

Minimal Residual Disease in Acute Myeloid Leukemia—Current Status and Future Perspectives

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Abstract In acute myeloid leukemia (AML), the achievement of a morphological complete remission (CR) is an important milestone on the road to cure. Still, the majority of patients who achieve a morphological CR will eventually relapse. Thus, morphological means are not sensitive enough to detect clinically relevant tumor burdens left behind after therapy. Over the last years, several methodologies, particularly multiparameter flow cytometry and polymerase chain reaction, have emerged that can detect, quantify, and monitor sub-microscopic amounts of leukemia cells (“minimal residual disease”, MRD). Newer techniques, such as next-generation sequencing, have not only changed our understanding of the molecular pathogenesis and clonal heterogeneity of AML but may also be used for MRD detection. Increasing evidence indicates that MRD could play an important role in dynamically refining disease risk and, perhaps, serve to fine-tune post-remission therapy in a risk-adapted manner, although the latter concept awaits validation through well-controlled trials. In this review, we discuss the current use of MRD

measurements during AML treatment and highlight future perspectives.

Keywords Acute myeloid leukemia · Minimal residual disease · Multiparameter flow cytometry · Real-time quantitative polymerase chain reaction · Next-generation sequencing · Prognostic and predictive value of MRD

Introduction

Many patients with acute myeloid leukemia (AML) will achieve a morphological complete remission (CR) with curative-intent induction chemotherapy [1–3]. Despite intensive post-remission therapies, however, fewer than half of the patients achieving a CR will be cured [1]. The likelihood of cure varies considerable across individual patients, highlighting the need for accurate methods to identify the patients at high risk of disease recurrence and direct post-remission

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therapy in a risk-adapted manner. There is now increasing evidence that levels of minimal residual disease (MRD) during the course of therapy can serve as an independent marker to identify such high-risk patients [5]. Besides achievement of a morphologically defined CR as prerequisite of cure, the term “molecular remission” had been introduced in the 2003 guidelines to refine treatment response in AML [4], and there is increasing evidence that levels of MRD after induction therapy are independently associated with risk of relapse and survival [5].

AML has a wide spectrum of cytogenetic and molecular abnormalities [2,6]. In some cases, leukemia-specific fusion genes (e.g., *PML-RARA* in t(15;17)(q22;q12), *RUNX1-RUNX1T1* in t(8;21)(q22;q22), *CBFB-MYH11* in inv(16)(p13.1q22) or t(16;16)(p13.1;q22), *MLL-MLLT3* in t(9;11)(p22;q23)) and/or clone-specific recurring gene mutations (e.g., *NPM1*, *DNMT3A*) or overexpression of genes, e.g., *WT1*, are present that can be used as molecular markers to detect and monitor MRD (Table 1). In recent years, two methods, multiparameter flow cytometry (MFC) and quantitative real-time polymerase chain reaction (RT-qPCR), have been developed to detect MRD in AML patients [1,2,5]. Increasing evidence indicates that the presence of MRD characterizes patients with a higher risk of relapse as compared to those who are MRD negative. Hence, MRD provides powerful prognostic information during treatment and follow-up that is in a statistical sense independent of pretreatment

characteristics such as cytogenetic or molecular abnormalities [5,7,8•]. Nonetheless, in AML, MRD as a tool to fine-tune risk assessment during post-remission therapy with adaption of treatment strategy is lagging behind acute lymphoblastic leukemia (ALL), acute promyelocytic leukemia (APL), or chronic myeloid leukemia (CML), in which MRD is now routinely used to guide treatment decisions at predefined checkpoints during therapy [9–13]. Below, we will review MRD assessment in AML by MFC and RT-qPCR, the proposed standardization of the methods and the potential use of MRD as a surrogate [14] for survival endpoints.

MRD Detection by MFC

Besides lineage determination to distinguish AML from ALL, immunophenotyping by MFC is a valuable tool to distinguish normal bone marrow (BM) from leukemia cells. Normal BM exhibits reproducible patterns of antigen expression during differentiation, whereas in the vast majority of AML patients (>90 %), leukemic cells are characterized by antigen-specific markers, which define a leukemia-associated aberrant immunophenotype (LAIP), thus allowing to monitor the proportion of cells expressing the LAIP in response to therapy [5]. Another approach is called “different-from-normal,” which uses a standard, fixed antibody panel to differentiate AML cells from normal hematopoietic cells or recovering

Table 1 Antibody combinations for the identification of leukemia-associated aberrant immunophenotypes as well as molecular targets for the measurement of minimal residual disease in acute myeloid leukemia

Class of leukemia-associated aberrant immunophenotypes	Examples (according to reference 5)	
Blast markers and myeloid lineage markers	CD34, CD45, CD117, CD133, HLA-DR CD13, CD15, CD33	
Lymphoid markers indicating cross lineage aberrant expression	CD2, CD7, CD8, CD19, CD22, CD56	
Myelomonocytic and megakaryocytic maturation markers	CD4, CD11b, CD14, CD36, CD64	
Markers for stem/progenitor cell subpopulations	CD34/CD117/CD38, CD45RA/CD123 + markers with aberrant or altered expression, e.g., CD33, CD123, CD135, CLL1, CD96, CD25, cross lineage markers	
Molecular targets	Frequency at diagnosis (%) in AML patients a (age >18 years)	Sensitivity
Fusion genes		
<i>PML-RARA</i>	5–8	10 ⁻⁴ 31
<i>RUNX1-RUNX1T1</i>	5–7	10 ⁻³ –10 ⁻⁴ 19
<i>CBFB-MYH11</i>	5	10 ⁻³ –10 ⁻⁴ 19
<i>MLL-MLLT3</i>	1.5 ^b	10 ⁻⁴ –10 ⁻⁵ 54
Gene mutation		
<i>NPM1</i>	35	10 ⁻⁵ –10 ⁻⁶ 24
<i>FLT3</i>	20–30	5 × 10 ⁻⁴ 75
<i>DNMT3A</i>	14–22	10 ⁻⁴ 85
Gene overexpression		
<i>WT1</i>	80	10 ⁻ –10 ⁻⁴ 26

^aFrequency according to reference 5

^bRoughly 50 % of *MLL*-rearranged AML

marrow after chemotherapy and is therefore independent from the initial LAIP. The latter approach has been successfully used in the setting of allogeneic hematopoietic stem cell transplantation (HSCT), fostered by the fact that immunophenotypic data from initial disease presentation was only available for a small subset of patients [15]. Visualization and analysis of immune-phenotypic data depends on manual inspection of cells on biaxial plots with multiple gating steps of selected cell populations. In an adequate sample, a MRD detection sensitivity of 10^{-3} to 10^{-4} can be achieved [5]. Currently, 6–10 differently labeled antibodies are used as standard for MFC-MRD detection [5,8,15]; analysis of immunophenotypic data by automated analysis algorithms (e.g., SPADE [16] or viSNE [17]) may facilitate interpretation and standardization of results in the future.

