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RESEARCH ARTICLE

Prevalence and clinical impact of CD56 and T-cell marker expression in acute myeloid leukaemia: A single-centre retrospective analysis

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Abstract

Flow cytometry-based immunophenotyping is a mainstay of diagnostics in acute myeloid leukaemia (AML). Aberrant CD56 and T-cell antigen expression is observed in a fraction subset of AML cases, but the clinical relevance remains incompletely understood. Here, we retrospectively investigated the association of CD56 and T-cell marker expression with disease-specific characteristics and outcome of 324 AML patients who received intensive induction therapy at our centre between 2011 and 2019. We found that CD2 expression was associated with abnormal non-complex karyotype, *NPM1* wild-type status and *TP53* mutation. CD2 also correlated with a lower complete remission (CR) rate (47.8% vs. 71.6%, $p = 0.03$). CyTdT and CD2 were associated with inferior 3-year event-free-survival (EFS) (5.3% vs. 33.5%, $p = 0.003$ and 17.4% vs. 33.1%, $p = 0.02$, respectively). CyTdT expression was also correlated with inferior relapse-free survival (27.3% vs. 48.8%, $p = 0.04$). In multivariable analyses CD2 positivity was an independent adverse factor for EFS (HR 1.72, $p = 0.03$). These results indicate a biological relevance of aberrant T-cell marker expression in AML and provide a rationale to further characterise the molecular origin in T-lineage-associated AML.

KEYWORDS
acute myeloid leukaemia, CD56, clinical impact, flow cytometry, T-cell marker

1 | INTRODUCTION

Acute myeloid leukaemia (AML) is a clonal disease characterised by the rapid proliferation of immature myeloid precursor cells in the bone marrow [1]. AML presents with a wide heterogeneity of morphological, immunophenotypic and genetic features, mirrored by a largely variable clinical course of the disease.

Immunophenotyping of leukaemic blasts by flow cytometry (FCM) is an essential diagnostic tool to discriminate AML from other leukaemic subtypes. Flow cytometric identification of leukaemia-associated immunophenotypes (LAIPs) in AML also plays an important role in the detection of measurable residual disease (MRD) [2]. In addition, the identification of specific cluster of differentiation (CD) markers, including CD33 or CD123, may guide targeted therapy of AML [3, 4]. FCM

allows the detection of aberrant myeloblastic immunophenotypes in up to 90% of AML cases [1]. Apart from typical myeloid and progenitor cell markers, a fraction of acute leukaemias express additional T- and/or B-cell markers (e.g., surface or cytoplasmic CD3, CD10, CD19, CD22, CD79a) and is classified as a mixed phenotype acute leukaemia (MPAL) [5, 6].

Several studies have reported an adverse prognostic association of CD56 and T-cell marker expression (cytoplasmic terminal deoxynucleotidyl transferase [cyTdT], CD2, CD7) in acute leukaemia, [7]–[11] while others have failed to show an association between immunophenotype and treatment outcome [7, 9, 12–14].

In this analysis, we aimed to investigate the prevalence and clinical impact of CD56 expression and other selected T-cell markers in adult AML patients intensively treated at our centre.

2 | MATERIALS AND METHODS

2.1 | Patients and methods

Flow cytometric analysis was performed on pre-therapeutic bone marrow ($N = 300$) or peripheral blood ($N = 24$) samples from patients diagnosed with AML at the University Hospital Münster between March 2011 and October 2019, who received intensive induction chemotherapy with cytarabine/daunorubicin, liposomal cytarabine/daunorubicin, or cytarabine/thioguanine/amsacrine (Table 1). The regional Institutional Review Board (2020-814-f-S) approved the study. Patients with acute promyelocytic leukaemia, myelodysplastic syndrome and patients with myelosarcoma without bone marrow involvement were excluded from the analysis.

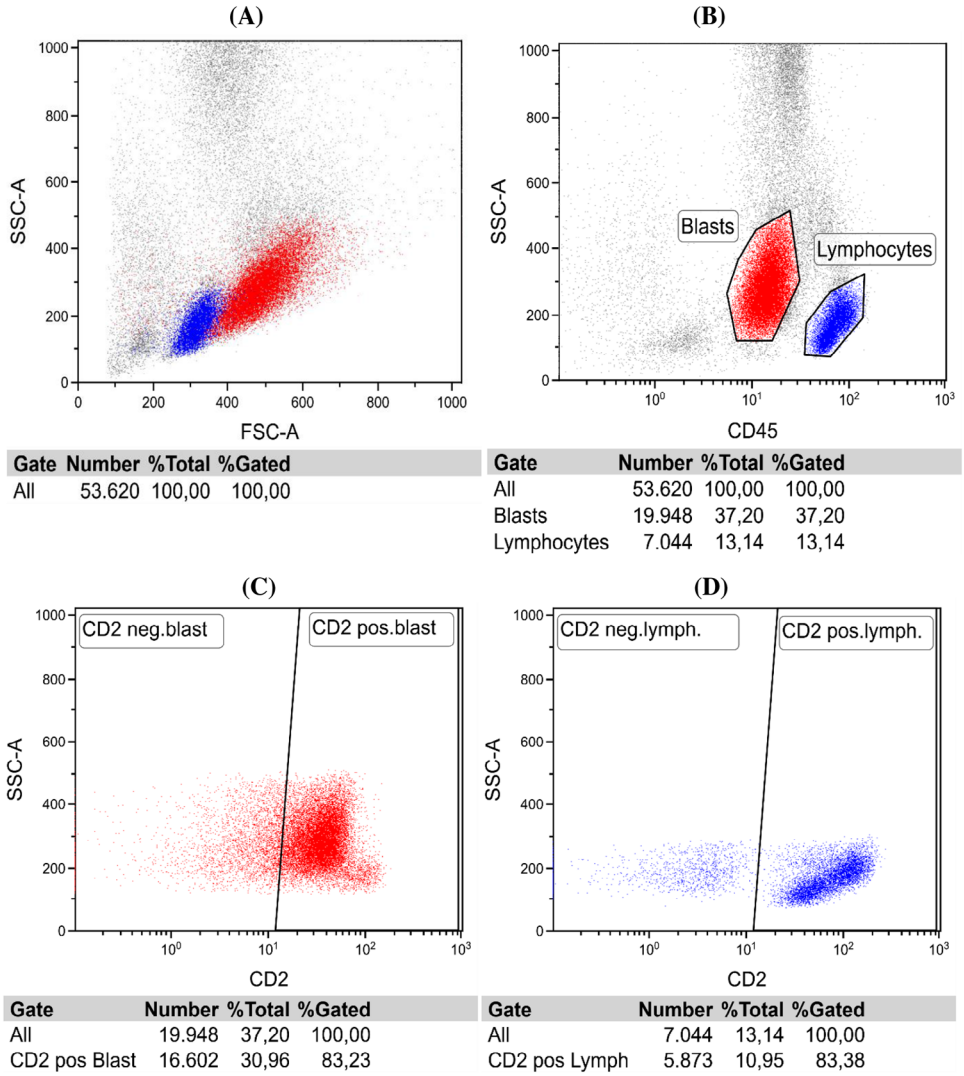
2.2 | Flow cytometric analysis of AML samples

