Response assessment in acute myeloid leukemia by flow cytometry supersedes cytomorphology at time of aplasia, amends cases without molecular residual disease marker and serves as an independent prognostic marker at time of aplasia and post-induction

In acute myeloid leukemia (AML), early *in vivo* assessment of treatment efficacy is highly relevant for further treatment stratification. In this study, we demonstrate that early detection of residual disease by flow cytometry during aplasia is an independent prognostic factor for event-free and overall survival, has improved sensitivity compared to morphological blast clearance, and delivers prognostic insight into patients missing a molecular measurable residual disease (MRD) marker.

Despite improvements in the use of pre-treatment genetic risk factors in AML, prediction of resistance and relapse probability is limited.[1] While most patients achieve a complete remission (CR) defined by cytomorphological assessment, we and others have previously shown that disease assessment below the level of cytomorphology can reflect disease activity more accurately.[2,3] This has prompted the European LeukemiaNet (ELN) to note that detection of MRD by flow cytometry provides a better assessment of remission status than cytomorphology.[4] Remission status is routinely assessed at time of post-induction; however, study groups and the current National Comprehensive Cancer Network guidelines have incorporated even earlier evaluation of treatment response by morphology (most commonly defined as day 14-21 after start of induction therapy) into treatment protocols.[5-7]

However, the relevance of response assessment by flow cytometry at this time point requires further exploration, since previous reports have given conflicting results.[3,5] We have demonstrated significant correlation of persistent disease with survival using a 3-color panel.[3] Even more recently, Chen *et al*. analyzed flow cytometric response in a cohort of 136 patients receiving intensive induction therapy.[5] While the correlation of flow status during aplasia with outcome was not investigated in their study, Chen *et al*. proposed limitations in the ability to predict CR using flow during aplasia.[5]

In contrast, although molecular MRD has not been proven to be of benefit at this early time point, post induction molecular MRD positivity has been proven to be highly predictive of relapse. However, routine molecular MRD assessment is only possible for a subset of patients (mainly those with mutated *NPM1* as well as those with *RUNX1-RUNX1T1* or *CBFB-MYH11* fusions), while response assessment by flow cytometry is possible in >95% of patients with AML. Although there have been encouraging reports of the additive value of molecular and flow MRD assessment,[6] the integration and potential additive value of response assessment by flow cytometry and molecular MRD is still not fully utilized.

Our study aims to address these two issues. Firstly, we compared response assessment by flow cytometry during aplasia to morphological assessment of blast clearance and evaluated the prognostic significance of flow status during aplasia and post-induction. Secondly, we elucidated whether flow status can complement molecular MRD assessment in our cohort.

Data from 166 consecutive patients receiving intensive induction chemotherapy for newly diagnosed AML

Table 1. Multivariate analysis for flow measurable residual disease status during aplasia and post Induction.

	HR (95% CI, *P*)	
	EFS	OS
Flow MRD during aplasia available (n=145)		
2017 ELN risk stratification by genetics	*P*<0.001	*P*=0.04
Favorable*	N/A	N/A
Intermediate	0.9 (0.6-1.5, *P*=0.9)	0.7 (0.3-1.9, *P*=0.5)
Adverse	2.8 (1.5-4.9, *P*=0.001)	1.7 (0.7-4.1, *P*=0.2)
Age (<60* *vs.* ≥60 years)	2.7 (1.6-4.3, *P*<0.001)	2.1 (1.0-4.2, *P*=0.04)
Flow status during aplasia (neg.* *vs.* pos.)	2.1 (1.3-3.4, *P*=0.003)	2.5 (1.2-5.3, *P*=0.02)
Flow MRD post Induction available (n=121)		
2017 ELN risk stratification by genetics	*P*=0.8	*P*=0.8
Favorable*	N/A	N/A
Intermediate	1.2 (0.6-2.3, *P*=0.7)	0.7 (0.2-2.0, *P*=0.7)
Adverse	1.3 (0.6-2.7, *P*=0.5)	0.8 (0.3-2.6, *P*=0.8)
Age (<60* *vs.* ≥60 years)	2.6 (1.4-4.6, *P*=0.002)	3.0 (1.2-7.5, *P*=0.02)
MRD status post induction (neg.**vs.* pos.)	2.3 (1.3-4.2, *P*=0.006)	2.0 (0.8-5.1, *P*=0.2)
Flow MRD during aplasia and post induction available (n=108)		
2017 ELN risk stratification by genetics	*P*=0.7	*P*=0.7
Favorable*	N/A	N/A
Intermediate	1.1 (0.5-2.2, *P*=0.8)	0.6 (0.2-2.0, *P*=0.4)
Adverse	1.4 (0.6-3.4, *P*=0.4)	0.8 (0.2-3.0, *P*=0.7)
Age (<60* *vs.* ≥60 years)	3.0 (1.5-5.7, *P*=0.001)	2.5 (0.9-7.2, *P*=0.08)
Combined MRD status (neg.* *vs.* pos.)	1.8 (1.2-2.7, *P*=0.006)	1.8 (0.9-3.6, *P*=0.07)

