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Rapid and accurate identification of acute promyelocytic leukemia with a novel multiparametric flow cytometric scoring system

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Dear Editors,

Acute promyelocytic leukemia (APL) is usually characterized by the distinctive balanced chromosomal translocation t(15;17)(q22;q21), generating the pathogenic PML::RARA oncoprotein [1]. All-trans retinoic acid (ATRA)-based combinations therapies represent the cornerstone of APL treatment, making APL the acute myeloid leukemia (AML) subtype with the highest cure rate [2]. Nevertheless, APL remains a hematological emergency due to its life-threatening coagulopathy and high rate of early death, with nearly 35% of APL patients dying before the first ATRA administration [3]. Therefore, rapid and accurate diagnosis is essential, ideally within 24 hours of clinical suspicion. Morphological examination of peripheral blood and/or bone marrow smears represents the initial step to raise suspicion of APL. In fact, current guidelines strongly recommend initiating ATRA therapy immediately upon morphological APL suspicion to significantly reduces the risk of early death. Additionally, the use of anti-PML immunofluorescence assays has been proposed as a rapid and reliable alternative for the APL diagnosis. However, nowadays, in almost every center definitive diagnosis relies on detection of the PML::RARA fusion transcript by reverse transcriptase–polymerase chain reaction (RT-PCR). Nevertheless, molecular and/or cytogenetic assays may take up 3 to 7 days, be inconclusive (especially in variant/cryptic rearrangements), or not available in all centers. Thus, multiparametric flow cytometry (MFC) remains an essential, routinely used tool for a rapid and accurate diagnostic workup of acute leukemias. Albeit many publications have reported a typical MFC-profile for APL leukemic cells [4], not all cases consistently conform to this typical pattern [5], prompting efforts to identify MFC marker combinations that maximize APL diagnostic accuracy. The most enduring and influential scoring system to predict the PML::RARA rearrangement remains Orfao et al.'s 1999 model [6].

In this retrospective study, we evaluated MFC data of 158 consecutive newly diagnosed APL diagnosed at the Hematology department of "Sapienza"-University of Rome over a 27-year period (1997-2024), using an extensive flow cytometry panel. BM cells were stained using a combination of mAbs recommended by the EuroFlow Consortium and by ELN [7-8]:

MPO/cCD79a/cCD3/CD45/CD34/CD117/HLADR/CD13/CD33/CD11b/CD15/CD2/CD99/CD9/CD14/CD4/CD56/CD123/CD65/CD133/CD16/CD19/CD38/CD5/CD7 (Società Italiana Chimici, SIC, Life Sciences, Rome, Italy; Becton Dickinson, San Jose, CA; Beckman Coulter, Brea, CA) (Figure 1A-1B). A positivity cutoff for CD34 of 10% was used [9]. Written informed consent was obtained according to local practice. All patients provided consent to treatment and to the use of their anonymized clinical data for scientific purposes. The study was conducted in accordance with the principles of the Declaration of Helsinki and complied with Italian ethical regulations. As a retrospective analysis using routinely collected clinical data, and in accordance with local regulations, formal approval from an ethics committee was not required.

The mean age at diagnosis was 53 years (SD, ±18 years) and 77 patients (49%) were male. A total of 38 patients (24%) were considered as morphological microgranular APL variants. Overall median expression percentages and positivity rate are displayed in Table S1. CD9, CD99, CD13, CD33, CD38 and MPO were constantly detected, with a high median expression percentages and mean fluorescence intensities (MFIs). CD117 resulted positive in 98% of APL samples. CD33 was expressed with a homogenous pattern in all 158 APL cases (100%), with a higher MFI compared to CD13, while CD13 had an heterogenous pattern of expression in 155 (98%). Conversely, CD11b, CD14 and CD65 were uniformly negative in all cases of APL, with HLA-DR and CD15 positive only in seven (4%) and four (3%) cases, respectively. CD34 resulted positive in 48 cases (30%) and positivity for CD2 was observed in 42 samples (27%), with most $CD34^+$ cases that were $CD2^+$ as well. CD9 and CD99 showed perfect flow cytometric correlation (ρ=1.0), as well as CD13 with CD33 (ρ=1.0), suggesting co-expression patterns among specific antigen pairs. Notably, moderately strong correlation was also observed between CD34 and CD2 (ρ=0.79), indicating their coordinated expression in a subset of cases. In fact, their contemporary positivity ($CD34^+/CD2^+$) was evidenced in 29 out of 38 morphologically variant APL cases (76%) (data not shown).

