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Molecular markers in acute myeloid leukaemia

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Abstract An increasing number of cytogenetic and molecular genetic aberrations have been identified in acute myeloid leukaemia (AML), highlighting the biological heterogeneity of the disease. Moreover, the characterisation of specific molecular abnormalities provides the basis for targeted therapies, such as all *trans* retinoic acid (ATRA) and arsenic trioxide treatment in acute promyelocytic leukaemia or tyrosine kinase inhibitors in AML with *FLT3* mutations. Several cytogenetic and molecular genetic changes have been shown to be prognostically relevant and have been acknowledged in the latest WHO classification of AML as separate entities. A detailed marker assessment at diagnosis is crucial for risk-stratification of AML patients, allowing the identification of those at high risk of relapse, who may benefit from early allogeneic stem cell transplantation. Finally, molecular markers are important for the detection of minimal residual disease after initial therapy and during long-term follow-up, which enables a more tailored treatment approach for individual AML patients.

Keywords AML · Cytogenetics · Molecular genetics · Minimal residual disease

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Pre-treatment prognostic factors

Cytogenetics

The leukaemic karyotype remains the most powerful predictor of prognosis in acute myeloid leukaemia (AML) and provides the framework for current risk-adapted treatment strategies adopted by different study groups (reviewed in [1]). Younger adult patients are generally categorised into 3 different risk groups based upon cytogenetics: favourable (about 20–25 %), intermediate (about 55–70 %), and adverse (about 10–20 %). While there is broad consensus about the favourable risk category, definition of intermediate and adverse risk slightly varies between the main collaborative groups for AML. Table 1 shows the revised Medical Research Council (MRC) cytogenetic risk group assignment based on the analysis of 5876 patients enrolled on the MRC AML10, 12, and 15 trials [2]. About 40–45 % of younger adult patients with AML have a normal karyotype and are generally allocated to the intermediate risk group (reviewed in [1]). Of note, adjusted risk classifications should be applied in elderly AML patients [3].

The favourable risk group includes 3 balanced translocations/inversions: t(15;17)(q22;q21)/*PML-RARA* in acute promyelocytic leukaemia (APL), as well as the core binding factor (CBF) leukaemias with t(8;21)(q22;q22)/*RUNX1-RUNX1T1* and inv(16)(p13.1q22)/t(16;16)(p13.1;q22)/*CBFB-MYH11*. Patients in this group usually show a complete remission (CR) rate above 90 %, with an overall survival (OS) of 55–85 % and therefore allogeneic stem cell transplantation (SCT) in first CR is generally not recommended for these patients [4, 5].

Additional cytogenetic abnormalities do not predict for poorer outcome in AML with favourable risk cytogenetics. Indeed in CBF leukaemia with inv(16)/t(16;16), presence

Table 1 Revised MRC AML cytogenetic classification applied in younger adults [1]

Risk group	Cytogenetic abnormality
Favourable	t(15;17)(q22;q21)
	t(8;21)(q22;q22)
	inv(16)(p13q22)/t(16;16)(p13;q22)
Intermediate	Entities not classified as favourable or adverse
Adverse	In the absence of favourable risk cytogenetic abnormalities:
	abn(3q) [excluding t(3;5)(q21–25;q31–35)], inv(3)(q21q26)/t(3;3)(q21;q26)
	add(5q)/del(5q), −5
	add(7q)/del(7q), −7
	t(11q23) [excluding t(9;11)(p21–22;q23) and t(11;19)(q23;p13)]
	t(9;22)(q34;q11)
	−17/abn(17p)
	Complex karyotype (≥4 unrelated abnormalities)

of additional abnormalities, particularly trisomy 22, is associated with a better outcome (reviewed in [1]).

Patients in the adverse risk group have a CR rate of only 60 % and a dismal OS of about 10–20 % [2, 6]. For these patients an allograft in first CR or experimental treatment strategies should be considered. The karyotypes −5/del(5q), −7, abn(3q), translocations involving the *MLL* locus at 11q23, and complex karyotypes are consistently included into the adverse risk categories of different AML study groups (reviewed in [1]).

MLL rearrangements have been generally considered to predict a poor outcome; however, different studies indicate that the prognostic impact of *MLL* translocations depends on the respective fusion partner. The t(6;11)(q27;q23) and t(10;11)(p12;q23), involving the *AF6* and *AF10* genes, respectively, were shown to confer inferior prognosis [2, 7, 8]. In contrast, the t(9;11)(p21–22;q23) involving *MLLT3* and t(11;19)(q23;p13) in which the fusion partner is the *ELL* or *ENL* gene depending the breakpoint on 19p are associated with a better outcome [2, 9].