CI: Confidence Interval; ELN: European LeukemiaNet; EFS: event-free survival; HR: Hazard Ratio; MRD: minimal measurable disease; n: number; neg.: negative; OS: overall survival; N/A: not available; pos.: positive. *Reference category.

(excluding acute promyelocytic leukemia) were analyzed for this retrospective study. Bone marrow samples were analyzed using a comprehensive antigen panel at diagnosis and during follow up. Patient selection, sample processing, quality control, and data availability are summarized in the *Online Supplementary Appendix*.

In 161 cases (97%), a leukemia-associated immunophenotype (LAIP) could be defined and flow assessment was, therefore, feasible. All patients received chemotherapy with either standard cytarabine/anthracycline combination ('7+3', n=35) or sequential high-dose cytarabine and mitoxantrone[7] (n=126) (*Online Supplementary Table S2*). Bone marrow assessment during aplasia was performed 7-9 days after completion of induction therapy (n=145 cases) and at time of post induction (n=121 cases) (*Online Supplementary Table S2*). A total of 108 patients (all achieving CR or CRi after induction therapy) had flow assessments available at both time points.

Gender, type of AML (*de novo vs.* secondary AML *vs.* therapy-related AML) or type of induction therapy ('7+3' *vs.* sequential high-dose cytarabine and mitoxantrone followed by pegfilgrastim) were not associated with flow status during aplasia. However, persistent disease by flow during aplasia was associated with increased age (56.9% *vs.* 35%; *P*=0.008) and refractory disease (36.9% *vs.* 6.3%; *P*<0.001) and at time of post induction (n=121 cases). As expected, patients with positive flow status during aplasia were more likely to have adverse risk according to Medical Research Council risk assessment (25% *vs.* 10%; *P*=0.024) and at time of post induction (n=121 cases) as well as by ELN risk criteria (38.5% *vs.*18.8%; *P*=0.001) and at time of post induction (n=121 cases) (*Online Supplementary Table S2*).

Next, we analyzed the influence of flow status during aplasia on outcome. Flow positivity during aplasia was associated with significantly shorter EFS (median EFS 6.0 *vs.* 19.1 months; *P*<0.001) (Figure 1A) and OS (median OS 21.2 months *vs.* median not reached; *P*=0.01) (*Online Supplementary Figure S2*). Median RFS was 13.8 months for flow positive *versus* 17.3 months for flow negative patients; however, this difference was not statistically significant (*P*=0.213) (*Online Supplementary Figure S2*).

Response assessment by flow cytometry during aplasia was able to identify two prognostically distinct groups among patients with morphological blast clearance[8] (median EFS 8.6 *vs.* 24.1 months; *P*=0.004) (Figure 1B), highlighting the increased sensitivity of residual disease detection by flow cytometry during aplasia.