Considering CD99 as a novel and promising marker for acute leukemias work-up [10], we selected 61 APL cases with available CD99 measurements and compared them to 97 consecutive newly diagnosed non-APL AML cases analyzed with comparable MFC panels over the 2014–2024 period (Table 1). The molecular and

cytogenetic profile of the non-APL AML cohort included 21 patients with isolated FLT3-ITD mutations (21.6%), 4 with concurrent FLT3-ITD and NPM1 mutations (4.1%), 4 with solely NPM1 mutations (4.1%), 12 cases (12.4%) with complex karyotypes, 4 with inv(16)/t(16;16) (4.1%), and 2 (2.1%) patients with KMT2A alterations, one KMT2A rearrangement [t(9;11)(p21;q23)] (1.0%) and one KMT2A-PTD (1.0%), respectively. Among the remaining cases, most exhibited a normal karyotype, while 8 cases (8.3%) showed various recurrent chromosomal abnormalities. Various antigens expressions dissimilarities emerged between APL and non-APL cohorts (Table 1). Therefore, several differences in MFC antigen positivity between APL and non-APL cases emerged from the univariate analysis (Table S2). To enhance discriminatory power, we evaluated co-expression patterns of some MFC antigens. Multivariate analysis (Table 2) identified that the three significant antigen pairs to discriminate between APL and non-APL diagnosis were: simultaneous CD9/CD99 positivity [OR 58.71 (95% CI, 5.82-8038.15)] ($p<0.001$), simultaneous CD34/CD2 positivity [OR 16.08 (95% CI, 1.47-389.78)] ($p=0.022$), and simultaneous HLA-DR/CD11b negativity [OR 38.78 (95% CI, 7.57-401.44)] ($p<0.001$). Thus, a weighted MFC diagnostic system score was developed, assigning differential point values to each of these three variables based on their OR. This score was named APLY (Acute Promyelocytic Leukemia flow cytometric identitY) and attributed two points to CD9/CD99 positivity and to HLA-DR/CD11b negativity and one point to CD34/CD2 positivity (Figure 1C). Based on the ROC curve analysis, a cutoff of 3 was selected to discriminate between APL and other AML subtypes. All 61 APL cases (100%) had a score ≥ 3, while only 11 out of 97 non-APL cases (11.3%) reached this threshold ($p<0.001$) (Table S3). These 11 non-APL cases, wrongly classified as APL by APLY score, exhibited heterogeneous molecular and cytogenetic profiles without a shared genetic signature, indicating that misclassification likely resulted from overlapping immunophenotypic features. Therefore, the APLY score achieved a sensitivity of 100.0% and a specificity of 88.4% for predicting an APL diagnosis. Its positive predictive value (PPV) was 84.7% and the negative predictive value (NPV) was 100%, with a consequent optimal F1 score of 91.8%. Subsequently, we applied on our population the historical and world-wide acknowledged Orfao et al.'s score with a score = 3 suggesting an APL diagnosis. Thirty-eight of 61 APL cases (62%) and 9 of 97 non-

APL cases (9%) reached this cutoff, yielding a sensitivity of 62.3% and a specificity of 90.5% (Table S3). In our cohort, the comparison with this pivotal model showed that sensitivity was higher for the APLY score (100.0% vs 62.3%) ($p<0.001$), with no meaningful difference in specificity (88.4%; vs 90.5%) ($p=0.64$). Similarly, the F1 score was significantly superior (0.92 vs 0.70) ($p<0.001$), highlighting the improved overall classification performance. Moreover, C-Index was higher for the APLY score [0.967 (95% CI, 0.925–0.998) vs 0.816 (95% CI, 0.685–0.947)], and the DeLong test demonstrated a highly significant difference between the AUCs ($Z=-4.412$, $p<0.001$) (Figure 1D). The Net Reclassification Improvement (NRI) was 0.356, indicating that 35.6% of patients were more accurately classified by the APLY score, with 23 APL cases correctly reclassified (37.7%) and only 2 non-APL cases incorrectly reclassified as APL (2.1%) compared to the Orfao et al.'s score.