However, interpretation of MFC for MRD detection is technically demanding and highly individualized, since it is dependent on the expertise and experience of the reference laboratory [8,15,18]. Increased accuracy and sensitivity of MRD detection can be obtained by incorporation of more antibodies and automated analysis algorithms for the detection of an abnormal cell [18]; albeit cost-effectiveness has so far not been systematically evaluated. Leukemic cells are quantified relative to other cells in the specimen; hence, the smallest cell cluster, judged as MRD positive, depends on the total number of analyzed cells. Consequently, if the cell quantity is limited (e.g., in aplasia during therapy), MRD sensitivity is reduced. In addition, sensitivity is largely a function of whether the same phenotype is present in normal immature cells. The analysis becomes more complex with increasing numbers of simultaneously applied antibodies. It is well recognized that there is limited consistency in results between individual laboratories [18]. Thus, beside standardization of immunophenotypic MRD assays analogous to RT-qPCR, as done for example in the Europe Against Cancer (EAC) program [19], extensive experience and regular cross validation is necessary to reproducibly identify LAIPs.

An important shortcoming is related to “antigenic shift” with a change of LAIP expression profile at relapse, which is a well-known problem and may occur in up to 91 % of the cases [20–22]. This “antigenic shift” is closely related to the time point of assessment with a very low frequency at an early time point, such as after induction therapy, and an increasing frequency over time, particularly during follow-up. Therefore, several consensus LAIP panels are needed for MFC-MRD especially at later time points during therapy to avoid false negative findings due to LAIPs with antigenic shift.

Nevertheless, advantage of MFC-MRD as compared to RT-qPCR is its applicability to the vast majority of AML patients and the very fast availability of results. An example of a screening antibody panel for MFC is outlined in Table 1.

MRD Detection by RT-qPCR

In general, RT-qPCR is an advanced PCR assay in combination with a fluorophore emitting light on amplification of the PCR product [23]. This RNA-based approach provides a sensitivity of 10^{-4} to 10^{-6} [5,24–26]. RT-qPCR measures mRNA expression levels of AML-specific genes relative to a control gene, which is used to control for quality factors during sample preparation, e.g., time between sample withdrawal and RNA preparation, cell concentration, and quality of reverse transcription that may influence cDNA yield. Besides providing comparability of different measurements, this allows documentation of sensitivity of each individual assay and elimination of poor quality samples. About 10 years ago, ABL was identified as the most stable and reliable gene in normal peripheral blood (PB) and BM and should therefore be used as control gene [27]. Within the EAC program, a framework for optimal conditions on RT-qPCR analysis including careful selection of probes and primers has been established through systematic parallel evaluation in an international network of expert laboratories, thereby enhancing assay sensitivity and specificity as well as interlaboratory comparability [19].

Very recently, the European LeukemiaNet developed a MRD-reporting software, where data from various RT-qPCR platforms can be imported, processed, and presented in an uniform manner to generate intuitively understandable reports allowing efficient data handling and harmonization of reporting [28••].

PML-RARA

MRD monitoring of the fusion gene *PML-RARA* by sequential RT-qPCR has been shown to be the strongest predictor of relapse in APL and, when coupled with preemptive therapy, provides a valid strategy to prevent overt hematological relapse [29]. This has led to the incorporation of MRD assessment in APL as a component of the standard response criteria [30]. In the context of several clinical trials, the standardized EAC RT-qPCR for *PML-RARA* has been shown to improve MRD detection rates as compared to conventional nested PCR assays [29,31]. Within a large UK study on 406 APL patients, mostly treated within the Medical Research Council (MRC) AML15 trial, the EAC RT-qPCR assay was the strongest predictive factor of relapse ($p < 0.0001$), even stronger than white blood cell (WBC) count at diagnosis [29]. Patients with detectable transcripts after the second course of standard therapy had a significant higher risk of relapse as compared to MRD-negative patients (relapse risk at 3 years, 19 vs. 8 %; $p = 0.003$). Evaluation at completion of the consolidation therapy revealed that BM was more sensitive as compared to PB (median difference, 1.5-log). Consequently, monitoring of BM after the end of therapy on a three-monthly basis has been recommended taking into account the assay sensitivity and

typical kinetics of relapse [30,32]. However, this approach is increasingly questioned with recently reported relapse rates in adult low-risk APL patients of 1.1 % at 3 years after therapy with arsenic trioxide (ATO) in combination with all-trans retinoic acid (ATRA) [33]. Nonetheless, MRD measurement remains an important tool to inform management of high-risk and relapsed APL patients, and MRD monitoring in these patients is highly recommended [5,30].

RUNX1-RUNX1T1

Several studies have noted the importance of MRD measurement by RT-qPCR in AML patients with t(8;21)(q22;q22) leading to the fusion transcript *RUNX1-RUNX1T1* [25,34,35,36–38]. Optimal outcomes are achieved when either a molecular remission or very significant reductions in *RUNX1-RUNX1T1* transcripts are achieved. However, significant heterogeneity within t(8;21)(q22;q22) leukemias is widely appreciated. While pretreatment variables associated with worse outcome have been recognized in AML with t(8;21)(q22;q22), including higher age, a high WBC count, deletion of the long arm of chromosome 9, nullisomy Y in male patients as well as the presence of *KIT* and/or *FLT3* mutations at diagnosis [39–46], a recent multicenter study in 100 AML patients with t(8;21)(q22;q22) (age range, 18–60 years) suggests that the level of MRD reduction outperforms high WBC and mutational status of *KIT/FLT3* to identify patients at high risk [35]. Hence, the authors suggested that MRD level, rather than secondary gene mutations, should be used for future treatment stratifications [35]. In particular, in the largest prospective study conducted to date on 163 patients, a >3-log reduction of transcript burden after the first induction therapy and a >4-log reduction after the first course of post-remission therapy were associated with cumulative incidences of relapse (CIR) of only 4 and 13 %, respectively [25]. Higher intensity regimens may lead to deeper log reductions after the first course of chemotherapy as has been shown for the addition of gemtuzumab ozogamicin (GO) to intensive chemotherapy [25] as well as for anthracycline dose intensification during intensive induction chemotherapy [47]. Patients, who received daunorubicin of 90 mg/m² showed a faster and deeper MRD reduction and achieved a higher proportion of complete molecular responses that translated into a reduced relapse rate as compared to those patients receiving 60 mg/m².

These findings were extended by Zhu et al. who evaluated a risk-directed therapy approach based on MRD in t(8;21)(q22;q22) AML patients. Within this study, 116 younger patients (range, 15–60 years) who achieved morphological remission with 1–2 courses of standard induction according to the “7+3” schema and then completed two cycles of intermediate-dose cytarabine-based consolidation therapy were examined. The lack of a major molecular response

(MMR, defined as a >3-log reduction in *RUNX1-RUNX1T1* transcript levels from baseline) after the second course of consolidation, or loss of MMR within 6 months, was used to categorize patients into high or low risk. Allogeneic HSCT was associated with reduced relapse risk and improved survival as compared to chemotherapy for high-risk patients (5-year CIR 22.1 vs. 78.9 %, $p < 0.0001$; 5-year disease-free survival (DFS) 61.7 vs. 19.6 %, $p = 0.001$; 5-year overall survival (OS) 71.6 vs. 26.7 %, $p = 0.007$), whereas it had no impact in low-risk patients (5-year CIR 14.7 vs. 5.3 %, $p = 0.33$) and was associated with inferior DFS relative to those treated with chemotherapy/autologous HSCT (70.3 vs. 94.7 %, $p = 0.024$) [34]. Although treatment assignment was not performed on a randomized manner, this study would support the idea of post-remission treatment adaptation according to MRD assessment in AML with t(8;21)(q22;q22).

