Prognostic significance of tetraspanin (CD81) expression in patients with acute lymphoblastic leukemia.

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INTRODUCTION Acute lymphoblastic leukemia (ALL) is a proliferation of lymphoid progenitor cells in the bone marrow, blood, and extramedullary sites. Risk stratification of ALL is based on age, white blood cell count, early therapeutic response, and minimal residual disease (MRD) which are found to play a role both in ALL prognosis and relapse prediction. Relapse remains a major obstacle to achieving a 100% overall survival rate, and relapsed B-ALL continues to be the leading cause of cancer-related death.

CD81 is an integral surface membrane protein that is involved in signal transduction in B cells, but its biological role in ALL is not well characterized. Relapse often results from the development chemoresistance. One of the mechanisms of chemoresistance involves ALL cell interactions with the bone marrow microenvironment. This is known as BM microenvironment induced chemoprotection , which is done by members of the tetraspanins family, mainly CD81 which are known to mediate microenvironmental interactions. Flow cytometry (FC) is a rapid, convenient, and generally applicable technique for detecting (MRD), it can differentiate leukemic cells from their normal immature Blineage counterparts, which are termed hematogones which are major obstacles in MRD detection, The ideal FC marker for detecting MRD in ALL should show a consistent pattern of expression in hematogones and an aberrant expression pattern in leukemic cells. and minimal overlap in expression between benign and leukemic cells, and its level of expression in leukemic cells should be stable over time .CD81 expression is aberrantly dim in a small series of ALL cases and this suggests the possible usefulness of CD81 expression in distinguishing ALL cells from hematogones, so it can be used as a minimal residual disease marker.

Aims So, this study aimed to measure the expression of CD 81 in adult ALL patients, to find the correlation between the marker and prognosis and survival in those patients, and the usefulness of using it as a marker for MRD detection and differentiating blast cells from its normal counterpart haematogones.

Settings and design: a prospective cohort study.

Patients and methods: This study done at Ain Shams University Hospital, Clinical Hematology, and Oncology Department, during the period between January 2021 and December 2022, with follow-up of the patients for 70 weeks. The study was carried out on 30 newly diagnosed B-ALL patients, at diagnosis and after induction chemotherapy (day 28).

Statistical analysis used: Data were tabulated and statistically analyzed using SPSS, version 20 (SPSS Inc., Chicago, IL).

INTRODUCTION

CD81 is an integral surface membrane protein that is a member of the tetraspanin family, it always has partners that vary according to cell type (e.g., CD19 in B cells to form a (CD19-CD21- CD81) multimolecular complex that is involved in signal transduction in B cells **[1].**

Although CD81 has been studied in some hematological malignancies such as multiple myeloma where its expression on plasma cells is associated with worse progression-free survival and overall survival, its prognostic value has not been addressed in acute lymphoid malignancies, such as ALL [2].

The prognosis of B-ALL remains dismal with relapse regardless of age, relapse remains a major obstacle to achieving a 100% overall survival rate, and relapsed B-ALL continues to be the leading cause of cancer-related death [3].

Risk stratification of ALL is based on age, white blood cell count, early therapeutic response, and minimal residual disease (MRD) which are found to play a role both in ALL prognosis and relapse prediction, key points of MRD monitoring and their clinical significance have been determined as well as MRD levels that allow detailed risk stratification of patients [4]

Relapse often results from the development of chemoresistance. One of the mechanisms of chemoresistance involves ALL cell interactions with the bone marrow microenvironment. This phenomenon is known as BM microenvironment-induced chemoprotection (BMC), which is done by members of the tetraspanins family mainly CD81 which are known to mediate microenvironmental interactions that have been extensively studied in solid tumors. Although the TSPAN family member CD81 is a minimal residual disease marker, its biological role in ALL is not well characterized [1], [5]So, this study aimed to measure the expression of CD 81 in adult B-ALL patients to find a correlation between this marker and prognosis and survival in those patients and to assess its role as a marker for MRD, in distinguishing the blast cells from its normal counterpart haematogones.

PATIENTS AND METHODS: this is a prospective cohort study that was carried out at the Ain Shams University Hospital, Clinical Hematology, and Oncology Department, during the period between January 2021 and December 2022, with follow-up of the patients for 70 weeks.