Delay in timely treatment is the main factor affecting the APL survival rate. Albeit molecular testing remains the gold-standard for APL diagnosis, rapid turnaround MFC data may support the prompt initiation of life-saving therapy. In this study, we demonstrated that APL cases could be rapidly and accurately identified by MFC using routine screening panels and we proposed a novel MFC scoring system (APLY score) for APL diagnosis. Our study emphasizes that CD9 and CD99 are always strongly expressed by all APL leukemic cells. While previous studies have revealed that nearly all APL samples (95-100%) exhibit homogeneous and moderate/bright expression of CD9 [11-12], to our best knowledge, our report represents the first study showing that CD99 and CD9 shares a uniform expression pattern in APL cases. In our APL cohort, CD99 was uniformly expressed on leukemic blasts and showed perfect concordance with CD9 ($\rho=1.0$). Consequently, we assessed CD9 and CD99 as a dual-antigen MFC signature ($CD9^+/CD99^+$) to improve diagnostic specificity. CD11b negativity in myeloid cells is a well-documented flow cytometric hallmark of APL [12]. Likewise, APL blasts characteristically often lack HLA-DR expression [13]. However, because isolated CD11b negativity or HLA-DR absence are not entirely specific for APL diagnosis, in our study we evaluated their simultaneous negativity to enhance APL diagnostic confidence. We found that this dual-negative ($HLA\text{-}DR^-/CD11b^-$) signature in 98.3% of APL cases compared to just 15.5% of non-APL

cases, closely mirroring Dong et al.'s report of 96% and 13%, respectively [13]. Although CD34 have been historically considered negative in APL cases over time studies have demonstrated that up to 20–40 % of APL cases may exhibit CD34 [5]. Moreover, CD34 expression in APL has been almost invariably associated with concurrent aberrant expression of CD2. This dual positivity ($CD34^+/CD2^+$) is more commonly observed in the microgranular APL variant. In our cohort, 28% of APL cases exhibited simultaneous $CD34^+/CD2^+$ expression, with a markedly higher frequency observed among cases with a variant morphology (76%). Similarly, Albano et al. reported that among morphologic variants, approximately 50–60% displayed a $CD34^+/CD2^+$ expression pattern [14]. Thus, the evaluation of $CD34^+/CD2^+$ expression and its inclusion into the APLY score enhances the identification of APL variant cases, since this positivity combination is anecdotal in non-APL AMLs. Therefore, in cases showing atypical or borderline immunophenotypic features, APLY score seems to substantially reduce diagnostic uncertainty, along with historical parameters such as high SSC, and the homogeneous versus heterogeneous expression patterns of CD33 and CD13, respectively.

Therefore, our results show that the combined use of three flow cytometric dual-antigen evaluations ($CD9^+/CD99^+$, $CD34^+/CD2^+$ and HLA-DR-/CD11b-) into a diagnostic MFC score (APLY score) yield excellent diagnostic performance, with an optimal sensitivity (100.0%) and specificity (88.4%) for discriminating those newly diagnosed AML cases in which molecular investigation to detect PML::RARA fusion rearrangements should promptly be performed. Remarkably, the APLY score (a straightforward, easy-to-apply scoring system), employing simple markers that should be accessible in any flow cytometry laboratory, correctly identified all APL cases in our cohort, underscoring its reliability as a front-line screening tool to facilitate the prompt ATRA administration as per clinical guidelines [15]. In particular, in peripheral centers or developing countries, where rapid molecular diagnosis of APL may be challenging, the APLY score could be a valuable robust screening tool considering that the required mAbs are widely accessible and suitable for evaluation with whichever type of cytometer available to date, supporting the reproducibility of this approach across different laboratory settings. Moreover, the comparison with the

historical Orfao et al.'s score [6] enabled to demonstrate that the inclusion of new and simple markers (such as CD99, CD9, CD2 and CD11b) yields a more accurate predictability of PML::RARA rearrangement and may be more easily applicable in routine clinical settings, requiring less expertise in MFC analysis and interpretation. External validation studies may further confirm these findings to integrate the APLY score into routine AML diagnostic workflows.