Complex karyotypes are associated with deletions of 5q, 7q and 17p (and *TP53* mutations), as well as gains of 8q, 11q, and 21q [10, 11]. While various definitions have been used to categorise complex karyotypes, a recent large MRC study which investigated the impact of complexity in AML with intermediate risk aberrations suggested that 4 or more abnormalities provide the most robust and prognostically relevant cut-off [2]. A very recent study involving 824 cases conducted by the Munich Leukemia Laboratory lends further support to standardise the definition of complex karyotype based on the presence of at least 4 unrelated cytogenetic abnormalities [12].

Besides the refinement of known adverse factors, also new cytogenetic risk entities have been proposed. In a study including almost 2000 younger adults with AML, a monosomal karyotype (defined as the presence of an autosomal monosomy with at least one other autosomal monosomy or structural chromosomal abnormality; excluding CBF AML) has been associated with a particularly unfavourable prognosis and suggested to be more predictive for adverse outcome than a complex karyotype [13]. The adverse prognostic significance of a monosomal karyotype has been confirmed by other studies [2, 12, 14]. However, the vast majority of patients with a monosomal karyotype had already been defined as high risk by the revised MRC cytogenetic classification and the criterion "monosomal karyotype" alone did not reliably classify patients as high risk [2, 12].

As cytogenetic classification in AML becomes more and more complex, large prospective studies are needed to evaluate the clinical utility of known risk categories and potential new risk factors. In particular, the prognostic impact of cytogenetic aberrations of low frequency, like t(v;11)(v;q23) other than t(9;11), del(7q), del(9q), or del(20q) need to be determined. Moreover, the prognostic relevance of additional genetic abnormalities in patients with balanced translocations and other chromosomal abnormalities remains unclear and requires further evaluation.

Molecular genetics

In recent years, a magnitude of molecular prognostic factors has been identified in AML. This is particularly important for cytogenetically normal (CN)-AML patients and has substantially improved characterisation and risk-stratification of this large, heterogeneous subgroup. To date, more than 90 % of AML patients can be categorised on the basis of either cytogenetic or molecular genetic characteristics.

Thus far, 3 molecular markers [mutations in the genes encoding nucleophosmin (NPM1), Fms-like Tyrosine kinase 3 (FLT3), and CCAAT Enhancer Binding Protein alpha (C/EBPα)] have been consistently demonstrated to be of independent prognostic significance in CN-AML patients and have been widely incorporated into trial risk assignments as well as in daily clinical practice. In addition, a number of studies suggest that detection of mutations in the *RUNX1* gene, partial tandem duplication of *MLL* (*MLL*-PTD), and overexpression of *EVI1* confer independent adverse prognostic information and could serve to further refine risk-stratification (reviewed in [15]). The prognostic relevance of other more recently identified molecular markers, such as mutations in *TET2* [16–18], *IDH1/2* [19–25], *DNMT3A* [26–32], *WT1* [33–35], *KIT* [36–39], *ASXL1* [40–44], *BCOR* [45], *BCORL1* [46], *PHF6*

[44, 47], as well as gene expression profiles (reviewed in [48]), microRNA signatures (reviewed in [49]), DNA methylation [50], or aberrant overexpression of single genes like *BAALC*, *ERG*, or *MN1* (reviewed in [15]) merits further investigation.

NPM1

Heterozygous mutations of the *NPM1* gene are the most frequent mutations in AML, detected in a third of cases, including approximately half with normal karyotype [51–54]. AML with mutated *NPM1* has been acknowledged by the 2008 WHO classification as a provisional separate entity [55]. Mutations of *NPM1* are regarded as a primary leukaemic lesion as they are stable throughout the course of the disease and are mutually exclusive with recurrent balanced translocations [56, 57]. They are associated with the presence of *FLT3*-ITD, *IDH1/2,* and *DNMT3A* mutations, but are rarely seen together with mutations of the *CEBPA* gene [54, 58].

Different *NPM1* mutations have been described, all being frameshift mutations by the insertion of 4 (rarely more) bases, in the majority of cases in exon 12 [51, 59]. These mutations lead to an aberrant cytoplasmic localisation of the nucleophosmin protein and impair the protein's nuclear shuttle function, which is important for activation and stabilisation of tumour suppressors such as p53 and ARF [60].