In multivariate analysis, flow positivity during aplasia

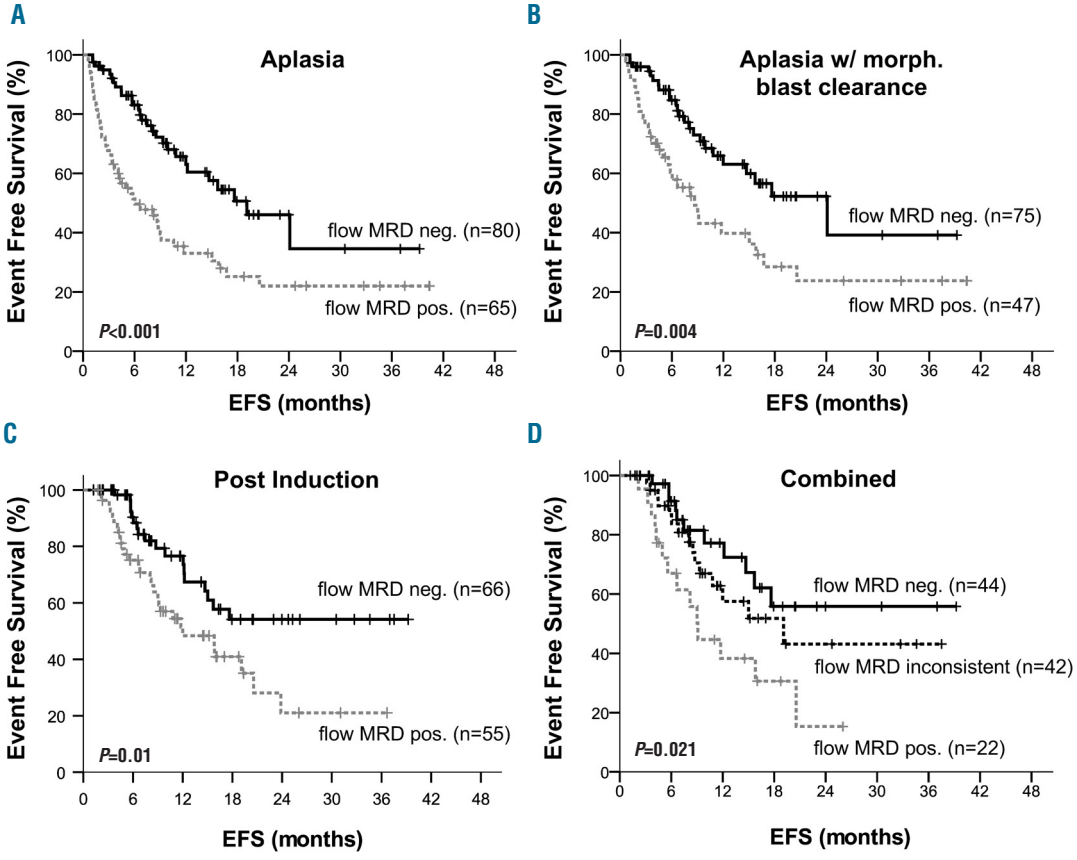

Figure 1. Survival stratified by flow measurable residual disease (flow MRD). (A) Event-free survival (EFS) stratified by flow status during aplasia: 65 patients were flow MRD positive (pos.) and 80 patients were flow MRD negative (neg.). Median EFS was 6.0 months for flow MRD pos and 19.1 months for flow MRD neg patients (*P*<0.001). (B) EFS stratified by flow status during aplasia for patients with morphological blast clearance: 47 patients were flow MRD pos and 75 patients were flow MRD neg. Median EFS was 8.6 months for flow MRD pos and 24.1 months for flow MRD neg patients (*P*=0.004). (C) EFS stratified by flow MRD post-induction: 55 patients were flow MRD pos and 66 patients were flow MRD neg. Median EFS was 12.0 months for flow MRD pos and not reached for flow MRD neg patients (*P*=0.01). (D) EFS stratified by combined MRD status: 22 patients were consistently flow MRD pos, 44 patients were flow MRD neg, and 42 patients had inconsistent flow MRD measurements. Median EFS was 9.1 months for flow MRD pos, not reached for flow MRD neg, and 19.1 months for flow MRD inconsistent patients (*P*=0.021). n: number.

