AIM OF THE WORK

To study CD81 expression on leukemic blast cells of childhood precursor B-acute lymphoblastic leukemia, as compared to benign B-cell progenitors (hematogones) and to evaluate its utility in minimal residual disease detection by flow cytometry.

PATIENTS

This study was conducted on:

1. Thirty two pediatric patients diagnosed as precursor B-acute lymphoblastic leukemia (at diagnosis and on day 28 post induction chemotherapy), presented to El Shatby children hospital and Alexandria main university hospital during the period between (22-2-2014 and 19-10-2014).

2. Thirty two patients doing bone marrow aspiration for conditions other than hematological malignancies, preferably those with increased hematogones (e.g., immune thrombocytopenic purpura, post-chemotherapy, some viral infections etc.) of matched age and sex as a control group.

Parents of all patients enrolled in the study were asked to sign an informed written consent before enrollment in the study after explaining the nature, steps and aim of the study. The approval of Medical Ethics Committee was obtained.

METHODS

All patients in the study were subjected to the following:

1- Full history taking including:

- Age.
- Gender.
- Presenting symptoms.
- Drug history.
- Medical history.
- Family History of leukemia or other malignancies.

2- Complete clinical examination including:

- Presence of general constitutional manifestations as fever, fatigue, bony aches and signs of bone marrow failure including pallor, purpura, ecchymosis or infection.
- Examination of the cervical, axillary and inguinal lymph nodes for the presence of lymphadenopathy.
- Abdominal examination to detect splenomegaly and hepatomegaly.

3- Abdominal Ultrasonography:

To assess the condition of the liver and spleen (for B-ALL cases only).

4- Routine laboratory investigations:

- **Complete blood count (CBC)** [112]**:** (for B-ALL cases and controls) venous blood samples were obtained under complete aseptic technique. About three mLs of venous blood were withdrawn from all subjects in lavender topped vacutainer blood collection tubes containing K_3 EDTA (Tri-Potassium ethylene diamine tetra acetic acid) for CBC. CBCs were performed on an automated cell counter ADVIA 2120 hematology system (Siemens Healthcare Diagnostics, Eschborn, Germany). Peripheral blood smears were spread, air dried, stained by Leishman's stain and microscopically examined to assess the peripheral differential blood cell counts, blast percentage and morphological examination [112]. The diagnosis of acute leukemia was primarily suspected by demonstrating the morphological characteristics of the blast cells if present peripherally, in addition to variable cytopenias. However, the definitive diagnosis was based on the detection of more than 20% blast cells in the bone marrow aspirate smears and flow cytometric immunophenotyping [112].

- **LDH and Uric acid [113]:** (for B-ALL cases and controls): Two mLs venous blood were withdrawn from all subjects in plain red topped vacutainer blood collection tubes to obtain serum samples for measurement of LDH and uric acid levels. These tests were automatically performed by the Chemistry auto analyzer Dimension RxL Max (Siemens Healthcare Diagnostics, USA) [113].

5- Bone marrow aspiration and examination [114]:

- Bone marrow aspiration was done for all cases and controls using Klima needle. Aspiration was done from the anterior or posterior superior iliac spines.

- Air-dried films from bone marrow aspirate specimens were stained by Leishman's stain and microscopically examined for morphological evaluation:

  - For B-ALL cases, the diagnosis was primarily based on the presence of 20% or more blast cells in the bone marrow aspirate (BMA) smears with the morphological characteristics of lymphoblasts, but the definitive diagnosis was established by flow cytometric immunophenotyping.

  - For the controls, BMA smears were microscopically examined for the presence of increased hematogones with their characteristic morphology [114].

6- Immunophenotyping by flow cytometry [115]:

The detailed characterization of hematopoietic cells is obtained by analyzing the expression of a given set of antigens in a cell population. In the present study, direct immunofluorescence staining was employed using specific directly conjugated fluorochrome-labeled monoclonal 28antibodies (McAbs) according to the manufacturer instructions. Immunophenotyping was done using **Becton Dickinson, FACSCalibur flow cytometer equipped with BD CellQuest Pro software (BD biosciences, San Jose, CA, USA)**[115]. Figure (13)

Figure (13): Becton Dickinson, FACSCalibur flow cytometer equipped with BD CellQuest Pro software.

A) Immunophenotyping for B-ALL cases at diagnosis [115]:

The initial diagnostic BMA specimens from all suspected acute leukemia cases were subjected to flow cytometric immunophenotyping (FCI) of the leukemic blast cells using the routine laboratory panel of monoclonal antibodies for the diagnosis of acute leukemia. This panel included [115]:

- A primary panel to distinguish AML from ALL and classify ALL in to B- or T-lineage. It comprised the following:
  - B-lymphoid markers: CD19.
  - T-lymphoid markers: CD2 and CD7.
  - Myeloid markers: CD13, CD33 and CD14.
  - Non-lineage specific markers: CD45, HLA-DR, CD34 and CD10.
- A second set of monoclonal antibodies was used to confirm the diagnosis of B-ALL in equivocal cases (Cyt CD22) or to further classify B-ALL in to various subtypes (Cyt μ).

B) Immunophenotyping for B-ALL cases on day 28 post-induction chemotherapy for MRD detection [115]:

Follow up BMA samples from B-ALL cases on day 28 post-induction chemotherapy were analyzed by FCI for MRD detection, using the routine laboratory panel for MRD detection that comprise the following McAbs: TDT, CD10, CD19, CD34 and CD38. This combination was shown to be quite pertinent to differentiate residual leukemic blasts from hematogones, based on the difference in fluorescence intensity displayed by both. According to immunophenotypic results, MRD was considered negative if ($< 0.01\%$ of the total BM mononuclear cells) and positive if ($\geq 0.01\%$) [115].

C) Immunophenotyping for controls for detection of hematogones [115]:

BMA samples from all controls were analyzed by FCI using the following panel of monoclonal antibodies: TDT, CD10, CD19, CD34 and CD38 to confirm the presence of hematogones by expressing the typical continuous spectrum of antigen expression that defines the normal B-cell maturation [115].

D) Analysis of CD81 expression on leukemic blasts of B-ALL and hematogones by flow cytometry [116]:

CD81 expression was analyzed on blast cells of BMA specimens of B-ALL cases at diagnosis and on day 28 post-induction chemotherapy and on hematogones in the controls BMA specimens by FC [116].

- Reagents and Equipments:

1. Lysing solution:

0.037 g Na EDTA + 1.0 g Potassium Bicarbonate ($KHCO_3$) + 8.3 g Ammonium Chloride (NH_4Cl) in 1 liter distilled water.

