The power and potential of integrated diagnostics in acute myeloid leukaemia

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Summary

The field of acute myeloid leukaemia (AML) diagnostics, initially based solely on morphological assessment, has integrated more and more disciplines. Today, state-of-the-art AML diagnostics relies on cytomorphology, cytochemistry, immunophenotyping, cytogenetics and molecular genetics. Only the integration of all of these methods allows for a comprehensive and complementary characterisation of each case, which is prerequisite for optimal AML diagnosis and management. Here, we will review why multidisciplinary diagnostics is mandatory today and will gain even more importance in the future, especially in the context of precision medicine. We will discuss ideas and strategies that are likely to shape and improve multidisciplinary diagnostics in AML and may even overcome some of today's gold standards. This includes recent technical advances that provide genome-wide molecular insights. The enormous amount of data obtained by these latter techniques represents a great challenge, but also a unique chance. We will reflect on how this increase in knowledge can be incorporated into the routine to pave the way for personalised medicine in AML.

Keywords: multidisciplinary diagnostics, acute myeloid leukaemia, precision medicine, AML diagnosis and management, artificial intelligence.

Current diagnostic workup in acute myeloid leukaemia

Cytomorphology

Cytomorphology is the indispensable starting point in the diagnostics of haematological diseases. This is also true from a historical perspective — the first classification efforts were based solely on cytomorphological and cytochemical features. In the past, present and future, cytomorphology was, is and will still be at the forefront of haematological diagnostics. It provides fast assessments of specimens and thus enables time- and cost-effective step-wise diagnostics. Abnormalities in cell morphology are readily identified by the trained haematologist and allow for distinction between normal and aberrant (and potentially leukaemic) cells. Evaluation of the percentage and relative distribution of erythropoiesis, granulopoiesis and monocytopoiesis identifies a range of haematological disorders. Cytochemical staining of non-specific esterase and myeloperoxidase and iron staining in many cases enable or facilitate cell lineage determination and evaluation of dysplasia. It is required for disease classification, validation of diagnoses, differential diagnostics and assessment of disease kinetics and response.

A bone marrow biopsy provides complementary information on cells in the tissue context, for example on cellularity and histotopography as well as the proportion and maturation of haematopoietic cells (Swerdlow *et al.*, 2017). In cases with fatty marrow or acute myeloid leukaemia (AML) with fibrosis, the blast count can only be reliably assessed in the biopsy specimen. Moreover, histologic evaluation aided by immunohistochemistry can validate classification, especially for cases of the subgroup of AML not otherwise specified (NOS), and facilitate the differentiation between myelodysplastic syndromes (MDS) and AML (Orazi, 2007).

Immunophenotyping

The presence and expression strength of antigens constitute the immunophenotype of a cell, which is indicative of cell lineage identity as well as the degree of maturation. Modern flow cytometers allow the parallel inspection of 8–10 markers. Leukaemic cells show aberrations in their immunophenotype, which can be broadly categorised as follows (Swerdlow *et al.*, 2017):

1 Expression of cross-lineage antigens (e.g. expression of lymphoid markers in AML cells, such as $CD19^+$ AML).
2 Asynchronous expression of maturational markers (e.g. concomitant expression of CD34 and CD11b in AML).

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3 Absent or decreased antigen expression of typical markers (e.g. HLA-DR-negative AML).
4 Overexpression of antigens (e.g. $CD33^{++}$ $CD34^{++}$ AML).

Flow cytometry is a crucial tool for detection, characterisation as well as quantification of healthy and malignant cell populations. Depending on the respective disorder, it plays an essential or supporting role for classification and differential diagnostics. In a myriad of haematological neoplasms, it has a pivotal function in the assessment of response, disease kinetics and especially in AML also in the detection of minimal/measurable residual disease (MRD). Immunophenotyping also encompasses the method of immunohistochemistry, which can be performed on bone marrow biopsy specimens. The stereotypical distribution patterns of myeloid leukaemias become apparent when the biopsy specimen is stained with an antibody directed against the blast marker CD34. Using suitable antibodies, a multitude of diagnostic questions can be addressed, for example on cell lineage identity and degree of maturation (Swerdlow *et al.*, 2017).

Cytogenetics

Cytogenetics encompasses the techniques of chromosome analysis and fluorescence *in situ* hybridisation (FISH). Chromosome analysis is performed by chromosome banding of metaphases. Non-malignant cells generally have a normal karyotype (46,XX or 46,XY), while the leukaemic karyotype might show acquired numerical or structural chromosomal aberrations. FISH relies on the use of fluorescent probes that are directed against specific chromosomal loci. This technique can be performed on interphase as well as on metaphase chromosomes. Probes can be either used to screen for known and/or suspected cytogenetic aberrations or, if directed against centromeres, to detect numerical aberrations. The use of so-called 24-colour FISH allows characterisation/validation of complex aberrations found in chromosome analysis after banding. While chromosome analysis enables a genome-wide, comprehensive evaluation, FISH provides a targeted, but fast approach. Subtypes of MDS and acute leukaemia are defined by specific cytogenetic aberrations. Beside its relevance for WHO classification, the most crucial role for cytogenetics in acute leukaemia is prognostic stratification. Cytogenetics is also important for the monitoring of disease kinetics, response assessment and the characterisation of clonal evolution.

Molecular genetics

Molecular genetics has rapidly evolved into an indispensable diagnostic discipline and has brought about major advances in our understanding of the molecular landscape of cancers, including AML. It has significantly contributed to optimisation of not only classification, but also of prognostication and residual disease monitoring. Moreover, it has aided the development of targeted therapeutics and is increasingly used as a therapeutic decision-making tool.

With respect to AML diagnostics, polymerase chain reaction (PCR)-based approaches as well as next-generation sequencing (NGS) represent the gold standard.