The study was carried out on 30 newly diagnosed B-ALL patients, at diagnosis and after induction chemotherapy (day 28).

Inclusion Criteria: • De novo B-ALL patients (no treatment panel started). • Ages >16 years.

Exclusion Criteria: • Patients out of age range • Patients with secondary B-ALL e.g., on top of MDS or CML, leukemic phase of NHL. • Biphenotypic leukemia. • Patients with concomitant autoimmune disease or other malignancy than B-ALL. • Relapsed B-ALL

l) Clinical examination and laboratory evaluation of patients at diagnosis: All the patients included in the study underwent the standard clinical examination (full history taking and physical examination), laboratory evaluation, and radiological studies including: 1. Full History Taking: with special emphasis on CNS manifestation. 2. Physical Examination: with special emphasis on lymph nodes (their nature, sizes, and distribution), and organomegaly. 3. Laboratory Investigations: a. Complete blood count using Sysmex XN1000, Abbot Diagnostics, USA with manual differential count using Leishman Stain. b. Liver and Kidney function tests using Integra 400 plus, Roche, USA. c. CSF cytology: for detection of CNS

disease. d. BM aspirate with morphologic examination. e. Cytogenetic analysis for karyotyping and Philadelphia chromosome. 4. Radiological studies: a. Chest X-ray, and C.T. for selected cases. b. Abdominal ultrasonography.

II) Flow cytometric analysis of patients at diagnosis and D28: Flow cytometric analysis was done at the Flow Cytometry Laboratory, Clinical Pathology Department, using a 10-color Navios flow cytometer Beckman colter, San Jose, CA, USA). Data was analyzed with CELL QUEST software). BM samples were obtained on EDTA vacationers (1-3ml) at diagnosis and D28. 1-At first diagnosis: -The routine panel was used for diagnosis in a stepwise manner: a primary panel for first identification whether it is myeloid, or B/ T lymphatic, and a secondary panel for cases diagnosed as B-ALL for further WHO subclassification. -CD81 expression was tested in the secondary panel. Monoclonal antibodies that were used in primary and secondary panels were as follows: -B-cell marker (CD19, CD20, CD79a). -T-cell marker (CD2, cytCD3, CD5, CD7, CD56). -Myeloid marker (CD33, CD13, CD14, CD117, CD64). -Others (CD34, HLA-DR-CD10, TDT, CD38, CD58, CD81). All reagents were obtained from Beckman Coulter (Hialeah, FL), except for CD81 was obtained from Dako. Proper instrument compensation and quality control were achieved using AutoSetup software, and flow-check and flow-set fluorospheres, respectively (Beckman Coulter, Brea, CA, USA). Previously titrated volumes of antibodies for surface and cytoplasmic staining of antigens were used. Sample Preparation and staining procedures: The whole blood staining method was performed first for surface markers and then cytoplasmic ones as follows: - Detection of Surface Markers by Direct Staining: • 12 X 75 mm plastic Falcon tubes were labeled with Lab no and the stained antibodies. • 10 µl monoclonal antibodies were dispensed into all appropriately labeled tubes. • 100-µl of the sample was added, and incubated in the dark for 20 minutes. • 3.5 ml lysing reagent was added to each tube, inverted once, and kept for 5 minutes. -Detection of Intracellular markers (Loken & Wells, 2000b): • 25 µl of paraformaldehyde as fixative was added (reagent 1), and incubated for 15 min • The tubes were centrifuged at 1500xg (3200 rpm) for 3 minutes. • The tubes were washed with 500 µl PBS, and the supernatant was discarded.• 25 µl of tween 20 as detergent (reagent 2) was added and incubated for 3 minutes. • 3.5 ml PBS was added, and centrifuged at 1500xg (3200 rpm) for 3 minutes.