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FIGURES and TABLEs

A	CD99% median (range)	CD99 MFI median (range)	CD9% median (range)	CD9 MFI median (range)	CD34% median (range)	CD2% median (range)	CD11b% median (range)	HLA-DR% median (range)	CD33% median (range)	CD33 MFI median (range)	CD13% median (range)	CD13 MFI median (range)	CD56% median (range)	CD38% median (range)	CD117% median (range)	CD133% median (range)	MPO% median (range)	CD4% median (range)	CD16% median (range)	CD15% median (range)
Total N = 158	100 (0-100)	63 (2-392)	100 (0-100)	80 (2-1254)	13 (0-100)	0 (0-100)	0 (0-100)	28 (0-100)	100 (0-100)	237 (5-2641)	100 (0-100)	285 (15-1800)	0 (0-100)	100 (63-100)	100 (1-100)	1 (0-100)	100 (0-100)	35 (0-100)	0 (0-19)	0 (0-100)
APLs N = 61	100 (97-100)	100 (42-392)	100 (100-100)	394 (51-1254)	1 (0-100)	0 (0-100)	0 (0-0)	0 (0-28)	100 (68-100)	380 (38-2641)	100 (69-100)	307 (42-113)	0 (0-77)	100 (100-100)	100 (26-100)	0 (0-100)	100 (100-100)	0 (0-100)	0 (0-0)	0 (0-10)
Non-APL AMLs N = 97	100 (0-100)	39 (2-392)	25 (0-100)	26 (2-380)	55 (0-100)	0 (0-100)	0 (0-100)	100 (0-100)	100 (0-100)	155 (5-750)	100 (0-100)	235 (15-1800)	0 (0-100)	100 (63-100)	100 (1-100)	100 (0-100)	48 (0-100)	84 (0-100)	0 (0-19)	0 (0-100)
P Value	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	*0.12*	***< 0.001****	*0.11*	*0.28*	***< 0.001****	*0.17*	*0.15*	***< 0.001****	***< 0.001****	***< 0.001****	*0.43*	***< 0.001****

B	CD99 n (%)	CD9 n (%)	C34 n (%)	CD2 n (%)	CD11b n (%)	HLA-DR n (%)	CD33 n (%)	CD13 n (%)	CD56 n (%)	CD38 n (%)	CD117 n (%)	CD133 n (%)	CD65 n (%)	CD14 n (%)	MPO n (%)	CD7 n (%)	CD4 n (%)	CD16 n (%)	CD15 n (%)
Total N = 158																			
neg	36 (23%)	45 (28%)	73 (46%)	135 (87%)	136 (88%)	77 (49%)	2 (1%)	4 (3%)	135 (85%)	0 (0%)	9 (6%)	81 (64%)	132 (96%)	131 (85%)	19 (12%)	131 (83%)	71 (45%)	155 (100%)	132 (85%)
pos	122 (77%)	113 (72%)	85 (54%)	21 (13%)	19 (12%)	81 (51%)	156 (99%)	154 (97%)	23 (15%)	157 (100%)	149 (94%)	45 (36%)	5 (4%)	23 (15%)	139 (88%)	27 (17%)	86 (55%)	0 (0%)	23 (15%)
Unknown	0	0	0	2	3	0	0	0	0	1	0	32	21	4	0	0	1	3	3
APLs N = 61																			
neg	0 (0%)	0 (0%)	40 (66%)	43 (70%)	61 (100%)	60 (98%)	0 (0%)	0 (0%)	57 (93%)	0 (0%)	0 (0%)	60 (98%)	59 (100%)	61 (100%)	0 (0%)	59 (97%)	48 (79%)	61 (100%)	61 (100%)
pos	61 (100%)	61 (100%)	21 (34%)	18 (30%)	0 (0%)	1 (2%)	61 (100%)	61 (100%)	4 (7%)	60 (100%)	61 (100%)	1 (2%)	0 (0%)	0 (0%)	61 (100%)	2 (3%)	13 (21%)	0 (0%)	0 (0%)
Unknown	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0
Non-APL AMLs N = 97																			
neg	36 (37%)	45 (46%)	33 (34%)	92 (97%)	75 (80%)	17 (18%)	2 (2%)	4 (4%)	78 (80%)	0 (0%)	9 (9%)	21 (32%)	73 (94%)	70 (75%)	19 (20%)	72 (74%)	23 (24%)	94 (100%)	71 (76%)
pos	61 (63%)	52 (54%)	64 (66%)	3 (3%)	19 (20%)	80 (82%)	95 (98%)	93 (96%)	19 (20%)	97 (100%)	88 (91%)	44 (68%)	5 (6%)	23 (25%)	78 (80%)	25 (26%)	73 (76%)	0 (0%)	23 (24%)
Unknown	0	0	0	2	3	0	0	0	0	0	0	32	19	4	0	0	1	3	3
P Value	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	*0.69*	*0.28*	***0.042****	*> 0.95*	***0.036****	***< 0.001****	*0.36*	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	*> 0.95*	***< 0.001****

*Statistically significant differences.