The PCR allows the specific amplification of known target sequences. By the method of quantitative PCR (qPCR), aberrations cannot only be detected but also sensitively monitored. Template amplification is measured in real time using fluorescent probes and quantification is performed relative to a standard. The input of cDNA, that is reverse transcribed (RT) RNA, permits transcript detection and quantification (RT-qPCR). The major advantage of PCR-based assays is their high sensitivity of up to 10^{-6}.

Next-generation sequencing, in contrast to older sequencing techniques (e.g. Sanger sequencing), offers the capability for massive parallelisation. This enables sequencing of hundreds of samples and/or genomic loci in one run. Panel-based sequencing represents the current state-of-the-art NGS methodology — such a panel could for example comprise all genes known to be associated with myeloid neoplasms that show diagnostic and/or clinical relevance today. Panel-based sequencing has led to better molecular characterisation in AML and it can be used also now for MRD.

Next-generation sequencing is a highly versatile platform and in the future new innovative NGS applications are likely to transition from research to routine diagnostics. With the technique of whole genome or whole exome (i.e. all protein-coding genes) sequencing (WGS/WES), sequence variations as well as numerical and structural aberrations can be detected. Sequencing the whole transcriptome (WTS/RNA-Seq) allows for genome-wide gene expression analysis, the detection of fusion transcripts and also for mutational analysis of expressed loci.

Optional diagnostic methods

Gene expression profiling

Gene expression profiling (GEP) has shown its potential to finally be integrated into routine diagnostic settings. Several studies had demonstrated that classification can benefit from GEP. Differentiation between AML and acute lymphoblastic leukaemia (ALL) can be realized based solely on expression profiles (Golub *et al.*, 1999; Haferlach *et al.*, 2010) and GEP-based approaches were able to reproduce classification of genetically defined subtypes, while at the same time providing insight into the underlying pathobiology (Schoch *et al.*, 2002; Debernardi *et al.*, 2003; Valk *et al.*, 2004; Haferlach *et al.*, 2010; Visani *et al.*, 2018). One study proposed the improvement of classification by introduction of GEP data for subclassification of AML with a normal karyotype (AML-NK) (Bullinger *et al.*, 2004). Here, GEP identified two distinct groups among AML-NK cases and stratification was of prognostic relevance, a finding that

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was validated in an independent study (Bullinger *et al.*, 2004; Radmacher *et al.*, 2006). Further research efforts have highlighted GEP's value for risk stratification (Valk *et al.*, 2004; Huang *et al.*, 2017; Visani *et al.*, 2018). With respect to AML therapy, gene expression profiles have been found to differ between responders and non-responders in induction therapy (Heuser *et al.*, 2005; Herold *et al.*, 2018) and can be used to predict drug sensitivity (Raponi *et al.*, 2008; Visani *et al.*, 2017; Tyner *et al.*, 2018). Despite all these promising studies, also reviewed in Visani *et al.* (2018), microarray-based GEP has never been implemented into routine diagnostics in AML — today's options with RNA-Seq will hopefully change this.

Epigenetic analysis

Similarly, epigenetics plays an important role in leukaemogenesis and AML disease biology (Wouters & Delwel, 2016). Partly, aberrant DNA methylomes with resulting gene expression deregulation in AML can be explained by recurrent aberrations in epigenetic regulators, such as *DNMT3A*, *ASXL1*, *TET2*, *KMT2A*, *IDH1* and *IDH2* (Wouters & Delwel, 2016). However, even in the absence of said somatic aberrations, distinct classes, defined by their DNA methylome, are discernible and of prognostic relevance (Figueroa *et al.*, 2010). Further research into the epigenome could lead to improved classification — especially in cases for which no leukaemia-driving (cyto-)genetic event can be identified. Epigenetic compounds (e.g. hypomethylating agents) are already an integral part of the therapeutic arsenal and AML diagnostics would be likely to benefit from inclusion of epigenetic analytics. However, there is, as of yet, no prospect of epigenetic analysis in the clinical routine.

The need for integrated diagnostics

From phenotype to genotype

The introduction of the French–American–British (FAB) classification, which was initially based solely on morphological characteristics (Bennett *et al.*, 1976; Bennett *et al.*, 1985), set the stage for modern AML diagnostics by providing objective criteria for patient stratification. The soon reached conclusion was that morphology alone cannot uncover the full range of heterogeneity in AML, which led to the introduction of immunophenotypic criteria to AML classification (Bennett *et al.*, 1991; Catovsky *et al.*, 1991). In parallel, several AML-specific cytogenetic aberrations have been described and some of them could be linked to a specific phenotype (Second MIC Cooperative Study Group, 1988).

For the following decade, cytomorphology and immunophenotyping in some cases together with cytogenetics were the basis for AML classification (Second MIC Cooperative Study Group, 1988). Meanwhile, new methods increasingly allowed molecular insight and FISH as well as molecular genetic methods have challenged the comprehensiveness of AML classification, yet again.

Since its introduction in 2001 (Jaffe *et al.*, 2001), the WHO classification has unified well established (cytomorphology, immunophenotyping, chromosome analysis) *and* molecular-orientated diagnostic disciplines (FISH, molecular genetics) for a comprehensive classification of haematological neoplasms. Today, 11 AML subtypes are defined by genetics (eight cytogenetically, three by gene mutations). The complete transition from the FAB to the WHO classification therefore also signifies the paradigm change from phenotype to genotype (Swerdlow *et al.*, 2017).

The WHO classification

Table I provides an overview of the different categories and subtypes of AML and related neoplasms. Further details on AML subtypes are given in the following paragraph. Here, we only focus on details of classification that need specific diagnostic approaches and sometimes directly lead to prognostic and therapeutic consequences.

Although genetics plays a crucial and partly entity-defining role for the classification of AML according to WHO, the presence of ≥20% blasts in peripheral blood or bone marrow is a requirement for AML diagnosis, making cytomorphology essential for AML classification.