2. Phosphate Buffered Saline (PBS):

Obtained in the form of tablets. (Gibco BRL, USA, cat No.18912-014). It is formed of:

- Potassium dihydrogen Phosphate: KH_2PO_4 0.1 mol/L
- Di sodium monohydrogen Phosphate: Na_2HPO_4 0.1 mol /L

One tablet was dissolved in 500 mLs distilled water to give pH 7 and was thus ready to use.

3. Monoclonal Antibodies (McAbs):

The following McAbs were used in relevant combinations for the diagnosis of B-ALL, MRD detection and hematogones detection as described before. The McAbs were conjugated with Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) (Table 5).

Table (5): Panel of McAbs used in the present study [115]

MoAbs	Type of antigen	Clone	Cat.No	Manufacturer
CD2 (PE)*	Cell surface	TP1/31	R300712	Immunostep,Spain
CD7 (FITC)*	Cell surface	7PE-100T	F0826	Immunostep,Spain
CD10 (PE)	Cell surface	SS2136	R0848	DAKO,Denmark
CD19(PE/FITC)	Cell surface	HD37/TC669	R0808/TC66901	DAKO,Denmark
CD14 (PE)	Cell surface	47-3D6	14F-100T	Immunostep,Spain
CD13 (FITC)	Cell surface	WM15	F215631	Immunostep,Spain
CD33 (PE)	Cell surface	HIM3-4	R270412	Immunostep,Spain
HLA-DR (FITC)	Cell surface	GRB-1	F160512	Immunostep,Spain
CD34 (PE)	Cell surface	BIRMA-K3	P7238	DAKO,Denmark
CD38 (FITC)	Cell surface	T16	F7053	Beckman Coulter,CA,USA
CD45 (FITC)	Cell surface	5B1	F130080202	Immunostep,Spain
TdT (FITC)	cytoplasmic	TdT-6	F332789	BD Biosciences,CA,USA
Cyt μ (PE)	cytoplasmic	UHB	P733156	Beckman Coulter, CA,USA
Cytoplasmic CD22 (FITC)	cytoplasmic	HIB22	F114495	Immunostep,Spain

CD81 (FITC)	Cell surface	JS-81	F551108	BD Biosciences, CA,USA

4. Falcon tubes: (BD Biosciences) (Ref 352052):

5 mLs polystyrene round – bottom tube, 12×75 mm style.

5. Flow cytometer:

BD, FACSCalibur flow cytometer equipped with BD CellQuest Pro software (BD biosciences, San Jose, CA, USA).

-Sample preparation:

- For B-ALL cases at diagnosis and controls; EDTA anti-coagulated BMA samples were used.

- For follow up specimens; BM monoclonal cells were separated using **Ficoll-Hypaque Density gradient centrifugation method as follows[115]:**

Three mLs of EDTA anti-coagulated BMA specimens were diluted with an equal volume of phosphate buffered saline (PBS). With a sterile pipette, three mLs Ficoll solution were placed in to a 15 mLs conical centrifuge tube and the diluted BMA (BMA/PBS mixture) was slowly layered over the Ficoll solution, then centrifuged for 30 minutes at 2000 rpm at room temperature, this results in three visible layers: a top layer of plasma, an interphase layer of mononuclear cells, the Ficoll layer and then a layer of (red cells, neutrophils and some platelets) forming a pellet at the bottom. The plasma was then removed and the mononuclear cell layer was placed into another centrifuge tube. Excess PBS (approximately three times the volume of mononuclear cell layer) was added to the mononuclear cell layer and mixed by gentle pipetting, then centrifuged at 2000 rpm for 10-15 minutes. The supernatant was removed and the cells were re-suspended in PBS, then the washing step was repeated once more.

- Procedure for CD81 staining:

- For analysis of CD81 in the initial diagnostic BMA specimens, the antibody combination used was CD81-FITC and CD34-PE. In cases that were negative for CD34, CD81-FITC was combined with CD10-PE.

- For the follow up and control specimens the antibody combination was CD81-FITC and CD10-PE.

Staining was performed, using a stain lyse wash technique, according to the manufacturer instructions as follows:

1. 100 µLs of EDTA anti-coagulated BM or BM mononuclear cells (containing about $1x10^6$ BM cells) were added to a Falcon tube.

2. 10 µLs of each antibody in the relevant combination (as described above) were added, mixed well by vortexing and incubated for 15-20 minutes in the dark at room temperature.

3. After incubation, the cells were washed twice with PBS (centrifugation was done at 2000 rpm for 2 minutes each at room temperature).

4. Two mLs lysing solution were added to each sample, mixed gently with a vortex mixer and incubated for 10 minutes at room temperature in the dark.

5. The tubes were then centrifuged for two minutes at 2000 rpm and the supernatant was discarded leaving approximately 50 µLs of fluid.

6. The cells were then washed twice with PBS as mentioned before. The supernatant was gently aspirated and discarded; leaving approximately 50µLs of fluid.

7. The pellet was resuspended in 0.3 mLs PBS for flow cytometric analysis. The flow cytometric analysis was performed immediately or the cells were fixed in 0.3 mLs 1% paraformaldehyde in PBS and stored in the dark at 2-8ºc until analysis, which was done within 24 hours maximally after staining.

8. A negative control tube of unstained cells, submitted to all the previous steps except the staining one was included with each sample to compensate for non-specific background of auto-fluorescence and to distinguish between fluorescent positive and fluorescent negative cell populations.

- Acquisition and data analysis:

Data were acquired on BD FACSCalibur flow cytometer using CellQuest Pro software.

For the initial diagnostic specimens and control cases, $1x10^4$ events were acquired, whereas for the follow up specimens $2x10^5$ events were acquired.

Gating was done around the leukemic blasts on the forward versus side scatter plots in the initial diagnostic specimens, while in the follow up and control specimens gating was done around the mononuclear cells on the forward versus side scatter plots. MFI of CD81 expression was determined on leukemic blasts and hematogones (on CD81 versus CD34 or CD10 two-parameter plots).

Statistical analysis of the data: [117]

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 [118]. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test or Monte Carlo

correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between the two studied groups was done using independent t-test. For abnormally distributed data, comparisons between two independent populations were done using Mann Whitney test while Kruskal Wallis test was used to compare between different groups and pair wise comparison was assessed using Mann-Whitney test. Correlations between two quantitative variables were assessed using spearman coefficients regarding normality of the data. Significance of the obtained results was judged at the 5% level.

Receiver operating characteristic curve (ROC) was plotted to analyze a recommended cutoff and to establish a lower threshold for normal expression of CD81 that can be used to differentiate leukemic blast cells from hematogones.

- Sensitivity is defined as; the fraction of people with the disease that the test correctly identifies as positive (True Positive percentage).
- Specificity is defined as; the fraction of people without the disease that the test correctly identifies as negative (True negative percentage).

