DISCUSSION

ALL is one of the most frequent childhood diseases in which there is malignant clonal proliferation of the progenitor lymphoid cells in the BM, with blocked differentiation at an early stage as a consequence of somatic mutations. These abnormal cells accumulate, supersede normal hematopoietic precursors, disrupt the normal BM cell distribution and finally appear in peripheral blood and they are capable of infiltration of extramedullary sites with disease progression. Moreover, ALL is a biologically heterogeneous disorder so that morphologic, immunologic, cytogenetic, biochemical, and molecular genetic characterizations of leukemia lymphoblasts are required to establish the diagnosis or to exclude other possible causes of BM failure and to classify ALL subtypes. This heterogeneity reflects the fact that leukemia may develop at any point of the multiple stages of normal lymphoid differentiation [32,119]. ALL can be classified into 4 groups: Pro-B-ALL, common B-ALL, pre-B-ALL and mature B-ALL [120]. B-ALL forms the majority of ALLs in children [121]. It represents 75% of all acute leukemias, with a peak incidence at 2 to 5 years of age [122].

Independent of the classical prognostic indicators like age, WBC count, immunophenotype, cytogenetic analysis and treatment response; MRD detected in early phases of therapy is shown to provide powerful prognostic information [82]. MRD corresponds to the very low number of cells remaining in the patient during or after the treatment; namely in the remission period and is not detectable by morphologic examination [77]. The morphologic threshold for unequivocal detection of residual disease in patients with precursor B-ALL is generally considered to be a blast count of 5% of total nucleated cells in bone marrow aspirate smears [59].

Flow cytometry is a rapid, convenient and generally applicable technique for detecting MRD in precursor B-ALL. It can be more than 100 times more sensitive than morphologic examination [79-81]. It is also used to differentiate lymphoblasts from their normal immature B-lineage counterparts, termed hematogones, due to the fact that they share morphologic and immunophenotypic features in particular the expression of CD10, CD34 and TdT, which could hamper MRD assessment in the regenerating phase occurring during chemotherapy and after treatment withdrawal, when normal precursors often expand [92,123]. The combination CD34/CD19/TdT/CD10 and CD38 is commonly used to differentiate these cell types, based on the difference in fluorescence intensity displayed by both. However, the immunophenotypic differences between hematogones and leukemic blasts in some cases may be subtle and extensive experience may be required for reliable interpretation of some antigens or for detecting very low level of disease [93].

MRD detection by flow cytometry depends on the presence of leukemia associated immunophenotype, which is based on the detection of aberrant expression of a number of antigens on lymphoblasts. This is very important especially in cases of common B-ALL (CD10+ve), in which there is major difficulty to differentiate blast cells from hematogones [59].

Studies to differentiate hematogones from blasts gained importance in recent years. However, because of the absence of specific and informative markers, normal lymphoid progenitors may be misinterpreted as MRD and the accuracy and reliability of FC-based

MRD measurement may be limited at BM regeneration phase [124]. The ideal FC marker for MRD detection in precursor B-ALL should show a consistent pattern of expression in hematogones and an aberrant expression pattern in leukemic cells in a high proportion of cases. There should be minimal overlap in expression between benign and leukemic cells and the level of expression in leukemic cells should be stable over time [93].

CD81 is an integral surface membrane protein that is a member of the tetraspanin family. In humans, CD81 is expressed in most tissues, including blood cells with the exception of erythrocytes and platelets [110]. CD81 forms a CD19-CD21-CD81 multi-molecular complex that is involved in signal transduction in B-cells [95, 96,125].

The aim of the present work was to study the expression of CD81 on leukemic blast cells of pediatric precursor B-ALL as compared to benign precursor B-cells (hematogones) and to evaluate its utility in the detection of minimal residual disease by FC (as extensive data regarding the use of this marker in MRD detection were not present in literature).

The current study was conducted on a total of 64 patients divided in to two groups.The first group included 32 patients diagnosed as precursor B -ALL, monitored at diagnosis and on day 28 after induction chemotherapy. Their ages ranged from 0.5 to 16 years with a median of 5 years and a male to female ratio of 1.2:1. The second group included 32 age and sex matched patients doing bone marrow aspiration for conditions other than hematological malignancies, whose bone marrows showed increased hematogones (ITP, postchemotherapy neuroblastoma and castleman's disease), which were confirmed by FC as controls. Moreno-madrid et al.[126] studied the presence of hematogones in the bone marrow of pediatric patients and defined them immunophenotypically by co-expression of CD10, CD38 and CD19 and by non-expression of surface immunoglobulins, these findings were consistent with our results. Moreover, they stated that hematogones were found in small concentrations in healthy people but their numbers were increased among children or adults undergoing various pathological processes as lymphomas, non neoplastic blood cytopenias, post-chemotherapy, post bone marrow transplant and HIV, being the most common in such situations [92,127], which explained and confirmed why we chose such population as control group.