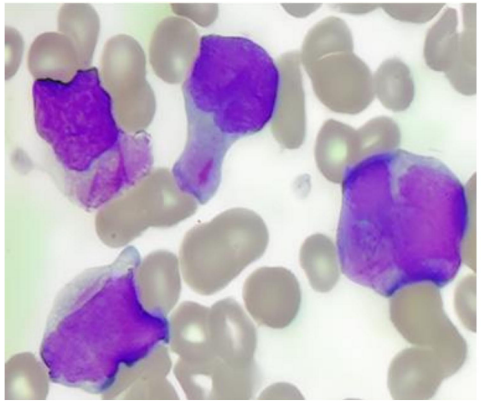
Table 1. Overall MFC antigen expression and mean fluorescence intensity (MFI) in all AML cases with available CD99 data. **A)** Median expression percentage and median MFI in all selected AML cases and comparison between APLs and Non-APL AMLs. **B)** Antigen positivity rates and comparison between APLs and Non-APL AMLs

Characteristics	Univariate Analysis				Multivariate Analysis	
	APL Cases (%) (N=61)	Non-APL AML Cases (%) (N=97)	OR (95% CI)	p Value	OR (95% CI)	p Value
Simultaneous CD34/CD2 Positivity	17/61 (27.9)	3/97 (3.1)	9.58 (3.18-38.06)	< 0.001	16.08 (1.47-389.78)	**0.022**
Simultaneous CD9/CD99 Positivity	61/61 (100.0)	34/97 (35.0)	226.39 (30.85-2888.01)	< 0.001	58.71 (5.82-8038.15)	**< 0.001**
Typical CD34/CD15 Pattern	42/61 (68.9)	21/97 (21.6)	7.75 (3.85-16.26)	< 0.001	4.44 (0.74-27.27)	0.10
Simultaneous HLA-DR/CD11b Negativity	60/61 (98.3)	15/97 (15.5)	214.68 (51.84-1993.64)	< 0.001	38.78 (7.57-401.44)	**< 0.001**
Simultaneous CD13-Heterogeneous and CD33-Homogeneous Pattern	58/61 (95.0)	64/97 (66.0)	8.68 (3.08-33.27)	< 0.001	3.56 (0.46-27.46)	0.21

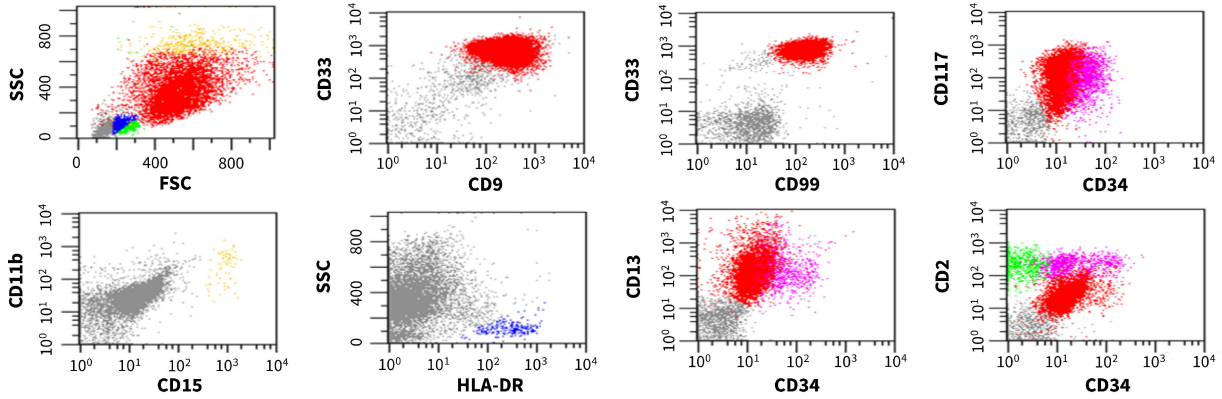
Table 2. Univariate and multivariate analyses of MFC antigen combinations for their significance in the differential diagnosis of APL. In multivariate analysis p values in bold are statistically significant

