Multilineage dysplasia (MLD) in acute myeloid leukemia (AML) correlates with MDS-related cytogenetic abnormalities and a prior history of MDS or MDS/MPN but has no independent prognostic relevance: a comparison of 408 cases classified as “AML not otherwise specified” (AML-NOS) or “AML with myelodysplasia-related changes” (AML-MRC)

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Running title: AML MRC in the 2008 WHO classification

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Abstract
The WHO classification of acute myeloid leukemia (AML) is hierarchically structured and integrates genetics, data on patients’ history, and multilineage dysplasia (MLD). The category “AML with MDS-related changes” (AML-MRC) is separated from “AML not otherwise specified” (AML-NOS) by presence of either MLD, MDS-related cytogenetics or history of MDS or MDS/MPN. To clarify whether MLD alone is clinically relevant, we analyzed 408 adult patients categorized as AML-MRC or AML-NOS. 3-year-EFS (median 13.8 months vs. 16.0 months) and 3-year-OS (45.8% vs. 53.9%) did not differ significantly between patients with MLD versus without. However, MLD correlated with pre-existing MDS (p<0.001) and MDS-related cytogenetics (p=0.035). Patients with MLD as sole AML-MRC criterion (AML-MLD-sole; n=90) had less frequently FLT3-ITD (p=0.032), and lower median age than AML-NOS (n=232), but EFS, OS, and WBC did not differ significantly. Contrarily, patients with AML-NOS combined with AML-MLD-sole (n=323) had better 3-year-EFS (16.9 vs. 10.7 months; p=0.005) and 3-year-OS (55.8% vs. 32.5%; p=0.001) than patients with history of MDS or MDS/MPN or MDS-related cytogenetics (n=85). Gene expression analysis showed distinct clusters for AML-MLD-sole combined with AML-NOS versus AML with MDS-related cytogenetics or MDS history. Thus, MLD alone demonstrated no independent clinical impact, while cytogenetics and MDS history were prognostically relevant.
INTRODUCTION

In 2008, the WHO published its revised classification of acute myeloid leukemia (AML).\(^1\) By paying attention to the clinical relevance of cytogenetic alterations\(^2-4\) and to the molecular heterogeneity of this complex disorder,\(^5,6\) the first category of AML was assigned to patients with recurrent genetic alterations.\(^1\) *CEBPA* and *NPM1* mutated AML were acknowledged as separate provisional entities. Due to its prognostic importance, it was recommended to always define the *FLT3* mutation status, particularly in cytogenetically normal AML.\(^7\)

Thus, genetic alterations play a major role in the WHO classification, but morphologic, cytochemical, immunophenotypic, and clinical information are also required for classification.\(^7\) One of these morphologic criteria is multilineage dysplasia (MLD) referring to cases with dysplastic features in at least two hematopoietic lineages. According to the WHO, cases with at least 20% of bone marrow or peripheral blasts which do not fulfill the criteria of the categories “AML with recurrent genetic abnormalities” or “therapy-related AML (t-AML)”, are classified as AML with myelodysplasia-related changes (AML-MRC) when at least one of the following prerequisites is fulfilled: a history of secondary AML (s-AML) arising from a previous MDS (myelodysplastic syndrome) or MDS/MPN (with overlapping myeloproliferative features), AML with MDS-related cytogenetic abnormalities, and/or AML with MLD. If none of these aspects is fulfilled, cases are classified as “AML, not otherwise specified” (AML-NOS). AML with <20% of myeloid blasts and more than 50% of erythroid precursors (the former FAB subtype M6)\(^8,9\) are classified as AML-NOS, regardless of the presence of dysplastic features.\(^1\) Therefore, in the new WHO classification, dysplasia alone can be sufficient to assign a case to the “AML-MRC” category.

Some previous studies in AML considered dysplasia to be prognostically relevant,\(^10-14\) while others were not able to confirm an independent clinical relevance of MLD.\(^15\) Our former analysis in 614 patients with *de novo* AML confirmed cytogenetics, age, and high lactate dehydrogenase (LDH) as independent prognostic parameters, while presence of dysplasia had no independent clinical impact.\(^16\) More recently, we investigated MLD for its prognostic impact in *NPM1* mutated AML, and again observed no independent influence on outcomes, while a mutated *FLT3* status represented a prognostically adverse parameter.\(^17\)

Thus, whether the presence of MLD has an independent prognostic meaning in AML or not, is still controversial,\(^18,19\) and it remains to be clarified whether the categorization of AML
cases as myelodysplasia-related or “MRC” solely based on the presence of MLD is justified according to biological and clinical aspects.

We here studied the clinical relevance of MLD\textsuperscript{20} in a cohort of 408 patients with AML at diagnosis who were corresponding to the AML-MRC or AML-NOS categories according to the WHO classification\textsuperscript{1} therewith excluding cases with the above mentioned criteria such as recurrent genetic alterations or t-AML. Patients were characterized by a comprehensive diagnostic assessment consisting of detailed cytomorphologic investigation, immunophenotyping, cytogenetics, molecular genetics, and gene expression profiling, to evaluate whether presence of MLD was associated with specific phenotype or genotype features.