• 10-µl Mo Ab was added for cytoplasmic intracellular staining and incubated in the dark for 20 minutes. • At the end of surface marker staining ± cytoplasmic staining, cells are re-suspended in 500 µl PBS and analyzed on a Flow Cytometer. -Events acquisition was continued to a minimum of 20,000 events, The cell population lies in a gate with low side scatter (SS) and dim CD45, the gated cells were analyzed and back gating was used to confirm their position in the forward scatter (FS). 2-The MRD panel at day 28 has been done including B-cell markers and immaturity markers based on the first diagnosis phenotype. -Events acquisition was continued to a minimum of 500,000 up to 1,000,000 events, to ensure gating viable events; continuously flowing events were selected using time as a gating parameter. - Leukemic events were defined at a dot plot in a predetermined region, the number of events was calculated by the machine, and at least 10 events should be present to be considered a cluster, sequential gating strategy was applied - Doublets were excluded in the forward scatter area/forward scatter peak plot and dead cells were eliminated by removing low forward scatter events. A dot plot of CD45/SSC was used to exclude granulocytes and restrict the gate to mononuclear cells. -The cell population with a primitive marker expression lies in a gate with low SS and dim. The panel that was used to establish the leukemic nature of the residual B cells and differentiate them from their normal counterpart (hematogones) included CD10PE, CD19PC5.5, CD20FITC, CD34PC7, CD38APC-A750, CD45ECD, CD58FITC, in addition to any aberrant marker detected at diagnosis (e.g., aberrant expression

of myeloid makers or T lymphoid markers). The intended strategy for identifying residual leukemic cells was to locate events that show abnormal maturation patterns, e.g., bright CD10, lack of CD20, abnormally bright CD34, abnormally bright CD58, heterogenous CD38 expression, or negative CD45 expression. The MRD was calculated by dividing the number of detected residual leukemic events by the number of total nucleated cells. A level of 0.01% was used as a positivity threshold for MRD. CD81 expression (using CD81APC) was evaluated on the residual leukemic events (on samples that proved to be MRD positive) and on hematogones, and was subjected to comparative analysis.

RESULTS:

This study included 30 adult patients with newly diagnosed de-novo B-ALL, the average age of the studied sample is 32.9 years old and ranges from (17 to 55) years old, and the percentage of males slightly more than females, the most common symptoms and signs in the studied patients were fever followed by lymphadenopathy then hepatosplenomegaly and bone pain, the mean Hb was 12 ± 21.8 (g/dL), the mean WBCs was 26.5 ± 30.9(x10^9/L), the mean platelets was 49.6 ± 67.8(x10^9/L) and the mean Peripheral blasts % was 54.2 ± 30,shows the Philadelphia chromosome in acute lymphoblastic patients, 15 patients were positive for the Philadelphia chromosome, 11 were negative for the Philadelphia chromosome, the study was not done for 4 patients, the immunophenotyping of 30 cases of Acute lymphoblastic leukemia, 25 cases was Pre-B-ALL, and 5 cases were common B-ALL.

Table (): The expression of CD81 initially at diagnosis N % CD81 Positive 30 100.0% Mean ± SD (minmax) Median (IQR) Percent of expression of CD81 97.8 ± 2 (93 - 100) 98 (97 - 100) MFI of CD81 23 ± 20.2 (2 – 102.

		N	%
CD81	**Positive**	30	100.0%
		Mean ± SD (min-max)	**Median (IQR)**
Percent of expression of CD81		97.8 ± 2 (93 - 100)	98 (97 - 100)
MFI of CD81		23 ± 20.2 (2 - 102)	17 (11 - 31)

		Survival (weeks)
Percent of expression of CD81 at diagnosis	**r**	.318
	P	.249
MFI of CD81 at diagnosis	**r**	-.121
	P	.668
MFI of CD81 on blast cells at D28	**r**	-.385
	P	.194

shows that there is no statistically significant correlation between the expression of CD81 and the survival of the patients.

		N	%
Fate	**dead**	15	50.0%
	alive	15	50.0%
Survival (weeks) *Mean ± SD (min-max)/ Median (IQR)*		26 ± 17.6 (4 - 68)	24 (10 - 30)
Outcome among live patients	**Complete remission**	9	60.0%
	Resistant	5	33.3%
	Relapsing	1	6.7%

Distribution of survival and outcomes in the studied acute lymphoblastic leukemia patients during the study period.

illustrates that half of the studied acute lymphoblastic leukemia patients died with survival ranging from (4 – 68) weeks. Among those who live, most of them had complete remission.