However, there are three exceptions, for which the AML diagnosis is made independent of blast count:

1 AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*.
2 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*.
3 APL (acute promyelocytic leukaemia) with t(15;17)(q22;q11-12); *PML-RARA*.

In the absence of subtype-defining aberrations, cases with AML can be categorised into AML with myelodysplasia-related changes, therapy-related neoplasms or AML, NOS.

Classification of AML with myelodyplasia-related changes depends on multidisciplinary diagnostics. In addition to the presence of ≥20% blasts, cases must exhibit MDS-related features which are defined as (i) a history of MDS or MDS/MPN (myelodysplastic/myeloproliferative neoplasm); (ii) MDS-related cytogenetic abnormalities; or (iii) multilineage dysplasia (as defined by the presence of ≥50% dysplastic cells in 2–3 haematopoietic lineages, assessed by bone marrow cytomorphology). Up to ~25% of *de novo* AML cases present with multilineage dysplasia (Haferlach *et al.*, 2003). The presence of multilineage dysplasia, however, does not influence the prognosis in AML with *NPM1* mutation (Falini *et al.*, 2010) or AML with biallelic *CEBPA* mutation (Bacher *et al.*, 2012), *NPM1* and biallelic *CEBPA* mutations thus take diagnostic precedence over multilineage dysplasia (Swerdlow *et al.*, 2017).

Prior exposure to cytotoxic therapy or radiotherapy identifies therapy-related neoplasms. A differentiation into

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Table I. Acute myeloid leukaemia (AML) and related precursor neoplasms according to the WHO classification (2017).

Subclassfication	Subtypes
AML with recurrent genetic abnormalities	• AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* • AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* • Acute promyelocytic leukaemia with *PML-RARA* • AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3* • AML with t(6;9)(p23;q34.1); *DEK-NUP214* • AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, *MECOM* • AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); *RBM15-MKL1* • Provisional entity: AML with *BCR-ABL1* AML with gene mutations • AML with mutated *NPM1* • AML with biallelic mutation of *CEBPA* • Provisional entity: AML with mutated *RUNX1*
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML, not otherwise specified	• AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukaemia • Acute monoblastic and monocytic leukaemia • Pure erythroid leukaemia • Acute megakaryoblastic leukaemia • Acute basophilic leukaemia • Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations associated with Down syndrome	• Transient abnormal myelopoiesis associated with Down syndrome • Myeloid leukaemia associated with Down syndrome

individual subtypes of myeloid neoplasms is not intended in the WHO classification, although it might be clinically meaningful from our perspective. This category includes not only cases with AML, but also with t-MDS or t-MDS/MPN-overlaps (Swerdlow *et al.*, 2017).

Cases that do not fulfil the diagnostic criteria of all AML subtypes mentioned above are by exclusion categorised as AML, NOS. Within this category, subclassification is based on morphological and immunophenotypical criteria, and most entities of this group are synonymous to the old FAB subtypes. With the potential exception of pure erythroid leukaemia, AML, NOS subclassification does not provide prognostic information *per se* based on its morphoplogy (Swerdlow *et al.*, 2017).

Multidisciplinarity is a prerequisite for an optimal diagnosis

Multidisciplinarity aids fast diagnosis as well as differential diagnosis of acute leukaemias (AML vs. ALL). While today two methods can be applied: cytomorphology including cytochemistry (turnaround time 2–4 h) and immunophenotyping (turnaround time 2–6 h), in many cases both methods are used in parallel, depending on the respective facility — which consolidates and validates findings from either method. However, if for morphological questions trephine biopsies including histochemistry and immunohistochemistry are the methods of choice, immunophenotyping is the quicker technique to be applied.

In addition to classification, risk stratification already at diagnosis is of great clinical importance. Cytogenetics and molecular genetics provide the most powerful information for prognosis. In order to determine the risk group of a given case according to the recommendations of the European LeukemiaNet (ELN), a comprehensive genetic characterisation is required that reaches significantly beyond the 11 genetic aberrations that define an entity (see also Table II). The importance of genetic evaluation both for classification and risk stratification firmly establishes chromosome analysis, FISH and molecular genetics in the diagnostic evaluation of every (suspected) case of AML.

In conclusion, state-of-the-art classification in AML in accordance with the WHO guidelines and also for ELN prognostication relies on the diagnostic disciplines of cytomorphology, immunophenotyping, cytogenetics and molecular genetics. A combined and interdisciplinary approach is needed to harmonise reports and increase the quality of any single method by implementation of knowledge already available from others. Of note, turnaround times are differing from some hours to a week.

The emerging molecular landscape of AML

In AML classification, it is foreseeable that more subtypes defined by specific molecular aberrations or their co-occurance will be introduced. Prerequisite for the recognition of an entity by the WHO is its clinical relevance and biological homogeneity, which makes it discernible not only by a given genetic aberration, but also by its clinical, morphological and/or immunophenotypical characteristics (Swerdlow *et al.*, 2017).

A comprehensive effort to gain deeper insight into the molecular landscape of AML led to the suggestion of 11 distinct classes — based solely on genetic features (Papaemmanuil *et al.*, 2016). Basis for this proposition was a study

Table II. Risk stratification according to European LeukemiaNet (ELN) recommendations (Döhner *et al.*, 2017).

Risk group	Genetic aberration
Favourable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with low *FLT3*-ITD allelic ratio (<0·5) Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1 and* high *FLT3*-ITD allelic ratio (≥0·5) Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD low allelic ratio (<0·5) (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favourable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* −5 or del(5q); −7; −17/abn(17p) Complex karyotype, monosomal karyotype Wild-type *NPM1 and* high *FLT3*-ITD allelic ratio (≥0·5) Mutated *RUNX1* Mutated *ASXL1* Mutated *TP53*

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with 1540 AML patients for which mutational and cytogenetic analysis was performed and correlated with clinical data. For mutational analysis, 111 cancer genes were targeted. At least one driver mutation was identified in 96% of cases. The majority of classes identified by this genetic approach equalled entities recognised by the WHO classification, while three represented novel genetic classes (compare Table III).