The following antibodies were used in routine clinical diagnostics: TdT (APC fluorochrome, clone E17-1519), CD45 (V500 fluorochrome, clone HI30), CD2 (PerCP Cy5.5 fluorochrome, clone RPA-2.10), CD3 (PerCP Cy5.5 fluorochrome, clone SK7 and V450 fluorochrome, clone UCHT1), CD4 (APC fluorochrome, clone SK3), CD7(PE fluorochrome, clone M-1701), CD8 (FITC fluorochrome, clone SK1) and CD56 (APC fluorochrome, clone NCAM16.2), all purchased from BD Biosciences (San Jose, CA, USA). Samples were investigated using six-colour panels on a FACS Canto II cytometer (BD Biosciences). Measurements were performed using FACS Diva software (BD Biosciences) and analysed using Kaluza Analysis flow cytometry software version 2.1 (Beckman Coulter, Kraemer Blvd. Brea, CA 92821 USA). A minimum of 50,000 events were recorded per sample. Flow cytometer performance was checked regularly using CS&T beads (BD Biosciences). Leukaemic blasts and lymphocytes were gated based on their CD45 expression and side scatter profile. For each marker, individual gates discriminating positive and negative lymphocyte populations were defined. The 'negative' lymphocyte population was considered as a negative internal control population. These gates were then applied to the blast pop-

TABLE 1 Pre-treatment patient characteristics.

Age, years, median (range)	**60 (18–84)**
Sex, *n*	324
Male, *n* (%)	193 (59.6)
Female, *n* (%)	131 (40.4)
AML type, n	324
De novo, *n* (%)	229 (70.7)
sAML, *n* (%)	73 (22.5)
tAML, *n* (%)	22 (6.8)
Cytogenetics, *n*	311
Normal, *n* (%)	140 (45.0)
Complex aberrant*, *n* (%)	56 (18.0)
Other abnormal, *n* (%)	115 (37.0)
t(8;21), *n*	303
Present, *n* (%)	8 (2.6)
Absent, *n* (%)	295 (97.4)
inv16/t(16;16), *n*	303
Present, *n* (%)	10 (3.3)
Absent, *n* (%)	293 (96.7)
FLT3-ITD, *n*	322
Present, *n* (%)	59 (18.3)
Absent, *n* (%)	263 (81.7)
NPM1 mutation, *n* (%)	322
Mutated, *n* (%)	95 (29.5)
Wild type, *n* (%)	227 (70.5)
NPM1/FLT3-ITD mutational status, *n*	322
$NPM1^{wt}/FLT3\text{-}ITD^{neg}$, *n* (%)	201 (62.4)
$NPM1^{mut}/FLT3\text{-}ITD^{pos}$, *n* (%)	33 (10.2)
$NPM1^{mut}/FLT3\text{-}ITD^{neg}$, *n* (%)	62 (19.3)
$NPM1^{wt}/FLT3\text{-}ITD^{neg}$, *n* (%)	26 (8.1)
TP53 mutation, *n*	58
Mutated, *n* (%)	9 (15.5)
Wild type, *n* (%)	49 (84.5)
ELN genetic risk*, *n*	307
Favourable, *n* (%)	59 (19.2)
Intermediate, *n* (%)	152 (49.5)
Adverse, *n* (%)	96 (31.3)
Type of induction treatment, *n*	324
TAA, *n* (%)	1 (0.3)
CPX 351, *n* (%)	4 (1.2)
"7+3", *n* (%)	319 (98.5)

Abbreviations: AML, acute myeloid leukaemia; sAML, secondary AML; tAML, therapy-related AML; *FLT3-ITD*, internal tandem duplication of the *FLT3* gene; *NPM1*, nucleophosmin 1; ELN European LeukaemiaNet; *TP53*, tumor protein p53; TAA, cytarabine, thyoguanine, amsacrine.
*According to ELN 2010 definitions.