As regards the hematological findings, serum LDH levels and uric acid levels in the present study, B-ALL cases at diagnosis showed a statistically significant difference ($p<0.001$) from the control group and from day 28 follow up BM specimens. This was attributed to adequate response to induction chemotherapy and a reduction in the tumor burden. Similarly, Hafiz et al.[128] and Fawzi et al.[129], found a statistically significant difference ($p<0.001$) between B-ALL cases at diagnosis and both the control group and follow up day 28 post-induction chemotherapy specimens, as regards their hematological findings and LDH levels. They also attributed that to effective induction chemotherapy and reduction in the tumor burden.

Immunophenotypically, common precursor B-ALL was the most commonly encountered subtype in our study constituting 50% of the cases followed by pre-B-ALL (31.3%) and the least frequently encountered subtype was pro-B-ALL (18.8%). Wenxiu et al. [130] stated that the most commonly encountered subtype in their study was also the common B-ALL subtype.

Regarding CD81 expression, we found that CD81 MFI was lower in leukemic blast cells of B-ALL cases than in hematogones of the control group, with a mean of 304.49 ± 202.18 and 2684 ± 502.1 respectively and the difference between them was statistically significant ($p<0.001$). Follow up BM specimens of all studied B-ALL cases based on the routine MRD panel were negative for residual B-ALL blasts and the TdT+/CD10+/CD19+ cells in these specimens were benign hematogones, which also showed bright CD81expression with a mean of 2434.11±309.16, like that of control specimens. This confirmed that CD81 showed bright expression on hematogones. We also screened CD81 expression on normal BM cells and found that hematogones had the brightest expression while lymphocytes, myeloid blasts and monocytes had moderate to weak expression and granulocytes had the weakest expression level of CD81.

In agreement with our findings were the findings reported by Muzzafar et al. [(93)] who studied the expression of CD81 by FC on benign precursor B-cells (hematogones) and leukemic blasts in precursor B-ALL and established its usefulness in MRD assays. They found that hematogones showed the brightest CD81 expression and leukemic blasts showed aberrantly decreased expression of CD81 and the difference between the range of MFI of immature hematogones and the range of MFI of leukemic blasts was statistically significant ($P<0.0034$). Moreover, they also mentioned that in cases which were negative for MRD benign immature hematogones showed bright CD81 expression. They also found similar findings to ours when they studied CD81 expression among normal BM cells.

Barrena et al. [(131)] characterized CD81 expression during normal B-cell maturation and 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+ hematogones. Moreover, CD81 was aberrantly underexpressed in nine (75%) out of twelve precursor B-ALL cases in their series.

Luo et al. [(103)] reported that FC of BM lymphoid cells showed that early B-cell progenitors expressed the highest level of CD81 and confirmed the lack of CD81 expression in other hematopoietic precursors which was consistent with our findings. They also mentioned that the differential expression of CD81 in lymphoid cell types in the bone marrow could potentially be exploited for diagnostic purposes in separating lymphoid from non-lymphoid acute and chronic leukemias and that this separation may be particularly useful in minimally differentiated leukemias, where other known immunophenotypic markers or cytogenetic abnormalities may be lacking.

In this context Muzzafar et al. [(93)] added that the explanation of aberrant CD81 underexpression in precursor B-ALL is still unknown. However, they suggested that the partially decreased CD81 expression in precursor B-ALL could affect CD19 signaling and/or the adhesion of leukemic blasts to the BM microenvironment.

The utility of tetraspanin molecules including CD81 in combination with other B-cell associated markers, not only for the diagnostic classification of B-cell neoplasias but also for the identification of aberrant phenotypes that could be used for monitoring of minimal disease levels, was also reported in several other studies (Sanchez et al, Lucio et al, Vidriales et al) [(132-134)].

As regards CD81 expression in other neoplastic hematological conditions, Luo et al.[103] reported that high levels of CD81 expression by FC was seen in diffuse large B-cell lymphoma (DLBCL) cases and in normal germinal centre B-cells, that could be used in the future in the risk stratification of these cases. In contrast, FC of plasma cell myeloma showed diminished expression of CD81 in myeloma cells. Zoller et al. [95] also mentioned that in some carcinomas, CD81 appeared to have anti-metastatic effects.

