Blood and Lymphatic Cancer: Targets and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

REVIEW

Strategies for minimal residual disease detection: current perspectives

This article was published in the following Dove Medical Press journal:
Blood and Lymphatic Cancer: Targets and Therapy

Giacomo Andreani
Daniela Cilloni

Department of Clinical and Biological Sciences, University of Turin, Turin, Italy

Abstract: Currently, the post-remission treatment in acute leukemia is based on the genetic profile of leukemic cells at diagnosis (ie, *FLT3* ITD positivity) and on the level of measurable residual disease (MRD) after induction and consolidation chemotherapy. Two methods are currently preferred for MRD evaluation in many centers: multiparameter flow cytometry and real-time quantitative PCR. Additional methods such as next-generation sequencing and digital PCR are under investigation, in an attempt to increase the sensitivity and thus allowing the detection of small clones. Many studies suggest that MRD positivity after chemotherapy is associated with negative prognosis, and the reappearance of MRD during follow-up allows impending relapse to be identified and consequently enables early intervention. Finally, MRD positivity before hematopoietic stem cell transplantation is predictive of the outcome. Although the significance of MRD in acute leukemia has been widely explored, the assessment of molecular MRD is not yet a routine practice. In this review, we describe the significance of MRD in different settings and the main markers and methods used for MRD detection.

Keywords: minimal residual disease, acute leukemia, multiparameter flow cytometry, real-time quantitative polymerase chain reaction, next-generation sequencing, digital PCR

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem cell disorders with different sensitivity to chemotherapy.[1] The molecular pathogenesis of AML has been studied for years by cytogenetic and molecular analyses.[1,2] Recurrent genetic abnormalities play an essential role in the pathogenesis of the disease and are well established prognostic markers. More recently, studies based on next-generation sequencing (NGS) clearly showed that leukemia is composed of different clones having different genetic profiles.[2,3] Frequently, a funding clone can be identified at diagnosis and additional small subclones can be detected only by very sensitive methods.[4,5] The small clones, undetectable at diagnosis, are capable of expansion over time and might be responsible for relapse. Furthermore, additional mutations may emerge in clones and subclones during chemotherapy and may change their sensitivity profile to different agents, thus favoring chemo-resistance and finally relapse.[4] The so-called "clonal evolution" explains the different mutational profiles frequently observed from diagnosis to relapse.

Although the majority of patients with de novo AML can undergo morphological remission after chemotherapy treatment, the rate of relapse is still very high.[1] A

Correspondence: Daniela Cilloni
Department of Clinical and Biological Sciences, University of Turin, 10043 Turin, Italy
Tel +39 11 902 6610
Fax +39 11 903 8636
Email daniela.cilloni@unito.it

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/BLCTT.S172693

number of studies clearly showed that the identification of persistence of a small amount of minimal residual disease, or more precisely measurable residual disease (MRD), is an important prognostic factor to establish the risk of relapse and, as a consequence, the post-remission therapy.[6,7]

Currently, the post-remission treatment is based on the genetic profile of leukemic cells at diagnosis and on the level of minimal residual disease after induction and consolidation chemotherapy, detected by multiparameter flow cytometry (MFC) in the majority of the studies.[8] In this review, an example is provided by the GIMEMA clinical trial AML1310 (ClinicalTrials.gov. Identifier NCT01452646) that stratified the post-remission therapy according to the presence of MRD. Intermediate-risk AML with positive MRD received allogeneic hematopoietic stem cell transplantation (allo-HSCT), while MRD-negative cases were treated only with consolidation chemotherapy. Although the results are still under analysis, it is reasonable that in the near future the decision between chemotherapy or chemotherapy plus allo-HSCT will be based not only on genetic risk at diagnosis but also on the presence of MRD.

The reappearance of MRD during follow-up allows identification of identify impending relapse and consequently enables early intervention.[7]

Despite the significance of MRD in acute leukemia having been widely explored, with an increasing number of studies demonstrating the positive prognostic value of MRD negativity, the assessment of molecular minimal residual disease is not yet a routine practice. Many studies are based on few genetic markers and the agreement on the methods and targets is still far from being reached. An attempt to standardize the methodology and to provide suggestion on when and how to monitor MRD has been recently published under the aegis of the European Leukemia Net (ELN).[9]

MRD indicates the presence of leukemia cells down to the levels of $1:10^4$ to $1:10^6$ white blood cells, compared with 1:20 in morphology-based measurement.