Gate	Number	%Total	%Gated
All	53.620	100,00	100,00

Gate	Number	%Total	%Gated
All	53.620	100,00	100,00
Blasts	19.948	37,20	37,20
Lymphocytes	7.044	13,14	13,14

Gate	Number	%Total	%Gated
All	19.948	37,20	100,00
CD2 pos Blast	16.602	30,96	83,23

Gate	Number	%Total	%Gated
All	7.044	13,14	100,00
CD2 pos Lymph	5.873	10,95	83,38

FIGURE 1 Gating strategy of the flow cytometric analysis of CD56 and T-cell marker expression on AML blasts. Dot-plots of exemplary flow cytometric data showing (A) separation of bone marrow cells by FSC-A versus SSC-A, (B) gating of blast (red) and lymphocyte populations (blue) according to SSC-A and CD45 expression, (C) how the 'negative' lymphocytic internal control population was defined for CD2, (D) CD-positive (CD2) blast population as defined by the non-negative lymphocyte gate established in (C).

ulation. For each marker the 'positive' blast population was defined by events in the patient's individual blast gate not mapping to the 'negative' lymphocyte gate (Figure 1).

2.3 | Statistical analysis

A blast population was considered marker-positive if 20% or more of the blast cells were detected in the 'positive' blast gate as defined above [15]. As a second part of our study, we analysed CD56- and selected T-cell marker expression as continuous variables without a predefined threshold of positivity. In this case we analysed continuous marker expression per 10% increase in expression. The expression of two different CD3 clones (SK7 and UCH1) was analysed together in the 'categorical' analysis. Time-to-event variables and complete remission (CR) were defined as previously described [16]. Three-year overall survival (OS), relapse-free survival (RFS), and EFS were estimated using the Kaplan–Meier estimator and compared using the log-rank test. Median follow-up time was calculated by the reverse Kaplan-Meier method. The 2010 European LeukaemiaNet (ELN) risk classification was used because information on biallelic and in-frame basic leucine zipper domain (bZIP) CCAAT enhancer binding protein alpha (*CEBPA*) mutations was not available for most cases. Baseline patient characteristics were compared using the Mann-Whitney test for continuous and the chi-square test or Fischer exact test, respectively. The Benjamini-Hochberg method was used for adjustment for multiplicity. Univariable analysis was performed using the Cox proportional hazards model for OS, RFS, EFS and a logistic regression model for CR. Multivariable analysis, including age, type of AML, risk according ELN 2010 classification and marker expression was performed using the Cox proportional hazards model or multivariable logistic regression. CD3 and CD8 markers were not included in univariable and multivariable analyses due to the

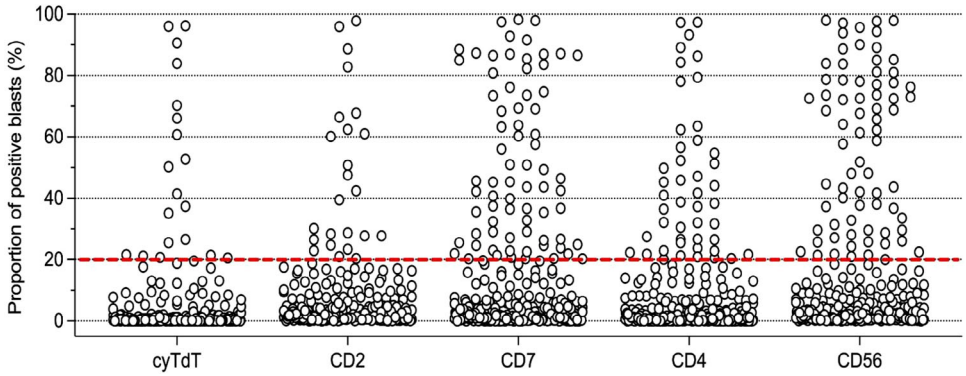


FIGURE 2 T-cell and CD56 marker positivity of blast populations. Violin plots displaying the proportion of positive blasts for indicated markers in percent (%) for individual patients (dots). The distributions of CD3 and CD8 markers are not shown due to the small number or absence of positive cases, respectively.

low number of patients with marker positivity. Missing data were not imputed. Two-sided p values < 0.05 were defined as significant. Statistical analyses were performed with IBM SPSS statistics, version 28.0 (IBM Corp., Armonk, NY, USA) and the R software package, version 4.0.2 (R Foundation, Vienna, Austria; https://www.r-project.org).

3 | RESULTS

3.1 | Baseline characteristics

A total of 324 AML patients (59.6% males and 40.4% females) receiving intensive AML induction therapy were identified. Median age at the time of diagnosis was 60 years (range 18–84 years). Note that 70.7% of patients had de novo AML, 22.5% had secondary AML (following MDS or MDS/MPN) and 6.8% had AML post cytotoxic therapy. Further details on pre-treatment characteristics are summarised in Table 1.

3.2 | Proportion of CD56 and T-cell marker positive cases of AML

CD56 and T-cell marker expression on ≥20% of leukaemic blasts was detected in 73 (22.5%) cases for CD56, 73 (22.5%) for CD7, 46 (14.2%) for CD4, 24 (7.5%) for CD2, 19 (5.9%) for cyTdT, 5 (1.6%) for surface CD3 and 0 cases for CD8, respectively. The overall proportion of marker positive blasts as well as a more detailed display of cases with blast positivity of ≥20% of leukaemic blasts is shown in Figure 2.

3.3 | Association of CD56 and T-cell marker expression with specific subgroups of AML

In univariable analyses of categorical marker positivity on ≥20% of leukaemic blasts (Table 2), cyTdT expression was significantly associated with older age ($p = 0.04$).

We also analysed the association of marker positivity with cytogenetic and molecular genetic features. CD2 positive AML patients had a higher frequency of abnormal karyotype ($p = 0.01$), inversion of chromosome 16 ($p < 0.0001$) and $NPM1^{neg}/FLT3\text{-}ITD^{wt}$ status ($p = 0.01$). CD56 expression was associated with t(8;21) ($p = 0.001$) and $NPM1^{mut}/FLT3\text{-}ITD^{wt}$ status ($p = 0.03$). CyTDT and CD2 expression were associated with $NPM1^{wt}$ cases ($p = 0.04$ and $p = 0.01$, respectively). We also found a significant association of CD2 with the prevalence of *TP53* mutations ($p = 0.03$), even though the screening for mutant *TP53* was only performed in a limited number of 58 patients (Table 2).