Figure 1. Morphologic, immunophenotypic characterization and Acute Promyelocytic Leukemia flow cytometric identitY (APLY) score of APL cases. **A)** Bone marrow smear morphology demonstrates abnormal promyelocytes with characteristic APL features, including abundant Auer rods and "Faggot" cells. **B)** Flow cytometry dot plots illustrate the immunophenotype of the abnormal APL population (in red). Partial CD34/CD2 co-expression on APL blast cells is displayed in fuchsia. Residual granulocytes are marked in yellow, residual T-lymphocytes in green, and residual B-lymphocytes in blue. **C)** APLY Score parameters and their assigned points used to identify APL cases based on immunophenotypic features. A score ≥3 strongly suggests APL diagnosis. **D)** Receiver operating characteristic (ROC) curve analysis of the APLY Score (Blue line) for the diagnosis of APL. The area under the curve (AUC) was 0.968 (95% CI, 0.925–0.998), pinpointing an excellent discriminatory ability of the APLY Score for the detection of PML::RARA fusion gene positivity. ROC analysis was used to determine the optimal cutoff value to discriminate PML::RARA-positive cases, balancing both sensitivity and specificity. ROC analysis was also performed foe the Orfao et al.'s Score, which showed a lower AUC of 0.816 (95% CI, 0.685–0.947). The DeLong test demonstrated a statistically significant difference between the two curves ($Z = -4.412$, $p<0.001$), confirming that the APLY Score provides additional and clinically relevant diagnostic value compared to the reference Orfao et al.'s Score.

A



B



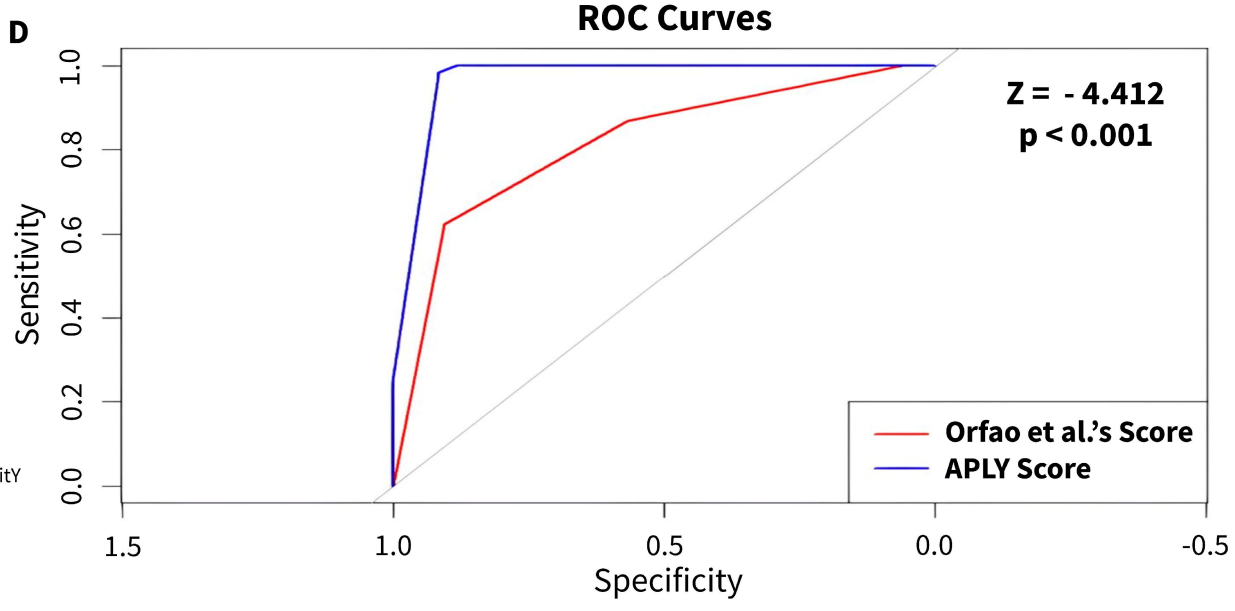
C

Parameter	Point/Value
Simultaneous CD9/CD99 Positivity	2
Simultaneous CD34/CD2 Positivity	1
Simultaneous HLA-DR/CD11b Negativity	2