RESULTS

The present study was conducted on thirty two pediatric patients with precursor B-ALL, monitored at diagnosis and on day 28 post-induction chemotherapy. Thirty two age- and sex-matched individuals performing bone marrow aspiration for conditions other than hematological malignancies, whose bone marrows showed increased hematogones were included in this study as a control group.

The control group included; 25 patients (78.12%) diagnosed as idiopathic thrombocytopenic purpura (ITP), two (6.25%) diagnosed as hemophagocytic syndrome (non-malignancy associated), three (9.37%) were in the post-chemotherapy phase of neuroblastoma, while only one patient (3.12%) had histiocytosis and another patient had castleman's disease (Table 6 and figure 14).

Table (6): The diagnoses of the controls enrolled in the present study (n = 32)

Diagnosis	No.	%
ITP	25	78.12
Hemophagocytic syndrome (non-malignancy associated)	2	6.25
Post-chemotherapy neuroblastoma	3	9.37
Histiocytosis	1	3.12
Castleman's disease	1	3.12

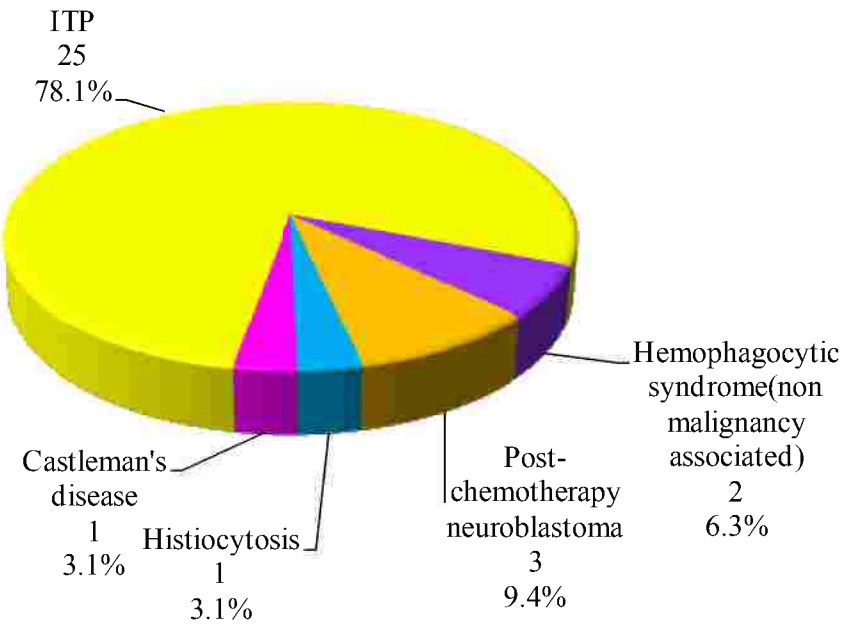


Figure (14): The diagnoses of the controls enrolled in the present study

Personal Data:

B-ALL cases group included 18 males and 14 females. Their ages ranged between 0.5-16 years with a mean of 6.66 ± 4.62, whereas the control group included 21 males and 11 females, whose ages ranged from 1.0-15 years with a mean of 5.11 ± 3.40 (Table 7 and figure 15). There were no statistically significant differences between the two groups regarding age and sex.

Table (7): Comparison between the two studied groups (B-ALL cases at diagnosis and controls) according to demographic data.

	B-ALL Cases (n = 32)		Controls (n = 32)		Test of sig.	P
	No.	%	No.	%		
Sex						
Male	18	56.3	21	65.6	χ^2= 3.090	0.079
Female	14	43.8	11	34.4		
Age (years)						
Min. – Max.	0.50 – 16.0		1.0 – 15.0		t = 1.527	0.132
Mean ± SD.	6.66 ± 4.62		5.11 ± 3.40			
Median	5.0		4.0			

χ^2: Chi square test
t: Student t-test

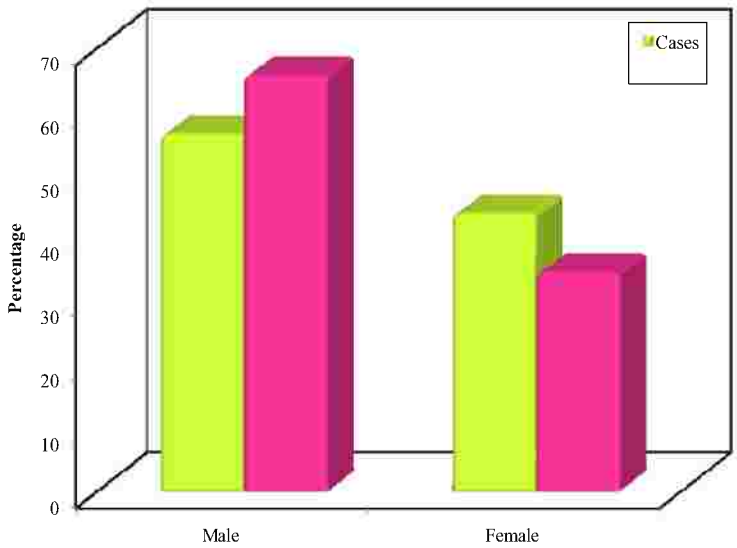


Figure (15): Comparison between the two studied groups (B-ALL cases at diagnosis and controls) according to sex

Clinical features of B-ALL cases at diagnosis:

Fever was the most frequently encountered clinical manifestation (93.8%), followed by fatigue (90.6%), then bone aches and pallor (87.5%), while purpura was the least frequently encountered clinical manifestation (40.6%). On examination, lymphadenopathy and splenomegaly were found in 27 cases (84.4%), whereas hepatomegaly was found in 14 cases only (43.8%) (Table 8 and figure 16).