However, a subset of patients (4%) fulfilled the criteria for two or more classes. This poses the question which aberration would take diagnostic precedence. In the end, 11% of patients remained unclassified (Papaemmanuil *et al.*, 2016). However, this is far better than the current AML, NOS category which includes 25–30% of AML patients (Swerdlow *et al.*, 2017). It is still a goal to find meaningful and relevant characteristics that allow for classification of all these unclassifiable cases in the future. This is likely to improve patient risk stratification, treatment and outcome.

Advances in diagnostic techniques have led to improvements of AML classification and will continue to do so. Among the innovative techniques that will likely drive optimisation of AML classification are WGS/WES and RNA-Seq. Both methods provide genome-wide and unbiased insight into chromosomal and sequence aberrations (WGS/WES) as well as into (aberrant) gene expression and sequence alterations for expressed genes (RNA-Seq). This not only reproduces already known but also might lead to identification of new leukaemia-driving or -promoting molecular aberrations with not only diagnostic, but also prognostic, predictive or therapeutic impact.

Why germline matters — Is the outbreak of AML predictable?

Germline mutations in *CEBPA*, *DDX41*, *RUNX1*, *ANKRD26*, *ETV6* and *GATA2* as well as inherited bone marrow failure and telomere syndromes predispose an individual to myeloid neoplasia. The recognition of distinct disease entities within the recently introduced category of 'myeloid neoplasms with germline predisposition' (Swerdlow *et al.*, 2017) will go a long way to increase our knowledge on hereditary factors that drive or promote AML pathobiology. In addition to that, only few risk factors have been identified, such as smoking (Fircanis *et al.*, 2014) or prior exposure to cytotoxic compounds or radiotherapy. Two recently published retrospective studies have dealt with the question whether one can predict the onset of AML within the general population. A predictive AML 'prodrome' could be identified by molecular genetic screening and the laboratory parameter of red cell distribution width (Abelson *et al.*, 2018; Desai *et al.*, 2018). Prospective studies will be necessary to determine whether screening for AML will one day be feasible and clinically meaningful.

Multidisciplinary diagnostics for prognosis and risk stratification

In addition to a patient's age and performance, genetics represent the single most relevant marker for risk stratification in AML (De Kouchkovsky & Abdul-Hay, 2016). Accordingly, the currently used risk stratification systems consider cytogenetic and molecular aberrations of high prognostic relevance. Two stratification models are well established: risk stratification recommended by the ELN (see also Table II) (Döhner *et al.*, 2017) and the risk model of the Medical Research Council (MRC) (Grimwade *et al.*, 2016), which is shown in Table IV.

Acute myeloid leukaemia with *NPM1* provides a case study for the prognostic importance of the genetic context. A recent study highlighted for example that the presence of *NPM1* mutations did not compensate for the negative impact of concomitant adverse cytogenetic aberrations. The study was restricted to patients without concomitant *FLT3*-ITD mutation or with a low *FLT3*-ITD allelic ratio (Angenendt *et al.*, 2019). A *DNMT3A* mutation is the most frequently occurring co-mutation in AML with mutated *NPM1* (Ivey *et al.*, 2016; Cappelli *et al.*, 2019). The prognostic impact of this *NPM1–DNMT3A* co-mutational

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Table III. Genetic subclassification of acute myeloid leukaemia (AML) according to Papaemmanuil *et al.* (2016).

Suggested genetic class	Class recognised by the WHO classification (2017)
inv(16)	AML with inv(16) or t(16;16); *CBFB-MYH11*
t(15;17)	APL with *PML-RARA*
t(8;21)	AML wit t(8;21); *RUNX1-RUNX1T1*
KMT2A fusions	AML with t(9;11); *KMT2A-MLLT3*
inv(3)	AML with inv(3) or t(3;3); *GATA2*, *MECOM*
t(6;9)	AML with t(6;9); *DEK-NUP214*
NPM1	AML with mutated *NPM1*
Biallelic *CEBPA*	AML with biallelic mutation of *CEBPA*
TP53-aneuploidy	
Chromatin-spliceosome	
IDH2^{R172}	

Table IV. Medical Research Council risk stratification according to Grimwade *et al.* (2016).

Risk group	Genetic aberration
Favourable	t(15;17)(q22;q21)/*PML-RARA* t(8;21)(q22;q22)/*RUNX1-RUNX1T1* inv(16)(p13q22)/t(16;16)(p13;q22)/*CBFB-MYH11* *NPM1* mutation (in absence of *FLT3*-ITD or *DNMT3A* mutation) Biallelic *CEBPA* mutation
Intermediate	Cytogenetic/molecular genetic abnormalities not classified as favourable or adverse
Adverse	In the absence of favourable risk cytogenetic/ molecular genetic abnormalities: abn(3q) [excluding t(3;5)(q21~25;q31~35)/ *NPM1-MLF1*] inv(3)(q21q26)/t(3;3)(q21;q26)/*GATA2/EVI1* add(5q)/del(5q), −5 t(5;11)(q35;p15.5)/*NUP98-NSD1* t(6;9)(p23;q34)/*DEK-NUP214* 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)/*BCR-ABL* −17/abn(17p)/*TP53* mutation Complex karyotype (≥4 unrelated abnormalities) *ASXL1* mutation *DNMT3A* mutation *FLT3*-ITD *MLL*-PTD *RUNX1* mutation

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pattern is itself determined by genetic interdependencies. Mutations that affect the glycines at position 12 or 13 of NRAS positively influence the prognosis in *NPM1–DNMT3A* AML (Papaemmanuil *et al.*, 2016), while an additional *FLT3*-ITD mutation has a negative prognostic impact (Papaemmanuil *et al.*, 2016; Cappelli *et al.*, 2019). Still, risk stratification according to current guidelines takes into account only *FLT3*-ITD (both ELN and MRC) or *DNMT3A* (MRC only) co-mutations (Grimwade *et al.*, 2016; Döhner *et al.*, 2017). With this exception, the complete genomic landscape can only partly be considered by both the ELN and MRC stratification system as it is too complex for routine application.