Two methods are currently preferred for MRD evaluation in many centers: MFC and real-time quantitative PCR (qPCR). Additional methods such as NGS and droplet digital PCR (ddPCR) are under investigation, in an attempt to increase the sensitivity and thus allow the detection of small clones. Each methodology differs in the sensitivity to detect the presence of MRD and in the number of patients who can potentially be monitored.

When applying molecular monitoring in AML patients, an important aspect should be considered, ie, the age-related clonal hematopoiesis. In the last few years, at least three different large studies carried out on thousands of healthy subjects of different ages investigated the presence of mutations in hematopoietic cells by using NGS technique.[10–12] The mutations detected in healthy subjects are typical of leukemic cells including *TET2*, *DNMT3A,* and *ASXL1*. These studies identified mutations with an allele frequency of more than 2% in an increasing proportion of subjects during aging. By the age of 70 years, more than 10% of the subjects have clonal hematopoiesis which is a risk factor for both the development of malignancies, which occur in 0.5%–1% of them per year, and cardiovascular events, with an increased risk of four times compared to age-matched controls.[11,12] The detection of mutations in healthy people suggests the existence of pre-leukemic stem cells. When additional mutations are acquired, leukemia might develop. Intensive chemotherapy selectively targets the malignant clone, thus allowing the pre-leukemic clone to survive.

Recently, the persistence of these mutations has been demonstrated during remission after chemotherapy without an evident impact on relapse, suggesting the persistence of pre-leukemic stem cells.[5] These data have an important impact on the significance of MRD and on the choice of the molecular marker to follow.

Molecular MRD

There are two main approaches for molecular MRD quantification: real-time PCR and DNA sequencing.[9] The PCR approach includes real-time PCR, digital PCR, and chimerism analysis. Real-time PCR represents, so far, the gold standard for MRD detection, although its application is limited to a selected number of patients (about 40%), carrying one or more suitable molecular markers.[13]

This limitation might be overcome by NGS that, at least in theory, can be applied to all leukemia-specific genetic markers. This approach can increase the percentage of patients who can be monitored by another 40%. However, this method still requires validation and standardization.[9]

Marker for molecular MRD

Fusion transcript in core binding factor (CBF) leukemias: CBFB-MYH11 and RUNX1-RUNX1T1

Despite the negative impact of MRD positivity in core binding factor (CBF) leukemias on relapse, no effect was clearly demonstrated on overall survival (OS) in multivariate analysis. This could be ascribed to the relatively high response rates of CBF leukemias to salvage therapy. In inv(16) patients, Yin et al[14] demonstrated that after induction chemotherapy, more than ten *CBFB-MYH11* copies /10^5 ABL copies in peripheral blood were

the most useful prognostic variables for relapse risk on multivariate analysis. MRD, after the end of therapy, was also informative with levels of transcript inferior to ten copies/10^5ABL copies in peripheral blood (PB) associated with a cumulative incidence of relapse at 5 years in 36% of cases compared to 78% for those not reaching this threshold. During follow-up, the presence of less than 50 copies/10^5ABL copies of transcript in bone marrow (BM) and ten copies in PB was associated with an OS at 5 years of 100% and 91%, respectively.[14] We should therefore keep in mind that low and stable levels of transcripts may be detectable by PCR for a relatively long period of time after chemotherapy without evidence of relapse.[15]

Similarly, for *RUNX1-RUNX1T1*, Yin et al[15] established that a threshold of less than 100 copies/10^5 *ABL* copies in PB and less than 500 copies /10^5 *ABL* copies in BM was associated with an OS at 5 years of 95% and 94%, respectively. The French group[16] analyzed 94 *RUNX1-RUNX1T1* positive patients during follow-up and showed that the molecular remission after the completion of consolidation therapy was not predictive. By contrast, the negativity in PB at the same time point predicts an OS at 4 years of 96% compared to 63% if positive. As for *CBFB- MYH11*, the MRD negativity at earlier time was not prognostically relevant.[16]

NPM1 mutation

The mutation of *NPM1* can be target for MRD by qPCR. *NPM1* has been found to be mutated in 50%–60% of AML patients with a normal karyotype.[21]

The presence of measurable *NPM1* transcripts in PB or in BM after at least two cycles of chemotherapy is associated with a high risk of relapse in many studies.[20] Ivey et al[17] reported a 3-year OS of 75% for patients with *NPM1* negativity in PB vs 24% for those with positive *NPM1*. Shayegi et al[18] reported a 3-year OS of 84% for negative patients at the same time point but measured in BM compared to 76% for those with low *NPM1* levels (*NPM1/ABL* <1%) and 45% with those who maintain positive levels with a *NPM1/ABL* ratio >1%. Similarly, Kronke et al[19] reported a 4-year OS of 90% for those who reached *NPM1* negativity in BM after two cycles of chemotherapy compared to 56% for those with *NPM1* positivity.