3.4 | Association of CD56 and T-cell marker expression with outcome measures

We then investigated the association of marker expression with the CR rate after induction treatment. CD2 positivity was associated with lower CR rates (47.8 vs. 71.6% for CD2, $p = 0.03$) (Table 2).

Survival estimates at 3 years were analysed with a median time of follow-up (interquartile range) of 4.53 (2.740–5.92) years. CyTdT positive patients had an inferior EFS (5.3% vs. 33.5%, $p = 0.003$) and an inferior RFS (27.3% vs. 48.8%, $p = 0.04$), no significant difference in OS was found (36.8% vs. 45.7%, $p = 0.11$) (Figure 3). Similarly, EFS was lower in CD2-positive patients (17.4% vs. 33.1%, $p = 0.02$). CD2 positive cases also showed a trend towards inferior 3-year OS (30.4% versus 46.6%, $p = 0.09$). CD3, CD4, CD7 and CD56 positivity were not significantly associated with outcome (Figure 3, Figure S1).

3.5 | Univariable and multivariable analysis for marker expression as categorical variable

In the univariable Cox proportional hazards model, blast positivity for cyTdT or CD2 as a categorical variable (≥20% vs. < 20%) was associated with inferior EFS (HR 2.02, $p = 0.003$ and HR 1.82, $p = 0.01$,

TABLE 2 Association of CD56 and T-cell marker expression on >20% of AML blasts and patients' characteristics.

Marker	cyTdT		CD2		CD4		CD7		CD56	
Proportion of positive blasts	≥20%	<20%	≥20%	<20%	≥20%	<20%	≥20%	<20%	≥20%	<20%
Proportion of positive blasts, *n* (%)	19 (5.9)	305 (94.1)	24 (7.5)	295 (92.5)	46 (14.2)	27.8 (85.8)	73 (22.5)	25.1 (77.5)	73 (22.5)	251 (77.5)
Age, years, median (range)	67	60	61	60	56	61	59	61	62	60
	(48–77)	(18–84)	(18–76)	(18–84)	(18–74)	(18–84)	(19–74)	(18–84)	(18–78)	(18–84)
p[a]	**0.04**		0.59		0.25		0.20		0.05	
Cytogenetics										
Normal, *n* (%)	8 (44.4)	132 (45.1)	4 (16.7)	134 (47.5)	16 (36.4)	124 (46.4)	31 (44.3)	109 (45.2)	23 (34.8)	116 (47.5)
Complex, *n* (%)	2 (11.1)	54 (18.4)	9 (37.5)	46 (16.3)	9 (20.5)	47 (17.6)	15 (21.4)	41 (17.0)	16 (24.2)	40 (16.4)
Other abnormal, *n* (%)	8 (44.4)	107 (36.5)	11 (45.8)	102 (36.2)	19 (43.2)	96 (36.0)	24 (34.3)	91 (37.8)	27 (40.9)	88 (36.1)
p[b]	0.67		**0.01**		0.63		0.76		0.16	
Risk according ELN 2010 classification										
Favourable	0 (0)	59 (20.5)	4 (16.7)	55 (19.8)	9 (22.0)	50 (18.8)	7 (10.3)	52 (21.8)	19 (28.4)	39 (16.3)
Intermediate	13 (68.4)	139 (48.3)	7 (29.2)	142 (51.1)	15 (36.6)	137 (51.5)	39 (57.4)	113 (47.3)	23 (34.3)	129 (54.0)
Adverse	6 (31.6)	90 (31.3)	13 (54.2)	81 (29.1)	17 (41.5)	79 (29.7)	22 (43.4)	74 (31.0)	25 (37.3)	71 (29.7)
p[b]	0.14		0.06		0.45		0.23		**0.03**	
t(8;21)										
Present, *n* (%)	0 (0)	8 (2.6)	0 (0)	8 (2.7)	0 (0)	8 (2.9)	0 (0)	8 (3.2)	7 (9.7)	1 (0.4)
Absent, *n* (%)	19 (100)	295 (97.4)	24 (100)	285 (97.3)	45 (100)	269 (97.1)	73 (100)	241 (96.8)	65 (90.3)	248 (99.6)
p[b]	0.52		0.46		0.45		0.24		**0.001**	
inv(16)/t(16;16)										
Present, *n* (%)	0 (0)	10 (3.3)	5 (20.8)	5 (1.7)	0 (0)	10 (3.6)	0 (0)	10 (4.0)	2 (2.8)	8 (3.2)
Absent, *n* (%)	19 (100)	293 (96.7)	19 (79.2)	288 (98.3)	45 (100)	267 (96.4)	73 (100)	239 (96.0)	70 (97.2)	241 (96.8)
p[b]	0.52		**<0.0001**		0.45		0.23		0.85	

(Continues)

TABLE 2 (Continued)