Several large-scale studies have highlighted the complexity of the genetic landscape in AML (Cancer Genome Atlas Research Network, 2013; Papaemmanuil *et al.*, 2016; Tyner *et al.*, 2018). However, the mutation status of only a few genes is taken into account by the currently used risk stratification models. This includes mutations in *NPM1*, *FLT3*-ITD, *CEBPA* (biallelic), *RUNX1*, *ASXL1* and *TP53* (Grimwade *et al.*, 2016; Döhner *et al.*, 2017). In addition, the MRC classification considers *KMT2A*-PTD and *DNMT3A* mutations (Grimwade *et al.*, 2016).

Although prognostic stratification today is far from trivial, current risk stratification systems are still oversimplified. Neither clinical nor patient-specific parameters are incorporated nor are interdependencies of genetic aberrations. Further studies suggest that gene expression analysis as well as DNA methylation analysis might also provide complementary prognostic information (Valk *et al.*, 2004; Bullinger *et al.*, 2010; Figueroa *et al.*, 2010; Tyner *et al.*, 2018).

It will be a challenge to determine and validate the influence of all possible parameters and to model a universally applicable risk stratification system will prove increasingly difficult — if not impossible. Personalised risk stratification might be the solution to this problem. For the training of risk prediction algorithms, large databases that match genomic with clinical data are required. Since the predictive accuracy of the trained algorithm correlates with sample size (Gerstung *et al.*, 2017), it should be an incentive to combine databases to reach a sample size that allows accurate prediction even for cases with rare (genetic) features. Data harmonisation and the use of a common data model (e.g. the OHDSI OMOP: https://www.ohdsi.org/) are integral to any effort to create a comprehensive unified database. Proof-of-concept for the utility of such a knowledge bank-based approach has been demonstrated recently (Gerstung *et al.*, 2017). The authors highlighted how a multistage model, trained on data of the AMLSG cohort (n = 1540) (Papaemmanuil *et al.*, 2016) and validated on the TCGA cohort (n = 186) (Cancer Genome Atlas Research Network, 2013), may aid the decision for or against allogeneic transplantation. A machine learning algorithm that was trained on the data of 3421 patients is currently under development and already outperforms ELN risk classification (Shreve *et al.*, 2019). We envision that web applications will make personalised risk predictions applicable within the clinical setting.

We envision several benefits by implementation of a patient-specific risk prediction into the clinical routine.

Firstly, such a personalised risk stratification algorithm can be designed to include not only genetic parameters, but

also patient-specific parameters, such as age, fitness and comorbidities as well as laboratory parameters of potential prognostic significance (e.g. white blood cell count).

Secondly, data on interdependencies of genetic aberrations can be considered by such an algorithm. In contrast to this, it would never be feasible to incorporate all possible genetic scenarios and co-mutation patterns into a generally applicable risk stratification model.

Thirdly, an accordingly designed and trained algorithm could identify the most relevant and targetable contributors to a patient's prognosis and thereby aid therapeutic decisions.

The power of integrated diagnostics as a therapeutic decision-making tool

In general, the therapeutic algorithm for AML can be divided into two separate phases:

1 Induction therapy with the goal to achieve a complete remission (CR).
2 Post-remission therapy with the goal to erase residual disease and prevent relapse.

For both scenarios and time points and also at relapse a comprehensive and individual diagnostic approach is needed. Applicable techniques include: morphology, cytogenetics, immunophenotyping and an increasing number of molecular genetic tests. All of the latter assays are needed at diagnosis, and morphology at a minimum for the definition of first CR.

However, several aspects are important:

1 Diagnostic parameters lead to risk classification that needs to guide further strategies including allogeneic stem cell transplantation (allo-SCT) in first CR in accordance with current guidelines (MRC, ELN, NCCN) (Grimwade *et al.*, 2016; Döhner *et al.*, 2017; Tallman *et al.*, 2019).
2 Diagnostic information increasingly leads to individualised treatment not only in *PML-RARA*-positive AML but also in *FLT3*-mutated AML. Other findings such as the detection of mutations of *IDH*, or *SF3B1*, *KIT* and others can influence choice of drugs. This is also true for the application of drugs such as the anti-CD33 monoclonal antibody gemtuzumab ozogamicin.
3 Age in combination with cytogenetic or molecular data influence treatment and drug choice, for example in including venetoclax, azacytidine or CPX-351 according to the guideline of the National Comprehensive Cancer Network (NCCN) (Version 3.2019) (Tallman *et al.*, 2019).
4 MRD parameters that can be followed by immunophenotyping and/or molecular assays (PCR, digital PCR, NGS) lead to individual follow-up strategies and treatment (Schuurhuis *et al.*, 2018).
5 At relapse, the genetic landscape may differ from that at first diagnosis and complete workup is recommended for best rescue therapy.

After induction treatment a cytomorphological evaluation of the bone marrow is performed to determine whether the patient has achieved a CR, which is defined, among other criteria, by <5% blasts in the bone marrow.

The criteria of CR and other response definitions as well as the definition of treatment failure and relapse are indicated in Table V.