APLY Score – Acute Promyelocytic Leukemia flow cytometric identitY

APL diagnosis is strongly suggested by **≥ 3 points**

D



SUPPLEMENTARY FIGURES and TABLEs

A	CD99% median (range)	CD99 MFI median (range)	CD9% median (range)	CD9 MFI median (range)	CD34% median (range)	CD2% median (range)	CD11b% median (range)	HLA-DR% median (range)	CD33% median (range)	CD33 MFI median (range)	CD13% median (range)	CD13 MFI median (range)	CD56% median (range)	CD38% median (range)	CD117% median (range)	CD133% median (range)	MPO% median (range)	CD4% median (range)	CD16% median (range)	CD15% median (range)
Total N = 255	100 (0-100)	63 (2-392)	100 (0-100)	189 (2-1622)	4 (0-100)	0 (0-100)	0 (0-100)	2 (0-100)	100 (0-100)	271 (5-2641)	100 (0-100)	276 (15-1800)	0 (0-100)	100 (63-100)	100 (0-100)	0 (0-100)	100 (0-100)	0 (0-100)	0 (0-19)	0 (0-100)
APLs N = 158	100 (97-100)	100 (42-392)	100 (88-100)	357 (51-1622)	1 (0-100)	0 (0-100)	0 (0-0)	0 (0-42)	100 (68-100)	382 (38-2641)	100 (68-100)	288 (33-1270)	0 (0-100)	100 (88-100)	100 (0-100)	0 (0-100)	100 (100-100)	0 (0-100)	0 (0-0)	0 (0-64)
Non-APL AMLs N = 97	100 (0-100)	39 (2-392)	25 (0-100)	26 (2-380)	55 (0-100)	0 (0-100)	0 (0-100)	100 (0-100)	100 (0-100)	155 (5-750)	100 (0-100)	235 (15-1800)	0 (0-100)	100 (63-100)	100 (1-100)	100 (0-100)	48 (0-100)	84 (0-100)	0 (0-19)	0 (0-100)
P Value	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***0.032****	***< 0.001****	***< 0.001****	*0.54*	***< 0.001****	*0.90*	*0.47*	***< 0.001****	***< 0.001****	***< 0.001****	*0.20*	***< 0.001****

B	CD99 n (%)	CD9 n (%)	C34 n (%)	CD2 n (%)	CD11b n (%)	HLA-DR n (%)	CD33 n (%)	CD13 n (%)	CD56 n (%)	CD38 n (%)	CD117 n (%)	CD133 n (%)	CD65 n (%)	CD14 n (%)	MPO n (%)	CD7 n (%)	CD4 n (%)	CD16 n (%)	CD15 n (%)
Total N = 255																			
neg	36 (23%)	45 (18%)	143 (56%)	208 (82%)	233 (92%)	168 (66%)	2 (1%)	4 (2%)	223 (88%)	0 (0%)	12 (5%)	174 (79%)	220 (98%)	228 (91%)	19 (7%)	228 (89%)	133 (61%)	252 (100%)	225 (89%)
pos	122 (77%)	210 (82%)	112 (44%)	45 (18%)	19 (8%)	87 (34%)	253 (99%)	251 (98%)	30 (12%)	242 (100%)	240 (95%)	45 (21%)	5 (2%)	23 (9%)	236 (93%)	27 (11%)	86 (39%)	0 (0%)	27 (11%)
Unknown	97	0	0	2	3	0	0	0	2	13	3	36	30	4	0	0	36	3	3
APLs N = 158																			
neg	0 (0%)	0 (0%)	110 (70%)	116 (73%)	158 (100%)	151 (96%)	0 (0%)	0 (0%)	145 (93%)	0 (0%)	3 (2%)	153 (99%)	147 (100%)	158 (100%)	0 (0%)	156 (99%)	110 (89%)	158 (100%)	154 (97%)
pos	61 (100%)	158 (100%)	48 (30%)	42 (27%)	0 (0%)	7 (4%)	158 (100%)	158 (100%)	11 (7%)	145 (100%)	152 (98%)	1 (1%)	0 (0%)	0 (0%)	158 (100%)	2 (1%)	13 (11%)	0 (0%)	4 (3%)
Unknown	97	0	0	0	0	0	0	0	2	13	3	4	11	0	0	0	35	0	0
Non-APL AMLs N = 97																			
neg	36 (37%)	45 (46%)	33 (34%)	92 (97%)	75 (80%)	17 (18%)	2 (2%)	4 (4%)	78 (80%)	0 (0%)	9 (9%)	21 (32%)	73 (94%)	70 (75%)	19 (20%)	72 (74%)	23 (24%)	94 (100%)	71 (76%)
pos	61 (63%)	52 (54%)	64 (66%)	3 (3%)	19 (20%)	80 (82%)	95 (98%)	93 (96%)	19 (20%)	97 (100%)	88 (91%)	44 (68%)	5 (6%)	23 (25%)	78 (80%)	25 (26%)	73 (76%)	0 (0%)	23 (24%)
Unknown	0	0	0	2	3	0	0	0	0	13	0	32	19	4	0	0	1	3	3
P Value	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	*0.28*	***0.040****	***0.005****	*> 0.95*	*0.079*	***< 0.001****	***0.035****	***< 0.001****	***< 0.001****	***< 0.001****	***< 0.001****	*> 0.95*	***< 0.001****

*Statistically significant differences.