CBFB-MYH11

Similar to *RUNX1-RUNX1T1*, MRD measurement of *CBFB-MYH11* resulting from inv(16)(p13.1q22) or t(16;16)(p13.1;q22) has been shown to be of clinical importance: Yin et al. defined relevant MRD checkpoints during treatment in a large series of 115 patients by identifying that an absolute copy number reduction in PB below 10 both after first induction and after first consolidation therapy was associated with a reduced CIR of 21 and 36 % as compared to 52 and 78 % above this copy number, respectively [25]. Other studies came to qualitatively similar conclusions [36,48–50]. In the study by Jourdan et al., $n = 98$ patients with AML and inv(16)(p13.1q22) or t(16;16)(p13.1;q22) were studied. As shown for AML with t(8;21)(q22;q22), multivariate analysis revealed higher MRD level (>3-log vs. ≤3-log) after the second consolidation as the only significant prognostic factor for RFS (HR, 0.40; range, 0.18–0.91) [35].

MLL-MLLT3

AML with *MLL-MLLT3* is rare in adults (about 2.0 % of the patients) but is more common in childhood AML (9–12 %) [51,52]. Adult patients have an intermediate survival and one that is superior as compared to other *MLL*-rearranged AML [53]. Scholl et al. established a sensitive RT-qPCR assay for different fusion types and showed that patients achieving a PCR-negative state had a very low probability of relapse (11 %) and a high OS at 4 years of 70 %, whereas those patients with RT-qPCR positive results all relapsed and died within 3 years [54,55]. These data had recently been expanded by Abildgaard et al. who reported on a RT-qPCR assay in combination with a locked nucleic acid probe for quantification of the most common break point region of the *MLL-MLLT3* fusion gene in pediatric patients [56].

***NPM1* Mutations**

Frameshift mutations of the *NPM1* gene are one of the most frequent molecular abnormalities in AML, particularly in patients with a normal karyotype [3,5]. To date, more than 50 different *NPM1* mutations have been reported [57]. The three most common variants (types A, B, and D) represent 90 % of all mutated cases and have been shown to be reliable markers for MRD detection with a high sensitivity [58]. The same assay can be adapted for cases with rare variants by replacing the mutation-specific primer, but these case-specific assays should be carefully tested in control samples (e.g., *NPM1* wild-type AML) to avoid nonspecific background amplification from the wild-type *NPM1* allele [59]. In contrast to other molecular aberrations (e.g., *FLT3*), *NPM1* mutations are typically stable during the course of the disease, which supports the notion that they are an early pathogenetic lesion in AML [60]. Similar to the findings in CBF-AML, RT-qPCR assessment of MRD can distinguish patients at high risk of relapse: in a study on 245 adult patients with *NPM1* mutated AML, relevant MRD checkpoints could be defined [61]. Achievement of RT-qPCR negativity after double induction therapy identified patients with a low CIR (6.5 % after 4 years; $p < 0.001$), translating into significant differences in OS (90 vs. 51 %, respectively; $p = 0.001$). After completion of therapy, CIR was 15.7 % in RT-qPCR-negative patients as compared to 66.5 % in RT-qPCR-positive patients ($p < 0.001$) [61]. These data are extended by the study of Hubmann et al. in whom a *NPM1* mutation cutoff level of 0.01 was associated with a CIR after 2 years of 77.8 % for patients with ratios above the cutoff versus 26.4 % for those with ratios below the cutoff [62]. Of note, in the randomized French ALFA-0701 trial showing the superiority of intensive chemotherapy in combination with GO over intensive chemotherapy alone, *NPM1*-MRD was predictive for response to therapy since more MRD-negative results were obtained in patients treated in the GO arm as compared to those treated in the control arm after induction therapy (39 vs. 7 %; $p = 0.006$) as well as at the end of treatment (91 vs. 61 %; $p = 0.028$) [63•]. This is one of the first randomized studies indicating that MRD assessment may serve as a surrogate for survival endpoints for the treatment under investigation.

Additionally, in a retrospective analysis performed by the German Study Alliance Leukemia, increasing levels of *NPM1* MRD were predictive of an impending relapse after chemotherapy (MRD increase > 1 % *NPM1*mut/ABL1) or allogeneic HSCT (MRD increase > 10 % *NPM1*mut/ABL1) [64].

Internal Tandem Duplication of the *FLT3* Gene

Though *FLT3* internal tandem duplication (*FLT3*-ITD) can be detected in roughly 25 % of all AML patients and is one of the

most affected gene mutations in AML, its suitability as a MRD marker has been questioned for several reasons: (1) its heterogeneity according to size, number of clones per patient, allelic ratio, and insertion site within the *FLT3* gene and (2) its proposed instability (reported on about 25 % of paired diagnosis-relapse samples) during the course of treatment, previously based on mainly semiquantitative methods with limited sensitivity [65–70]. However, these results may, to a certain degree, be hampered by insufficient sensitivity of the applied methods. In most cases, the mutation originally detected at diagnosis is also present at relapse, often at a higher allelic ratio than at diagnosis [68,69,71]. The other cases, where a *FLT3*-ITD is acquired at relapse, may represent clonal disease progression [60,71]. Newer techniques are aimed to improve the sensitivity of *FLT3*-ITD detection, such as RT-qPCR with patient-specific primers [72]. However, this approach is time-consuming since each *FLT3*-ITD needs a clone-specific primer/probe designed from the junctional sequence. Technically, this may not be possible for every case due to the constraints of the sequence at the junction. In addition, patients with a low allelic *FLT3*-ITD may not be obtainable for direct sequencing since the wild-type sequence is competitively amplified. Recently, another PCR-based assay to detect *FLT3*-ITD MRD has been reported. Within this assay, primers oriented in the opposite direction were used; hence, amplification occurred only if a *FLT3*-ITD was present. Again, this approach is technically limited, since short *FLT3*-ITDs (less than 30–40 bases) will not be detected due to insufficient primer annealing space, which may apply to roughly 25 % of all *FLT3*-ITD cases [73,74]. Both approaches are therefore not ready to be implemented in clinical routine care. Of note, next-generation sequencing (NGS) has been shown to be useful for MRD assessment, particularly in patients with *FLT3*-ITD [75]. Yet, major challenge is caused by the mass of data generated, including data storage, data analysis, and interpretation of the results. Finally, even with the decreasing costs for NGS studies, these tests remain expensive and require expertise to accurately analyze and decipher the complex data [76]. Taken together, though *FLT3*-ITD mutation testing should be mandatory in all AML patients at diagnosis as well as at relapse for prognostic purposes and for guiding therapeutic decisions, it currently has little utility for MRD monitoring.