	Mean ± SD (min-max)	Median (IQR)
Percent of expression of CD38 on the blast cells	78.3 ± 21.3 (34 - 100)	87 (65 - 98)
MFI of CD38	10.4 ± 2 (7 - 15)	10 (9 - 12)
percent of expression of CD 58 on the blast cells	94.3 ± 11.4 (37 - 100)	98 (94 - 99)
MFI of CD 58	89.8 ± 11.9 (65 - 103)	90 (87 - 100)
Percent of expression of CD10 on the blast cells	92.2 ± 8.4 (78 - 100)	97 (85 - 99)
MFI of CD10 on blast cells	60.7 ± 12.8 (34 - 99)	70 (60 - 87)
Percent of expression of CD81 on the blast cells	70.3 ± 19.3 (29 - 100)	87 (65 - 98)
MFI of CD81 on the blast cells.	1.8 ± 2.2 (0 - 10)	1.6 (0 - 3)

Shows the immunophenotyping analysis at D28, and the immunophenotyping of these residual blast cells as described in the table regarding the expression of CD 81, CD38, CD10, and CD58

	Complete remission		resistant and relapsing		P
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	
Percent of expression of CD81	96.9 ± 2.7	98 (98 - 100)	98.7 ± 1.3	98 (98 - 100)	.202
MFI of CD81 at initial	19.5 ± 12.1	17 (10 - 23)	21.2 ± 18.3	17 (10 - 23)	.737
Percent of expression of CD38	79 ± 24.7	67 (65 - 98)	77.3 ± 17.4	67 (65 - 98)	.988
MFI of CD38	10.9 ± 2.6	10 (8 - 12)	9.7 ± 1.9	10 (8 - 12)	.502
Percent of expression of CD 58	90.1 ± 20.2	98 (95 - 99)	97.3 ± 2	98 (95 - 99)	.409
MFI of CD 58	87 ± 14.1	95 (87 - 99)	94 ± 6.8	95 (87 - 99)	.521

Percent of expression of CD10	94.4 ± 6.9	98 (85 - 100)	93.4 ± 8.3	98 (85 - 100)	463
MFI of CD10	69.8 ± 17.1	69 (65 - 80)	72.1 ± 9.5	69 (65 - 80)	.952
MRD after 1 month	0.004 ± 0.004	0.04 (0 - 0.7)	0.292 ± 0.362	0.04 (0 - 0.7)	.007 *
MFI of CD81 on blast cells	1.21 ± 1.25	1.76 (0 - 3.9)	1.919 ± 1.946	1.76 (0 - 3.9)	.619
MFI of CD81 on haematogones	116 ± 19.2	100 (94.5 - 150)	117.1 ± 27.1	100 (94.5 - 150)	.412

) shows that there is no statistically significant difference in all flow cytometry analysis results between those with complete remission, and those with resistant and relapsing outcomes except for MRD at D28, where there is a significant increase in those with resistant and relapsing outcomes.