Patients with an isolated *NPM1* mutation have a more favourable outcome, with higher CR rates, reduced risk of relapse and longer OS [52–54, 61]. However, in most studies this favourable prognosis was only seen in patients with wildtype *FLT3*. Moreover, the prognostic impact of *NPM1* might depend on the presence of additional *IDH1/2* mutations [44]. A favourable outcome associated with *NPM1* was also observed in elderly AML patients [62] as well as in relapsed AML cases [63].

FLT3

About 20–27 % of AML arising in younger adults and 30–50 % of those with normal karyotype harbour in-frame internal tandem duplications (ITD) of the class III receptor tyrosine kinase FLT3 [64–66]. FLT3 is expressed on normal haematopoietic progenitor cells and is important for cell differentiation, proliferation, and survival [67]. Most *FLT3*-ITD mutations occur in exons 14 and 15, affecting the juxtamembrane domain which regulates the kinase activity of the receptor [68]. The duplications, which differ in length and insertion sites, lead to constitutive activation of the receptor and promote proliferation of leukaemic cells [69]. CN-AML patients with *FLT3*-ITD show a significantly inferior outcome compared to patients without the mutation [65, 66, 70, 71]. This is predominantly due to a higher relapse rate in cases with *FLT3*-ITD, whereas the initial response to chemotherapy is similar. The prognosis is particularly poor for patients with a high *FLT3*-ITD allelic ratio which may stem from homozygous mutations resulting from acquired uniparental disomy, as well as for patients harbouring the ITD integration site in the beta1-sheet of the tyrosine kinase domain (TKD)-1 [72] and for cases with coexisting mutations of *WT1* [34, 73].

The prognostic significance of another, less frequent class of *FLT3* mutations involving amino acid substitutions in the TKD (mostly at codons D835 and I836) is still unclear and might depend on the presence of other mutations [74–77]. Both ITD and TKD point mutations of *FLT3* are associated with a high white blood cell count and increased LDH levels at diagnosis [71]. *FLT3* mutations are more frequent in *PML-RARA*+ APL and are associated with the clinically aggressive microgranular variant subtype [78–80].

CEBPA

The gene *CEBPA* is mutated in approximately 5–10 % of AML, mostly CN-AML cases [54, 81]. In addition, germline *CEBPA* mutations have been reported in familial AML [82]. C/EBPα is a key transcription factor involved in myelopoiesis [83]. Mutations cluster in the amino- and carboxy-terminal regions, with the former leading to expression of a truncated isoform of C/EBPα (p30) and loss of the full length protein (p42) [81]. Carboxy-terminal mutations affect regions involved in mediating dimerisation and DNA-binding. Interestingly, in the majority of patients with *CEBPA* mutations, both alleles are involved, combining an upstream mutation in one allele with a downstream mutation in the other. Significant insights into the biology of *CEBPA* mutations have been provided by murine models, which have shown how loss of p42 expression (mimicking biallelic amino-terminal *CEBPA* mutations) or compound heterozygous mutations affecting amino- and carboxy-terminal regions affect haematopoiesis and give rise to AML [84]. While early studies reported that *CEBPA* mutation predicts a relatively favourable outcome in AML, it has subsequently been shown that this effect is accounted for by the subset of patients with biallelic mutations, especially those who lack *FLT3*-ITD [58, 85]. Similar to *NPM1*, *CEBPA*-mutated AML has been recognised as a provisional entity in the 2008 WHO classification [55].

Different studies have investigated outcome of patients with *NPM1*, *FLT3*, and *CEBPA* mutations and their mutual influence on prognosis [52–54, 58, 85]. Importantly, AML with wildtype *FLT3* and mutated *NPM1* or biallelic *CEBPA* mutations is associated with a relatively favourable

prognosis similar to CBF leukaemia (see Fig. 1) and therefore patients with these molecularly defined subsets are not considered candidates for an allograft in first CR [54, 58, 86]. The genotypes "mutated *NPM1* without *FLT3*-ITD" and "mutated *CEBPA*" in CN-AML have been incorporated into the favourable risk category of the recent European LeukemiaNet classification [87].