***DNMT3A* Mutations**

The complexity of the leukemic genome has been highlighted by the discovery of mutations in genes important for epigenetic gene transcription regulation. *DNMT3A* mutations can be found in 15–25 % of AML patients, particularly in AML with normal cytogenetics [77,78] and are thought to be a “founder” mutation since they are present in early preleukemic hematopoietic stem cells [79•]. This has been confirmed in two pivotal

population-based studies showing an increasing incidence of clonal hematopoiesis with increasing age and *DNMT3A* as the most frequently mutated gene [80,81]. In AML patients, about 60 % of *DNMT3A* mutations affect residue R882 [82,83]. Several studies evaluated the stability of *DNMT3A* mutations in paired diagnosis and relapse material [60,84]. In the largest analyses, Hou et al. studied sequentially 316 samples from 138 patients, including 35 patients with distinct *DNMT3A* mutations and 103 patients without mutations at diagnosis. At relapse, all initially *DNMT3A*-mutated cases showed the same mutation, whereas all other patients remained negative at relapse [84]. Differential MRD assessment using *DNMT3A*, *NPM1*, *FLT3*, and *KIT* has been performed by Ploen and co-workers, who developed a multiplex allele-specific quantitative PCR assay for the sensitive detection of *DNMT3A* mutations affecting residue R882 [85]. Analysis of DNA from 298 diagnostic AML samples revealed *DNMT3A* mutations in 45 AML patients (15 %); the mutation was stable in 12 of 13 patients presenting with relapse or secondary myelodysplastic syndrome. Interestingly, persistent levels of *DNMT3A* mutations could also be found in remission samples from 14 patients up to 8 years after initial AML diagnosis, whereas MRD levels of concurrent mutations at diagnosis, such as *NPM1* and *FLT3*, became negative. Furthermore, cell sorting demonstrated the presence of *DNMT3A* mutations in leukemic blast cells, but also in T and B cells from the same patients [85]. These data suggest that *DNMT3A* mutations in these cases are rather markers for clonal hematopoiesis than AML-defining events. Thus, long-term persistence of *DNMT3A* may simply reflect the turn back from overt leukemia to clonal but otherwise clinically normal hematopoiesis. Based on the current data, the suitability of *DNMT3A* as a MRD marker within standard AML treatment is questioned; it may be of interest in clinical trials using epigenetically active drugs in combination therapy as well as single agent maintenance therapy (e.g., NCT01757535).

MRD Detection by Measuring Expression of *WT1*

WT1 is overexpressed in roughly 80 % of AML patients, thus making it an attractive target for MRD monitoring [26]. However, its value for MRD monitoring has been debated due to (1) the difficulty to discriminate the residual expression of leukemic cells from background expression, since the expression of *WT1* is not leukemia-specific and (2) differences of the applied assays. In 2009, the ELN consortium systematically evaluated nine different *WT1* RT-qPCR assays leading to the recommendation of a standardized assay as well as proposed threshold levels of 50 and 250 *WT1* copies/ 10^4 *ABL* copies for MRD detection in PB and BM samples, respectively [26]. The predictive value of MRD assessment had then been analyzed within a cohort of 129 AML patients treated with conventional “7+3”-based chemotherapy with diagnostic *WT1* levels

exceeding 2×10^4 copies/ 10^4 *ABL* copies, allowing the discrimination of at least a 2-log reduction as compared to the pretreatment level. A greater *WT1* reduction after the first induction chemotherapy was associated with a reduced relapse risk (hazard ratio [HR] 0.54 per log reduction; range, 0.36 to 0.83; $p=0.004$), independent from age, WBC count, or cytogenetic risk group. Reduction of *WT1* below the threshold limits defined in normal controls by the end of consolidation also predicted a reduced relapse risk ($p=0.004$). However, the degree of *WT1* overexpression in most AML patients was too modest to afford a highly sensitive universal marker for sequential MRD monitoring, when background levels of expression observed in normal PB and BM were taken into account [26]. In addition, PB seems to be more informative due to the much higher background expression in normal BM [5,63••]. Nevertheless, several studies have shown a correlation between detectable *WT1* MRD and clinical outcome [86–89]. Very recently, as already mentioned above, Lambert et al. evaluated the differential prognostic significance of *WT1* and *NPM1* MRD level assessed by RT-qPCR in 278 adult AML patients (age range, 50–70 years) treated in the ALFA-0701 trial [63••]. Positive *WT1* MRD (defined as >0.5 % in PB) after induction and at the end of consolidation treatment were both significantly associated with a higher risk of relapse (HR=3.15 [1.78–5.58], $p<0.0001$; HR=3.41 [1.62–7.17], $p=0.001$, respectively) and shorter OS (HR=3.23 [1.64–6.37], $p=0.0007$; HR=4.64 [1.38–15.62], $p=0.013$, respectively). However, the impact of the addition of GO to chemotherapy was not reflected by *WT1* MRD levels, whereas *NPM1* MRD levels tracked along with the treatment efficacy in terms of better molecular remissions in those patients treated with GO as adjunct to intensive chemotherapy. This discrepancy on the background of a significant beneficial impact of GO on all survival endpoints further questions the clinical use of *WT1* MRD assessment. In summary, *WT1* expression for MRD assessment should be restricted to cases where MRD assessment is otherwise not possible.

MRD-Directed Therapies

To date, the efficacy of close MRD-directed therapy is best supported for patients with APL [29,31,32]. For non-APL AML, MRD-based decision-making has not yet been embraced for routine clinical practice [5], but in genetically defined subsets of AML, such as CBF-AML and AML with *NPM1* mutations close MRD assessment has entered clinical trials triggering intensification of post-remission therapy in case of persistent MRD (e.g., NCT02013648, NCT00893399). Furthermore, the more general applicable approach of LAIPs detection with MFC has been integrated into a number of currently recruiting trials with MRD-triggered treatment intensification (EudraCT Number: 2013-002843-26, NCT02349178, NCT01452646, NCT01462578, NCT01677949).

Table 2 Impact of minimal residual disease status at different time points during induction and consolidation treatment on cumulative incidence of relapse