Table (8): Distribution of the studied B-ALL cases at diagnosis according to their clinical presentations (n = 32)

Clinical presentation	No.	%
Fever	30	93.8
Fatigue	29	90.6
Bone aches	28	87.5
Pallor	28	87.5
Lymphadenopathy	27	84.4
Splenomegaly	27	84.4
Hepatomegaly	14	43.8
Purpura	13	40.6

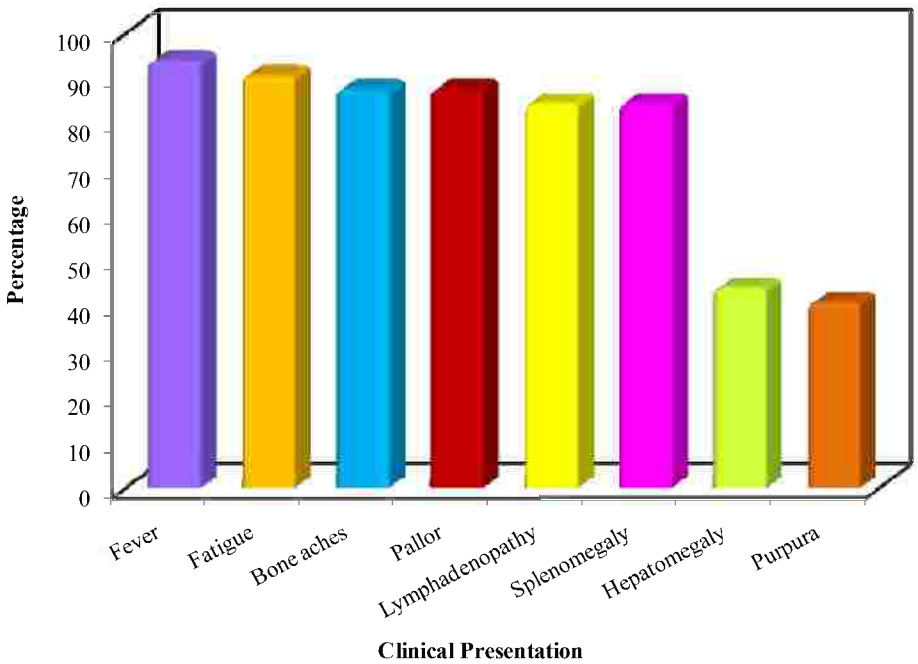


Figure (16): Distribution of the studied B-ALL cases at diagnosis according to clinical presentations

Hematological Profile:

A) B-ALL cases at diagnosis:

B-ALL cases at diagnosis had WBC counts ranging from 1.76 to 162.8 ($\times 10^9$/L) with a mean of 27.51±35.7 ($\times 10^9$/L), hemoglobin levels ranging from 3.8 to 10.5 g/dl with a mean of 6.59±1.86 g/dl and platelet counts ranging from 10 to 315 ($\times 10^9$/L) with a mean of 62.44±68.21 ($\times 10^9$/L). As regards their bone marrow blast percentage, it ranged from 61 to 98 % with a mean of 88.03±8.39%. The blast percentage in the peripheral blood ranged from 2 to 90% with a mean of 49.84±30.95%.

B) Control group:

The control group had WBC counts ranging from 4.28 to 14.4 ($\times$109/µl) with a mean of 8.25 ± 2.67 ($\times 10^9$/L), hemoglobin levels ranging from 5.90 - 15.20 g/dl with a mean of 10.68 ± 1.61 g/dl and platelet counts ranging from 5.0 - 783.0 ($\times 10^9$/L) with a mean of 66.59 ± 151.06 ($\times 10^9$/L). As regards their bone marrow blast percentage, it ranged from 1.0 to 4.0 % with a mean of 2.76 ± 1.13 %. None of the controls had blast cells in the peripheral blood.

All hematological parameters except platelet counts showed a statistically significant difference ($p < 0.001$) from those of the control group (Table 9 and figures 17-19).

Table (9): Comparison between the hematological profiles of B-ALL cases at diagnosis and that of the control group

	B-ALL cases at diagnosis (n = 32)	Controls (n = 32)	Test of sig.	p
WBCs ($\times 10^9$/L)				
Min. – Max.	1.76 - 162.80	4.28 - 14.40	Z = 2.981*	0.003*
Mean ± SD.	27.51 ± 35.70	8.25 ± 2.67		
Median	13.25	7.48		
Hb (g/dL)				
Min. – Max.	3.80 - 10.50	5.90 - 15.20	t= 9.418*	<0.001*
Mean ± SD.	6.59 ± 1.86	10.68 ± 1.61		
Median	6.15	10.85		
Platelets ($\times 10^9$/L)				
Min. – Max.	10.0 - 315.0	5.0 - 783.0	t= 0.142	0.888
Mean ± SD.	62.44 ± 68.21	66.59 ± 151.06		
Median	39.50	20.50		
Bone Marrow Blasts (%)				
Min. – Max.	61.0 - 98.0	1.0 – 4.0	Z = 6.895*	<0.001*
Mean ± SD.	88.03 ± 8.39	2.76 ± 1.13		
Median	90.50	3.0		

Z: Z for Mann Whitney test
t: Student t-test
*: Statistically significant at $p \leq 0.05$

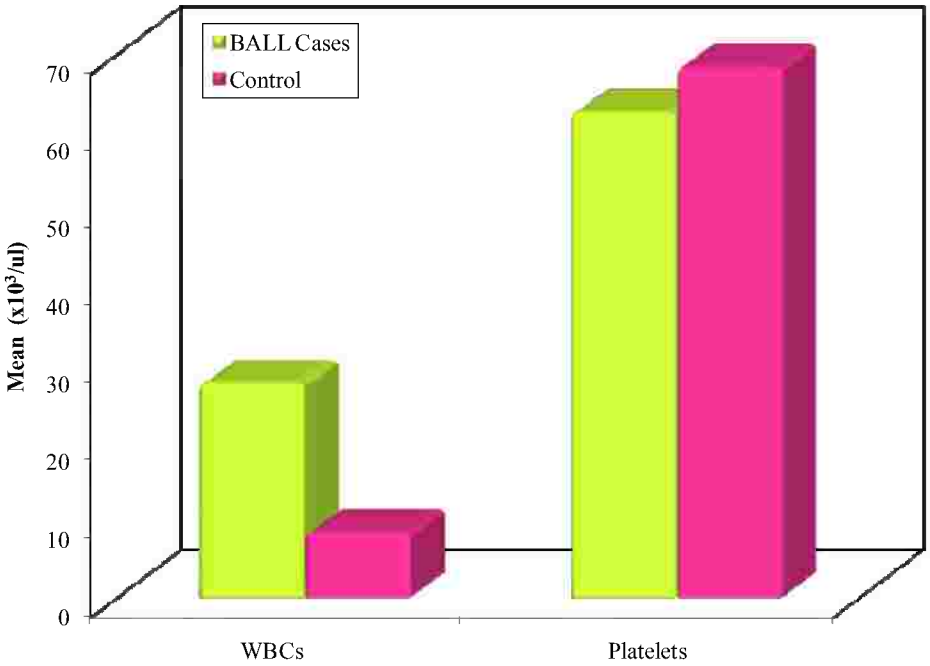


Figure (17): Comparison between B-ALL cases at diagnosis and controls according to WBC ($\times 10^9$/L) and platelet ($\times 10^9$/L) counts