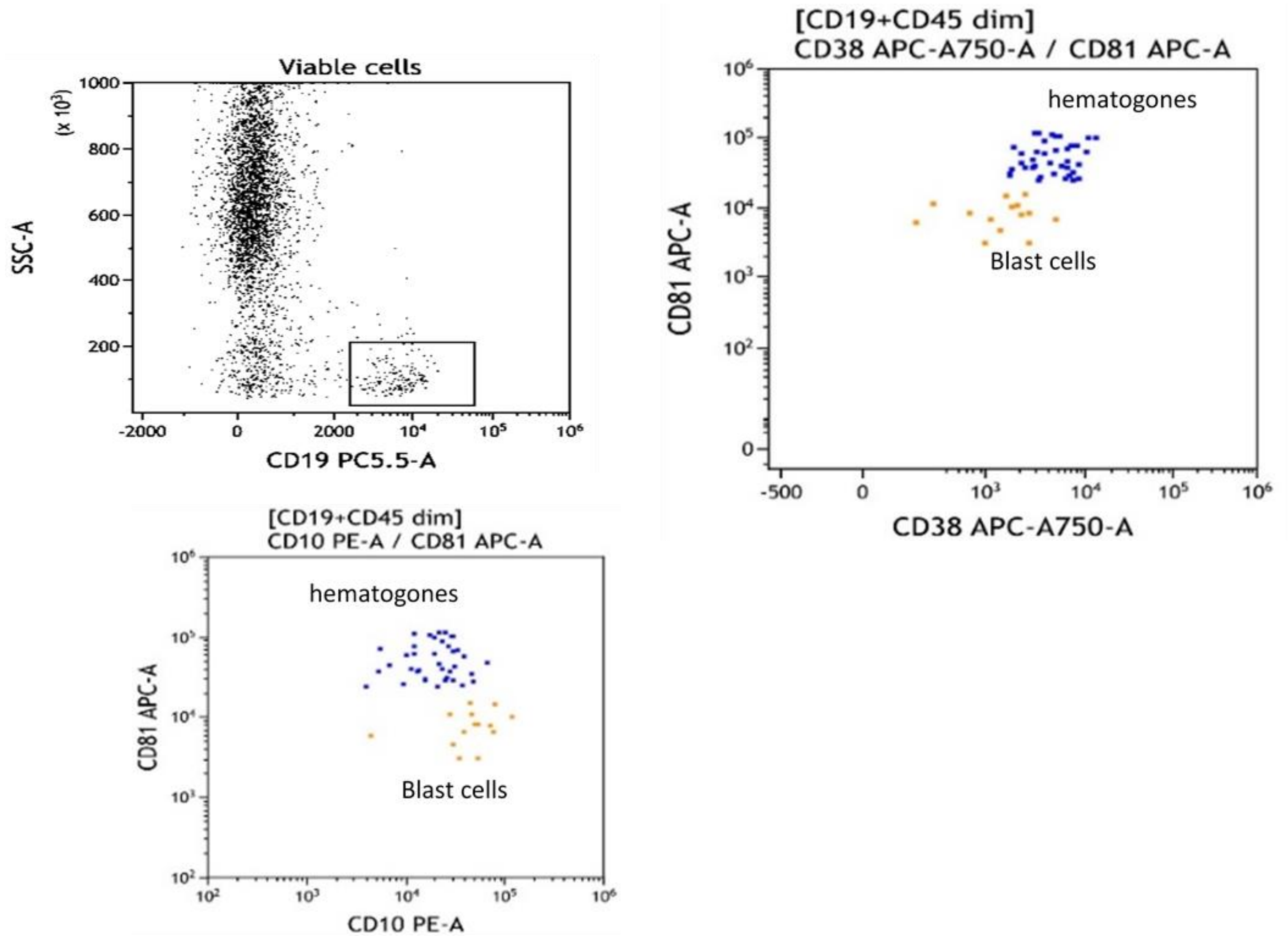

Fig (3): 3 dot plots showing the sequential gating for detection of minimal residual cells using CD19, CD10, CD81, CD38, and CD45.

DISCUSSION

In our study, CD81 was assessed at initial at first diagnosis, it was positive in all the studied cases, mean percent of expression of CD81 was 97.8 ± 2 SD (93 – 100) on the blast cells, with MFI at initial diagnosis was 23 ± 20.2 SD (2 - 102), with no correlation between the expression of CD 81 and another prognostic marker as the age of the patients, WBCs count, blast count, HB, or platelets count, there is no statistically significant difference in the percent of expression of CD 81 results between dead and alive patients, also there is no statistically significant correlation between the expression of CD81 and survival of patients, there was a non-significant difference between the MFI of CD81 on blast cells at initial and on MRD which was measured after one month, the same results was obtained by [6]

our result is contrary to the result obtained by **Hussein O., et al at 2020** who found that CD81+ expression has a potential role as a prognostic marker; where its expression has a higher incidence of relapse and decreased OS & DFS than patients with CD81- expression, but their work on different sample type may explain this, whereas their study was done on AML patients **[7]**

Also, the current study is contrary to a study done by **Boyer et al., 2016,** who found that Patients with AML expressing CD81 had elevated leukocyte count (P=0.02) and were more likely classified as intermediate or adverse risk (P<0.001**)[8]**

Clearance of leukemic cells from bone marrow at the end of the induction is an important factor in assessing the risk of disease relapse, The MRD can be detected by several techniques, including the MFC of aberrant immunophenotype. in ALL, detection of residual blast cells and its demarcation from normal counterpart hematogones is great challenge, in this part of our work, we measure CD81 after 1 month as an MRD marker, at the end of induction chemotherapy, and comparing its result with its expression on the hematogones which are the main obstacles in MRD measurement, with comparing these results with other markers for MRD CD38, CD58,and CD10.