	Induction therapy		Early consolidation	Late consolidation	Patients in first CR	Proportion analyzed in MRD study
MCF						
Reference [98] Age >60 years Cut point 0.1 %	Course 1 MRD- CIR 2 years 60 % n=145 MRD+ CIR 2 years 79 % n=140	Course 2 MRD- CIR 2 years 73 % n=179 MRD+ CIR 2 years 82 % n=99	MRD- CIR 2 years 23 % n=97 MRD+ CIR 2 years 68 % n=24		n=455	63 % unselected population
Reference [8] Age 18–60 years Cut point 0.1 %	MRD- CIR 2 years 27 % n=109 MRD+ CIR 2 years 44 % n=55	MRD- CIR 2 years 37 % n=141 MRD+ CIR 2 years 68 % n=42			n=208	79 % unselected population
Reference [99] Age 18–60 Cut point 0.1 %	MRD- CIR 2 years 11 % n=45 MRD+ CIR 2 years 55 % n=81				n=126	Selected population
RT-qPCR CBF RUNX1-RUNX1T1						
Reference [25] Cut point 3–4-log reduction ^b	MRD- CIR 2 years 4 % n=28 MRD+ CIR 2 years 32 % n=31		MRD- CIR 2 years 13 % n=18 MRD+ CIR 2 years 49 % n=20		n=158	38 % unselected population
Reference [34*] Cut point 3-log reduction				Chemo/auto-HSCT MRD- CIR 2 years 5 % n=29 MRD+ CIR 2 years 79 % n=26 Allo-HSCT MRD- CIR 2 years 15 % n=18 MRD+ CIR 2 years 22 % n=40	n=113	Selected population
CBFB-MYH11						
Reference [25] Cut point <10 copies	MRD- CIR 2 years 21 % n=31 MRD+ CIR 2 years 50 % n=30		MRD- CIR 2 years 36 % n=36 MRD+ CIR 2 years 78 % n=9		n=106	57 % unselected population
Reference [48] Age 18–60 Cut point Negativity			MRD- CIR 2 years 20 % n=29 MRD+ CIR 2 years 47 % n=20	MRD- CIR 2 years 10 % n=14 MRD+ CIR 2 years 69 % n=35	n=49	Selected population
CBF-AML						
Reference [35*] Age 18–60 Cut point 3-log reduction			MRD- CIR 2 years 21 % n=124 MRD+ CIR 2 years 54 % n=52		n=176	Selected population
NPM1						
Reference [61] Age 18–60 Cut point Negativity		MRD- CIR 2 years 0 % n=26 MRD+ CIR 2 years 47 % n=111 MRD- CIR 2 years 42 % n=55 MRD+ CIR 2 years 91 % n=24		MRD- CIR 2 years 11 % n=62 MRD+ CIR 2 years 68 % n=67	n=137	Selected population
Reference [62] Age 18–80 Cut point 3-log reduction					n=79	Selected population
					n=61	Selected population

Table 2 (continued)

	Induction therapy	Early consolidation	Late consolidation	Patients in first CR	Proportion analyzed in MRD study
Reference [63••] Age 18–80 Cut point 3-log reduction	MRD– CIR 2 years 21 % <i>n</i> =15 MRD+ CIR 2 years 55 % <i>n</i> =46		MRD– CIR 2 years 45 % <i>n</i> =32 MRD+ CIR 2 years 67 % <i>n</i> =9		
Reference [64] Age 18–80 Cut point 1 % chemo/auto 10 % allo-HSCT			Chemo/auto-HSCT MRD– CIR 2 years 38 % <i>n</i> =65 MRD+ CIR 2 years 75 % <i>n</i> =15 Allo-HSCT MRD– CIR 2 years 13 % <i>n</i> =38 MRD+ CIR 2 years 77 % <i>n</i> =9	<i>n</i> =127	Selected population

In reference 49 and 50, no data for cumulative incidence of relapse are available *allo* allogeneic, *auto* autologous, *chemo* chemotherapy, *CIR* cumulative incidence of relapse, *HSCT* hematopoietic stem cell transplantation, *MRD* minimal residual disease ^aMRD assessment after course 1 *n*=101 and after course 2 *n*=25 ^bCut point after induction therapy, 3-log reduction; cut point after consolidation therapy, 4-log reduction

A widely cited study in 216 pediatric AML patients has used a comprehensive risk stratification strategy based on genetic abnormalities at diagnosis and MRD findings to direct decisions on the second induction course and subsequent therapy [90]. At diagnosis, patients were provisionally classified according to their underlying genetic abnormalities; responses to each course of therapy, as assessed by morphologic and flow MFC-MRD, determined the final risk classification. Those patients with a MRD level of ≥ 1 % after induction I were classified as high risk. Intended treatment consisted in low-risk patients (*n*=68) of five courses of chemotherapy, whereas high-risk patients (*n*=79), as well as standard-risk patients (*n*=69) with matched-related donors, were eligible for allogeneic HSCT (performed in 48 high- and 8 standard-risk patients). Comparison with historical controls suggested that this approach could improve outcomes.

In an observational series of 10 adult AML patients who were treated for increasing *NPM1* MRD levels with 5-azacytidine (5-AZA), Sockel and co-workers reported promising results: after a median follow-up time of 10 months (range 2–12 months) from initiation of 5-AZA treatment only three patients developed a hematologic relapse [91]. A molecular response with a ≥ 1 -log decrease in the MRD level was observed in 7 of the 10 patients [91]. The impact of 5-AZA therapy in AML patients with an impending hematological relapse and evaluable MRD marker (such as t(8;21), inv(16), and *NPM1*) is currently explored in a prospective phase II clinical trial (NCT01462578). In addition, since mutant *NPM1* can induce an antileukemic T cell response, it could serve as a target in the setting of immunotherapy [92]. Future strategies might therefore additionally use adoptive immunotherapy in case of reoccurrence of mutated *NPM1* after HSCT or administration of donor lymphocyte infusion (DLI) in patients after allogeneic HSCT [93].

MRD as a Predictor of Outcome After Transplant

The general notion that MRD-positive patients should immediately be candidates for an allogeneic HSCT has put into question by studies showing high relapse rates even after allogeneic HSCT in pre-transplant MRD-positive patients [15,94]. Bastos-Oreiro and co-workers evaluated the prognostic impact of MFC-MRD before and after allogeneic HSCT. MRD-negative patients (defined as ≤ 0.1 % LAIPs) at the time point of allogeneic HSCT had significant lower rates of relapse (15 vs. 66 %, *p*=0.045) and better OS (83 vs. 52 %, *p*=0.021) after 1 year as compared to MRD-positive patients [94]. Consistently, in the study presented by Walter et al., a marked impact of MRD positivity before allogeneic HSCT with relapse rates as high as 60 to 70 % has been shown [15]. However, the benefit of further therapy to reduce the level of MRD before allogeneic HSCT and the level of MRD that

would preclude the likelihood of cure after allogeneic HSCT are currently unknown. Additional intensive chemotherapy augments the risk for organ toxicity or life-threatening infections and may not always reduce the leukemia burden because of resistant leukemia cells. Nevertheless, a recent study on pediatric leukemia patients with positive MRD reported relatively high survival rates after allogeneic HSCT (5-year OS, 66.7 vs. 80.4 % for MRD positive vs. negative AML patients), suggesting that the negative effect of MRD had been partially offset by allogeneic HSCT [95]. Although MRD is associated with a several fold increased risk of relapse after allogeneic HSCT, up to 20–30 % of patients with MRD at the time of transplantation experience prolonged DFS; i.e., some MRD-positive patients will be salvaged with either myeloablative or nonmyeloablative conditioning allogeneic HSCT [15]. Therefore, MRD-positive AML patients should not be excluded from a potentially curative allogeneic HSCT.

Of course, based on these data, one could question whether, in fact, MRD-negative patients actually require allogeneic HSCT for long-term relapse-free survival.