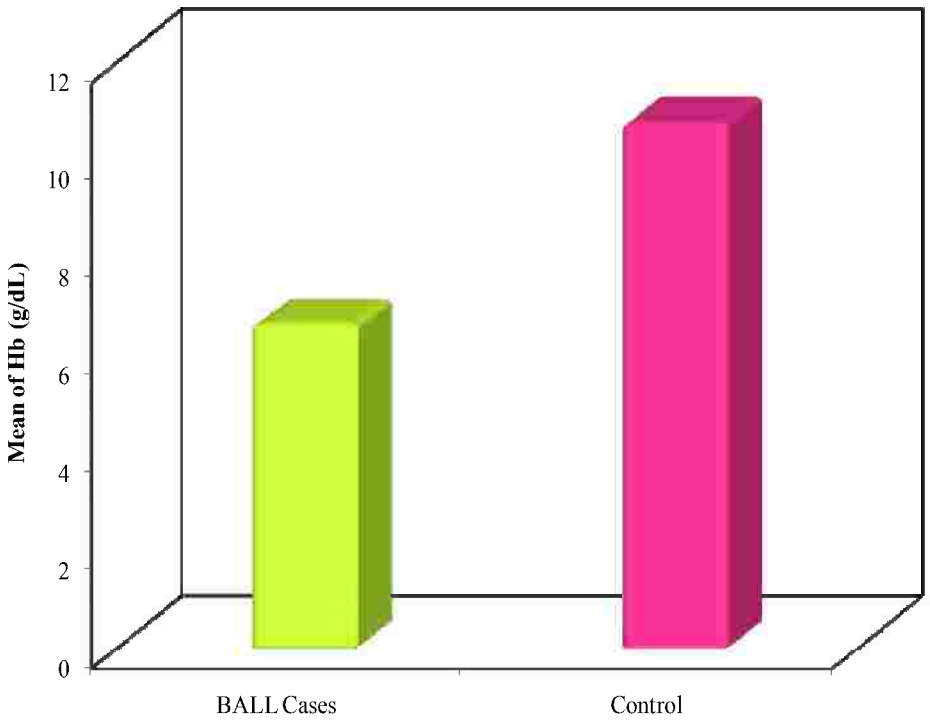


Figure (18): Comparison between B-ALL cases at diagnosis and controls according to hemoglobin concentration (g/dl)

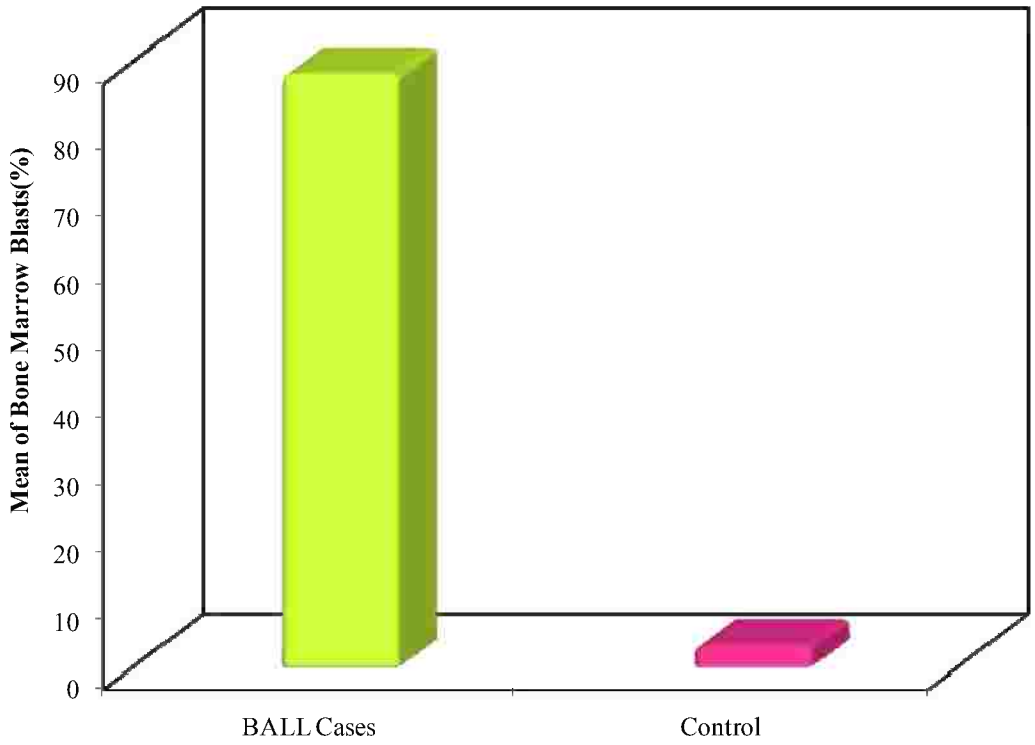


Figure (19): Comparison between B-ALL cases at diagnosis and controls according to bone marrow blast percentage (%)

C) B-ALL cases on day 28 post-induction chemotherapy:

Follow up of B-ALL cases on day 28 post-induction chemotherapy showed WBC counts ranging from 2.11-10.8 ($\times 10^9$/L) with a mean of 5.73±1.91 ($\times 10^9$/L), hemoglobin levels ranging from 6.5-12 g/dl with a mean of 9.96±1.35 g/dl and platelet counts ranging from 143-650 ($\times 10^9$/L) with a mean of 310.6±97.76 ($\times 10^9$/L). As regards their bone marrow blast percentage, it ranged from 1.0-4.0% with a mean of 2.53±0.86%.

There were statistically significant differences ($p<0.001$) between B-ALL cases at diagnosis and follow up day 28 post-induction chemotherapy regarding all hematological parameters (Table 10 and figures 20-22).

Table (10): Comparison between the hematological profile of B-ALL cases at diagnosis and on day 28 post-induction chemotherapy

B-ALL Cases	At diagnosis (n = 32)	Follow up day28 (n = 30)#	Test of sig.	p
WBCs ($\times 10^9$/L)				
Min. – Max.	1.76 - 162.80	2.11 – 10.80	Z = 4.403*	<0.001*
Mean ± SD.	27.51 ± 35.70	5.73 ± 1.91		
Median	13.25	5.55		
Hb (g/dL)				
Min. – Max.	3.80 - 10.50	6.50 – 12.0	t= 8.108*	<0.001*
Mean ± SD.	6.59 ± 1.86	9.96 ± 1.35		
Median	6.15	10.15		
Platelets ($\times 10^9$/L)				
Min. – Max.	10.0 - 315.0	143.0 – 650.0	t= 11.652*	<0.001*
Mean ± SD.	62.44 ± 68.21	310.60 - 97.76		
Median	39.50	305.0		
Bone Marrow Blasts (%)				
Min. – Max.	61.0 - 98.0	1.0 – 4.0	Z = 6.808*	<0.001*
Mean ± SD.	88.03 ± 8.39	2.53 ± 0.86		
Median	90.50	2.50		