Integrated diagnostics should assist treatment decision especially in patients above the age of 65. This is underlined by a recent study that showed improved outcome for patients ≥65 years with adverse cytogenetics under azacytidine treatment compared to conventional therapy (which included 7 + 3 chemotherapy, low-dose cytarabine and best supportive care). For a subset of patients the study also evaluated the influence of molecular aberrations on therapy outcome. While patients carrying *TP53* and *NRAS* mutations benefitted from azacytidine treatment, patients with *FLT3* and *TET2* mutations had better outcome under conventional therapy regimens (Döhner *et al.*, 2018).

Response monitoring and MRD

The detection of residual disease in haematological neoplasms has been improved in parallel to therapy optimisation. Sensitivities of 1:20 (cytomorphology) (Schuurhuis *et al.*, 2018), or 1:100 (FISH) (Ravandi *et al.*, 2018) were never thought to be sufficient to reliably monitor diseases kinetics in AML. This is also reflected by the abbreviation 'MRD', which was initially defined as minimal residual disease, and is of today more correctly defined as measurable residual disease. Sensitivities of 10^{-4} and 10^{-6} are needed to assess residual disease, and this can be achieved by using state-of-the-art molecular approaches or multiparameter flow cytometry (MFC) with 8–10 colours (Schuurhuis *et al.*, 2018). For MFC two differing approaches are implemented in MRD diagnostics: the different-from-normal approach (DfN) and the leukaemia-associated immunophenotype (LAIP). The latter has to be determined at diagnosis, since it is patient-specific. With modern flow cytometry a LAIP can be identified in up to 90% of cases (Swerdlow *et al.*, 2017). The DfN approach focuses on leukaemia- rather than on patient-specific aberrant markers and allows flow cytometric MRD monitoring even if the leukaemic immunophenotype at diagnosis is unknown.

Molecular MRD monitoring is dependent on suitable markers, which can be categorised as follows (Schuurhuis *et al.*, 2018):

1 Fusion gene transcripts (*PML-RARA* for APL).
2 Somatic mutations (e.g. *NPM1*).
3 Aberrant gene expression (e.g. *WT1* and *EVI1*).

However, when choosing a marker for molecular MRD assessment, several limitations have to be taken into consideration. Some potential MRD markers cannot be measured with the required sensitivity of 10^{-4} to 10^{-6} (e.g. *WT1* gene expression levels). There are also various biological situations

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Table V. Definitions of response, treatment failure and relapse in acute myeloid leukaemia (AML) according to Döhner *et al.* (2017), excerpt of the table on 'Response criteria in AML' in 'Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel'.

Category	Definition
Response	
CR without minimal residual disease (CR_{MRD-})	If studied pretreatment, CR with negativity for a genetic marker by RT-qPCR, or CR with negativity by multiparameter flow cytometry
Complete remission (CR)	• Bone marrow blasts <5% • Absence of circulating blasts and blasts with Auer rods • Absence of extramedullary disease • ANC ≥ 1·0 × 10^9/l (1000/μl) • Platelet count ≥100 × 10^9/l (100 000/μl)
CR with incomplete haematologic recovery (CR_i)	All CR criteria except for residual neutropenia (<1·0 × 10^9/l [1000/μl]) or thrombocytopenia (<100 × 10^9/l [100 000/μl])
Morphologic leukaemia-free state (MLFS)	• Bone marrow blasts <5% • Absence of blasts with Auer rods • Absence of extramedullary disease • No haematologic recovery required
Partial remission (PR)	All haematologic criteria of CR; decrease of bone marrow blast percentage to 5–25%; and decrease of pretreatment bone marrow blast percentage by at least 50%
Treatment failure	
Primary refractory disease	No CR or CR_i after two courses of intensive induction treatment; excluding patients with death in aplasia or death due to indeterminate cause
Death in aplasia	Deaths occurring ≥7 days following completion of initial treatment while cytopenic; with an aplastic or hypoplastic bone marrow obtained within 7 days of death, without evidence of persistent leukaemia
Death from indeterminate cause	Deaths occurring before completion of therapy, or <7 days following its completion; or deaths occurring ≥7 days following completion of initial therapy with no blasts in the blood, but no bone marrow examination available
Relapse	
Haematologic relapse (after CR_{MRD-}, CR, CR_i)	Bone marrow blasts ≥5%; or reappearance of blasts in the blood; or development of extramedullary disease
Molecular relapse (after CR_{MRD-})	If studied pretreatment, reoccurrence of MRD as assessed by RT-qPCR or by multiparameter flow cytometry

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that complicate the use of molecular aberrations for MRD monitoring (Schuurhuis *et al.*, 2018):

1 At relapse, some genetic loci are prone to chromosomal losses or gains (e.g. *FLT3*-ITD/TKD mutations, *EVI1*).
2 The somatic origin of a given mutation might be unclear, because of their recurrence in the germline (e.g. *RUNX1*, *CEBPA*).
3 Clonal haematopoiesis of indeterminate potential (CHIP)-associated gene mutations often persist (Busque *et al.*, 2012; Jaiswal *et al.*, 2014; Xie *et al.*, 2014; Genovese *et al.*, 2015; Steensma *et al.*, 2015), even during CR (*DNMT3A*, *ASXL1*, *TET2*) (Jongen-Lavrencic *et al.*, 2018; Höllein *et al.*, 2018b)

Only the *RUNX1-RUNX1T1*, *PML-RARA* and *CBFB-MYH11* rearrangements as well as the *NPM1* mutation are currently fully recommended as sole MRD markers by the ELN (Schuurhuis *et al.*, 2018).