**PATIENTS, MATERIALS AND METHODS**

**Patients**

Our retrospective study was based on 408 adult patients with AML diagnosed between August 2005 and February 2009 as AML with MDS related changes (AML-MRC) or AML, not otherwise specified (AML-NOS) according to the WHO guidelines.\textsuperscript{1} All patients were referred to the MLL Munich Leukemia Laboratory for diagnosis of AML. Prerequisites to be entered into this study were adequate material for morphologic assessment, complete cytogenetic and molecular genetic information, and availability of clinical data. Thus, 461 patients were assigned to the AML-MRC or AML-NOS categories according to the WHO. After in-depth morphologic analysis, 53 cases had to be excluded as dysplastic features were not evaluable following WHO guidelines due to insufficient numbers of evaluable cells in the respective hematopoietic lineages as a result from a very high degree of blasts percentage, insufficient smears not allowing adequate analysis, or hypocellularity. Thus, 408 AML patients with complete clinical and laboratory data were remaining. Patients with a classification as “AML with mutated $NPM1$” or “mutated $CEBPA$”, both representing provisional separate entities according to the WHO classification, were not excluded from analysis. The study was performed in accordance with the Declaration of Helsinki and informed consent was obtained in all cases. The study was approved by the Institutional Review Board of Munich Leukemia Laboratory. A subgroup of patients ($n=208/408$) had been included in our previous study on MLD in $NPM1$ mutated AML.\textsuperscript{17}
Cytomorphology

Morphologic analysis of all 408 AML cases was independently performed by different investigators (MM, KM and UB) and subsequently validated (TH). Bone marrow and peripheral blood smears were stained using the May-Grünwald-Giemsa (MGG) method. Cytochemistry was done with myeloperoxidase (MPO) and non-specific esterase (NSE). Dysplasia was assessed following the definitions of Goasguen et al. and the WHO 2008 classification:

- **Dysgranulopoiesis** (DysG) was defined as ≥50% of at least 10 polymorphonuclear neutrophils (PMN) being agranular or hypogranular, or with hyposegmented nuclei (pseudo Pelger-Huet anomaly). At least 25 cells were evaluated, but usually 100 cells were counted. MPO deficiency in the PMN was defined as ≥50% MPO-negative cells in at least 10 PMN after strong positivity of eosinophils or other PMN was confirmed.
- **Dyserythropoiesis** (DysE) was defined as ≥50% of dysplastic features in at least 25 erythroid precursors: megaloblastoid aspects, karyorrhexis, nuclear particles, or multinuclearity. **Dysmegakaryopoiesis** (DysM) was diagnosed when ≥3 megakaryocytes or ≥50% in at least six cells showed dysplastic features such as microkaryocytes, multiple separated nuclei, or very large single nuclei. According to the WHO, multilineage dysplasia (MLD) was defined by dysplastic features in at least two hematopoietic lineages. Trilineage dysplasia (TLD) was diagnosed when DysG, DysE, and DysM in combination were detectable.

Cases which were evaluable in only one or two hematopoietic lineages (and, for example, one was called dysplastic and the other one non-dysplastic) had to excluded from analysis (n=53, see above). As the WHO 2008 classification does not differentiate between MLD and TLD, we included cases in which at least two lineages were evaluable and both were dysplastic or both were not, since they still were evaluable for presence of MLD.

Cytogenetic analysis

Chromosome banding analysis in combination with fluorescence in situ hybridization techniques was performed in all 408 patients according to standard procedures. Patients were categorized as having “AML with an MDS-related cytogenetic abnormality” according to WHO definitions: complex aberrant, -7/del(7q), -5/del(5q), isochromosome i(17q), -
13/del(13q), del(11q), del(12p) (i.e. translocations involving 12p), del(9q), idic(X)(q13),
t(11;16), t(3,21), t(1;3), t(2;11), t(5;7), t(5;17), t(5;10), t(3;5). For prognostic evaluation based
on cytogenetics all cases were assigned to the “favorable”, “intermediate”, or “adverse” risk
categories following the revised MRC (Medical Research Council) criteria according to
Grimwade et al.23,24

Molecular mutation screening and immunophenotyping

In addition, PCR screening was done for the following molecular aberrations: NPM1
(nucleophosmin) mutations,25 FLT3-ITD (internal tandem duplications of the FLT3 gene),26
NRAS mutations,27 partial tandem duplications of the MLL gene (MLL-PTD),28 and CEBPA
mutations.29 Immunophenotyping with multiparameter flow cytometry (MFC) was performed
as previously described.30

Gene expression profiling

Microarray analysis was performed on 96 cases using HG-U133 Plus 2.0 microarrays
(Affymetrix, Santa Clara, CA). The samples were prepared as previously reported.31,32 Gene
expression raw data were processed according to the manufacturer’s recommendations.
After quality control, raw data were normalized using the robust multi-array average
normalization algorithm as implemented in the R-package affy version 1.22.0.33 For
supervised statistical analyses, samples were grouped accordingly and for each disease
entity differentially expressed genes were calculated by means of t-statistics. Resulting p-
values were adjusted for multiple testing.34 To visualize similarity of gene expression
patterns, principal component analyses were applied. Transformed gene expression data
were analyzed using Partek Genomics Suite Version 6.4 (Partek Inc., St. Louis, MO).
Microarray data can be downloaded at Gene Expression Omnibus, GEO, under accession
number GSE21261.35

Statistical analysis

Mean differences were analyzed using the t-test. Chi-square test / Fisher’s Exact Test were
applied in case of contingency tables. Overall survival (OS) was defined as the time from
diagnosis to death or date of the last follow-up. Event free survival (EFS) was defined as the time from diagnosis to date of failure (no CR, relapse, death) or date of the last follow-up. The probabilities of OS and EFS were estimated using the Kaplan-Meier method. The log-rank test was used to compare risk factor categories in survival analysis. Cox proportional hazard regression models were performed to investigate treatment results and the risk factors affecting time to event. All tests were 2-sided, accepting $p \leq 0.05$ as indicating a statistically significant difference. These p-values were not adjusted for multiple testing. Statistical analyses were performed using SPSS software version 14.0.1 (Chicago, IL).

RESULTS

Clinical characteristics and outcomes

Cases were equally distributed by sex (males, n=212; 52.0%; females, n=196; 48.0%). The median age was 69.8 years (range, 18.3 - 88.1 years); the median WBC was $8.9 \times 10^9$/L (range, 0.14 - 600.0 x10$^9$/L). Median follow-up was 13.9 months (95% CI, 11.8 - 16.0 months).