In the current study, percent of expression of CD81 on the blast cells, the mean was 70.3 ± 19.3 (29 - 100), and the median was 87 (65 - 98), MFI of CD81 on the blast cells, the mean was 1.8 ± 2.2 (0 - 10), the median was 1.6 (0 - 3), that the mean MFI of CD81 on haematogones was 110.6 ± 25.5, this means that CD81 is good MRD marker in differentiating the blast cells from haematogones, this result near to

the results in the study done by **Muzzafar et al.,** Who found that the mean MFI of CD81 on haematogones was 114, also, he found CD81 expression to be aberrantly dim in a small series of ALL cases and suggested the possible usefulness of CD81 expression in distinguishing ALL cells from hematogones **[6]**

The same result was also obtained by **Nagant et al**., who found that CD81 was expressed on both hematogones and lymphoblasts, with 100% positivity for both populations but easily discrimination can be done by assessing the MFI for both, it was dimer in the blast than hamatogones **[9]**

Barrena et al also examined CD81 expression in a variety of B-lineage neoplasms as part of a broader study of tetraspanin molecule expression in benign and neoplastic B cells. They demonstrated uniformly high levels of CD81 expression in early-stage CD34+/CD10+ and late-stage CD34–/CD10+ hematogenous, he also found that CD81 was aberrantly under-expressed in 9 (75%) of 12 B-ALL cases in their series **[10]**

In the current study, there is a statistically significant difference between the MFI of CD38 level in hematogones cells and blast cells, this is near to the result obtained by **Muzzafar et al**., who found that CD38 expression was also easily interpretable and highly sensitive, with 89% of specimens positive for MRD demonstrating aberrant CD38 expression, this confirms that CD38 is another extremely useful marker for B-ALL MRD detection, with very similar properties to CD81 hematogones which show uniform bright expression, They showed that CD81 was complementary to CD38 in MRD detection, as more than half of the specimens with non-diagnostic CD38 levels showed aberrant dim CD81 expression and 127 (95.5%) of 133 positive specimens could be identified using a combination of CD81 and CD38 expression **[6]**, the same results obtained by Xia et al, who found that CD38 were under-expressed in lymphoblast versus hematogones **[11]**

In the present study, there is a statistically significant difference between the MFI of CD58 level in hematogones cells and blast cells, **also Veltroni et al.,** showed that CD58 expression was significantly higher in blasts than in hematogones, CD58 has expressed in 99.4% of B ALL cases and 93.5% of these showed over-expression compared to normal **[12]**

Nagant et al. found that 90% (35/39) of patients with B-ALL expressed CD58 and only 53% of subjects with hematogones expressed CD58. Additionally, they found that the intensity of CD58 expression by hematogones was significantly lower than on lymphoblast **(Nagant et al., 2018).**

This study shows that there is a statistically insignificant difference between the MFI of CD10 level in hematogones cells and blast cells which is near to the results in the study done by Chernysheva et al.,

who found no significant difference in the demarcation of hematogones from blast cells on using CD 10 as a single marker.

CD 81 is good MRD marker in the detection of the residual blast cells and differentiating it from normal hematogones, giving results near CD38, and CD58, it can be added to the MRD panel for ALL.

In the current study, there is no statistically significant difference in all flow cytometry analysis results between dead and alive patients. Also, there is no statistically significant correlation between the Percent of expression of flow cytometry analysis results and the survival of patients, in our study, there is no statistically significant difference in all flow cytometry analysis results between those with complete remission, resistant, and relapsing patients.

In conclusion,

- **CD 81 is a good MRD marker for the detection of the residual blast cells and differentiating it from normal hematogones, it can be added to the MRD panel for B-ALL,**
- **No role is detected as a prognostic marker in patients of B-ALL, it needs to be studied with large sample size for comprehensive assessment of its role.**

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