We further studied CD81 expression among different immunologic subtypes of B-ALL. We found that CD81 MFI was lowest in common B-ALL (with a mean of 243.80 ± 119.77) followed by pre-B-ALL (with a mean of 263.90 ± 104.78) and it was highest in pro-B-ALL (with a mean of 534.0 ± 335.17). There was a statistically significant difference between pro-B-ALL and common B-ALL (p=0.002) and between pro-B-ALL and pre-B-ALL (p=0.005). However, no statistically significant difference was found between common and pre-B-ALL. No definitive explanations were found for the different expression levels of CD81 among the different subtypes; however this could be attributed to the synergistic effect of CD81 with other markers.

We also found that CD81 MFI was lower among CD10 positive cases when compared to CD10 negative cases, with a mean of 251.53 ± 112.51 and 534.0 ± 335.17 respectively, however, the difference was not statistically significant. CD34 positive cases showed slightly higher CD81 MFI than CD34 negative cases, with a mean of 307.36 ± 220.02 and 294.26 ± 131.93 respectively, but the difference was also not statistically significant most likely owing to the relatively few samples studied.

In contrast to our findings Barrena et al. [131] reported that CD34 positive precursor B-ALL cases displayed a significantly reduced CD81 expression than CD34 negative cases. The discrepancy between our findings and that of Berrana et al. could be attributed to our small sample size.

In this context Muzzafar et al. [93] reported that the frequency of aberrantly decreased CD81 was higher among precursor B-ALL cases that were CD34 positive than among CD34 negative cases.

Luo et al. [103] measured CD81 and CD10 expression in cell suspensions from DLBCL tumor specimens by FC analysis and found that all tumor cells showed high levels of CD81 expression independent of the level of CD10 expression, although they mentioned that CD81 expression was regulated developmentally in a similar nature to CD10, being high in both early B-cell progenitors in the bone marrow and in the germinal center.

In the present work, two cases who were further followed up after day 28, revealed relapse after consolidation by routine FC analysis. On performing CD81, BM leukemic blasts revealed dim expression of CD81 (similar to that of the diagnostic specimens), which was confirmed by the conventional MRD panel, although the conventional morphological examination could not accurately specify that these cells were malignant blasts and not normal hematogones. This evidence highlights the specificity of CD81 in differentiation between normal hematogones and actual blast cells. The persistence of low CD81 MFI in these cases might reflect the stability of CD81 as a marker of neoplastic disease.

This was in agreement with Muzzafar et al [93] who analyzed CD81 expression in residual blasts in de novo cases, relapsed cases and various cases at the end of induction, after consolidation as well as during maintenance chemotherapy and found that in nearly all cases, leukemic blasts retained their aberrantly decreased expression of CD81 at all stages of therapy, which showed that MFI of CD81 expression was stable in 91% of their cases with sequential sampling.

An interesting finding in one of these relapsed cases was showing positivity for NG2 marker, which is an established marker of disease aggressiveness and could explain the cause of this relapse.

That findings were important to mention as several other studies (Van Lochem et al, Van Wering et al, Borowitz et al, Chen et al. [135-138]) reported that phenotypic shifts are well known to occur in precursor B-ALL with some markers between diagnostic and post-chemotherapy or relapse specimens on leukemic blasts and on background hematogones in up to 72% of cases.

One case was unexpectedly found to have high CD81 MFI at diagnosis (MFI=1304), it was thus decided not to include this case in the analysis in order not to disturb the homogeneity of our sample, but this case was thoroughly investigated and had no remarkable clinical or hematological findings. On immunophenotyping this case showed common B-ALL phenotype with aberrant expression of CD5 and was CD34 negative. However, this case died before ending the induction therapy so we weren't capable of follow up on day 28 post-induction chemotherapy. No definitive explanation could be found for this high CD81 MFI. However, this needs to be thoroughly investigated on a larger sample size to find out a definitive explanation for this result. In this context muzzafar et al. [93] reported that five cases had unexpectedly high CD81 MFI and three of them were negative for CD34.

In the present study there was no correlation between CD81 MFI on blast cells and different studied hematological parameters; as WBC count at diagnosis, blast counts in the peripheral blood or in the bone marrow. Although no data concerning the correlation of CD81 MFI on blast cells with any of these hematological parameters were found in literature, we could attribute these findings to the relatively few samples studied.