Classification following the WHO classification of 2008

For classification of cases following the 2008 WHO classification,$^1$ the following parameters had to be evaluated in all 408 cases: total blast count $\geq$20%, presence of MDS-related cytogenetic changes, a history of previous MDS (or MDS/MPN), and presence of MLD. Taking these parameters into account, this resulted in 175 cases that were assigned to the category “AML-MRC” (42.9%) and the remaining 233 patients were categorized as “AML-NOS” (57.1%). Most cases qualified by one criterion for classification as AML-MRC: 90 cases (90/175; 51.4%) showed solely MLD (AML-MLD-sole), 25 cases (14.3%) had MDS related cytogenetics, and 11 cases (6.3%) had a history of MDS (n=9) or MDS/MPN (n=2). The remaining 49 cases (28.0%) demonstrated more than one criterion for the category AML-MRC: 44 cases (44/49; 89.8%) had a combination of MLD plus MDS-related cytogenetics or plus an MDS or MDS/MPN history, while 5 cases showed other combinations (Table 1).
**Morphologic findings**

Dysplasia in one hematopoietic lineage was seen in 130/408 cases (31.9%). In 110 cases (27.0%), we detected dysplasia in two cell lineages, while 36/408 cases (8.8%) had TLD. Thus, a total of 146 cases (35.8% from the whole cohort) had MLD.

**Cytogenetics and molecular genetics**

Cytogenetics was available in all 408 cases. According to the refined MRC criteria, most cases (n=366; 89.7%) were assigned to the intermediate prognostic group which was due to the high proportion of normal karyotypes (n=281; 68.9% of all cases), whereas 42 cases (10.3%) were classified as adverse risk. Cytogenetics of the cohort was detailed in table 2. “Other” chromosomal abnormalities present were those different from deletions of the chromosomes 5 or 7, translocations, or complex karyotypes. As patients from the category “AML with recurrent genetic abnormalities” according to the WHO had to be excluded from our study, there were no favorable risk cases (Table 2).

Molecular screening revealed NPM1 mutations as the most frequent aberrations (203/397; 51.1%). This was followed by mutations of FLT3-ITD (89/398; 22.4%), NRAS (24/187; 12.8%), CEBPA (17/225; 7.6%), and MLL-PTD (24/399; 6.0%) of cases investigated, respectively (Table 3).

**Immunophenotyping**

Immunophenotyping was performed in 324/408 cases (155 AML cases with MLD and 169 cases without MLD, respectively). While the expression of most antigens did not differ between both groups, HLA-DR showed lower levels of expression in cases with MLD (positive cells: 31.13 +/- 1.71% vs. 40.19 +/- 1.79%; p<0.001). Other antigens with weaker expression in MLD cases included CD117 (25.49 +/- 1.92% vs. 31.53 +/- 1.75%; p=0.023), CD135 (8.92 +/- 1.27% vs. 13.47 +/- 1.53%; p=0.023), CD38 (66.71 +/- 2.01% vs. 72.26 +/- 1.57%; p=0.029), and TdT (4.28 +/- 0.86% vs. 8.43 +/- 1.26%; p=0.07), while Lactoferrin was more strongly expressed in MLD cases (14.63 +/- 1.36% vs. 8.79 +/- 0.85%; p<0.001).

**Comparison of different subgroups**
To determine whether the different subgroups as defined above were clinically relevant, we performed comparisons of different parameters: These included biologic characteristics (age, WBC), genetic features as assessed by chromosome banding/FISH, molecular alterations, the immunophenotype, as well as gene expression studies. Clinical outcomes were compared regarding OS and EFS.

**Comparison of patients according to presence and number of dysplastic cell lineages**

We compared EFS and OS between the different subgroups as defined by presence and number of dysplastic cell lineages. The median EFS did not differ significantly between the different cohorts with 18.4 months (95% CI, 14.1 - 22.7 months) in those without dysplasia, and 12.2 months (95% CI, 7.1 – 17.2 months), 11.6 months (95% CI, 6.2 - 16.9 months), and 17.4 months (95% CI, 10.3 - 24.5 months) in those with dysplasia being present in one, two, or three hematopoietic lineages, respectively. Similar, OS did not differ significantly between these cohorts (Table 4).

MLD was seen in a higher frequency in AML cases with a history of MDS or MDS/MPN when compared to others (28/42; 66.7% vs. 119/366; 32.5%; p<0.001). In addition, we observed a higher frequency of MLD in cases with MDS-related cytogenetic abnormalities when compared to others (27/55, 49.1% vs. 120/353; 34.0%; p=0.035).

Outcomes were compared between AML patients with MLD (MLD+; n=147) versus patients without MLD (MLD-; n=261). The median EFS (MLD+: 13.8, 95% CI, 9.4 - 18.2 months; MLD-: 16.0 months; 95% CI, 12.3 - 19.7 months) and 3-year-OS (MLD+: 45.8%, MLD-: 53.9%) showed no significant differences (Figure 1 a-b). We performed the same analysis in the subgroup of patients with normal karyotype. Also, no differences in outcome of AML patients with MLD (MLD+; n=91) versus patients without MLD (MLD-; n=190) were observed. The median EFS (MLD+: 17.3, 95% CI, 12.7 – 21.8 months; MLD-: 18.4 months; 95% CI, 13.5 – 23.3 months, p=0.333.) and 3-year-OS (MLD+: 59.2%, MLD-: 57.0, p=0.202.) also did not differ.

**Comparison of patients with AML-MRC solely due to MLD versus patients with AML-NOS**
Next, we analyzed whether MLD as a sole criterion for the classification of cases as AML-MRC (“AML-MLD-sole”) was clinically relevant. Patients from this subgroup (n=90) were characterized by ≥20% blasts, presence of MLD, but no history of MDS or MDS/MPN, and no evidence of MDS-related cytogenetic abnormalities. This subgroup was compared to the AML-NOS patients (referring to cases with blast counts ≥20% or patients with more than 50% of erythroid cells in combination with presence of ≥20% of blasts of total bone marrow cells regardless of MLD presence, and to cases without the MRC criteria; n=233).