Z: Z for Mann Whitney test
t: Student t-test
*: Statistically significant at $p \leq 0.05$
#: Two cases died during induction chemotherapy

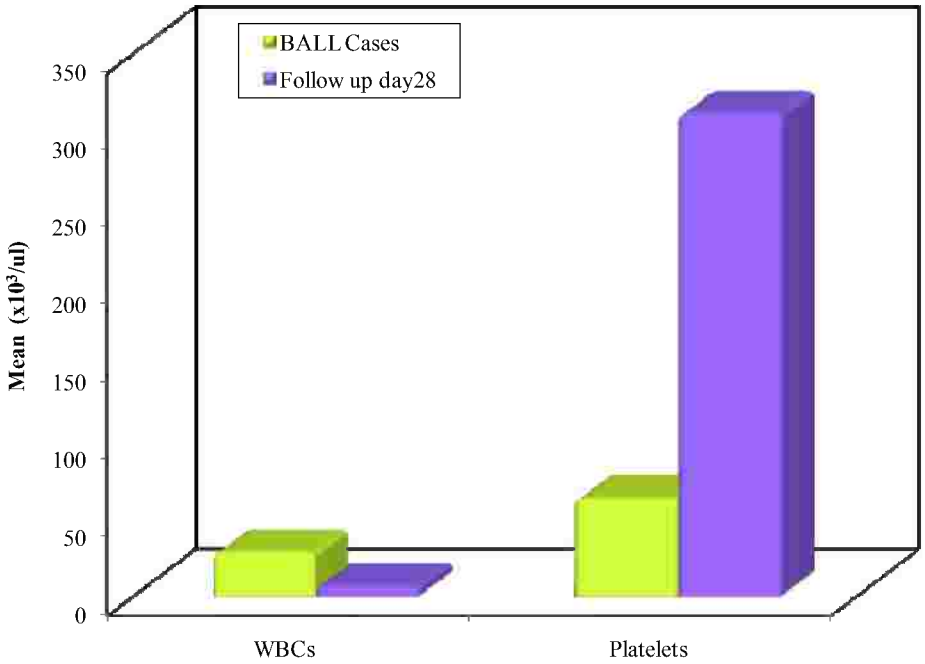


Figure (20): Comparison between B-ALL cases at diagnosis and on follow up day 28 post-induction according to WBC ($\times 10^9$/L) and platelet counts ($\times 10^9$/L)

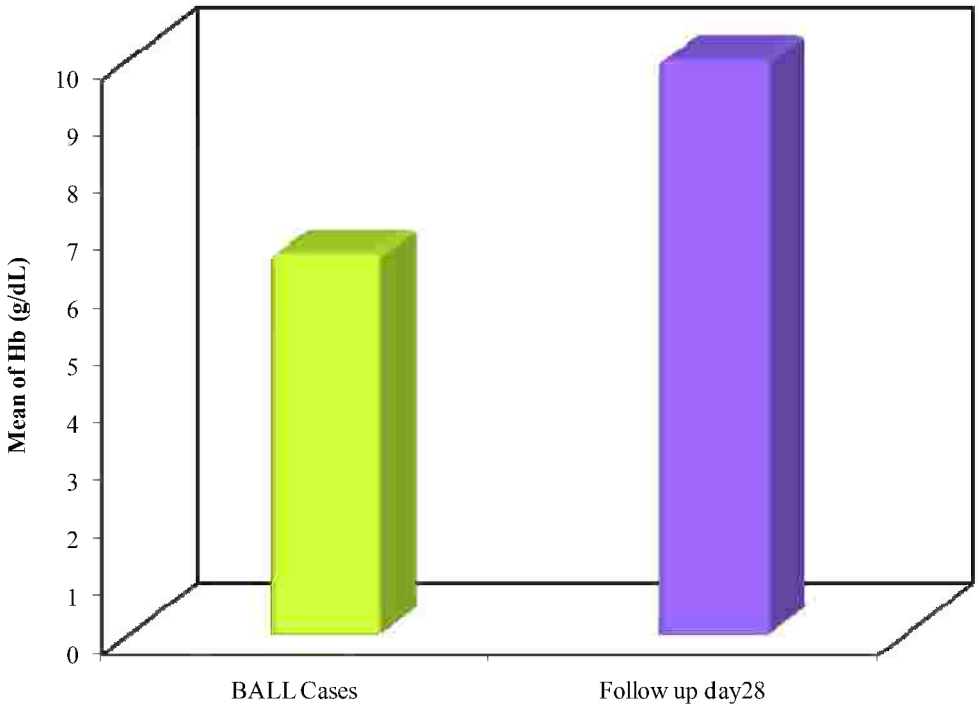


Figure (21): Comparison between B-ALL cases at diagnosis and on follow up day 28 post-induction according to hemoglobin concentration (g/dl)

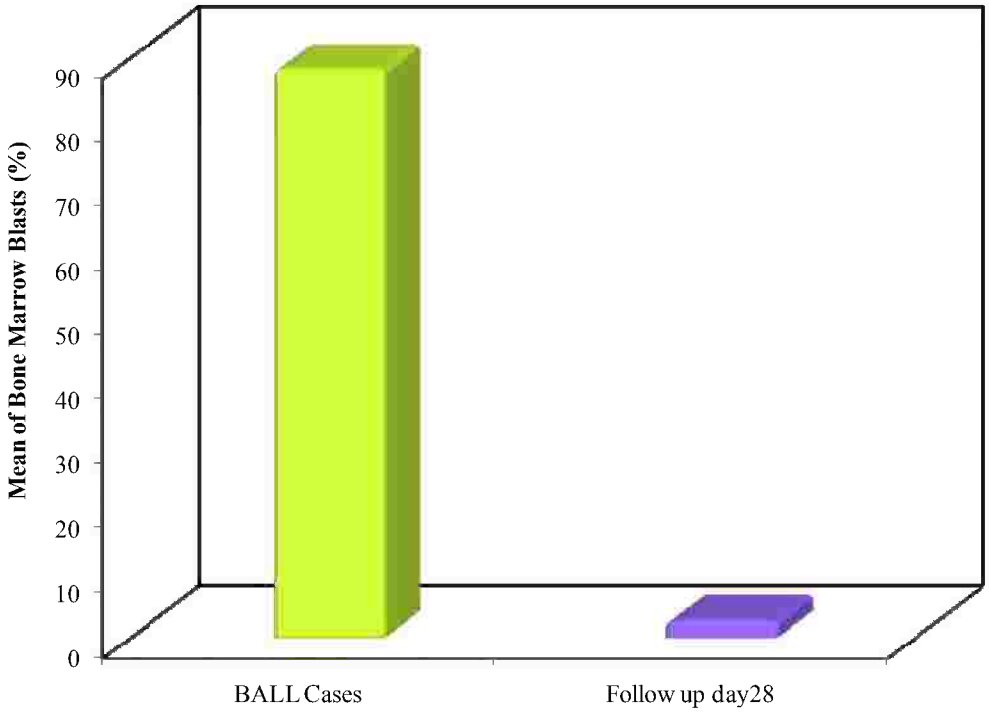


Figure (22): Comparison between B-ALL cases at diagnosis and on follow up day 28 post-induction according to bone marrow blast percentage (%)

Blood Chemistry:

Uric acid and lactate dehydrogenase (LDH) were done for B-ALL cases (at diagnosis and on day 28 post-induction chemotherapy) and for the control group (Tables 11-12 and figures 23 and 24).