Quantitative PCR represents the gold standard for molecular assessment of the MRD status. All PCR-based approaches require the use of aberration- and often also patient-specific assays. For qPCR, which depends on standards for relative quantification, this increases the labour intensity of the technique. In digital PCR (dPCR), the compartmentalisation of the reaction volume permits a binary fluorescence signal read out (signal or no signal) after PCR and thus absolute quantification (Sykes *et al.*, 1992; Vogelstein & Kinzler, 1999). Compared to qPCR it offers several advantages: in addition to an improved signal-to-noise ratio and the independence from standards, potentially present PCR inhibitors and PCR efficiency have a much smaller influence on the measurement (Huggett *et al.*, 2015; Quan *et al.*, 2018). Based on its properties, dPCR is a suitable and feasible method for sensitive MRD monitoring (Cilloni *et al.*, 2019) and is likely to prove its value in the clinical setting.

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Next-generation sequencing, with sensitivities of approx. 1% mutational load (Schuurhuis *et al.*, 2018), is a valuable tool to identify potential MRD markers at diagnosis, but not yet for their monitoring. However, efforts have been made to increase sensitivity by optimising experimental parameters and bioinformatic algorithms (Thol *et al.*, 2018). This will allow for reliable NGS-based MRD quantification in the future.

Already, there are a few examples of how MRD status informs therapeutic decisions. Pre-emptive therapy for APL patients with MRD positivity strongly reduced relapse risk (Grimwade *et al.*, 2009). Patients who underwent allo-SCT have been shown to benefit from MRD monitoring and pre-emptive therapy in the case of a positive MRD status (Schroeder *et al.*, 2013). Moreover, molecular monitoring of patients with t(8;21)/*RUNX1-RUNX1T1* or with mutated *NPM1* after induction and consolidation therapy identified those at high risk of relapse and thus beneficiaries of allo-SCT (Zhu *et al.*, 2013) and/or high-dose cytarabine (Krönke *et al.*, 2011; Shayegi *et al.*, 2013; Ivey *et al.*, 2016; Höllein *et al.*, 2018a).

Currently, MRD status is monitored either by MFC or molecular approaches. A recent study, however, found that combining both methods strongly improved prediction of relapse risk. When residual disease was detected using either method, relapse risk was ~50%. When MRD positivity was ascertained by both methods, the relapse risk was 73·3% (Jongen-Lavrencic *et al.*, 2018). This highlights the complementarity of both methods and strongly argues for synergistic multidisciplinary diagnostics in MRD detection.

In addition, the study showed that all identified mutations were suitable MRD markers, with the exception of CHIP-associated genes: *DNMT3A*, *TET2* and *ASXL1*. Molecular MRD markers were identified by the authors at diagnosis using a panel of 54 genes associated with myeloid neoplasms (Jongen-Lavrencic *et al.*, 2018). If this was to be validated in broader prospective studies, molecular MRD detection for almost every AML patient would be feasible, since 96% of patients carry at least one driver mutation (Papaemmanuil *et al.*, 2016).

In order to firmly establish MRD diagnostics in AML, standardisation of molecular and immunophenotypic MRD assessments is an absolute must. This would allow for the definition of valid and reliable response criteria, for the identification of clinically meaningful MRD thresholds and for determination of the clinical utility of MRD for different AML subtypes. First standardisation attempts are under way, such as the UK NEQAS pilot project for minimal residual disease evaluation in AML by flow cytometry (http://www.ukneqasli.co.uk/eqa-pt-programmes/flow-cytometry-programmes/minimal-residual-disease-for-aml-by-flow-cytometry-pilot-not-accredited/)

Today's needs and future directions for integrated diagnostics in AML

Just 15 years ago the diagnostic state-of-the-art in AML included only cytomorphology, immunophenotyping and metaphase chromosome banding analysis. We have since experienced a paradigm change from phenotype to genotype and an ever-increasing importance of multidisciplinary diagnostics. Today, FISH and molecular techniques are indispensable. All diagnostic disciplines are needed to inform and/or assist classification, prognostication, therapeutic decision and monitoring of residual disease. Table VI gives an overview of the respective essential diagnostic tool set.

Data obtained by integration of all of the different diagnostic disciplines are immense, especially since the introduction of NGS. All guidelines published need a minimum of diagnostic information not only at diagnosis but also for MRD measurement and at relapse. Today, this includes genetic data at all time points. However, up to now, the general approach was a targeted one, for example screening a patient for entity-defining genetic aberrations. In the future, diagnostics will entail data on the global genomic and transcriptomic level, instead of focusing on single aberrations.

Why are next steps needed and how can they be implemented?

1. Capabilities of NGS increase and prices will go down.
2. Turnaround time of NGS-based methods is below seven days and can already influence first-line treatment.
3. MRD diagnostics will be possible and can follow individual findings in nearly all patients.
4. WGS, WES and WTS will be feasible for routine use in the next five years and will outperform methods such as chromosome banding analysis, FISH, array comparative genomic hybridisation (CGH) or panel testing using NGS at diagnosis and at relapse to define the complete landscape of AML and foster personalised treatment.

Depending on the respective reimbursement structures and countries, the costs for methods such as cytogenetics, FISH, and especially molecular testing differ over a broad range. It is beyond the intention of this review to discuss this in detail. However, in the future methods like WGS and WTS will challenge the gold standards from today not only with respect to reproducibility and sensitivity but also with respect to turnaround times and costs. Parallel studies are needed to define the respective advantages and drawbacks.

All data available now, but even more data from WGS/WES and transcriptomics represent a great challenge, as the obtained information for one patient, let alone the genetic and gene expression landscape of AML in general, will be beyond human comprehension. However, it also provides us with the unique opportunity to translate the advance in knowledge into

Table VI. Mandatory diagnostic techniques in 2020.