The AML-MLD-sole patients showed a significant lower median age (62.8 years; range, 20.4-85.0) than those with AML-NOS (66.0 years; range, 18.3-88.1; p=0.043). Median WBC did not differ significantly between both subgroups (AML-MLD-sole: 21.8 x 10^9/L; range 0.4-370.0 x 10^9/L; AML-NOS: 11.4 x 10^9/L; range 0.14 - 600.0 x 10^9/L; p=0.279).

In gene expression analysis, 24 cases with AML-MLD-sole were compared to 56 cases with AML-NOS. Using the top-500 genes sorted according to the t-statistic, no significant differences were observed according to the underlying expression profile for these two groups. As observed in a principal component analysis, the patients largely intercalated with each other (Fig. 7a, and suppl. data) meaning that there were no clear expression signatures to distinguish between AML-MLD-sole and AML-NOS.

Regarding molecular mutations, the AML-MLD-sole cohort exhibited an equal incidence of NPM1 mutations (49/203, 57.6%; vs. 144/203, 62.3%, n.s.) whereas there was a significantly lower frequency of FLT3-ITD (15/89, 17.4% vs. 68/89, 29.4%, p=0.032) than in those with AML-NOS. NRAS mutations were slightly more frequent in the AML-MLD-sole than in the AML-NOS cohort (8/24, 17.8% vs. 8/24 8.7%; n.s.). MLL-PTD and CEBPA mutations were similarly distributed (Table 3).

Median EFS did not differ significantly between the AML-MLD-sole and AML-NOS subgroups (17.5 months, 95% CI, 7.8 - 27.2 months vs. 16.1 months, 95% CI, 11.7 - 20.6 months), nor did 3-year OS (56.8% vs. 55.4%), indeed survival curves were overlapping (Figure 2a-b). Thus, the only parameter differing significantly between both cohorts was the frequency of FLT3-ITD mutations, while biologic characteristics and clinical outcomes showed no significant differences.
Comparison of patients with AML-MRC due to cytogenetics or due to MDS or MDS/MPN history with the remaining patients of the total cohort

Subsequently, patients with MDS-related cytogenetic abnormalities (n=55) and those with AML arising from previous MDS or MDS/MPN (n=42) were each compared to the respectively remaining patients of the total cohort (n=353 and n=366, respectively). Here, presence of MDS-related cytogenetics was associated with shorter median EFS (6.8 vs. 16.0 months, p=0.007) and OS (17.4 months vs. not reached, p=0.008) (Figure 3a-b). Also, MDS or MDS/MPN history was associated with shorter median EFS (12.7 vs. 16.6 months, p=0.075) and OS (15.0 months vs. not reached, p=0.018, respectively) when compared to all others.

Comparison of patients with AML-MRC due to cytogenetics and/or MDS or MDS/MPN history to patients classified as AML-MRC only due to the presence of MLD

Next, we combined patients with AML-MRC due to cytogenetics and/or MDS or MDS/MPN history to one cohort (n=85). When compared to the subgroup of AML-MLD-sole (n=90, i.e. those with MLD but without MDS-related cytogenetic changes and without MDS or MDS/MPN history), the cohort with the characteristic cytogenetics or MDS or MDS/MPN history had significantly higher age (median 69.8 vs. 62.8 years; p=0.002) and lower WBC (median 6.3 vs. 21.8; p=0.001). They further had a lower frequency of NPM1 mutations (10/81; 12.3% vs. 49/85; 57.6%; p<0.001). No significant differences were detected for all other mutations. Median EFS was significantly worse in the subgroup of AML-MRC due to cytogenetics and/or MDS or MDS/MPN history compared to the AML-MLD-sole group (10.7 months vs. 17.5 months; p= 0.019), the same effect was seen for median OS (16.8 months vs. not reached; p= 0.009) (Figure 4a-b).

Comparison of patients with AML-MRC due to cytogenetics and/or MDS or MDS/MPN history to patients classified as AML-NOS

The comparison of the above established new cohort with AML-MRC due to cytogenetics and/or MDS or MDS/MPN history (n=85) to the AML-NOS patients showed a trend to higher age (median 69.8 vs. 66.0; p=0.049) and significantly lower WBC (median 6.3 vs. 11.4; p<0.001). Regarding molecular markers, we found a significantly lower frequency of NPM1
mutations and FLT3-ITD in this group when compared to the MLD-NOS patients (p<0.001). All other molecular markers did not differ significantly. Patients with AML-MRC due to cytogenetics and/or MDS or MDS/MPN history had both a worse EFS and OS (10.7 months vs. 16.1 months; p= 0.012; 16.8 months vs. not reached, 0.002, respectively) compared to the patients in AML-NOS (Figure 5a-b).

Comparison of patients with AML-NOS combined with patients with MLD as sole MRC-criterion to AML-MRC due to cytogenetics and/or MDS or MDS/MPN history

As being explained above, we had not been able to define any clinical and prognostic differences between patients with AML-MLD-sole and those with AML-NOS, but observed significant differences when both are combined and then compared to the cohort resulting from the combination of patients with MDS-related cytogenetics and MDS or MDS/MPN history. Therefore, we established a novel cohort (n=323) which was composed of AML-NOS (n=233) and AML-MLD-sole patients of the MRC group (n= 90). We tested this newly defined group against all remaining MRC-cases (n=85) being composed of AML-MRC based on myelodysplasia-related cytogenetics (n=47) and patients with a preceding MDS or MDS/MPN (n= 33) or both criteria in combination (n=5).

First, significant differences were observed with respect to age and WBC: Median age of the new cohort composed of AML-NOS plus AML-MLD-sole was significantly lower (65.6 years, range, 18.3 - 88.1 years) compared to AML-MRC based on cytogenetics and/or an MDS or MDS/MPN history (69.8 years, range, 28.5 - 85.1 years; p=0.011). Median WBC was 13.8 x 10^9/L (range, 0.14 - 600.0) in the AML-NOS plus AML-MLD-sole patients being significantly higher when compared to the second cohort (6.3 x 10^9/L; range, 0.8 - 11.5; p<0.001).