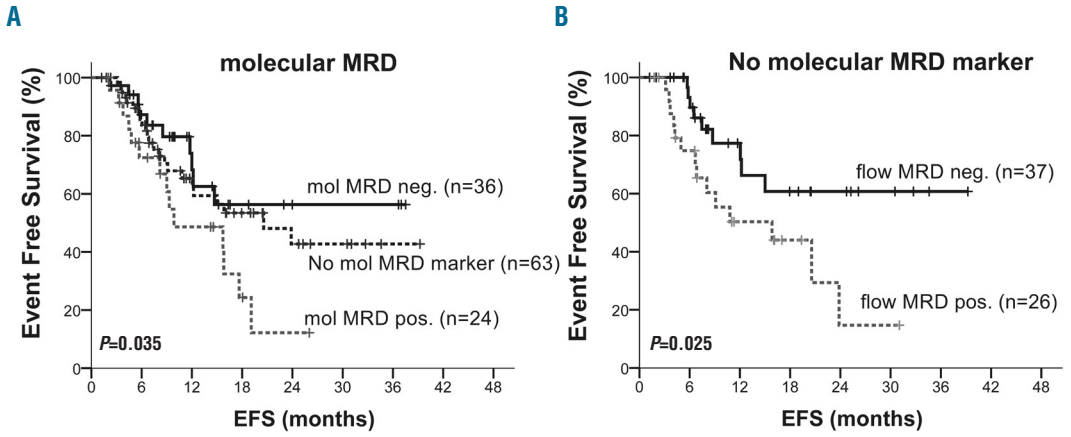

Figure 2. Survival stratified by molecular measurable residual disease (mol MRD). (A) Event-free survival (EFS) stratified by mol MRD: 24 patients were molecular MRD positive (pos.), 36 patients were mol MRD negative (neg.), and 63 cases lacked a suitable mol MRD marker. Median EFS was 9.9 months for mol MRD pos *versus* median not reached for mol MRD neg patients (P=0.035 for mol MRD pos. *vs.* mol MRD neg.). For patients who lacked a suitable molecular marker post-induction, median EFS was 20.6 months. (B) EFS stratified by flow MRD status for patients without mol MRD marker: 26 patients were flow MRD pos *versus* 37 patients were flow MRD neg. Median EFS was 15.8 months for flow MRD pos and not reached for flow MRD neg patients (P=0.025). n: number.

was an independent risk factor, along with age and ELN risk classification, and was associated with significantly shorter EFS (HR 2.1; P=0.003) (Table 1) and OS (HR 2.5; P=0.02) (Table 1). Our data confirm that flow status during aplasia has higher prognostic impact than morphological assessment in adult AML, thus questioning the role of morphology during aplasia. Finally, flow status during aplasia retained prognostic significance even if patients who died during the first three months after diagnosis were excluded, highlighting the relevance independently from early treatment complications during induction (*Online Supplementary Results*).

We then analyzed the detection of MRD by flow cytometry after induction therapy, in patients achieving morphological CR or CR with incomplete hematologic recovery (CRi) (n=121). Flow MRD positivity post induction was associated with significantly shorter EFS (12.0 *vs.* median not reached; P=0.01) (Figure 1C). This was also true for relapse-free survival (RFS), which was significantly shorter for flow MRD positive patients (median RFS 10.3 *vs.* median not reached; P=0.007) (*Online Supplementary Figure S2*). Flow MRD positivity after induction therapy was associated with a higher rate of CRi (28.6% *vs.* 12.1%; P=0.02) (*Online Supplementary Table S2*). In multivariate analysis, flow MRD post induction also showed independent prognostic value for EFS (HR 2.3; P=0.006) (Table 1) but not OS (HR 2.0; P=0.2) (Table 1), which might be due to limited follow up in our cohort (median 13.7 months).