To improve the diagnostic utility of CD81 as a marker for MRD detection, we tried to establish a lower threshold for CD81 expression by hematogones, using ROC curve analysis. A value of 1134 for CD81 MFI was shown to have highest sensitivity and specificity for separation between leukemic blasts and hematogones, where hematogones had values greater than 1134; while blasts had values lower than or equal to 1134. This cutoff value in the present study was close to that established by Muzzafar et al [93]. Who established a cutoff value for CD81 MFI, in which blast cells of most cases had values equal to or less than 1000 and hematogones had values more than 1000. The results of ROC curve analysis in the present study can't be generalized, due to the insufficient sample size used and also because the random sampling method was not applied.

The results of the current study together with those of other relevant studies indicated that CD81 could be a robust marker for differentiating leukemic blast cells in precursor B-ALL from hematogones and could be a useful addition to the flow cytometric panels used for MRD assessment.

SUMMARY

Acute lymphoblastic leukemia (ALL) is a malignant clonal disorder which originates in a single B- or T-lymphocyte progenitor cell. This results in proliferation and accumulation of blast cells in the bone marrow (BM), which suppress hematopoiesis and disrupts the normal BM cell distribution, then appears in the peripheral blood. ALL represents approximately 80% of childhood leukemias among which precursor B-ALL represents 85%. ALL shows a sharp peak among children aged from 2 to 5 years with slight male predominance.

Conventional methods for diagnosis of ALL include mainly peripheral blood and BM examination together with immunophenotyping by flow cytometry. In addition to diagnosis, flow cytometric immunophenotyping (FCI) is used in minimal residual disease (MRD) detection as it is rapid, relatively sensitive and quantitative. MRD is defined as the lowest detectable level of disease in patients in complete clinical remission which can only be detected by highly sensitive techniques such as multi-parametric flow cytometry or PCR. MRD is considered the major predictor for relapse of leukemia. Evaluation of MRD can be problematic in conditions where hematogones are abundant in the BM; this is frequently seen in BM specimens of children after therapy of ALL. This difficulty originates from the close resemblance between hematogones and lymphoblasts as regards their morphological and immunophenotypic features.

Since leukemic blasts often show aberrant gain or loss of surface antigens or changes in the pattern or intensity of antigen expression giving rise to an immunophenotypic profile distinct from normal cells, the ideal FC marker for MRD detection should show a consistent pattern of expression on hematogones and an aberrant expression pattern on leukemic cells with minimal overlap in expression between both. In addition, the level of expression on leukemic cells should be stable over time.

CD81 known as TAPA-1, a member of the tetraspanin family, found on most human tissues except RBCs and platelets. It forms a CD19-CD21-CD81 multi-molecular complex that is involved in signal transduction in B-cells.

The aim of the present work was to study the expression of CD81 on leukemic blast cells of pediatric precursor B-ALL as compared to hematogones and to evaluate its utility in MRD detection by FC. The present study was conducted on a total of 64 patients divided into two groups. The first group included 32 patients diagnosed as precursor B-ALL, monitored at diagnosis and on day 28 after induction chemotherapy, while the second group included 32 age and sex matched patients doing BMA for conditions other than hematological malignancies; whose BMs showed increased hematogones, which was confirmed by FCI as controls. The selection of cases was based on their clinical features, morphology and immunophenotyping of the leukemic cells.

Analysis of CD81 expression by FCI was done for B-ALL patients at diagnosis and on day 28 post-induction to detect its usefulness in differentiating leukemic blasts from hematogones and in MRD detection. Moreover, its expression was compared among different subtypes of B-ALL, as well as for the control group.

It was found that CD81 MFI was lower in leukemic blasts of B-ALL cases than in hematogones of control group ($p<0.001$). With exception of the two cases that died during the induction phase of chemotherapy, all cases entered in complete remission on day 28, showing negative MRD and most of them had increased hematogones by the routine MRD panel. This was confirmed by high MFI CD81 on day 28 post-induction chemotherapy specimens. Beyond day 28 two relapsed cases showed persistently low CD81 MFI that might indicate its stability on leukemic blasts after chemotherapy. A statistically significant difference in CD81expression was found among different subtypes of B-ALL, in which common B-ALL showed the least expression. ROC curve analysis was performed in an attempt to establish lower threshold for CD81 expression by hematogones. A cutoff value of 1134 for CD81 MFI was found to have the highest sensitivity and specificity for separation between leukemic blast cells and hematogones, where leukemic blasts were ≤1134 and hematogones were >1134.

Based on the results of the present study, it can be concluded that CD81 could be a robust marker for differentiating leukemic blast cells in precursor B-ALL from hematogones and a useful addition to the flow cytometric panels used for assessment of MRD.