Clinical relevance of an objective flow cytometry approach based on limit of detection and limit of quantification for measurable residual disease assessment in acute myeloid leukemia. A post-hoc analysis of the GIMEMA AML1310 trial

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Supplemental methods

AML1310 study design and MRD assessment.

Patients with newly-diagnose AML were eligible for the GIMEMA AML1310 trial provided they met the following criteria for eligibility: age 18 to 60.9 years; (ii) AML other than M3; (iii) WHO performance status 0-3; (iv) adequate liver (serum bilirubin level ≤ 2 UNL; AST and ALT ≤ 3 UNL) and renal (serum creatinine ≤ 2 UNL) functions; (v) LVEF ≥ 50% by echocardiogram; (vi) absence of severe concomitant neurological or psychiatric diseases and congestive heart failure or active uncontrolled infections; (vii) signed informed consent. Patients with therapy-related AML were not considered eligible. Exclusion criteria included blast crisis of chronic myeloid leukemia, AML supervening after other chronic myeloproliferative diseases or antecedent myelodysplastic syndromes of more than six months duration and other progressive malignant diseases.

Study procedures included upfront evaluation included bone marrow (BM) aspirate for morphology, cytogenetics, molecular genetics and MFC analysis. The baseline MFC assessment was a necessary step, not only for diagnostic purposes, but also to identify leukemia associated immunophenotypes (LAIP). Identification of baseline LAIPs was the essential requirement for monitoring MRD after therapy. Patients were studied at diagnosis for the presence of RUNX1-RUNX1T1 or CBFβ/MYH11 rearrangements, defining core binding factor (CBF) leukemias, and for NPM1, FLT3 and c-KIT mutations. Molecular analysis, LAIPs assessment and post-consolidation MRD determinations were centralized at Laboratorio di Diagnostica Integrata Oncoematologica “OPPO”, at Tor Vergata University Hospital of Rome, whereas conventional karyotype was carried out at local institutions. Response to treatment was assessed on BM and peripheral blood, according to the recommendations of an international working group. Patients who did not achieve complete remission (CR), CR incomplete (CRi) or partial remission (PR) after the first induction course or CR/CRi after two induction courses were considered as treatment failures. At the established time-point, BM MRD was determined by a high-sensitivity 8–color MFC assay. BM samples were processed following the Lyse-wash-stain-wash procedure. This includes bulk lysis of red blood cells followed by washing with PBS, resuspension of the pellet in a smaller volume allowing for increased cell concentration and staining of the cells with MoAb cocktail. The bulk lysis leads to a higher reproducibility since the labelling conditions are reproducible and the volume is constant for a given quantity of cells. Lysis was performed by Ammonium chloride (NH4Cl) because of its minimal effects on cell physical properties. The threshold for discriminating MRD negative from MRD positive cases was set at 3.5x10-4 (0.035%) RLC and the selected time-point was the post-consolidation phase,

once the hematologic recovery was complete. MRD assessment was carried on the total number of mononuclear cells (MNC). The AML1310 trial was designed at a time when ELN 2010/2017 and NCCN 2018 recommendations were not yet published. Therefore, when the trial regulatory path was concluded, we started recruiting and stratifying patients according to contemporary classification, that was the NCCN 2009 version 1.27 For the purpose of our AML1310 study, 4 categories of risk were identified: favorable- (NCCN-FR) or poor-risk (NCCN-PR) patients, who were submitted to AuSCT or ASCT, respectively; intermediate-MRD negative (NCCN-IR-Neg) or positive (NCCN-IR-Pos) patients, who were to receive AuSCT or ASCT, respectively. Moreover, we enucleated a fifth group of patients belonging to the intermediate-risk category, in whom we failed to identify any LAIP (NCCN-IR-no-LAIP category); these patients were allocated to the AuSCT post-consolidation option. ASCT and AuSCT were to be performed within three months of the end of the consolidation course.