For patients who obtain a *NPM1* negativity in PB but remain positive in BM after the end of treatment, the ELN recommendations suggest to closely monitor the mutation in PB and BM every 4 weeks for at least 3 months.[9]

PML-RARA

In acute promyelocytic leukemia, the most significant MRD end point is the achievement of PCR negativity for *PML-RARA* at the end of consolidation treatment, independently from the therapeutic strategy, all-trans retinoic acid (ATRA) associated with chemotherapy, or ATRA and arsenic trioxide. *PML-RARA* negativity at the end of consolidation treatment is associated with a low risk of relapse and a high probability of long-term survival.[22,23]

As for CBF leukemia, we should consider that measurable levels of *PML-RARA* during active treatment should not trigger a treatment change. The usefulness of serial PCR-based MRD monitoring during treatment is still under investigation.

The Wilms' tumor gene (WT1)

The usefulness of *WT1* quantitative assessment, using q-PCR, as a marker for MRD detection in AML has been demonstrated many years ago.[24–30] *WT1* is overexpressed in about 80%–90% of the patients.[24,25] The persistence of *WT1* overexpression after treatment is always indicative of MRD.[24–30] *WT1* can thus be considered as the most universal marker of AML. In a European study, it was shown that both the level of *WT1* reduction from baseline after induction or consolidation therapy and the clearance of the transcript to normal values are highly predictive of relapse.[25] Many studies suggested that the persistence of abnormal values of *WT1* after induction or consolidation treatment has an impact on the probability of relapse. An increase of *WT1* levels during follow-up always predicts the leukemia recurrence.

Importantly, *WT1* is overexpressed independently of the genetic lesion(s) in the leukemic cells. This could generate suspicion because it is evident that *WT1* is not specific for one particular leukemic clone. By contrast, considering the recent advances coming from NGS studies that clearly indicate that there is a clonal selection in acute leukemias, the usefulness of a molecular marker able to track all the leukemic clones independently from all genetic lesions is unquestionable.

The main advantages of *WT1* assay are that it can be measured in PB, the method has been standardized,[25] the assessment of the results is not dependent on human expertise, in contrast to flow cytometric analysis of MRD, and it can detect the emergence of leukemic clones that are genetically or phenotypically different from those detected at diagnosis.

In the recent recommendations of the ELN by Schuurhuis et al,[9] the authors claimed that *WT1* mRNA quantitation should not be used as minimal residual disease marker in AML, due to low sensitivity and specificity, unless no other MRD markers, including flow cytometric ones, are available in the patient. Despite these recommendations, in the last 15 years, many scientific papers have been published showing

that *WT1* is a reliable marker of MRD that is able to predict relapse with a high level of accuracy.[24–30]

The fear of the lack of *WT1* sensitivity is mainly based on the fact that there is a background of expression in healthy subjects.[25] In addition, the absolute value of *WT1* in AML at diagnosis is not always two logs higher than in normal samples.[28] This led to a sort of skepticism toward *WT1* that, however, cannot be justified by the evidence provided by prospective and retrospective studies.[24,25,28]

Additional molecular markers

BCR-ABL

The 2016 WHO diagnostic guidelines included *BCR-ABL* positive trait as a provisional entity. The vast majority of the patients are characterized by p190 transcript, which is uncommon in chronic myeloid leukemia patients.[31]

Since *BCR-ABL* positive AML is a rare subtype of leukemia, very little data are available on the outcome and the prognostic value of *BCR-ABL*-based MRD detection.

IDH1/IDH2

Since the evidence that *IDH1/IDH2* genes can be mutated in about 10%–20% of AML cases, many groups are investigating the possibility of using *IDH1/2* as a marker for MRD detection.