Marker	cyTdT		CD2		CD4		CD7		CD56	
FLT3-ITD										
Present, *n* (%)	2 (10.5)	57 (18.8)	1 (4.2)	56 (19.1)	8 (17.8)	51 (18.4)	17 (23.3)	42 (16.9)	7 (9.7)	52 (20.9)
Absent, *n* (%)	17 (89.5)	246 (81.2)	23 (95.8)	237 (80.9)	37 (82.2)	226 (81.6)	56 (76.7)	207 (83.1)	65 (90.3)	197 (79.1)
p^b	0.52		0.09		0.92		0.35		0.05	
NPM1										
Mutated, *n* (%)	0 (0)	95 (31.4)	1 (4.2)	93 (31.7)	12 (26.7)	83 (30.0)	22 (30.1)	73 (29.3)	27 (37.5)	67 (26.9)
Wild type, *n* (%)	19 (100)	208 (68.6)	23 (95.8)	200 (68.3)	33 (73.3)	194 (70.0)	51 (69.9)	176 (70.7)	45 (62.5)	182 (73.1)
p^b	**0.04**		**0.01**		0.72		0.89		0.11	
NPM1/FLT3-ITD										
$NPM1^{wt}/FLT3\text{-}ITD^{neg}$, *n* (%)	17 (89.5)	184 (60.7)	23 (95.8)	175 (59.7)	27 (60.0)	174 (62.8)	47 (64.4)	154 (61.8)	44 (61.1)	157 (63.1)
$NPM1^{mut}/FLT3\text{-}ITD^{pos}$, *n* (%)	0 (0)	33 (10.9)	1 (4.2)	31 (10.6)	2 (4.4)	31 (11.2)	13 (17.8)	20 (8.0)	6 (8.3)	27 (10.8)
$NPM1^{mut}/FLT3\text{-}ITD^{neg}$, *n* (%)	0 (0)	62 (20.5)	0 (0)	62 (21.2)	10 (22.2)	52 (18.8)	9 (12.3)	53 (21.3)	21 (29.2)	40 (16.1)
$NPM1^{wt}/FLT3\text{-}ITD^{pos}$, *n* (%)	2 (10.5)	24 (7.9)	0 (0)	25 (8.5)	6 (13.3)	20 (7.2)	4 (5.5)	22 (8.8)	1 (1.4)	25 (10.0)
p^b	0.10		**0.01**		0.45		0.20		**0.03**	
***TP53*, *n* (%)**										
Mutated	1 (100)	8 (14.0)	4 (50)	5 (10.0)	1 (25.0)	8 (14.8)	4 (22.2)	5 (12.5)	4 (36.4)	5 (10.6)
Wild type	0 (0)	49 (86.0)	4 (50)	45 (90.0)	3 (75.0)	46 (85.2)	14 (77.8)	35 (87.5)	7 (63.6)	42 (89.4)
p^c	0.27		**0.03**		0.63		0.55		0.05	
Complete remission, *n* (%)	8 (47.1)	195 (70.9)	11 (47.8)	189 (71.6)	35 (85.4)	168 (66.9)	42 (64.6)	161 (70.9)	50 (76.9)	152 (67.3)
p^b	0.10		**0.03**		0.20		0.47		0.16	

Note: Significant *p* values are marked in bold. *p*-Values shown after adjustment for multiplicity according to Bejnamini-Hochberg method.

Abbreviations: AML, acute myeloid leukaemia; ELN, European LeukaemiaNet; *FLT3-ITD*, internal tandem duplication of the *FLT3* gene; *NPM1* nucleophosmin 1; *TP53*, tumor protein p53; CR, complete remission.

[a]Mann–Whitney test; [b]chi-square- test; [c]Fisher exact test.