MRD as a Surrogate Endpoint

Beyond a rapid blast cell clearance assessed by morphology after induction therapy [96,97], achievement of MRD negativity or marked transcript level reduction after first or second induction therapy seems to be of high prognostic impact (Table 2) [8, 25, 62, 63, 64, 98, 99]. Thus, a rapid decline in MRD levels after induction therapy reflects a highly chemosensitive disease with a per se favorable prognosis. However, relapse rates in patients with MRD negativity or marked transcript level reduction after induction therapy range between 0 and 73 % with a median of 27 %. Therefore, at least in one quarter of these patients, the label “low risk” is misleading, raising the question of specificity and sensitivity of the assay. In addition, Table 2 also illustrates a further shortcoming due to a broad variety of cut points used in these studies. This simply reflects the fact that in each study, the cut point was established and optimized within and for the reported cohorts without any validation process resulting in very low external validity, which is further put into question due to the highly selected patient populations. Therefore, a common international attempt to move forward standardization of MRD assessment is mandatory for future use of MRD in routine practice.

Beyond routine practice, MRD may increasingly be used to optimize efficacy assessment of specific treatment components such as standard consolidation therapy and to facilitate drug development. Exemplarily, Burnett et al. recently stated that consolidation therapy with 1.5 g/m² bid for 3 days is equivalent effective as the former used standard dose of 3 g/m² bid for 3 days mainly based on similar OS rates [100]. However, despite comparable OS rates in both arms, the strong trend

($p=0.06$) towards a higher relapse rate with the lower dosage of 1.5 g/m² points in a somewhat different direction [7]. In this situation, MRD assessment and systematic comparison of MRD levels in the two treatment arms would have added important scientific arguments. Currently, two studies are available showing that MRD may serve in the future as an early efficacy read out and may therefore be used as a surrogate [14] for survival endpoints. One study in CBF-AML revealed that higher anthracycline dosage (DNR 90 mg/m² for 3 days) during standard induction therapy was associated with a significant reduction in MRD levels and longer survival as compared to patients receiving the lower anthracycline dosage (DNR 60 mg/m² for 3 days) [47]. In addition, Lambert et al. showed a marked additional reduction of MRD levels by GO in conjunction to intensive chemotherapy in the French ALFA 0701 with a clear beneficial impact on OS [63]. These examples illustrate that early biomarker endpoints as a surrogate marker are of high interest to provide an early readout of clinical efficacy. Such early readouts will allow designing reasonable adaptive drug development plans with the aim to speed up the process of the transition of new drugs into routine patient care. Quantifying residual disease by MFC or RT-qPCR has the potential to serve as an early biomarker surrogate for survival endpoints but has so far not been evaluated in a confirmatory prospective manner.

Conclusions

Our progress in unraveling the genetic and immunophenotypic heterogeneity of AML has allowed us to identify a number of aberrations for MRD assessment. Despite the ability to achieve remission in most patients, AML remains a lethal cancer with most patients eventually dying of their disease; as outcomes are variable, however, better risk assessment and subsequent dynamic adaptation of the therapy may ultimately improve outcomes. MRD measured quantitatively is a strong prognostic tool and used increasingly to guide MRD-adapted therapy. Recent studies suggest the possibility that MRD-guided modification of post-remission treatment intensity based on MRD status may be used to optimize outcomes and that MRD assessment should be implemented in clinical trials as an early readout tool for clinical efficacy. However, the process to regularly include MRD-based treatment decision-making and clinical efficacy measure in non-APL AML into clinical practice has just begun.

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Compliance with Ethics Guidelines

Conflict of interest Sabine Kayser, Roland B. Walter, Wendy Stock, and Richard F. Schlenk each declare no potential conflicts of interest.

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References

Papers of particular interest, published recently, have been highlighted as:

- Of importance
- Of major importance

1. Burnett A, Wetzler M, Löwenberg B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol*. 2011;29(5):487–94.
2. Ferrara F, Schiffer CA. Acute myeloid leukaemia in adults. *Lancet*. 2013;381(9865):484–95.
3. Döhner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453–74.
4. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the international working group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. *J Clin Oncol*. 2003;21(24):4642–9.
5. Grimwade D, Freeman SD. Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "Prime Time"? *Blood*. 2014;124(23):3345–55.
6. Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368(22):2059–74.
7. Schlenk RF. Post-remission therapy for acute myeloid leukemia. *Haematologica*. 2014;99(11):1663–70.
8. Terwijn M, van Putten WL, Kelder A, et al. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study. *J Clin Oncol*. 2013;31(31):3889–97. **Results of a large clinical trial evaluating the role of immunophenotyping in MRD monitoring.**
9. Baccarani M, Deininger MW, Rosti G, et al. European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood*. 2013;122(6):872–84.
10. Vora A, Goulden N, Wade R, et al. Treatment reduction for children and young adults with low-risk acute lymphoblastic leukaemia defined by minimal residual disease (UKALL 2003): a randomised controlled trial. *Lancet Oncol*. 2013;14(3):199–209.
11. Vora A, Goulden N, Mitchell C, et al. Augmented post-remission therapy for a minimal residual disease-defined high-risk subgroup of children and young people with clinical standard-risk and intermediate-risk acute lymphoblastic leukaemia (UKALL 2003): a randomised controlled trial. *Lancet Oncol*. 2014;15(8):809–18.
12. Brüggemann M, Raff T, Kneba M. Has MRD monitoring superseded other prognostic factors in adult ALL? *Blood*. 2012;120(23):4470–81.
13. Brüggemann M, Raff T, Flohr T, et al. Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. *Blood*. 2006;107(3):1116–23.
14. Buyse M, Molenberghs G, Paoletti X, et al. Statistical evaluation of surrogate endpoints with examples from cancer clinical trials. *Biom J*. 2015 Feb 12. doi: 10.1002/bimj.201400049. [Epub ahead of print].
15. 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):1813–21.
16. Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. *Trends Immunol*. 2012;33(7):323–32.
17. Amir el AD, Davis KL, Tadmor MD, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol*. 2013;31(6):545–52.
18. Feller N, van der Velden VH, Brooimans RA, et al. Defining consensus leukemia-associated immunophenotypes for detection of minimal residual disease in acute myeloid leukemia in a multi-center setting. *Blood Cancer J*. 2013;3, e129.
19. Gabert J, Beillard E, van der Velden VH, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—a Europe Against Cancer program. *Leukemia*. 2003;17(12):2318–57.
20. Baer MR, Stewart CC, Dodge RK, et al. High frequency of immunophenotypic changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B study 8361). *Blood*. 2001;97(11):3574–80.
21. Langebrake C, Brinkmann I, Teigler-Schlegel A, et al. Immunophenotypic differences between diagnosis and relapse in childhood AML: implications for MRD monitoring. *Cytometry B Clin Cytom*. 2005;63(1):1–9.
22. Voskova D, Schoch C, Schnittger S, Hiddemann W, Haferlach T, Kern W. Stability of leukemia-associated aberrant immunophenotypes in patients with acute myeloid leukemia between diagnosis and relapse: comparison with cytomorphologic, cytogenetic, and molecular genetic findings. *Cytometry B Clin Cytom*. 2004;62(1):25–38.
23. Kubista M, Andrade JM, Bengtsson M, et al. The real-time polymerase chain reaction. *Mol Aspects Med*. 2006;27(2-3):95–125.
24. Chou WC, Tang JL, Wu SJ, et al. Clinical implications of minimal residual disease monitoring by quantitative polymerase chain reaction in acute myeloid leukemia patients bearing nucleophosmin (NPM1) mutations. *Leukemia*. 2007;21(5):998–1004.
25. Yin JA, O'Brien MA, Hills RK, et al. Minimal residual disease monitoring by RT-qPCR in core-binding factor AML allows risk-stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. *Blood*. 2012;120(14):2826–35.
26. 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):5195–201.
27. Beillard E, Pallisgaard N, van der Velden VH, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-