Median EFS was significantly better in the AML-NOS plus AML-MLD-sole patients when compared to the second cohort (16.9 months vs. 10.7 months; p= 0.005), the same effect was seen for median OS (not reached vs. 16.8 months; p= 0.001) (Figure 6a-b).

In gene expression analysis, 80 cases of the combined group AML-NOS plus AML-MLD-sole were compared against 16 AML-MRC cases based on cytogenetics and/or a MDS history. According to the t-statistic and adjusted for multiple testing the underlying expression profiles for these two groups showed significant differences (Supplemental Spreadsheet). While we had not been able to define any significant differences of gene expression patterns of
patients with AML-MLD-sole versus AML-NOS (see above, Figure 7a), these newly defined groups (AML-NOS + AML-MLD-sole versus AML-MRC due to cytogenetics/MDS history) formed two separate clusters based on statistically significant differential gene expression (Figure 7b). Genes with higher expression in AML-MRC cases were involved in cellular processes such as regulation of transcription (PSIP1, SOX15, LYL1), signal transduction (KIT, JAK3, SYDE1, TIGD5), or chromatin modification (HDAC7). Genes with lower expression in AML-MRC included genes with known relevance in cell development, DNA-dependent regulation of transcription and nuclear mRNA splicing (HDAC8, POU4F2, HIPK2, SFRS11, MEIS1, HOXA1, HOXA5, HOXB6, HOXB7, NKX3-1). Additional information on the top-500 significantly differentially expressed probe sets and their functional annotation is available in the online supplement.

Analysis of prognostic factors

Univariate analysis was performed for WBC, gender, age, presence of MLD, history of MDS or MDS/MPN, MDS-related cytogenetics, NPM1, FLT3-ITD, CEBPA, MLL-PTD and NRAS mutations to evaluate the prognostic significance of these distinct risk parameters. Among the above mentioned parameters only higher age, a history of MDS or MDS/MPN, the presence of MDS related cytogenetics, and absence of an NPM1 mutation were associated with significantly worse EFS. A significantly worse OS in univariate analysis was only seen for cases with higher age, the presence of MDS related cytogenetics, and cases without an NPM1 mutation. In multivariate analysis, higher age was the only parameter independently related to worse EFS (p<0.001) and OS (p<0.001), whereas absence of an NPM1 mutation only was associated with a significantly shorter OS (p=0.037) (Table 5).

DISCUSSION

It was one major achievement of the WHO classification of 2001 to consider genetic features as a main category for the classification of AML. In addition, the WHO focused on morphologic, immunophenotypic, and other clinical data. Multilineage dysplasia was introduced to encompass AML cases with MDS-like features, including unfavorable cytogenetic abnormalities and an unfavorable response to therapy. Based on the difficulties to define whether MLD has independent prognostic significance in AML, or not this
subgroup was renamed in the updated WHO classification of 2008 as "AML with myelodysplasia-related changes" (AML-MRC), and the criteria were expanded to include a history of MDS or MDS/MPN or specific cytogenetic findings.\textsuperscript{1,7}

However, the categorization of AML cases solely based on dysplastic features is still being controversially discussed: Wandt \textit{et al.} performed an investigation of 1766 patients with \textit{de novo}, s-AML and t-AML (excluding patients with acute promyelocytic leukemia, APL) and demonstrated a significant association of MLD with unfavorable cytogenetic profiles. Moreover, presence of MLD was negatively correlated with achievement of complete remission in univariate analysis. Nevertheless, in multivariate analysis, age, cytogenetics, and the \textit{NPM1} mutant/\textit{FLT3}-ITD status retained prognostic significance, while MLD had no independent prognostic power.\textsuperscript{15} Similarly, we had previously analyzed an independent series of 614 patients with \textit{de novo} AML recruited between 1992 and 1999 for the prognostic implications of dysplasia. Trilineage dysplasia correlated with unfavorable cytogenetics, but presence or absence of dysplasia failed to demonstrate independent prognostic relevance. In multivariate analysis, only cytogenetics, age, and elevated LDH had an independent prognostic impact.\textsuperscript{16} In the analysis of Yanada \textit{et al.} including 109 adult AML cases, significant differences were observed between outcomes of patients with recurrent genetic abnormalities, AML with MLD, t-AML, and AML-NOS according to the WHO (2001) criteria. Cytogenetic risk and patient age maintained their prognostic value in multivariate analysis, whereas the prognostic significance of MLD and prior therapy was lost after adjusting for cytogenetic risk and age.\textsuperscript{37} More recently, we investigated the prognostic meaning of MLD in a cohort of 318 patients all with \textit{NPM1} mutated AML. Again, we were not able to determine any independent influence of MLD for outcomes in this distinct subgroup, while the \textit{FLT3}-ITD status remained the sole prognostically relevant parameter in multivariate analysis.\textsuperscript{17} These studies thus led to the conclusion that dysplasia showed correlations with other adverse parameters such as unfavorable karyotypes, but had no independent impact on outcomes, when other clinically relevant parameters were included in multivariate analyses.