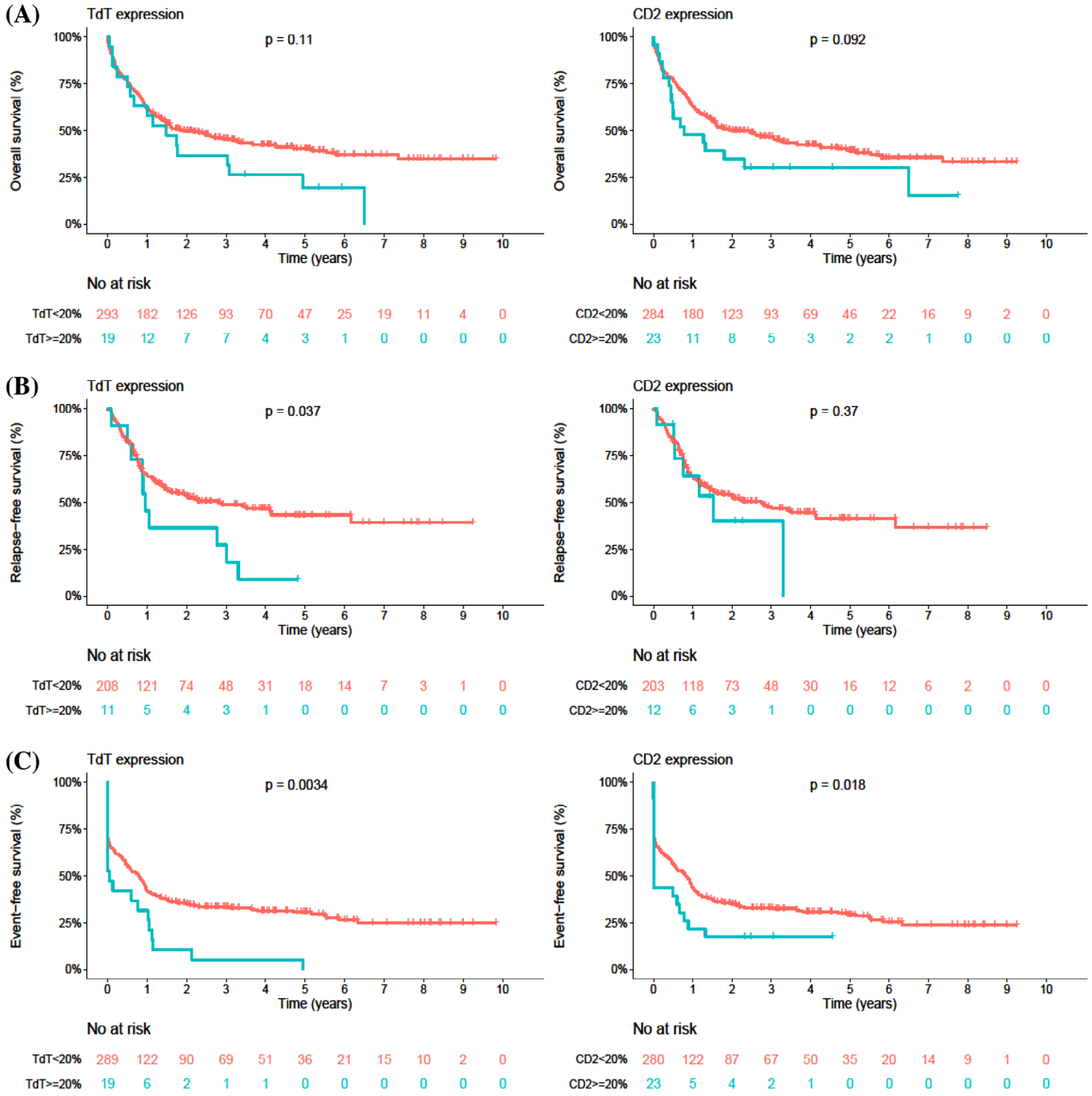


FIGURE 3 T-cell and CD56 marker positivity and outcome. Kaplan–Meier curves showing (A) overall survival, (B) relapse-free survival and (C) event-free survival according to cyTDT and CD2 expression. Survival curves for CD3, CD4, CD7 and CD56 are shown in supplementary Figure 1.

respectively) (Table 3). In addition, cyTdT expression correlated with decreased RFS (HR 1.97, $p = 0.04$). Both, cyTdT and CD2 expression were associated with lower CR rates in the univariable logistic regression model (odds ratio [OR] 0.36 with $p = 0.04$ and OR 0.37 with $p = 0.02$, respectively), but not in multivariable analysis. In contrast, CD4 expression was associated with improved CR rates in univariable and multivariable regression models (OR 2.88 and 4.31, $p = 0.02$ and $p = 0.008$, respectively). CD7 and CD56 markers showed no correlation with outcome (Table 3).

3.6 | Univariable and multivariable analysis of marker expression as a continuous variable

Marker analysis as a continuous variable confirmed most of the results described above for categorical variables. In univariable analysis, CD2 positivity correlated with slightly inferior OS (HR 1.09, $p = 0.05$). Both, cyTdT and CD2 were significantly associated with experiencing an event in the univariable analysis, (HR 1.13, $p = 0.004$ and HR 1.14, $p = 0.002$, respectively). Higher cyTdT expression was associated with

a decreased RFS in the univariable analysis (HR 1.13, $p = 0.05$). The correlation of CD2 with lower EFS was confirmed in the multivariable model (HR 1.72, $p = 0.03$) (Table S1). Similarly, CD2 expression as a continuous variable was associated with a lower CR rate only in the univariable logistic regression model (OR 0.95, $p = 0.01$) but not in multivariable analysis. CD4 expression was associated with a higher CR rate in univariable and multivariable analyses (OR 1.03, $p = 0.05$ and OR 1.23, $p = 0.005$, respectively).

4 | DISCUSSION

In this retrospective study, we analysed the association of expression of selected T-cell markers and CD56 with various patient characteristics and outcome measures in a single-centre cohort of 324 AML patients receiving intensive induction therapy regimens.

Aberrant marker expression is a hallmark of AML and has been observed ever since immunophenotyping was established to discriminate lineages of acute leukemias. Several markers are considered to be lineage exclusive, including such as CD3 for T cells and, when highly expressed, CD19 for B cells. This has led to the definition of MPALs, which display markers of more than one lineage, often referred to as leukaemias of ambiguous lineage according to the WHO classification [17] or biphenotypic acute leukaemia according to the EGIL classification [6]. In adult patients, MPAL is characterised by frequent resistance to therapy and poorer outcome as compared to typical AML or ALL [18]. In our cohort of AML patients treated with intensive induction therapy, we identified five cases with CD3 surface expression that, retrospectively, should have been classified as T cell/myeloid MPAL. As these 5 cases were initially diagnosed and treated as AML, they were included in this retrospective analysis.

For all other T-/NK-cell markers the clinical significance of aberrant expression in AML is still incompletely understood. We therefore set out to look for prognostic associations for CD2, CD4, CD7, CD8, CD56 and cyTdT expression.

Most data on aberrant marker expression in AML were generated between 1990 and 2010, when predominantly two- to four-colour flow cytometric staining panels were used. This made the accurate characterisation of abnormal subpopulations much more challenging as compared to modern six or eight-colour flow cytometric panels [19]. Methodological differences in staining protocols and definitions of antigen-positivity may have contributed to the controversial results published previously [7, 9, 13]. These differences include the use of a wide variety of monoclonal antibody panels composed of individual and divergent clones of antigen-directed antibodies, the type of flow cytometer used, individual gating strategies, varying sizes of patient populations studied and single versus multi-institutional designs of the analyses.

Our data showed that patients with cyTdT positive AML had a significantly lower RFS and EFS in univariable, but not in multivariable analyses, suggesting that cyTdT expression is secondary to other adverse features of AML. TdT plays a role in DNA synthesis and contributes to the diversity of immunoglobulins and T-cell receptors in immature lymphocytes and thymic lymphocytes. TdT is a widely used marker in the diagnosis of precursor lymphoblastic neoplasms, but it is also expressed in some AML subtypes, particularly in AML with minimal differentiation and AML without maturation [20]. However, there is no consensus on the clinical implications of TdT positivity in AML. Previous publications from the 1990s to early 2000s showed no impact of TdT expression on survival [7, 9, 21] or even found an association with improved prognosis [22]. More recently, another publication reported an association between TdT expression and shorter RFS in AML with an intermediate risk-karyotype.[20] TdT may also play a role in both *FLT3-ITD* and *NPM1* mutagenesis in AML [23–25]. It was suggested that TdT primes replication slippage through N-nucleotide addition, which, in turn, can create a NPM1 mutation. Borrow et al. observed that TdT is not expressed at high levels in *NPM1*-mutated AML and suggested that TdT levels may be downregulated secondary to the acquisition of the *NPM1* mutation [24]. TdT expression was also correlated with *NPM1* negativity in our analysis.