Uric acid and LDH levels were significantly higher ($p<0.001$) in B-ALL cases at diagnosis when compared to both control group and day 28 samples.

Table (11): Comparison between the two studied groups (B-ALL cases at diagnosis and controls) according to uric acid (mg/dl) and lactate dehydrogenase (LDH) (IU/L)

	B-ALL Cases (n = 32)	Controls (n = 32)	Test of sig.	p
Uric acid (mg/dl)				
Min. – Max.	6.50 - 19.0	4.0 – 7.0	t= 9.341*	<0.001*
Mean ± SD.	10.68 ± 3.19	5.28 ± 0.72		
Median	9.60	5.15		
LDH (IU/L)				
Min. – Max.	280.0 - 1170.0	259.0 – 450.0	Z = 4.822*	<0.001*
Mean ± SD.	535.84 ± 200.52	365.06 ± 46.60		
Median	450.0	374.50		

t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table (12): Comparison between the two studied groups (cases at diagnosis and follow up 28 day) according to uric acid (mg/dl) and LDH (IU/L)

B-ALL cases	At diagnosis (n = 32)	Follow up day 28 (n = 30)#	Test of sig.	p
Uric acid (mg/dl)				
Min. – Max.	6.50 - 19.0	3.0 – 6.50	t= 10.089*	<0.001*
Mean ± SD.	10.68 ± 3.19	4.59 ± 0.87		
Median	9.60	4.50		
LDH (IU/L)				
Min. – Max.	280.0 - 1170.0	100.0 - 298.0	Z = 6.685*	<0.001*
Mean ± SD.	535.84 ± 200.52	221.43 ± 53.71		
Median	450.0	221.0		

t: Student t-test
Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$
#: Two cases died during induction chemotherapy

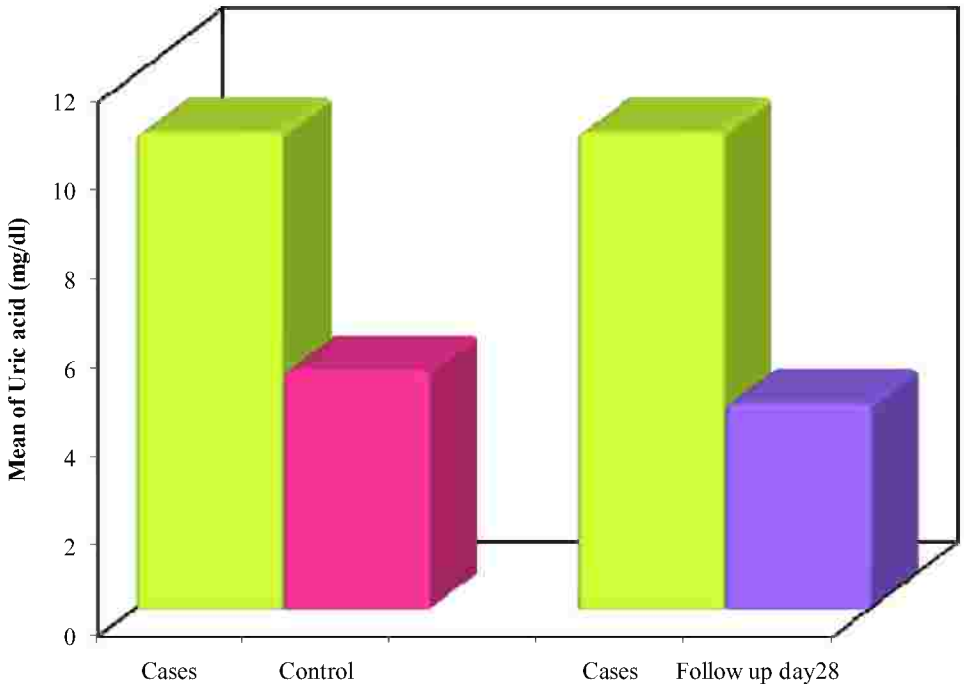


Figure (23): Comparison between the two studied groups (B-ALL cases at diagnosis and controls and follow up day 28 samples) according to uric acid (mg/dl)

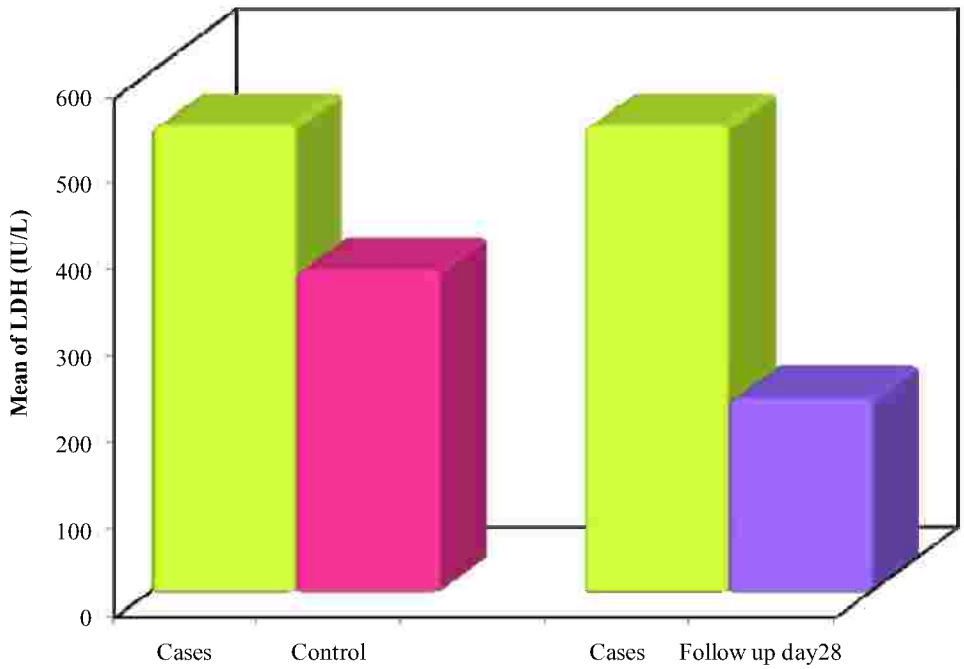


Figure (24): Comparison between the two studied groups (B-ALL cases at diagnosis and controls and follow up day 28 samples) according to lactate dehydrogenase (LDH) (IU/L)

Morphological Features:

A) B-ALL cases at diagnosis:

Bone marrow aspirate (BMA) smears from all cases of B-ALL included in the current study were stained with leishman's stain and microscopically examined. Lymphoblasts represented the majority of bone marrow cells in most cases. They varied from small blasts with scant cytoplasm, condensed nuclear chromatin and indistinct nucleoli to larger cells with moderate amounts of light blue to blue grey occasionally vacuolated cytoplasm, dispersed nuclear chromatin and multiple variably prominent nucleoli. The nuclei were round, irregular or convoluted (Figure 25).