Diagnostic technique	Diagnosis	Prognosis	Choice of therapy	Measurable residual disease
Cytomorphology	X		X	
Immunophenotyping	X		X	X
Cytogenetics	X	X	X	
Molecular genetics	X	X	X	X

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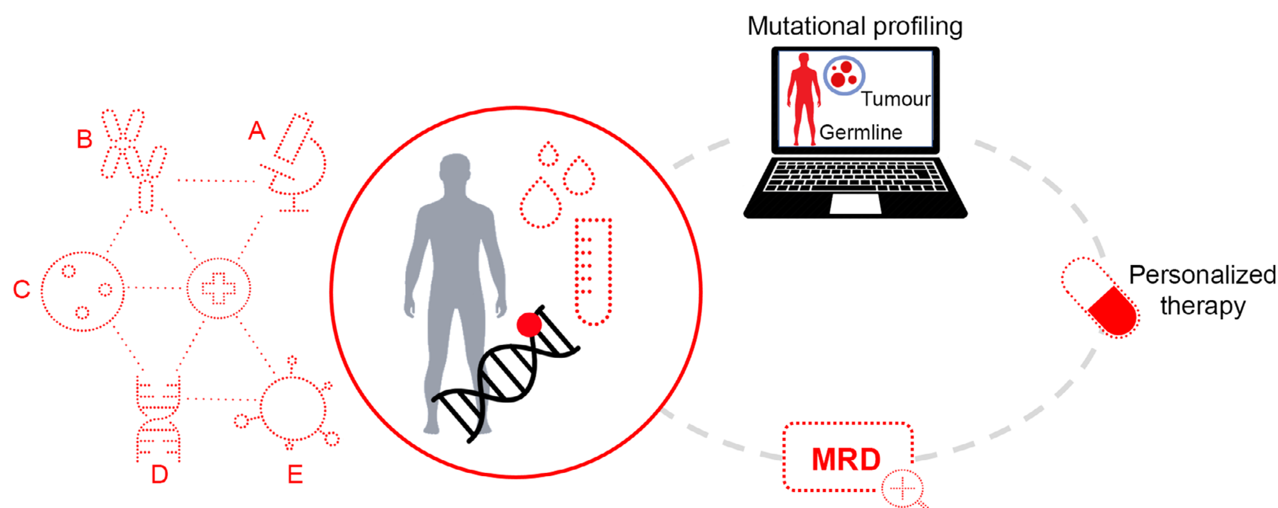

Fig 1. Precision medicine will be driven by multidisciplinary diagnostics and targetable genetic aberrations. Icons depict the diagnostic disciplines of (A) cytomorphology, (B) chromosome banding analysis, (C) FISH, (D) molecular genetics and (E) immunophenotyping. Only by combining findings of all diagnostic techniques a comprehensive characterisation of the underlying pathobiology can be attained. Mutational profiling plays a key role in identifying acute myeloid leukaemia drivers and targetable genetic aberrations, while carefully distinguishing between somatic and germline aberrations. Personalised therapies will significantly contribute to improved outcome. In the future, it will not suffice to describe leukaemia at initial diagnosis; instead multidisciplinary diagnostics will be required to monitor disease and response kinetics, clonal dynamics as well as residual disease iteratively. This ensures that every patient is treated adaptively and in the best possible way. Graphic by Dr. Wencke Walter, MLL Munich Leukemia Laboratory.

improved classification and prognostication and pave the way for precision medicine in AML in the truest sense of the term.

To reach this goal, physicians and scientists will need assistance to make sense of the data, identify clinically meaningful disease patterns as well as leukaemia-driving or -defining events and aid therapeutic decisions. This all cannot be done without streamlined workflows, automation of sample handling, databases, and a complex armamentarium of software tools for interpretation. Said assistance will for sure include artificial intelligence and cloud computing. It will bring up new challenges and solutions for data security and interpretation will also lead to ethical discussions how to handle germline findings. These data then need to be translated into reports understandable for doctors and patients.

As artificial intelligence using deep learning algorithms is on its way for routine applications also in diagnostics, several interesting approaches are ongoing, including AI-based image analysis of blood and bone marrow smears or the drawing of karyograms based on captured metaphases. So far none of these approaches have been used for routine diagnostics and they should be studied in prospective trials in comparison to gold standard approaches.

In the next five years the initial workup will not change: the diagnostic basis is and will be determined by cytomorphology, immunophenotyping and genetic analysis. The therapeutic aim is and ever will be to provide the best possible treatment for every patient, possibly a cure, while avoiding unnecessary risks and toxicities. However, owing to the heterogeneity of the disease, in the end the ideal approach to reach these ambiguous therapeutic aims will differ for every patient. This requires a deep understanding of the individual pathobiology attained by integrated diagnostics and continuous monitoring (see also Fig 1).

Conclusion

We envision a future where artificial intelligence with oversight by trained haematologists and scientists will find the best therapeutic algorithm and drugs for every patient — be it the participation in one or several (basket) studies, the choice for or against allogeneic SCT, the treatment with targeted therapeutics or the ideal sequence of therapeutic regimens. At the same time, special consideration must not only be given to a patient's genetic setup but also to treatment guidelines as well as to known and validated interdependencies between genetic aberrations and the influence of individual genetic aberrations or aberration patterns on drug sensitivity. Furthermore, AI can assist the detection of MRD and relapse prediction.

In perspective, all these goals will only be achievable if we use and integrate all diagnostic and technological tools from today, combine their information and in parallel test new options such as WGS, WTS and the implementation of AI and automation into our future thinking and doing.

Never before were the options in AML diagnostics so close to meet the needs, and cure for every single patient might be possible if we test all new options and challenge all state-of-the-art workflows without prejudice.

Conflict of interest

TH is part owner and founder of the MLL Munich Leukemia Laboratory. IS is employed by the MLL Munich Leukemia

Laboratory. The MLL offers diagnostic services for leukaemias and lymphomas, including cytomorphology, cytochemistry, immunophenotyping, cytogenetics, FISH, and a broad spectrum of molecular assays. In addition, MLL runs several research studies based on a combination of methods for routine use and also including whole genome sequencing and whole transcriptome sequencing.

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