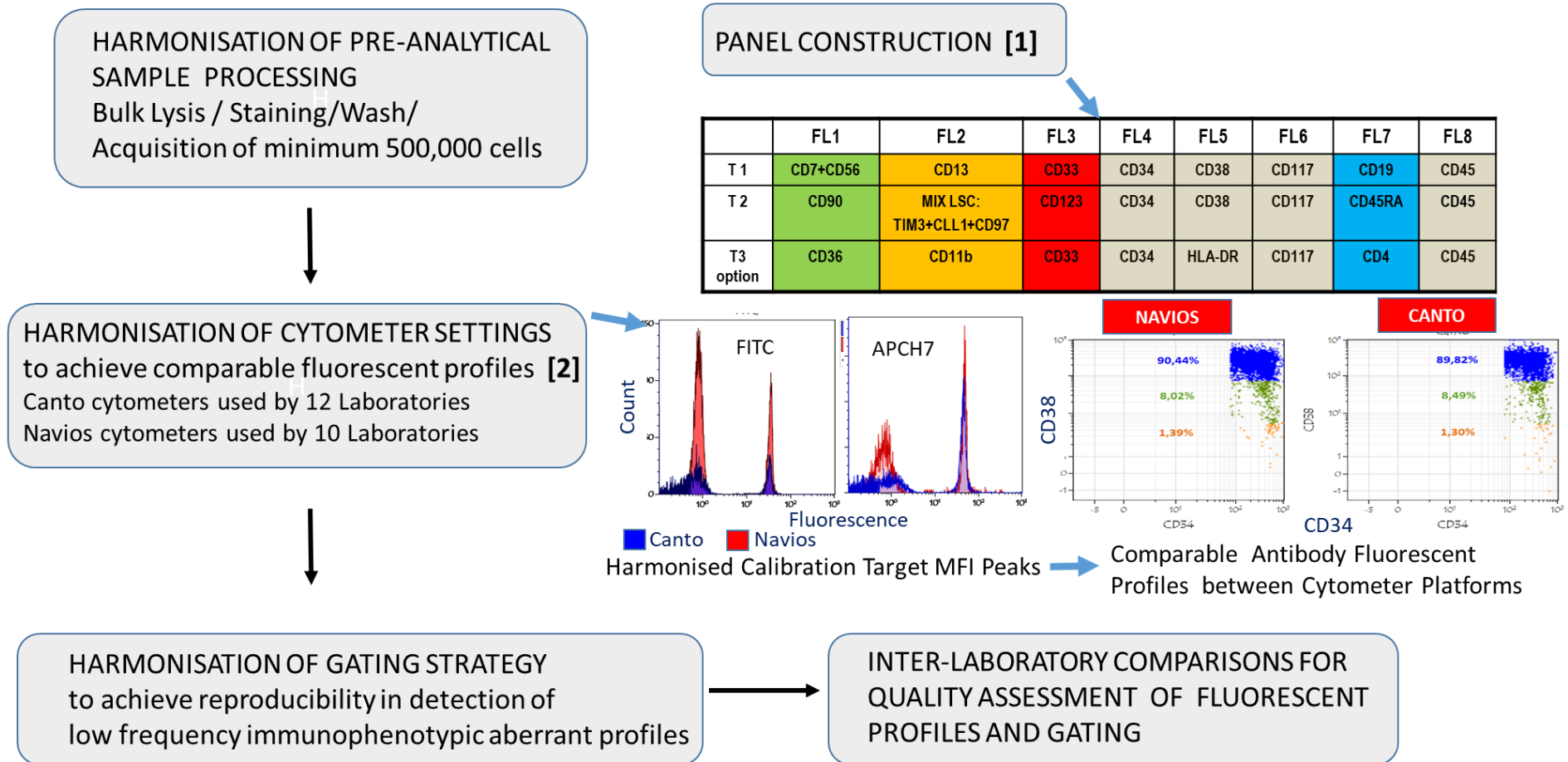


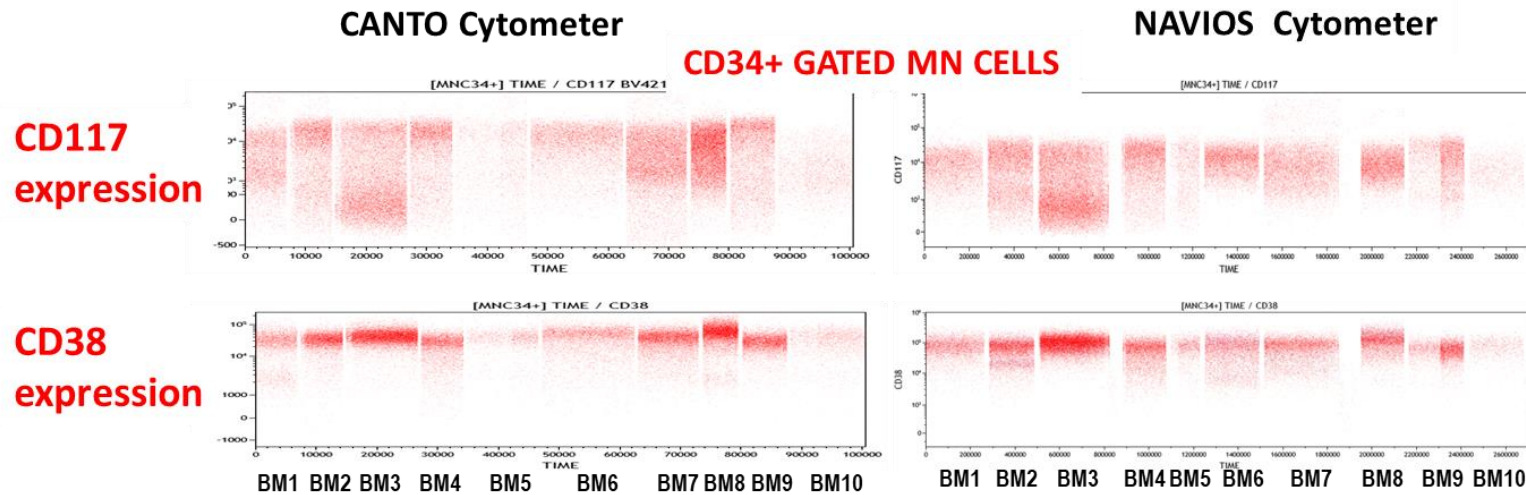
Figure 3

A Schema of a Flow Cytometric AML MRD multi-center Harmonisation Strategy



B Examples of Inter-Laboratory Quality Assessments