Others, in contrast, were able to determine a significant adverse impact of morphologic features in AML on clinical outcomes: The morphologic and histopathologic studies from Arber \textit{et al.} in 300 AML patients with \(\geq 20\%\) of bone marrow myeloblasts (AML and RAEB-T) revealed significantly worse survival in patients with MLD, while there were no significant differences in prognosis between AML arising from MDS and \textit{de novo} AML with MLD.\textsuperscript{13} However, control cases in the Arber study were more heterogeneous (including e.g.
recurrent cytogenetic aberrations) as compared to the present study in which control cases were recruited from AML-NOS and AML-MRC strictly in accordance with the WHO classification.\textsuperscript{1} Earlier, Goasguen et al. suggested an association of dysgranulopoiesis with lower complete remission rates in \textit{de novo} AML,\textsuperscript{10} while Gahn et al. reported an unfavorable prognostic impact of dysplastic features in AML at least in patients with favorable or intermediate cytogenetic risk profile.\textsuperscript{11} Very recently, Weinberg et al. evaluated the clinical, pathologic, cytogenetic, and molecular features in a relatively small cohort of 100 AML patients using 2008 WHO criteria and described significantly worse survival outcomes and CR rates in patients from the AML-MRC cohort (n=48) when compared to patients with AML-NOS.\textsuperscript{14} According to the authors, these results gave support to the previously observed clinical significance of multilineage dysplasia (MLD) when strictly defined by WHO 2008 criteria.\textsuperscript{1;7;14}

At this time the reasons for these diverging results,\textsuperscript{14} which are in contrast to the studies from Wandt et al.,\textsuperscript{15} Yanada et al.,\textsuperscript{37} and our own previous works\textsuperscript{16;17} (all failing to demonstrate an independent prognostic impact for dysplasia in AML) remain unclear. We aimed at clarifying whether the recent WHO category “AML with myelodysplasia-related changes” if based solely on the criterion of multilineage dysplasia was justified from biologic and clinical aspects. Therefore, we followed an algorithm based on the WHO classification from 2008\textsuperscript{1} and first excluded from our analysis patients with recurrent genetic alterations or t-AML and were then investigating a study cohort of 408 AML cases. According to the WHO 2008 definitions, these were assigned to the AML-MRC category due to MLD, and/or MDS or MDS/MPN history and/or myelodysplasia-related cytogenetics either as sole criterion or in combinations. All remaining patients were grouped to the AML-NOS category.

As we were not able to determine any prognostic impact of the presence of MLD, we compared patients who were categorized as AML-MRC solely based on MLD with AML-NOS patients: no significant differences regarding the biologic profile (age, WBC), and the clinical outcomes in terms of OS and EFS were detectable. Also with gene expression profiling, no significant differences were found between both patient cohorts. The only significant difference was a lower frequency of \textit{FLT3}-ITD in the cohort being classified as AML-MRC solely based on presence of MLD (p=0.032) but this does not translate into clinical different behaviour.

Our results were in contrast to the results of two previous studies (Weinberg et al.\textsuperscript{14} and Gahn et al.\textsuperscript{11}). In detail, Weinberg et al. performed separation of 100 patients with AML
according to WHO 2008 criteria: The AML-MRC cohort (n=48) was based on presence of MLD (n=41), and/or MDS-related cytogenetic abnormalities (n=14), and/or MDS history (n=16), while three patients had therapy-related myeloid neoplasms. When compared to the AML-NOS patients (n=39), the AML-MRC patients showed significantly worse OS, progression free survival (PFS), and lower CR rate. In a multivariate analysis, the cytogenetic risk group (p=0.001), age (p=0.037), evidence of FLT3-ITD (p=0.047), and the AML-MRC category (p=0.041) had an independent prognostic impact. However, Weinberg et al. did not consider presence of MLD separately in statistical analysis but focused only on the more comprehensive AML-MRC category. Therefore, it is difficult to draw final conclusions whether presence of MLD alone would have sustained an independent prognostically relevant position in multivariate analysis in a larger AML-MRC cohort.

Earlier, Gahn et al. analyzed a total of 102 patients with newly diagnosed AML for a potential clinical impact of dysplasia. Patients with dysgranulopoiesis had significantly shorter EFS than those without (p=0.025) and a CR rate of 56% versus 74% (p=0.04). In patients with unfavorable karyotypes, presence or absence of dysgranulopoiesis had no additional prognostic impact, while those with favorable karyotypes and evidence of dysgranulopoiesis experienced a significantly lower continuous complete remission rate when compared to those without (p=0.03). Of note, evidence of dysplastic features in megakaryopoiesis or erythropoiesis had no significant impact on remission rates. Presence of trilineage dysplasia (TLD) was correlated with a non-significant trend to lower CR rate only (55% in patients with TLD versus 65% in those without), while MLD was not considered in this analysis. Apparently, multivariate analysis had not been performed in the study from Gahn et al., which renders a direct comparison with our data as well difficult.

As we here have been unable to find significant differences between patients with AML-MRC solely based on MLD and AML-NOS patients, we subsequently combined patients from the AML-NOS and the AML-MRC solely due to MLD categories, and compared them to the patients with an MDS history and/or myelodysplasia-related cytogenetics. Comparison of these newly established cohorts revealed significant differences regarding to age (p=0.011) and WBC (p<0.001), and found as well different EFS (p=0.005) and OS (p=0.001). Significant differences were additionally shown by gene expression profiling, as distinct clusters were observed for combined patients with AML-MLD-sole with AML-NOS when compared to those with MDS-related cytogenetics and/or MDS or MDS/MPN history. Finally,
patients with an MDS history or with MDS-related cytogenetic abnormalities showed significantly worse EFS when compared to the other remaining cases of the total cohort.

Thus, while we were not able to determine a significant impact of the presence of MLD as sole criterion for the “AML-MRC” category, myelodysplasia-related cytogenetics and a history of previous MDS or MDS/MPN were biologically and prognostically highly relevant. These results suggest that separate categories “AML with myelodysplasia-related changes” solely based on MLD and “AML-NOS” has no fundamental underlying biologic basis. Instead, we would like to suggest to restrict the AML-MRC category to cases with a history of MDS or MDS/MPN or myelodysplasia-related cytogenetics completely irrespective of MLD findings, while cases solely being defined by morphologic criteria (MLD) should be combined with the AML-NOS category. As a consequence, this would result in only two distinct groups and avoid the interobserver variability of grading dysplastic features. This will finally represent the differences in the biology and prognosis of these AML cohorts and therefore would improve the reproducibility of risk-stratification in the setting of clinical trials. We would like to suggest to consider these aspects for further evaluations and implement data in a revised WHO classification of AML.
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Author contributions
MM, UB, and TW analyzed the data and wrote the manuscript. MM, UB, and KM did morphologic analysis. TW collected clinical data and contributed to statistics and figures. CH was responsible for chromosome banding analysis and fluorescence in situ hybridization (FISH). SS was responsible for molecular analysis. WK was responsible for immunophenotyping and involved in statistical analysis. AK performed GEP studies; biomathematic analyses were done by HK and MD. TH was the principal investigator of the study and validated cytomorphologic analysis and clinical data. All authors contributed to write this manuscript and agreed with the final version before submission.