Until now, it is not clear whether *IDH1/IDH2* become negative during remission, and contrasting data are reported in literature.[32,33] There are studies reporting the stability and suitability of *IDH* as a marker of MRD.[32,34] Recently, Petrova t al[35] published the evaluation of MRD in 90 patients, 22% of them with *IDH1/IDH2* mutations. They based the assessment on NGS and ddPCR. Many patients presented additional mutations such as *NPM1* or *MLL-PDT* and this allowed clinicians to conclude that *IDH1/2* correlated with the treatment response. Despite this, they found that the approach based on *IDH1/2* is less sensitive than *NPM1* in predicting relapse but more sensitive than *MLL*-partial tandem duplication (*PTD*).

Brambati et al explored the possibility of detecting *IDH1/2* after transplantation to better identify the risk of relapse.[34] They concluded that longitudinal monitoring of these mutations can be extremely useful in the allo-transplantation setting, a context in which these alterations can be considered markers of undesired residual pre-leukemic host hematopoiesis.

Multiparameter flow cytometry

Lots of effort has been devoted in an attempt to obtain the standardization of MFC.[36] For years, the detection of the leukemic population has been based on a panel of antibodies targeting early markers such as CD34 and CD117, markers of myeloid-lineage such as CD33, and myeloid differentiation antigens like CD11b, CD13, CD14, CD15 or lymphoid antigens including CD2, CD7, CD19, or CD56.

Two different approaches have been used to measure MRD with MFC: the leukemia-associated immunophenotype (LAIP) approach which characterizes the LAIP at diagnosis and tracks the identified population of blast cells during follow-up.[37] A second approach is based on "different from normal" approach (DfN), which is based on the identification of aberrant differentiation/maturation profiles at any time point. The DfN approach has the advantage that it can be applied even in the absence of the immunophenotype at diagnosis and can detect the immunophenotype shifts,[38] which is caused by the appearance of new clones. Immunophenotype shifts may emerge from leukemia evolution or clonal selection.[39]

The use of a large panel of antibodies can overcome the problem of using LAIP rather than DfN, as it is able to cover both the aspects.

Recently, the ELN recommendations suggest the term "LAIP-based DfN approach" for this combination strategy that can provide characterization of leukemic cells at diagnosis, MRD evaluation, and detection of new aberrancies not present at diagnosis.[9] For all these reasons, the ELN researchers recommend to use at least eight colors.

Differently from what has been demonstrated for qPCR-based MRD assessment, the evaluation of MRD by MFC is not recommended in PB for the lower frequency of leukemic cells.

Another important limitation of MFC was the absence of a commonly accepted threshold of negativity that is able to distinguish between MRD-positive and -negative cases. As 0.1% was found to be relevant in many published studies, the ELN recommends using this threshold. However, an MRD below 0.1 can be consistent with the persistence of residual leukemic cells. Several studies demonstrated the prognostic value of MRD to be below 0.01%,[40,41] showing that this threshold can identify patients with a very good prognosis. Independent validation of these very low levels may be highly relevant in future.

MRD in the setting of allogeneic stem cell transplantation

Many published data support the notion that the presence of MRD immediately prior to allo-HSCT is a strong, independent predictor of post-transplant outcomes in AML.[42]

The MRD positivity has been defined as one of the stronger predictive factors both in the ablative and non-myeloablative transplants.[43]

In a large study carried out in 279 AML patients, Zhou et al investigated MRD before and after HSCT.[44] It was shown that patients with MRD positivity before transplantation have a high relapse risk regardless of whether or not they clear MRD with conditioning chemotherapy.[44]

Studies in *NPM1*-mutated patients confirmed the impact of MRD pre-transplantation on the OS.[45] It was shown that only those patients who achieved at least a suboptimal reduction of *NPM1* levels after chemotherapy showed an improved OS after HSCT.

Bill et al[46] analyzed a cohort of 51 *NPM1*-mutated patients who received HSCT. Mutated *NPM1* MRD-positive patients, measured by ddPCR, had higher cumulative incidence of relapse and shorter OS. They demonstrated that *NPM1* MRD positivity, measured by ddPCR before allogeneic stem cell transplantation, is associated with worse prognosis independent of other known prognostic markers. Similar results have been described by Kayser et al[47] in a series of 67 patients. More recently, to extend the evaluation of MRD to all the patients treated with HSCT, an NGS approach has been used in a cohort of 116 patients in complete remission who were treated with HSCT.[48] The MRD was measured before transplantation, and it was demonstrated, in multivariate analysis, that MRD positivity was an independent negative predictor of relapse.