Inter-laboratory QA - similar marker expression profiles for 10 distributed BMs [3]



Inter-laboratory QA (22 laboratories) - reproducibility for rare event detection [4]

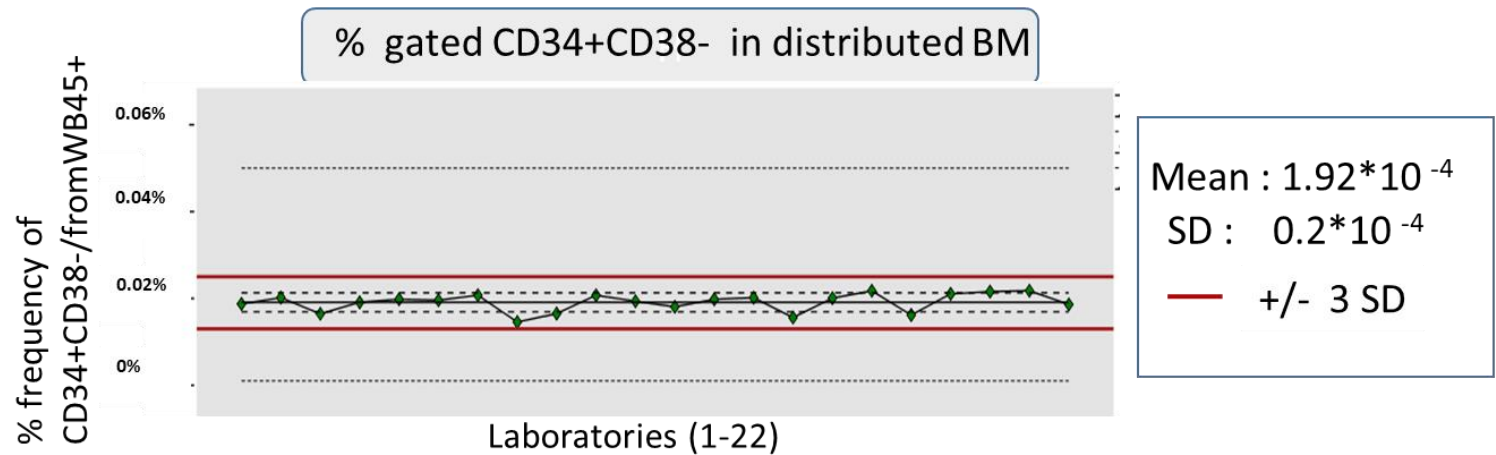


Figure 4

Figure 4