Disclosures
CH, WK, SS, and TH are part-owners of the Munich Leukemia Laboratory (MLL). MM, TW, KM, and AK are employed by the Munich Leukemia Laboratory (MLL). UB, HK, and MD have nothing to disclose.
Reference List


15. Wandt H, Schakel U, Kroschinsky F et al. MLD according to the WHO classification in AML has no correlation with age and no independent prognostic relevance as analyzed in 1766 patients. *Blood* 2008;111(4):1855-1861.


20. Miesner M, Weiss T, Macijewski K et al. AML with multilineage dysplasia (MLD) correlates with MDS-related cytogenetic abnormalities and a prior history of MDS or MDS/MPN but has no independent prognostic relevance: a comparison of 461 cases classified as "AML not otherwise specified" or "AML with myelodysplasia-related changes" as defined by the 2008 WHO classification. [abstract] *Blood* 2009;114(11):338-339. Abstract 823.


**TABLES**

**Table 1:** Patients with AML-MRC: Breakdown by WHO qualification criteria.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLD - sole</td>
<td>90 (51.4%)</td>
</tr>
<tr>
<td>MDS related cytogenetics (MRC) - sole</td>
<td>25 (14.3%)</td>
</tr>
<tr>
<td>History of MDS or MDS/MPN - sole</td>
<td>11 (6.3%)</td>
</tr>
<tr>
<td>MLD + pre-existing MDS</td>
<td>22 (12.6%)</td>
</tr>
<tr>
<td>MLD + MDS related cytogenetics</td>
<td>22 (12.6%)</td>
</tr>
<tr>
<td>MLD + MDS related cytogenetics + pre-existing MDS/MPN</td>
<td>2 (1.1%)</td>
</tr>
<tr>
<td>MDS related cytogenetics + pre-existing MDS/MPN</td>
<td>3 (1.7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>175 (100.0%)</strong></td>
</tr>
</tbody>
</table>
Table 2: Risk stratification according to cytogenetics in 408 patients from the study cohort according to Grimwade.23,24

<table>
<thead>
<tr>
<th>Refined Medical Research Council criteria23,24</th>
<th>No of cases</th>
<th>Subcategories</th>
<th>No of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate risk group</td>
<td>366 (89.7%)</td>
<td>normal karyotype</td>
<td>281 (68.9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>other non-complex</td>
<td>85 (20.8%)</td>
</tr>
<tr>
<td>Adverse risk group</td>
<td>42 (10.3%)</td>
<td>complex alterations</td>
<td>21 (5.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-7/add(7q)</td>
<td>11 (2.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>del(5q)/-5/add(5q)</td>
<td>6 (1.5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Others*</td>
<td>4 (1.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>408 (100%)</td>
<td></td>
<td>408 (100%)</td>
</tr>
</tbody>
</table>

* Cases with "others" were represented by deletions, other aberrations and cases with independent clones as classified based on Medical Research Council criteria.
Table 3: Incidence of molecular mutations in AML subgroups AML-NOS, AML-MRC with MDS-related cytogenetics and/or MDS/MPN history, and AML-MLD-sole.

<table>
<thead>
<tr>
<th>Mutation (no of patients analyzed*)</th>
<th>Patients with mutation</th>
<th>AML-NOS (n=233)</th>
<th>AML-MRC due to cytogenetics and/or MDS or MDS/MPN history (n=85)</th>
<th>AML-MLD-sole (n=90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>* from our cohort of 408 patients</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPM1 (n=397)</td>
<td>203 (51.1%)</td>
<td>144 (62.3%)</td>
<td>10 (12.3%)</td>
<td>49 (57.6%)</td>
</tr>
<tr>
<td>FLT3-ITD (n=398)</td>
<td>89 (22.4%)</td>
<td>68 (29.4%)</td>
<td>6 (7.4%)</td>
<td>15 (17.4%)</td>
</tr>
<tr>
<td>NRAS (n=187)</td>
<td>24 (12.8%)</td>
<td>8 (8.7%)</td>
<td>8 (16.0%)</td>
<td>8 (17.8%)</td>
</tr>
<tr>
<td>MLL-PTD (n=399)</td>
<td>24 (6.0%)</td>
<td>10 (4.3%)</td>
<td>6 (7.4%)</td>
<td>8 (9.3%)</td>
</tr>
<tr>
<td>CEBPA (n=225)</td>
<td>17 (7.6%)</td>
<td>9 (7.1%)</td>
<td>3 (6.7%)</td>
<td>5 (9.3%)</td>
</tr>
</tbody>
</table>

The p-values result from the comparison of the frequency of molecular markers between the three cohorts (patients with AML-NOS, AML-MRC due cytogenetics and/or MDS history, or AML-MLD-sole).
Table 4: Dysplastic features in 408 patients with AML (DysG: dysgranulopoiesis; DysE: dyserythropoiesis; DysM: dysmegakaryopoiesis; MLD: multilineage dysplasia).