Epigenetic modifiers

TET2 Mutations of the *TET2* gene have been found in various myeloid malignancies and are detected in 10–20 % of AML [17, 18, 88, 89]. *TET2* mutations are very heterogeneous and spread throughout the entire coding sequence [17, 18, 88]. They are loss-of-function mutations, in most cases nonsense and frameshift mutations leading to truncated translation. The detection of mutations in both *TET2* copies and loss of heterozygosity in different myeloid neoplasms suggest a role of *TET2* as a tumour suppressor gene [16, 90, 91]. In vitro and in vivo studies have demonstrated a role for *TET2* in myeloid differentiation and self-renewal of stem and progenitor cells [89, 92, 93]; however, the precise mechanisms and downstream effects of *TET2* are yet unknown. The TET2 protein is an enzyme catalysing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine which results in demethylation of DNA [94] and *TET2* mutations have been shown to impair this enzymatic function [92].

The prognostic relevance of *TET2* remains controversial—some studies suggest an adverse impact of *TET2* mutations on outcome in certain AML subgroups [17, 18]; in other studies no prognostic significance of *TET2* was found [95].

IDH Mutations of the gene encoding the isocitrate dehydrogenase IDH1 in AML were discovered by whole genome sequencing [19] and are detected in 8–16 % of cases with normal karyotype [19, 21]. Another 12–15 % (which do not harbour an IDH1 mutation) show mutations in IDH2 [20, 96], the mitochondrial homolog of IDH1. Both enzymes convert isocitrate to α-ketoglutarate; however, the mutated proteins exhibit a gain-of-function leading to aberrant accumulation of the oncometabolite 2-hydroxyglutarate (2-HG) [96, 97]. There is a functional overlap between IDH1/2 and TET2 and the two classes of mutations are mutually exclusive [17, 18, 89]. Patients with *IDH1/2* and *TET2* mutations show a similar epigenetic signature and global DNA hypermethylation [89]. Moreover, it was demonstrated that 2-HG which is generated as a result of mutations in IDH1/2 inhibits TET2 function [89].

Like for *TET2*, the impact of *IDH* mutations on survival of AML patients is not clear yet. Some studies have observed no difference in outcome with respect to the *IDH* mutation status [20, 21, 25], others have demonstrated a poor prognostic impact in certain AML subgroups [22–24]. Recent studies suggest that the impact of *IDH2* on

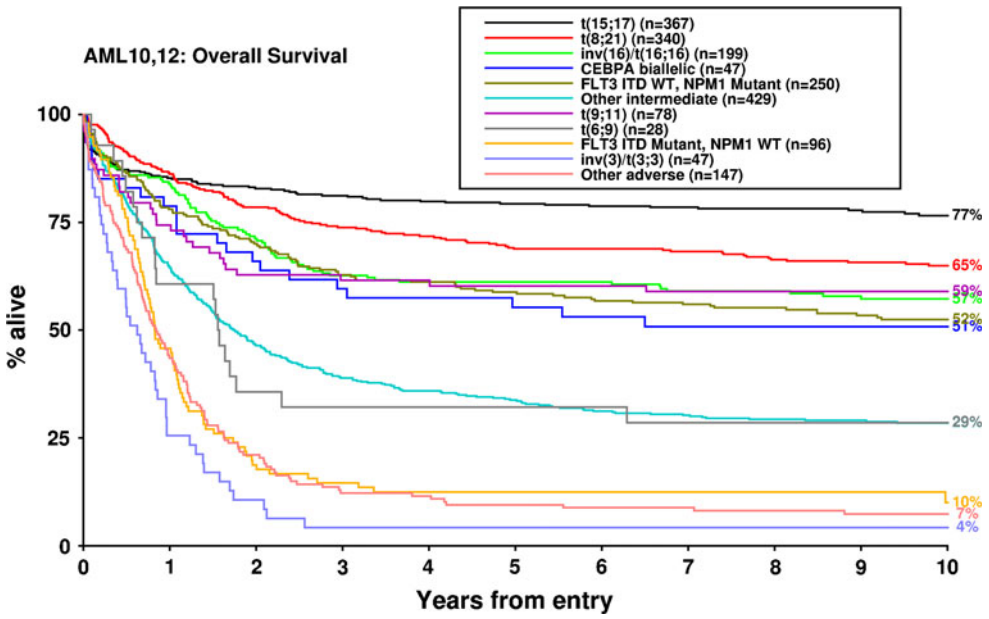

Fig. 1 Outcome of younger adults with AML according to cytogenetic and molecular abnormalities. Overall survival for younger adults treated in the MRC AML10 and AML12 trials screened for NPM1, CEBPA and FLT3-ITD mutations [58, 61] for whom cytogenetic data were available. Cases were classified in hierarchical fashion with t(15;17)(q22;q21), t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), t(9;11)(p21–22;q23), t(6;9)(p23;q34) and inv(3)(q21q26)/t(3;3)(q21;q26) at the top of the hierarchy, then *CEBPA* biallelic mutations, *NPM1*^{mut}/FLT3-ITD^{neg}, *NPM1*^{wt}/FLT3-ITD^{pos}, other intermediate and other adverse cytogenetic abnormalities

prognosis depends on the mutation site, with the *IDH2* R140 mutation being an independent favourable prognostic factor in AML patients [44, 98, 99].