- transcriptase polymerase chain reaction (RQ-PCR)—a Europe against cancer program. *Leukemia*. 2003;17(12):2474–86.
28. Ostergaard M, Nyvold CG, Jovanovic JV, et al. Development of standardized approaches to reporting of minimal residual disease data using a reporting software package designed within the European LeukemiaNet. *Leukemia*. 2011;25(7):1168–73. **This quality control study reports on a software suitable for efficient handling of qPCR data, generation of MRD reports and harmonization of MRD data.**
 29. 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):3650–8.
 30. Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood*. 2009;113(9):1875–91.
 31. Santamaria C, Chillón MC, Fernández C, et al. Using quantification of the PML-RARalpha transcript to stratify the risk of relapse in patients with acute promyelocytic leukemia. *Haematologica*. 2007;92(3):315–22.
 32. Grimwade D, Jovanovic JV, Hills RK. Can we say farewell to monitoring minimal residual disease in acute promyelocytic leukaemia? *Best Pract Res Clin Haematol*. 2014;27(1):53–61.
 33. Lo-Coco F, Avvisati G, Vignetti M, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med*. 2013;369(2):111–21.
 34. Zhu HH, Zhang XH, Qin YZ, et al. MRD-directed risk stratification treatment may improve outcomes of t(8;21) AML in the first complete remission: results from the AML05 multicenter trial. *Blood*. 2013;121(20):4056–62. **The authors evaluated a risk-directed therapy approach based on MRD in t(8;21)(q22;q22) AML patients.**
 35. Jourdan E, Boissel N, Chevret S, et al. Prospective evaluation of gene mutations and minimal residual disease in patients with core binding factor acute myeloid leukemia. *Blood*. 2013;121(12):2213–23. **Results of a prospective MRD clinical study in core-binding leukemia.**
 36. Hoyos M, Nomdedeu JF, Esteve J, et al. Core binding factor acute myeloid leukemia: the impact of age, leukocyte count, molecular findings, and minimal residual disease. *Eur J Haematol*. 2013;91(3):209–18.
 37. Leroy H, de Botton S, Gardel-Duflos N, et al. Prognostic value of real-time quantitative PCR (RQ-PCR) in AML with t(8;21). *Leukemia*. 2005;19(3):367–72.
 38. Weissner M, Haferlach C, Hiddemann W, Schnittger S. The quality of molecular response to chemotherapy is predictive for the outcome of AML1-ETO-positive AML and is independent of pre-treatment risk factors. *Leukemia*. 2007;21(6):1177–82.
 39. Schlenk RF, Benner A, Krauter J, et al. Individual patient data-based meta-analysis of patients aged 16 to 60 years with core binding factor acute myeloid leukemia: a survey of the German Acute Myeloid Leukemia Intergroup. *J Clin Oncol*. 2004;22(18):3741–50.
 40. Nguyen S, Leblanc T, Fenaux P, et al. A white blood cell index as the main prognostic factor in t(8;21) acute myeloid leukemia (AML): a survey of 161 cases from the French AML Intergroup. *Blood*. 2002;99(10):3517–23.
 41. Schoch C, Haase D, Haferlach T, et al. Fifty-one patients with acute myeloid leukemia and translocation t(8;21)(q22;q22): an additional deletion in 9q is an adverse prognostic factor. *Leukemia*. 1996;10(8):1288–95.
 42. Schnittger S, Kohl TM, Haferlach T, et al. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. *Blood*. 2006;107(5):1791–9.
 43. Cairoli R, Beghini A, Grillo G, et al. Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. *Blood*. 2006;107(9):3463–8.
 44. Boissel N, Leroy H, Brethon B, et al. Incidence and prognostic impact of c-Kit, FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). *Leukemia*. 2006;20(6):965–70.
 45. Paschka P, Marcucci G, Ruppert AS, et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. *J Clin Oncol*. 2006;24(24):3904–11.
 46. Allen C, Hills RK, Lamb K, et al. The importance of relative mutant level for evaluating impact on outcome of KIT, FLT3 and CBL mutations in core-binding factor acute myeloid leukemia. *Leukemia*. 2013;27(9):1891–901.
 47. Prebet T, Bertoli S, Delaunay J, et al. Anthracycline dose intensification improves molecular response and outcome of patients treated for core binding factor acute myeloid leukemia. *Haematologica*. 2014;99:e185–7.
 48. Corbacioglu A, Scholl C, Schlenk RF, et al. Prognostic impact of minimal residual disease in CBF-MYH11-positive acute myeloid leukemia. *J Clin Oncol*. 2010;28(23):3724–9.
 49. Schnittger S, Weissner M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFMYH11+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood*. 2003;102(8):2746–55.
 50. 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):4413–22.
 51. Forestier E, Heim S, Blennow E, et al. Cytogenetic abnormalities in childhood acute myeloid leukaemia: a Nordic series comprising all children enrolled in the NOPHO-93-AML trial between 1993 and 2001. *Br J Haematol*. 2003;121(4):566–77.
 52. Byrd JC, Mrózek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood*. 2002;100(13):4325–36.
 53. Krauter J, Wagner K, Schäfer I, et al. Prognostic factors in adult patients up to 60 years old with acute myeloid leukemia and translocations of chromosome band 11q23: individual patient data-based meta-analysis of the German Acute Myeloid Leukemia Intergroup. *J Clin Oncol*. 2009;27(18):3000–6.
 54. Scholl C, Breitingner H, Schlenk RF, et al. Development of a real-time RT-PCR assay for the quantification of the most frequent MLL/AF9 fusion types resulting from translocation t(9;11)(p22;q23) in acute myeloid leukemia. *Genes, Chromosomes Cancer*. 2003;38(3):274–80.
 55. Scholl C, Schlenk RF, Eiwen K, et al. The prognostic value of MLL-AF9 detection in patients with t(9;11)(p22;q23)-positive acute myeloid leukemia. *Haematologica*. 2005;90(12):1626–34.
 56. Abildgaard L, Ommen HB, Lausen B, Hasle H, Nyvold CG. A novel RT-qPCR assay for quantification of the MLL-MLL3 fusion transcript in acute myeloid leukaemia. *Eur J Haematol*. 2013;91(5):394–8.
 57. Falini B, Sportoletti P, Martelli MP. Acute myeloid leukemia with mutated NPM1: diagnosis, prognosis and therapeutic perspectives. *Curr Opin Oncol*. 2009;21(6):573–81.
 58. Gorello P, Cazzaniga G, Alberti F, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia*. 2006;20(6):1103–8.
 59. Schnittger S, Kern W, Tschulik C, et al. Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide

- important prognostic information in AML. *Blood*. 2009;114(11):2220–31.
60. Krönke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood*. 2013;122(1):100–8.
 61. 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):2709–16.
 62. Hubmann M, Köhnke T, Hoster E, et al. Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in NPM1-mutated patients identifies those at high risk of relapse. *Haematologica*. 2014;99(8):1317–25.
 63. Lambert J, Lambert J, Nibourel O, et al. MRD assessed by WT1 and NPM1 transcript levels identifies distinct outcomes in AML patients and is influenced by gemtuzumab ozogamicin. *Oncotarget*. 2014;5(15):628–6288. **This is one of the first randomized studies indicating that MRD-assessment may serve as a surrogate for survival endpoints for the treatment under investigation.**
 64. Shayegi N, Kramer M, Bornhäuser 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.
 65. Kayser S, Levis MJ. FLT3 tyrosine kinase inhibitors in acute myeloid leukemia: clinical implications and limitations. *Leuk Lymphoma*. 2014;55(2):243–55.
 66. Schnittger S, Schoch C, Kern W, Hiddemann W, Haferlach T. FLT3 length mutations as marker for follow-up studies in acute myeloid leukaemia. *Acta Haematol*. 2004;112(1-2):68–78.
 67. Palmisano M, Grafone T, Ottaviani E, et al. NPM1 mutations are more stable than FLT3 mutations during the course of disease in patients with acute myeloid leukemia. *Haematologica*. 2007;92(9):1268–9.
 68. Shih LY, Huang CF, Wu JH, et al. Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood*. 2002;100(7):2387–92.
 69. Kottaridis PD, Gale RE, Langabeer SE, et al. Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood*. 2002;100(7):2393–8.
 70. Cloos J, Goemans BF, Hess CJ, et al. Stability and prognostic influence of FLT3 mutations in paired initial and relapsed AML samples. *Leukemia*. 2006;20(7):1217–20.
 71. Levis M. FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? *Hematology Am Soc Hematol Educ Program*. 2013;2013:220–6.
 72. Schiller J, Praulich I, Krings Rocha C, Kreuzer KA. Patient-specific analysis of FLT3 internal tandem duplications for the prognostication and monitoring of acute myeloid leukemia. *Eur J Haematol*. 2012;89(1):53–62.
 73. Grunwald MR, Tseng LH, Lin MT, et al. Improved FLT3 internal tandem duplication PCR assay predicts outcome after allogeneic transplant for acute myeloid leukemia. *Biol Blood Marrow Transplant*. 2014;20(12):1989–95.
 74. Lin MT, Tseng LH, Beierl K, et al. Tandem duplication PCR: an ultrasensitive assay for the detection of internal tandem duplications of the FLT3 gene. *Diagn Mol Pathol*. 2013;22(3):149–55.
 75. Thol F, Kölking B, Damm F, et al. Next-generation sequencing for minimal residual disease monitoring in acute myeloid leukemia patients with FLT3-ITD or NPM1 mutations. *Genes, Chromosomes Cancer*. 2012;51(7):689–95.
 76. Mardis ER. The \$1,000 genome, the \$100,000 analysis? *Genome Med*. 2010;2:84.
 77. Ley T, Ding L, Walter M, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363(25):2424–33.
 78. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet*. 2011;43(4):309–15.
 79. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506(7488):328–33. **Identification of the very first mutation in the process of leukemia development.**
 80. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488–98.
 81. Genovesi G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371(26):2477–87.
 82. Gaidzik VI, Schlenk RF, Paschka P, et al. Clinical impact of DNMT3A mutations in younger adult patients with acute myeloid leukemia: results of the AML Study Group (AML5G). *Blood*. 2013;121(23):4769–77.
 83. Marcucci G, Metzeler KH, Schwind S, et al. Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. *J Clin Oncol*. 2012;30(7):742–50.
 84. Hou HA, Kuo YY, Liu CY, et al. DNMT3A mutations in acute myeloid leukemia: stability during disease evolution and clinical implications. *Blood*. 2012;119(2):559–68.
 85. Pløen GG, Nederby L, Guldberg P, et al. Persistence of DNMT3A mutations at long-term remission in adult patients with AML. *Br J Haematol*. 2014;167(4):478–86.
 86. Lasa A, Carricondo M, Estivill C, et al. WT1 monitoring in core binding factor AML: comparison with specific chimeric products. *Leuk Res*. 2009;33(12):1643–9.
 87. Cilloni D, Gottardi E, Fava M, et al. Usefulness of quantitative assessment of the WT1 gene transcript as a marker for minimal residual disease detection. *Blood*. 2003;102(2):773–4.
 88. Cilloni D, Messa F, Arruga F, et al. Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral blood samples collected after chemotherapy. *Haematologica*. 2008;93(6):921–4.
 89. Nowakowska-Kopera A, Sacha T, Florek I, Zawada M, Czekalska S, Skotnicki AB. Wilms' tumor gene 1 expression analysis by real-time quantitative polymerase chain reaction for monitoring of minimal residual disease in acute leukemia. *Leuk Lymphoma*. 2009;50(8):1326–32.
 90. Rubnitz JE, Inaba H, Dahl G, et al. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. *Lancet Oncol*. 2010;11(6):543–52.
 91. Sockel K, Wermke M, Radke J, et al. Minimal residual disease-directed preemptive treatment with azacitidine in patients with NPM1-mutant acute myeloid leukemia and molecular relapse. *Haematologica*. 2011;96(10):1568–70.
 92. Greiner J, Ono Y, Hofmann S, et al. Mutated regions of nucleophosmin 1 elicit both CD4(+) and CD8(+) T-cell responses in patients with acute myeloid leukemia. *Blood*. 2012;120(6):1282–9.
 93. Hofmann S, Götz M, Schneider V, et al. Donor lymphocyte infusion induces polyspecific CD8(+) T-cell responses with concurrent molecular remission in acute myeloid leukemia with NPM1 mutation. *J Clin Oncol*. 2013;31(3):e44–7.
 94. Bastos-Oreiro M, Perez-Corral A, Martínez-Laperche C, et al. Prognostic impact of minimal residual disease analysis by flow cytometry in patients with acute myeloid leukemia before and after allogeneic hemopoietic stem cell transplantation. *Eur J Haematol*. 2014;93(3):239–46.

95. Leung W, Pui CH, Coustan-Smith E, et al. Detectable minimal residual disease before hematopoietic cell transplantation is prognostic but does not preclude cure for children with very-high-risk leukemia. *Blood*. 2012;120(2):468–72.
96. Wheatley K, Burnett AK, Goldstone AH, et al. A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. *Brit J Haematol*. 1999;107(1):69–79.
97. Kern W, Haferlach T, Schoch C, et al. Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial. *Blood*. 2003;101(1):64–70.
98. 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):4123–31.
99. San Miguel JF, Vidriales MB, López-Berges C, et al. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. *Blood*. 2001;98:1746–51.
100. Burnett AK, Russell NH, Hills RK, et al. Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the medical research council AML15 trial. *J Clin Oncol*. 2013;31(27):3360–8.