<table>
<thead>
<tr>
<th>Dysplastic lineages</th>
<th>No of cases</th>
<th>Parameter</th>
<th>No of cases</th>
<th>No of cases</th>
<th>EFS (p)</th>
<th>OS (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No dysplasia</td>
<td>132 (32.3%)</td>
<td>No</td>
<td>MLD</td>
<td>261 (64.0%)</td>
<td>0.333</td>
<td>0.202</td>
</tr>
<tr>
<td>One dysplastic cell lineage</td>
<td>129 (31.9%)</td>
<td>DysG</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DysE</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dys M</td>
<td>83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two dysplastic cell lineages</td>
<td>111 (30.0%)</td>
<td>DysG + DysE</td>
<td>12</td>
<td>MLD</td>
<td>147 (36.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DysG + DysM</td>
<td>79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DysE + DysM</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trilineage dysplasia (TLD)</td>
<td>36 (8.8%)</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>408 (100%)</td>
<td>No</td>
<td></td>
<td></td>
<td>408 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

DysG: dysgranulopoiesis; DysE: dyserythropoiesis; DysM: dysmegakaryopoiesis; MLD: multilineage dysplasia, i.e. two or three lineages show dysplastic features.
Table 5: Univariate and multivariate analysis of risk factors in study cohort.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EFS (p)</th>
<th>OS (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>WBC</td>
<td>0.674</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td>0.746</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Presence of MLD</td>
<td>0.334</td>
<td>-</td>
</tr>
<tr>
<td>History of MDS or MDS/MPN</td>
<td>0.078</td>
<td>-</td>
</tr>
<tr>
<td>MDS-related cytogenetic changes</td>
<td>0.008</td>
<td>0.136</td>
</tr>
<tr>
<td>NPM1 mutated</td>
<td>0.005</td>
<td>0.110</td>
</tr>
<tr>
<td>FLT3-ITD mutated</td>
<td>0.457</td>
<td>-</td>
</tr>
<tr>
<td>CEBPA mutated</td>
<td>0.853</td>
<td>-</td>
</tr>
<tr>
<td>MLL-PTD mutated</td>
<td>0.094</td>
<td>-</td>
</tr>
<tr>
<td>NRAS mutated</td>
<td>0.807</td>
<td>-</td>
</tr>
</tbody>
</table>

WBC: white blood counts; MLD: multilineage dysplasia; EFS: event free survival; OS: overall survival
FIGURE LEGENDS

Figure 1: Event free survival (EFS) (a) and overall survival (OS) (b) in AML with and without multilineage dysplasia (MLD) according to Kaplan-Meier.

Figure 2: EFS (a) and OS (b) of AML-MRC solely due to MLD (“AML-MLD-sole”) versus AML, not otherwise specified (AML-NOS).

Figure 3: EFS (a) and OS (b) in AML with MDS-related cytogenetics versus without MDS-related cytogenetics.

Figure 4: EFS (a) and OS (b) in AML-MRC due to cytogenetics and/or MDS- or MDS/MPN-history versus AML-MRC solely due to the presence of MLD.

Figure 5: EFS (a) and OS (b) in AML-MRC due to cytogenetics and/or MDS- or MDS/MPN-history versus AML, not otherwise specified (AML-NOS).

Figure 6: EFS (a) and OS (b) in AML-MRC due to cytogenetics and/or MDS- or MDS/MPN-history versus AML-NOS combined with AML-MLD-sole.

Figure 7: Microarray analysis. In this supervised Principal Component Analysis (PCA) each patient is represented by a colored sphere. Ellipsoids are drawn with two-fold standard deviations. a) The gene expression signature is given for the top-500 probe sets differentially detected between 24 cases with AML-MLD-sole and 56 cases displaying AML-NOS. b) The gene expression signature is given for the top-500 probe sets differentially expressed between 80 cases AML-NOS plus AML-MLD-sole and 16 AML-MRC cases based on cytogenetics and/or a MDS history. Detailed information on the significantly differentially expressed probe sets and their functional annotation is available online.
FIGURE

Figure 1:

a)  
Event Free Survival

no MLD (n=261)  
with MLD (n=147)

p=0.333

Months

0 12 24 36 48

b)  
Overall Survival

no MLD (n=261)  
with MLD (n=147)

p=0.202

Months

0 12 24 36 48

Figure 2:

a)  
Event Free Survival

AML-MLD-sole (n=90)  
AML-NOS (n=233)

p=0.694

Months

0 12 24 36 48

b)  
Overall Survival

AML-MLD-sole (n=90)  
AML-NOS (n=233)

p=0.869

Months

0 12 24 36 48
Figure 3:

a) [Graph showing survival curves for MDS-related cytogenetics (n=55) vs. no MDS-related cytogenetics (n=353).]

p=0.007

b) [Graph showing survival curves for no MDS-related cytogenetics (n=353) vs. MDS-related cytogenetics (n=55).]

p=0.008

Figure 4:

a) [Graph showing survival curves for AML-MLD-sole (n=90) vs. AML-MRC due to cytogenetics/MDS history (n=85).]

p=0.019

b) [Graph showing survival curves for AML-MLD-sole (n=90) vs. AML-MRC due to cytogenetics/MDS history (n=85).]

p=0.009
Figure 5:

![Graph a) AML-NOS (n=233) and AML-MRC due to cytogenetics/MDS history (n=85)](image1)

p=0.012

![Graph b) AML-NOS (n=233) and AML-MRC due to cytogenetics/MDS history (n=85)](image2)

p=0.002

Figure 6:

![Graph a) AML-NOS + AML-MLD-sole (n=323) and AML-MRC due to cytogenetics/MDS history (n=85)](image3)

p=0.005

![Graph b) AML-NOS + AML-MLD-sole (n=323) and AML-MRC due to cytogenetics/MDS history (n=85)](image4)

p=0.001
Figure 7:

a) b)
Multilineage dysplasia (MLD) in acute myeloid leukemia (AML) correlates with MDS-related cytogenetic abnormalities and a prior history of MDS or MDS/MPN but has no independent prognostic relevance: a comparison of 408 cases classified as "AML not otherwise specified" (AML-NOS) or "AML with myelodysplasia-related changes" (AML-MRC)

Miriam Miesner, Claudia Haferlach, Ulrike Bacher, Tamara Weiss, Katja Macijewski, Alexander Kohlmann, Hans-Ulrich Klein, Martin Dugas, Wolfgang Kern, Susanne Schnittger and Torsten Haferlach