Apart from potential prognostic implications of *IDH* and *TET2*, the finding of alterations in enzymatic activities and epigenetic changes in a large proportion of AML patients might lead to the development of new targeted therapies for this patient group.

DNMT3A Mutations of *DNMT3A* occur in about 14–18 % of AML, including 20–35 % with normal karyotype and are associated with a poorer prognosis [26–32]. *DNMT3A* mutations are associated with the presence of *NPM1*, *FLT3* and *IDH* mutations [26, 30]. Several different loss-of-function mutations have been found in all exons of *DNMT3A*, most frequently a missense point mutation at amino acid R882. DNMT3A is a methyltransferase that converts cytosine to 5-methylcytosine and generates de novo DNA methylation. Aberrant methylation patterns have been implicated in tumourigenesis and tumour progression [100]. The functional consequences of *DNMT3A* mutations in leukaemia are barely understood. It is suggested that mutations result in a decreased methyltransferase activity of DNMT3A by a dominant-negative mechanism [26, 101]. A recent in vivo study revealed that *Dnmt3a*-null haematopoietic stem cells have an increased self-renewal capacity and lose their differentiation potential, which was accompanied by hypomethylation of several genes implicated in leukaemogenesis [102]. However, also marked focal hypermethylation was found in the absence of *Dnmt3a* and it thus remains unclear which biological function of Dnmt3a contributes to the *Dnmt3a*-null phenotype. Interestingly, knockout of *Dnmt3a* alone was not sufficient to initiate leukaemia [102].

Minimal residual disease (MRD) assessment

Response to initial chemotherapy is a strong prognostic factor in AML (reviewed in [15]). Using cytogenetic analysis including FISH, polymerase chain reaction (PCR) or flow cytometry, the depth of remission and risk of relapse can be determined much more accurately than with conventional cytological methods. With PCR- and flow cytometry-based techniques, the presence of sub-microscopic levels of residual leukaemia can be detected with a sensitivity of up to 1 AML cell in 10^4–10^6 bone marrow cells (reviewed in [103]).

MRD assessment has been applied in 2 different ways: first, at early timepoints to determine the depth of the initial therapy response to better risk-stratify patients and guide postremission therapy; and second, sequential monitoring utilising leukaemia-specific markers to identify an impending relapse, enabling early treatment intervention to prevent progression of the disease.

Real-time quantitative PCR

The method with highest sensitivity for MRD analysis is real-time quantitative PCR (RT-qPCR) for AML-specific target fusion genes or mutations. So far, assays have been successfully performed for *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *MLLT3-MLL*, *DEK-CAN*, and *NPM1* mutations (reviewed in [103]). Given the increasing number of molecular markers identified in AML, at least half of patients harbour a leukaemia-specific target that can potentially be used for PCR-based MRD monitoring. However, not every marker is equally suitable for MRD assessment. For example, the use of *FLT3*-ITD seems to be limited, since mutational status may differ between leukaemic subclones such that the *FLT3*-ITD associated with the predominant clone at diagnosis may not be detectable at the time of disease relapse [104–106]. *CEBPA* mutations are difficult to track due to their heterogeneity and MRD detection of *CEBPA* has only been performed in one study including 4 patients so far [107]. Another restriction for MRD utility is the background amplification level of markers in the normal bone marrow, which has been a relevant drawback for monitoring of *MLL*-PTD [108].

The use of MRD assessment to guide therapy is most advanced in APL, where a standardised RT-qPCR assay has been developed and MRD monitoring is recommended by international treatment guidelines [109, 110]. Longitudinal monitoring of *PML-RARA* has been shown to be a strong prognostic factor for clinical relapse [110, 111]. Moreover, MRD-guided pre-emptive therapy reduced the rate of overt clinical relapse and was associated with improved survival, particularly in patients with high risk disease [111–113]. In CBF leukaemias, use of RT-qPCR to assess kinetics of disease response predicts subsequent risk of relapse [114–116]. Although, the clinical utility of sequential MRD monitoring in *RUNX1-RUNX1T1*-associated leukaemias has been questioned due to early reports involving qualitative PCR assays in which fusion transcripts could be detected in patients in long-term remission, subsequent studies using RT-qPCR have shown that relapse of CBF leukaemia is predicted by a rising transcript level.