Finally, many papers reported the value of *WT1* monitoring in the setting of HSCT, both before HSCT to assess the quality of the remission and therefore predict the outcome and after HSCT to predict relapse.[48–52] The data reported in this setting are quite strong and led several centers to adopt *WT1*-based pre-emptive immunotherapy with cyclosporine discontinuation and/or donor lymphocyte infusion in patients with increasing *WT1* values after transplantation.[53,54]

Finally, although many studies reported that the *CBFB-MYH11* fusion transcript can persist at low level in patients during long-term remission after chemotherapy, only a few studies with small samples have addressed the detection of the *CBFB-MYH11* fusion transcripts after allo-HSCT.[55]

More recently, Tang et al reported 53 high-risk adult AML patients with inv(16) who received allo-HSCT.[56] During follow-up, seven patients experienced relapse. All relapses occurred in patients who either did not achieve major molecular response within the first 3 months or who lost major molecular response (MMR) in the first 3 months from transplantation.[56]

Digital PCR

New techniques are on the horizon for the detection of small leukemic clones. A promising approach is based on digital PCR. Digital PCR is a breakthrough technology designed to provide absolute nucleic acid quantification. It is particularly useful in detecting low amounts of target; therefore, it is highly sensitive in detecting MRD. It is estimated that ddPCR can detect up to 0.001% mutated allele frequency.[57]

This technique can overcome some difficulties faced by conventional PCR. With ddPCR, a sample is partitioned in single nucleic acid molecules. As a result of the partitioning of the sample into some sort of "bubbles", each bubble will contain zero or one molecule. After PCR amplification, nucleic acids may be quantified by counting the bubbles containing PCR end products. The main disadvantages for now are the cost of the analysis, the limited availability of the instruments that are not routinely introduced into diagnostic laboratories, and the lack of standardization.

In conventional PCR, the starting copy number is proportional to the number of PCR amplification cycles. Digital PCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and providing absolute quantification.

Few studies have been published on MRD by ddPCR. We have already mentioned the study by Petrova et al[35] based on *IDH1/2* mutation. This technique has also been explored by Brambati et al[34] in the setting of allogeneic bone marrow transplantation to identify the reappearance of small mutated clones of the recipient. In the same setting, Bill et al[46] reported the prognostic significance of *NPM1* positivity by ddPCR. A new assay based on digital PCR technique composed of multiplex pools of insertion-specific primers that selectively detect mutated but not wild-type *NPM1* has been described by Mencia-Trinchant et al.[58]

Next-generation sequencing

On the basis of the concept that MRD evaluation requires more than one technology and more than one marker, NGS is presently under investigation. Since there is a high degree of genotypic and phenotypic heterogeneity in AML, each patient should have a unique signature to be used to track MRD after therapy. Whole genome- or exome sequencing-based identification of clones and subclones in patients at diagnosis allows MRD to be followed by individualized monitoring. The usefulness of an NGS-based MRD assay is not only the prognostic stratification for relapse risk but also for the identification of different drugable targets for a

personalized therapy.[59] Prospective studies of the use of NGS in AML as a marker of MRD in the pre- and post-transplant setting are ongoing.

The main advantages of NGS technology are the reduced DNA sequencing time and cost, and the remarkably increased data-production capacity. NGS technologies rely on different methods for DNA template preparation, massively parallel reading of sequenced millions of short DNAs, real-time image capturing, alignment of sequences, sequence assembly, and variant detection. Each method has specific advantages: read length, accuracy, run time, and throughput. The main disadvantages of using NGS are shorter read length and the fact that its ability to detect MRD depends on the depth of sequencing and on the type of computational algorithms used. These aspects can complicate the process of standardization of the method for MRD detection.

Recently Onecha et al[60] explored the possibility of using deep sequencing MRD approach. They analyzed 190 patients affected by AML. A total of 211 (80%) single nucleotide variations and 46 (20%) indels were detected using the NGS custom panel. They followed the aberrations during follow-up with the same approach and were able to demonstrate that MRD status (MRD levels >0.1%) at post-induction was associated with a significantly lower rate of OS (33% vs 78%), while MRD-positive status after induction chemotherapy (MRD levels >0.025%) was associated with shorter OS (33% vs 81%) and significantly shorter DFS.

Levis et al[61] recently published a sensitive and specific MRD assay for *FLT3*-ITD mutations using NGS. They demonstrated a relationship between the mutation burden, as detected by their assay, and OS.