CD2 is a T-lymphocyte cell-adhesion molecule found on the surface of T-cells and NK cells. The prognostic significance of CD2 in AML is uncertain in the literature. Some studies have shown a negative impact of CD2 expression on remission rates [11, 26]. In our analysis, CD2 expression also correlated with a lower CR rate, a lower EFS and a trend towards a lower OS. However, as these associations except for EFS could not be confirmed in multivariable analyses, CD2-positivity may be a consequence rather than a cause of other unfavourable features in AML. CD2 expression was positively correlated with inv(16)[27]$^{-}$[29] and with *NPM1*^{wt}, which is well known from previous publications [30]. We also found that the CD2 marker was associated with an abnormal non-complex karyotype. Possibly, the presence of CD2 expression observed in cases with inv(16) somewhat neutralised a generally negative impact of CD2 expression on outcome measures, as described in one previous study [31].

CD4 is most commonly recognised as a marker of T-helper cells. However, it is also expressed at lower levels on monocytes and CD34-positive progenitors, including phenotypically very immature populations and more committed myelomonocytic precursors [19]. The prognostic role value of CD4 expression in AML is unclear. A recent study showed that CD4 expression was correlated with an unfavourable prognosis in *NPM1*^{wt}/*FLT3-ITD*^{neg} and cytogenetically normal AML [32]. In contrast, CD4 positivity in our analysis was an independent prognostic marker for achieving a CR after induction therapy in univariable and multivariable analyses, but, surprisingly, did not translate into favourable survival rates.

The CD56 positivity of blasts in our analysis was associated with the t(8;21)/*RUNX1::RUNX1T1* translocation (Table 2) as previously described [33]. An adverse association of CD7 and CD56 expression with outcome has been reported previously in several publications [14, 34–36], but was not confirmed in our analysis. This discrepancy may be explained by using of two- or four-colour flow cytometry and in relatively small numbers of patients [14, 35]. In contrast to previous studies, we applied a gating strategy that included an internal negative

TABLE 3 Univariable and multivariable analyses of associations between marker positivity of ≥20% of blasts and outcome measures.

	Univariable analysis			Multivariable analysis		
	HR/OR	95% CI	*p*-Value	HR/OR	95% CI	*p*-Value
Complete remission after induction therapy						
Age (per 10-year increase)	0.98	0.94–1.02	0.36			
Type of AML			**0.02**			0.30
tAML versus de novo	1.01	0.81–1.25		1.71	0.54–6.08	
sAML versus de novo	0.85	0.75–0.97		0.71	0.37–1.35	
ELN 2010			<**0.001**			<**0.001**
Intermediate versus favourable risk	0.76	0.66–0.88		0.06	0.003–0.30	
Adverse versus favourable risk	0.64	0.55–0.75		0.02	0.001–0.12	
cyTdT expression ≥20 versus < 20%	0.36	0.13–0.99	**0.04**	0.61	0.21–1.76	0.23
CD2 expression ≥20 versus < 20%	0.37	0.15–0.86	**0.02**	0.49	0.18.1.31	0.23
CD4 expression ≥20% versus < 20%	2.88	1.25–7.86	**0.02**	4.31	1.52–15.55	**0.008**
CD7 expression ≥20% versus < 20%	0.75	0.42–1.36	0.33			
CD56 expression ≥20% versus < 20%	1.62	0.87–3.16	0.14	1.33	0.63–2.92	0.34
Overall Survival	**6					
Age (per 10-year increase)	1.37	1.21–1.57	<**0.001**	1.45	1.25–1.68	<**0.001**
Type of AML			0.19			0.16
tAML versus de novo	1.27	0.71–2.24		0.80	0.43–1.52	
sAML versus de novo	1.23	0.88–1.72		0.80	0.55–1.16	
ELN 2010			<**0.008**			<**0.001**
Intermediate versus favourable risk	1.34	0.83–2.14		1.46	0.90–2.36	
Adverse versus favourable risk	2.35	1.45–3.82		2.68	1.61–4.45	
cyTdT expression ≥ 20% versus < 20%	1.52	0.91–2.53	0.11	1.10	0.65–1.89	0.61
CD2 expression ≥ 20% versus < 20%	1.53	0.93–2.53	0.10	1.30	0.77–2.18	0.16
CD4 expression ≥ 20% versus < 20%	1.08	0.72–1.62	0.72			
CD7 expression ≥ 20% versus < 20%	1.01	0.72–1.43	0.96			
CD56 expression ≥ 20% versus < 20%	1.14	0.81–1.62	0.45			
Relapse-free survival						
Age (per 10-year increase)	1.30	1.10–1.52	**0.002**	1.30	1.10–1.54	**0.002**
Type of AML			0.54			
tAML versus de novo	1.07	0.52–2.22				
sAML versus de novo	1.15	0.73–1.82				
ELN 2010			0.13			0.08
Intermediate versus favourable risk	1.33	0.80–2.23		1.32	0.78–2.23	
Adverse versus favourable risk	1.56	0.88–2.77		1.67	0.93–2.98	
cyTdT expression ≥ 20% versus < 20%	1.97	1.03–3.78	**0.04**	1.51	0.78–2.95	0.14
CD2 expression ≥ 20% versus < 20%	1.42	0.66–3.05	0.37			
CD4 expression ≥ 20% versus < 20%	1.35	0.83–2.18	0.23			
CD7 expression ≥ 20% versus < 20%	0.73	0.44–1.19	0.21			
CD56 expression ≥ 20% versus < 20%	1.32	0.85–2.06	0.22			
Event-free survival						
Age (per 10-year increase)	1.21	1.08–1.35	<**0.001**	1.22	1.08–1.37	**0.001**
Type of AML			0.19			0.40
tAML versus de novo	1.14	0.69–1.88		0.83	0.48–1.44	
sAML versus de novo	1.23	0.89–1.69		0.86	0.61–1.23	