While detection of persistent disease by flow cytometry at either aplasia and post-induction are associated with poor survival,[3,9,10] implementation into treatment decisions warrants a high degree of diagnostic certainty. A reasonable approach to achieve this is to mandate confirmation of flow positive status. Thus, we adapted a combined approach for this analysis. Persistent flow positivity at both time points identified patients with particularly short EFS (median EFS 9.1 months) (Figure 1D), whereas patients with flow negativity at both time points had a significantly better outcome in our cohort (median not reached; P=0.021). Combined flow status was an independent predictor of EFS (HR 1.8; P=0.006) (Table 1).

Finally, we analyzed whether response assessment by flow cytometry could complement molecular assessment of persistent disease. We identified those patients in our cohort with an eligible molecular marker for routine clinical assessment (n=61) (*Online Supplementary Methods* for further details). These included *NPM1* mutations (n=44), CBFβ-MYH11 (n=10), and *RUNX1-RUNX1T1* fusions (n=6). Post-induction, 24 patients were molecular MRD positive and 36 patients were molecular MRD negative. Molecular MRD positivity was associated with significantly shorter EFS (9.9 *vs.* median not reached; P=0.035) (*Online Supplementary Figure S2A*).

For all cases without an available molecular MRD marker (97 of 161 patients), flow positivity during aplasia (P=0.004; available in 89 cases) (*Online Supplementary Figure S3A*), post induction (P=0.025; available in 63 cases) (Figure 2B), or as combined status (P=0.048, available in 54 cases) (*Online Supplementary Figure S3B*) was associated with a significantly shorter EFS and flow positivity remained an independent risk factor in multivariate analysis (HR 2.5; P=0.016) (*Online Supplementary Table S3*).

Taken together, our analysis demonstrates that flow positivity during aplasia is an independent prognostic factor identifying patients with shorter EFS and OS, and showed improved sensitivity for detection of AML compared to morphological blast clearance. Patients with positive flow status during aplasia were more likely to have refractory disease, and those patients who did achieve a remission despite flow positivity at time of aplasia were significantly more likely to achieve CRi rather than a full CR. While most MRD assays are being implemented in clinical situations of complete (morphological) remission, our data support the use of flow MRD assays during aplasia.

Furthermore, combining flow assessment during aplasia with flow assessment post-induction identified patients who displayed particularly short EFS. Finally, we demonstrated that response assessment by flow cytometry delivers prognostic insight into patients missing a molecular MRD marker by showing robust prognostic separation in this subgroup.

Going forward, our study identifies early flow positivity as an independent prognostic marker for relapse which might contribute to individual treatment stratification and outcome. While these findings need to be evaluated prospectively, potential implications might include: (i) early initiation of stem cell donor search; (ii) participation within clinical trials; and (iii) vigilant monitoring schemes. The combination of two time points of flow assessment, at time of aplasia and at time of post-induction, allows relapse to be predicted with an even higher certainty. Finally, we were able to demonstrate that flow status correlates with survival in patients lacking an established molecular MRD marker. Prospective studies need to validate our findings and evaluate the relevance of flow-based treatment algorithms on outcome. Future trials will show whether application of response assessment by flow cytometry into therapeutics will translate into improved outcomes, and whether flow status can serve as a surrogate end point in AML.

Thomas Köhnke,[1,2] Veit Bücklein,[1,2] Sandra Rechkemmer,[1] Stephanie Schneider,[1,3] Maja Rothenberg-Thurley,[1,4,5] Klaus H. Metzeler,[1,4,5] Maria-Cristina Sauerland,[6] Wolfgang Hiddemann,[1,4,5] Karsten Spiekermann[1,4,5] and Marion Subklewe[1,2,4,5]

[1]Laboratory for Leukemia Diagnostics, Department of Medicine III, University Hospital, LMU Munich, Munich; [2]Translational Cancer Immunology, Gene Center, LMU Munich, Munich; [3]Institute of Human Genetics, LMU Munich, Munich; [4]German Cancer Consortium (DKTK), Heidelberg; [5]German Cancer Research Center (DKFZ), Heidelberg and [6]Institute of Biostatistics and Clinical Research, University of Münster, Münster, Germany

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Correspondence: MARION SUBKLEWE
marion.subklewe@med.uni-muenchen.de
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