Conclusion

In acute leukemias, the detection of MRD is highly informative of the outcome of therapies, including HSCTs. The majority of the studies on MRD reported data based on MFC and real-time qPCR. Many attempts are ongoing to improve the sensitivity and to standardize the currently available techniques. This aspect is important to reach a common agreement on the threshold of MRD that triggers therapeutic decisions. New molecular targets are under investigation with encouraging results. Furthermore, the availability of new molecular targeted drugs that can potentially fully eradicate the residual small clones stimulates the interest in the detection of residual leukemic cells. New promising approaches are on the horizon to enlarge the spectrum of patients who can be monitored for the persistence of leukemic clones, including NGS.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Döhner H, Weisdorf DJ, Bloomfield CD, Leukemia AM. Acute myeloid leukemia. *N Engl J Med Overseas Ed*. 2015;373(12):1136–1152.
2. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med*. 2016;374(23):2209–2221.
3. Grimwade D, Ivey A, Huntly BJ. Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance. *Blood*. 2016;127(1):29–41.
4. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150(2):264–278.
5. Jongen-Lavrencic M, Grob T, Hanekamp D, et al. Molecular minimal residual disease in acute myeloid leukemia. *N Engl J Med*. 2018;378(13):1189–1199.
6. Walter RB, Othus M, Burnett AK, et al. Resistance prediction in AML: analysis of 4601 patients from MRC/NCRI, HOVON/SAKK, SWOG and MD Anderson Cancer Center. *Leukemia*. 2015;29(2):312–320.
7. Hourigan CS, Gale RP, Gormley NJ, Ossenkoppele GJ, Walter RB, et al. Measurable residual disease testing in acute myeloid leukaemia. *Leukemia*. 2017;31(7):1482–1490.
8. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.
9. Schuurhuis GJ, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2018;131(12):1275–1291.
10. Genovese G, Jaiswal S, Ebert BL, et al. Clonal hematopoiesis and blood-cancer risk. *N Engl J Med*. 2015;372:1071–1072.
11. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488–2498.
12. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N Engl J Med*. 2017;377(2):111–121.
13. Krauter J, Görlich K, Ottmann O, et al. Prognostic value of minimal residual disease quantification by real-time reverse transcriptase polymerase chain reaction in patients with core binding factor leukemias. *J Clin Oncol*. 2003;21(23):4413–4422.
14. Yin JA, O'Brien MA, Hills RK, Daly SB, Wheatley K, Burnett AK. Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. *Blood*. 2012;120(14):2826–2835.
15. Yin JA, Frost L. Monitoring AML1-ETO and CBFbeta-MYH11 transcripts in acute myeloid leukemia. *Curr Oncol Rep*. 2003;5(5):399–404.
16. Willekens C, Blanchet O, Renneville A, et al. Prospective long-term minimal residual disease monitoring using RQ-PCR in RUNX1-RUNX1T1-positive acute myeloid leukemia: results of the French CBF-2006 trial. *Haematologica*. 2016;101(3):328–335.
17. Ivey A, Hills RK, Simpson MA, et al. UK National Cancer Research Institute AML Working Group. Assessment of minimal residual disease in standard-risk AML. *N Engl J Med*. 2016;374(5):422–433.
18. Shayegi N, Kramer M, Bornhauser M, et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. *Blood*. 2013;122(1):83–92.
19. Krönke J, Schlenk RF, Jensen KO, et al. Monitoring of minimal residual disease in *NPM1*-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. *J Clin Oncol*. 2011;29(19):2709–2716.
20. Balsat M, Renneville A, Thomas X, et al. Postinduction minimal residual disease predicts outcome and benefit from allogeneic stem cell transplantation in acute myeloid leukemia with *NPM1* mutation: a study by the acute leukemia French association group. *J Clin Oncol*. 2017;35(2):185–193.

21. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352(3):254–266.
22. Grimwade D, Jovanovic JV, Hills RK, et al. Prospective minimal residual disease monitoring to predict relapse of acute promyelocytic leukemia and to direct pre-emptive arsenic trioxide therapy. *J Clin Oncol*. 2009;27(22):3650–3658.
23. Platzbecker U, Avvisati G, Cicconi L, et al. Improved outcomes with retinoic acid and arsenic trioxide compared with retinoic acid and chemotherapy in non-high-risk acute promyelocytic leukemia: final results of the randomized Italian-German APL0406 trial. *J Clin Oncol*. 2017;35(6):605–612.
24. Inoue K, Ogawa H, Yamagami T, et al. Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. *Blood*. 1996;88(6):2267–2278.
25. Cilloni D, Gottardi E, de Micheli D, et al. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia*. 2002;16(10):2115–2121.
26. Østergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. *Br J Haematol*. 2004;125(5):590–600.
27. Lapillonne H, Renneville A, Auvrignon A, et al. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. *J Clin Oncol*. 2006;24(10):1507–1515.
28. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized *WT1* assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol*. 2009;27(31):5195–5201.
29. Ino K, Fuji S, Tajima K, et al. Clinical utility of Wilms' tumor 1 monitoring in patients with myeloid malignancy and prior allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2017;23(10):1780–1787.
30. Marjanovic I, Karan-Djurasevic T, Ugrin M, et al. Use of Wilms tumor 1 gene expression as a reliable marker for prognosis and minimal residual disease monitoring in acute myeloid leukemia with normal karyotype patients. *Clin Lymphoma Myeloma Leuk*. 2017;17(5):312–319.
31. Neuendorff NR, Burmeister T, Dörken B, Westermann J. BCR-ABL-positive acute myeloid leukemia: a new entity? Analysis of clinical and molecular features. *Ann Hematol*. 2016;95(8):1211–1221.
32. Jeziskova I, Razga F, Toskova M, et al. Quantitative detection of *IDH2* mutation for minimal residual disease monitoring in patients with acute myeloid leukemia and its comparison with mutations in *NPM1* gene. *Leuk Lymphoma*. 2013;54(4):867–870.
33. Chou W-C, Peng K-Y, Lei W-C, et al. Persistence of mutant isocitrate dehydrogenase in patients with acute myeloid leukemia in remission. *Leukemia*. 2012;26(3):527–529.
34. Brambati C, Galbiati S, Xue E, et al. Droplet digital polymerase chain reaction for DNMT3A and IDH1/2 mutations to improve early detection of acute myeloid leukemia relapse after allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2016;101(4):e157–e161.
35. Petrova L, Vrbacky F, Lanska M, Zavrelova A, Zak P, Hrochova K. IDH1 and IDH2 mutations in patients with acute myeloid leukemia: Suitable targets for minimal residual disease monitoring? *Clin Biochem*. 2018;61:34–39.
36. van Dongen JJM, Lhermitte L, Bottcher S, et al. EuroFlow Consortium (EU-FP6, LSHBCT 2006-018708). EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia*. 2012;26(9):1908–1975.
37. Al-Mawali A, Gillis D, Hissaria P, Lewis I, Incidence LI. Incidence, sensitivity, and specificity of leukemia-associated phenotypes in acute myeloid leukemia using specific five-color multiparameter flow cytometry. *Am J Clin Pathol*. 2008;129(6):934–945.
38. Baer MR, Stewart CC, Dodge RK, et al. High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B Study 8361). *Blood*. 2001;97(11):3574–3580.
39. Zeijlemaker W, Gratama JW, Schuurhuis GJ. Tumor heterogeneity makes AML a "moving target" for detection of residual disease. *Cytometry B Clin Cytom*. 2014;86(1):3–14.
40. Freeman SD, Virgo P, Couzens S, et al. Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukemia. *J Clin Oncol*. 2013;31(32):4123–4131.
41. Walter RB, Buckley SA, Pagel JM, et al. Significance of minimal residual disease before myeloablative allogeneic hematopoietic cell transplantation for AML in first and second complete remission. *Blood*. 2013;122(10):1813–1821.
42. Buckley SA, Wood BL, Othus M, et al. Minimal residual disease prior to allogeneic hematopoietic cell transplantation in acute myeloid leukemia: a meta-analysis. *Haematologica*. 2017;102(5):865–873.
43. Araki D, Wood BL, Othus M, et al. Allogeneic hematopoietic cell transplantation for acute myeloid leukemia: time to move toward a minimal residual disease-based definition of complete remission? *J Clin Oncol*. 2016;34(4):329–336.
44. Zhou Y, Othus M, Araki D, et al. Pre- and post-transplant quantification of measurable ("minimal") residual disease via multiparameter flow cytometry in adult acute myeloid leukemia. *Leukemia*. 2016;30(7):1456–1464.
45. Balsat M, Renneville A, Thomas X, et al. Postinduction minimal residual disease predicts outcome and benefit from allogeneic stem cell transplantation in acute myeloid leukemia with *NPM1* mutation: a study by the acute leukemia French association group. *J Clin Oncol*. 2017;35(2):185–193.
46. Bill M, Grimm J, Jentzsch M, et al. Digital droplet PCR-based absolute quantification of pre-transplant NPM1 mutation burden predicts relapse in acute myeloid leukemia patients. *Ann Hematol*. 2018;97(10):1757–1765.
47. Kayser S, Benner A, Thiede C, et al. Pretransplant NPM1 MRD levels predict outcome after allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia. *Blood Cancer J*. 2016;6(7):e449.
48. Candoni A, de Marchi F, Zannier ME, et al. High prognostic value of pre-allogeneic stem cell transplantation minimal residual disease detection by WT1 gene expression in AML transplanted in cytologic complete remission. *Leuk Res*. 2017;63:22–27.
49. Candoni A, Tiribelli M, Toffoletti E, et al. Quantitative assessment of *WT1* gene expression after allogeneic stem cell transplantation is a useful tool for monitoring minimal residual disease in acute myeloid leukemia. *Eur J Haematol*. 2009;82(1):61–68.
50. Nomdedéu JF, Esquirol A, Carricondo M, et al. Bone marrow WT1 levels in allogeneic hematopoietic stem cell transplantation for acute myelogenous leukemia and myelodysplasia: clinically relevant time points and 100 copies threshold value. *Biol Blood Marrow Transplant*. 2018;24(1):55–63.
51. Rautenberg C, Pechtel S, Hildebrandt B, et al. Wilms' tumor 1 gene expression using a standardized European leukemianet-certified assay compared to other methods for detection of minimal residual disease in myelodysplastic syndrome and acute myelogenous leukemia after allogeneic blood stem cell transplantation. *Biol Blood Marrow Transplant*. 2018;24(11):2337–2343.
52. Guolo F, Minetto P, Clavio M, et al. Combining flow cytometry and *WT1* assessment improves the prognostic value of pre-transplant minimal residual disease in acute myeloid leukemia. *Haematologica*. 2017;102(9):e348–e351.
53. di Grazia C, Pozzi S, Geroldi S, et al. Wilms Tumor 1 expression and pre-emptive immunotherapy in patients with acute myeloid leukemia undergoing an allogeneic hemopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2016;22(7):1242–1246.
54. Ogawa H, Tsuboi A, Oji Y, et al. Successful donor leukocyte transfusion at molecular relapse for a patient with acute myeloid leukemia who was treated with allogenic bone marrow transplantation: importance of the monitoring of minimal residual disease by WT1 assay. *Bone Marrow Transplant*. 1998;21(5):525–527.