(Continues)

TABLE 3 (Continued)

	Univariable analysis			Multivariable analysis		
	HR/OR	95% CI	*p*-Value	HR/OR	95% CI	*p*-Value
ELN 2010			**<0.001**			**<0.001**
Intermediate versus favourable risk	1.74	1.15–2.62		1.72	1.13–2.62	
Adverse versus favourable risk	2.39	1.55–3.69		2.49	1.58–3.91	
cyTdT expression ≥ 20% versus < 20%	2.02	1.26–3.24	**0.003**	1.53	0.93–2.51	0.07
CD2 expression ≥ 20% versus < 20%	1.82	1.14–2.92	**0.01**	1.72	1.06–2.80	**0.03**
CD4 expression ≥ 20% versus < 20%	0.826	0.56–1.22	0.33			
CD7 expression ≥ 20% versus < 20%	0.97	0.71–1.34	0.86			
CD56 expression ≥ 20% versus < 20%	1.00	0.73–1.37	1.00			

Note: Odds ratios (OR) greater or less than 1.0 indicate higher or lower CR rates, respectively, for the first category listed. Hazard ratios (HR) greater or less than 1.0 indicate an increased or decreased risk, respectively, of an event for the higher values of the continuous variables and the first category listed of the categorical variables. Significant *p* values are marked in bold.

Abbreviations: AML, acute myeloid leukaemia; sAML; secondary AML; tAML, therapy-related AML; ELN, European LeukaemiaNet.

control population, represented by the 'negative' lymphocyte gate in a large cohort of patients using six-colour flow cytometry.

Several limitations of our study should be considered when interpreting the results. First, it represents a retrospective analysis of clinical diagnostic data derived from evolving staining patterns that have been modified over time. However, we completely re-analysed the raw data available for each patient following a uniform gating scheme, which largely eliminates inter-observer-induced variability in the determination of blast positivity. Second, the clinical panels did not include negative isotype antibody controls, so low-level blast positivity in some cases could not be definitively distinguished from false positive unspecific signals. To overcome this limitation, we have put a major effort in careful gating according to the 'negative' lymphocyte population defined for each of the markers analysed. Another limitation is that only selected markers were stained in the clinical samples used for our analysis, whereas additional markers, including CD1a, CD5 and CD16, may have been of additional value in this context.

In the current literature, at least two leading hypotheses exist to explain the development of acute leukaemias with shared differentiation potential with myeloid and T-lymphoid lineages. A first model assumes the transformation of normal haematopoietic progenitors with retained bi-lineage T-lymphoid and myeloid potential [37]. Preclinical and clinical data suggested that T-cell progenitors may represent the cellular origin of a significant number of human AML cases. Previous studies have shown that T-cell-precursors such as thymic CD4/CD8 double negative cells, retain a robust myeloid differentiation capacity [38–40]. Such lymphoid progenitor-derived myeloid leukaemic cells still depend on this T-cell program, which simultaneously confers persistent myeloid/T-lymphoid plasticity [41, 42].

A second hypothesis proposed the induction of aberrant transdifferentiation by leukaemogenic mutations. For example, activation of NOTCH1 signaling in mouse bone marrow cells caused aberrant T-cell differentiation independent of thymic microenvironmental signals [37, 43]. Similarly, mutations inactivating *RUNX1* in haematopoietic progenitors can cause aberrant myeloid differentiation [44].

Another recent study proposed a molecularly distinct subtype of acute leukaemia with shared myeloid and T-cell lymphoblastic features, termed acute myeloid/T-lymphoblastic leukaemia (AMTL) [37]. This proposed diagnostic entity overlaps with early T-cell precursor (ETP) T-ALL and T- MPALs and also includes a subset of leukaemias currently classified as AML with aberrant expression of T-cell marker(s). The proposed classification of AMTL as a distinct entity would enable a more accurate prospective diagnosis and permit the development of improved therapies for patients who are inadequately treated with current approaches [37].

In summary, our data show that the expression of selected T-cell markers and CD56 is associated with both, favourable and unfavourable cytogenetic aberrations, molecular features, and several outcome measures in patients with AML. Aberrant expression of each individual T-cell marker probably is not sufficient to determine the prognosis and risk group for AML. Most likely, the prognostic impact can only be estimated in conjunction with additional molecular and cytogenetic features. Future studies on larger cohorts may help to compile sufficient numbers of T-cell marker positive AMLs to better define the prognostic role of individual and combinatorial marker positivity, and to unravel the molecular origins and targetable lesions of T-cell-marker positive AML.

AUTHOR CONTRIBUTIONS

KW and CS designed the study. IS and KW characterized the UKM cohort and re-analysed flow cytometry files. IS performed statistical studies and analysed the data. KW and CS contributed to data analysis. IS and KW wrote the manuscript. All authors collected flow cytometric and clinical patient data, interpreted the data and revised the manuscript.

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DATA AVAILABILITY STATEMENT

All data have been included in the manuscript. Further information may be obtained upon reasonable request to the corresponding author.

ETHICS STATEMENT

The study was approved by the Ethics Committee Westfalen-Lippe (2020-814-f-S).

PATIENT CONSENT STATEMENT

For this retrospective study, we received the local ethics approval statement, so no separate declaration of consent from the individual patient was required.

CLINICAL TRIAL REGISTRATION

The authors have confirmed clinical trial registration is not needed for this submission.

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