Supplementary Table S1. Overall MFC antigen expression and mean fluorescence intensity (MFI) in all AML cases. **A)** Median expression percentage and median MFI in all analyzed AML cases and comparison between APLs and Non-APL AMLs. **B)** Antigen positivity rates and comparison between APLs and Non-APL AMLs.

Antigen Positivity on Blast-Cells (Cut-off ≥ 20%)	Univariate Analysis			
	APL Cases (%) (N=61)	Non-APL AML Cases (%) (N=97)	OR (95% CI)	p Value
CD99	61/61 (100.0)	61/97 (62.9)	73.00 (9.95-9309.48)	**< 0.001**
CD9	61/61 (100.0)	52/97 (53.6)	106.60 (14.59-13585.72)	**< 0.001**
CD34 (Cut-off ≥ 10%)	21/61 (34.4)	64/97 (66.0)	0.26 (0.13-0.50)	**< 0.001**
CD2	18/61 (29.5)	3/97 (3.1)	11.24 (3.77-44.36)	**< 0.001**
HLA-DR	1/61 (1.6)	80/97 (82.5)	0.01 (0.00-0.02)	**< 0.001**
CD13	61/61 (100.0)	93/97 (95.9)	5.92 (0.61-789.79)	0.14
CD33	61/61 (100.0)	95/97 (97.9)	3.22 (0.26-447.05)	0.40
CD11b	0/61 (0.0)	19/97 (19.6)	0.03 (0.0-0.24)	**< 0.001**
CD15	0/61 (0.0)	23/97 (23.7)	0.02 (0.00-0.18)	**< 0.001**
CD56	4/61 (6.6)	19/97 (19.6)	0.32 (0.09-0.86)	**0.023**
CD133	1/61 (1.6)	44/97 (45.4)	0.01 (0.00-0.05)	**< 0.001**
CD4	13/61 (21.3)	73/97 (75.3)	0.09 (0.04-0.19)	**< 0.001**
CD7	2/61 (3.3)	25/97 (25.8)	0.12 (0.02-0.39)	**< 0.001**
CD117	61/61 (100.0)	88/97 (90.7)	13.20 (1.62-1713.79)	**0.010**
CD65	0/61 (0.0)	5/97 (5.2)	0.34 (0.03-1.78)	0.22
CD14	0/61 (0.0)	23/97 (23.7)	0.02 (0.00-0.18)	**< 0.001**
MPO (Cut-off ≥ 10%)	61/61 (100.0)	78/97 (80.4)	32.59 (4.21-2912.55)	**< 0.001**

Supplementary Table 2. Univariate analysis for MFC antigens association with the differential diagnosis of APL. The p values in bold are statistically significant.

A

Orfao et al.'s Score (Points)	Total (N=158) n (%)	APLs (N=61) n (%)	Non-APL AMLs (N=97) n (%)	P-Value
0	6 (3.8)	0 (0.0)	6 (6.2)	**< 0.001**
1	57 (36.0)	8 (13.1)	49 (50.5)	
2	48 (30.4)	15 (24.6)	33 (34.0)	
3	47 (29.7)	38 (62.3)	9 (9.3)	

B

APLY Score (Points)	Total (N=158) n (%)	APLs (N=61) n (%)	Non-APL AMLs (N=97) n (%)	P-Value
0	55 (34.9)	0 (0.0)	55 (56.7)	**< 0.001**
1	0 (0.0)	0 (0.0)	0 (0.0)	
2	31 (19.6)	0 (0.0)	31 (32.0)	
3	4 (2.5)	1 (1.6)	3 (3.1)	
4	52 (32.9)	44 (72.2)	8 (8.2)	
5	16 (10.1)	16 (26.2)	0	

Supplementary Table 3. Overall distribution of AML cases according to diagnostic scoring systems. **A)** Distribution of all AML samples based on their Orfao et al.'s Score, with a comparative analysis between APL and non-APL AML cases. **B)** Distribution of all AML samples based on their APLY Scores, with a comparative analysis between APL and non-APL AML cases.