Table 1S. Total number of events acquired according to LOD/LOQ coding and details on MNC and CD45 expressing cells denominators

	Level	Overall
Number. of MNC acquired (median [range])		559'197 [100'450-1'561'221]
Number of CD45 expressing cells acquired (median [range])		538'527 [88'040-1'548'172]
Number of positive events (median [range])		89 [0-38'061]
Percentage of positive events (median [range])		0.0161 [0.0000-9.0152]
LOD on CD45 expressing cells (median [range])		0.0037 [0.0013-0.0227]
LOQ on CD45 expressing cells (median [range])		0.0093 [0.0032-0.0568]
Positivity according to LOD LOQ on CD45 expressing cells (%)	LOD^{neg}	74 (28.4)
	$LOD^{pos}LOQ^{neg}$	43 (16.5)
	LOQ^{pos}	144 (55.1)

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; MNC, mononuclear cells.

Table 2S. Interactions between the "relative" 0.035% and "absolute" LOD/LOQ MRD estimates

Level	MRD Negative*	MRD Positive**	P
CD45-Positive LOD^{neg}	74 (48.1%)	0 (0.0%)	<0.001
CD45-Positive $LOD^{pos}LOQ^{neg}$	41 (26.6%)	2 (1.9%)	
CD45-Positive LOQ^{pos}	39 (25.3%)	105 (98.1%)	

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; MRD, measurable residual disease.
*Residual leukemic cells <0.035%
**Residual leukemic cells >0.035%

Figure 1S. Overall Survival analysis of MRD<0.035% and MRD≥0.035% patients according to the LOD-LOQ status in the NCCN intermediate risk group. MRD<0.035%LOD^{neg}/LOD^{pos}LOQ^{neg} patients had a longer duration of OS as compared to MRD<0.035%LOQ^{pos} and MRD≥0.035%LOQ^{pos} (p=0.0146).

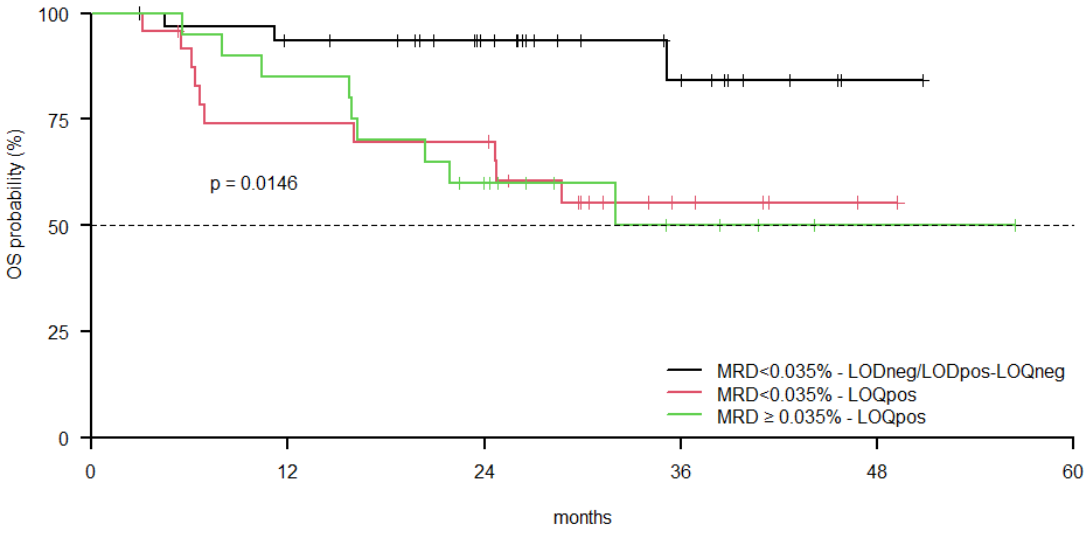

Figure 2S. Interaction of the 3 LOD-LOQ categories with post-remissional treatment (AuSCT, ASCT and no graft). LOD^{neg}/LOD^{pos}-LOQ^{neg} patients submitted to AuSCT had the best 2-years OS (88.9%) as compared to all the other possible combinations of LOD-LOQ and treatment (p=0.026).

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