55. Elmaagacli AH, Beelen DW, Kroll M, Trzensky S, Stein C, Schaefer UW. Detection of CBFbeta/MYH11 fusion transcripts in patients with inv(16) acute myeloid leukemia after allogeneic bone marrow or peripheral blood progenitor cell transplantation. *Bone Marrow Transplant*. 1998;21(2):159–166.
56. Tang FF, Xu LP, Zhang XH, et al. Monitoring of post-transplant *CBFB-MYH11* as minimal residual disease, rather than *KIT* mutations, can predict relapse after allogeneic haematopoietic cell transplantation in adults with inv(16) acute myeloid leukaemia. *Br J Haematol*. 2018;180(3):448–451.
57. Hindson CM, Chevillet JR, Briggs HA, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods*. 2013;10(10):1003–1005.
58. Mencia-Trinchant N, Hu Y, Alas MA, et al. Minimal residual disease monitoring of acute myeloid leukemia by massively multiplex digital PCR in patients with NPM1 mutations. *J Mol Diagn*. 2017;19(4):537–548.
59. Shapiro RM, Kim DDH. Next-generation sequencing-based minimal residual disease monitoring in patients receiving allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia or myelodysplastic syndrome. *Curr Opin Hematol*. 2018;25(6):425–432.
60. Onecha E, Linares M, Rapado I, et al. Novel deep targeted sequencing method for minimal residual disease monitoring in acute myeloid leukemia. *Haematologica*. Epub 2018 Aug 19.
61. Levis MJ, Perl AE, Altman JK, et al. A next-generation sequencing–based assay for minimal residual disease assessment in AML patients with *FLT3* -ITD mutations. *Blood Adv*. 2018;2(8):825–831.