Several studies have investigated *NPM1* mutations as targets for MRD detection using DNA- or RNA-based quantitative PCR assays [117–121]. It has been shown that *NPM1*-specific monitoring independently predicted the risk of relapse [119]. Moreover, tracking of *NPM1* has been successfully applied in the post-transplantation setting and predicted relapse earlier than assessment of chimaerism [120].

Besides leukaemia-specific mutations or fusion genes, aberrantly overexpressed genes have been considered as potential MRD markers, provided that they are at least 1,000-fold higher expressed than in normal cells and not modulated by regeneration after chemotherapy (reviewed in [103]). The main focus in this regard has been on *WT1*, which is overexpressed in about 70 % of AML cases. Early *WT1* monitoring has been shown to independently predict the risk of relapse within cytogenetic risk groups; however, high background expression of *WT1* in normal haematopoietic cells limits the sensitivity of *WT1* assays and makes it inappropriate for long-term follow-up [122].

Flow cytometry

For patients who have no suitable molecular MRD marker, monitoring by flow cytometry may be applicable. This technique is based upon the identification of a leukaemia-associated aberrant immunophenotype (LAIP) at diagnosis that is uncommon in normal haematopoietic cells and in regenerating bone marrow. In the majority of cases, LAIPs can be identified in the blast population, which comprise cross-lineage expression of lymphoid antigens, asynchronous antigen expression, and up- or downregulation of antigens (reviewed in [103]). For MRD detection, either a fixed panel of antibodies or patient-specific antibodies may be used. As the leukaemic immunophenotype frequently changes in AML at relapse, it is recommended to use antibody panels which detect all LAIPs present at diagnosis in order to ensure the stable detection of at least one LAIP during the course of the disease (reviewed in [123]). These antigen shifts may be caused by the selection of leukaemic subclones during chemotherapy which were not detectable at diagnosis or to a lesser extent by clonal evolution of the blast population. A major problem of using flow cytometry for MRD detection is the large assay variability between different laboratories. Nevertheless, several studies have demonstrated the independent prognostic significance of MRD assessment with flow cytometry after induction and consolidation therapy [124–126]. Thus far, sensitivity of MRD detection using flow cytometry is significantly lower than that of RT-qPCR, which limits the method to early MRD assessment. However, this will be probably overcome with the use of advanced multicolour technologies in the future.

Both for PCR- and flow cytometry-based MRD approaches, large prospective trials are needed to evaluate the clinical utility of MRD in non-APL AML. In this regard, it is particularly important to further standardise the different MRD assays and to equalise time points and cut-offs for MRD detection. Most likely, MRD assessment will provide a useful tool for risk-stratification during therapy and follow-up and may improve outcome for AML patients.

Concluding remarks

The identification of molecular markers in AML has greatly improved our understanding of disease pathogenesis and facilitated the discrimination of biologically and clinically distinct subgroups. Cytogenetic analysis and molecular screening for *NPM1*, *FLT3* and *CEBPA* mutations have been widely adopted into the routine diagnostic work-up of AML, informing treatment decisions including the application of allogeneic SCT in first CR. The increasing number of molecular markers identified as a result of recent high throughput sequencing initiatives has presented a considerable challenge to establish the prognostic significance of particular constellations of mutations which may be relatively uncommon, demanding analysis of very large numbers of homogeneously treated patients. Nevertheless, it seems likely that these technologies will lead to the identification of new molecular markers with independent prognostic impact, which will improve prediction of an individual patient's response to therapy. It is anticipated that further subgroups of patients who may benefit from particular (targeted) therapeutic approaches will be identified. In addition, technical improvements in flow cytometry with an increased number of available colours and the capacity to identify leukaemic stem cell populations, coupled with the development of an expanding range of RT-qPCR assays for leukaemia-specific molecular targets, make it highly plausible that longitudinal tracking of MRD can be applied in virtually every case of AML in the near future. These approaches could provide powerful tools to individually tailor a patient's treatment throughout the entire course of the disease. This prospect will be extremely pertinent to the design of the next wave of large scale clinical trials which will need to establish the most informative molecular markers and determine whether the use of MRD monitoring to guide therapy is clinically useful, leading to meaningful improvements in patient survival.

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