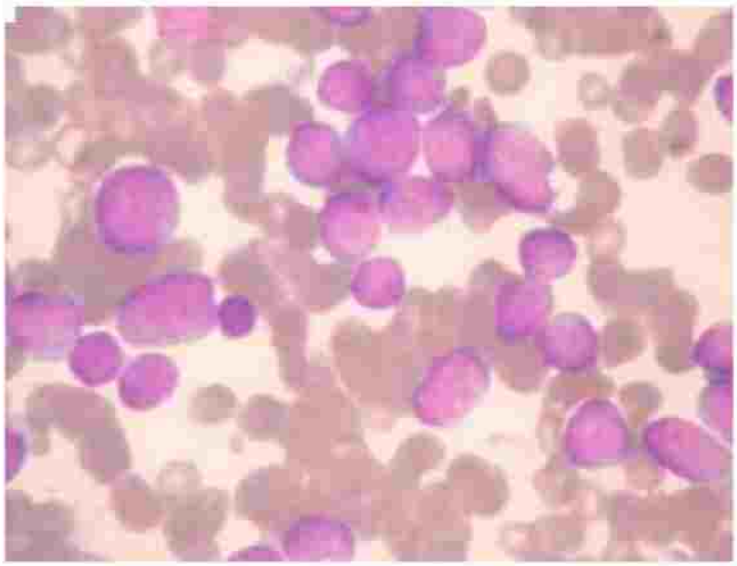


Figure (25): Bone marrow aspiration smear of precursor B-ALL case showing lymphoblasts

B) Control group:

Selection of these controls was based on having abundant hematogones in their bone marrows. Leishman stained BMA smears from all controls were examined microscopically. In most cases hematogones were present in sufficient numbers to be recognized. They were small to medium in size with small cells predominating. The nucleus was round or oval and the nuclear chromatin was condensed but homogeneous. Nucleoli were absent or small and indistinct. There was scant or no cytoplasm; when present, cytoplasm was moderately to deeply basophilic and devoid of inclusions, granules or vacuoles. There was often a spectrum of sizes and morphologic features from hematogones to mature lymphocytes (Figure 26). Although the morphology of hematogones differs from that of lymphoblasts, in some cases a portion of hematogones exhibited a close resemblance with lymphoblasts.

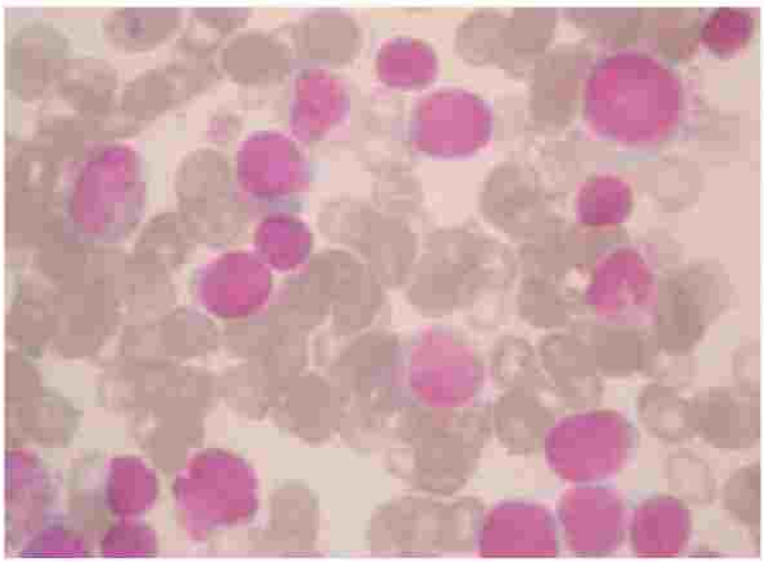


Figure (26): Bone marrow aspiration smear of an ITP case showing abundant hematogones.

C) Follow up of B-ALL cases on day 28:

Follow up BMA smears from all B-ALL cases on day 28 post-induction chemotherapy were stained by leishman's stain and microscopically examined. In almost all cases bone marrow aspirate smears showed regeneration of the normal tri-lineage hematopoiesis. Most cases showed prominent hematogones. In many cases hematogones exhibited features morphologically indistinguishable from lymphoblasts causing diagnostic confusion. (Figure 27)

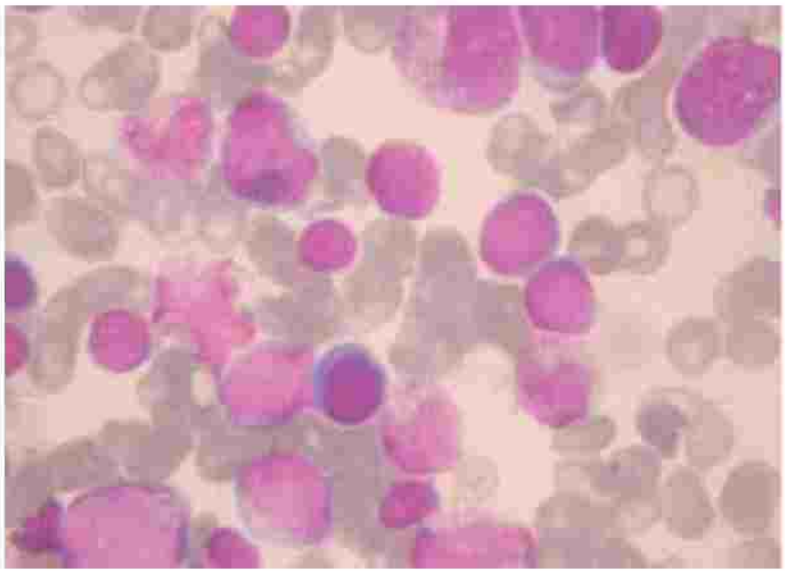
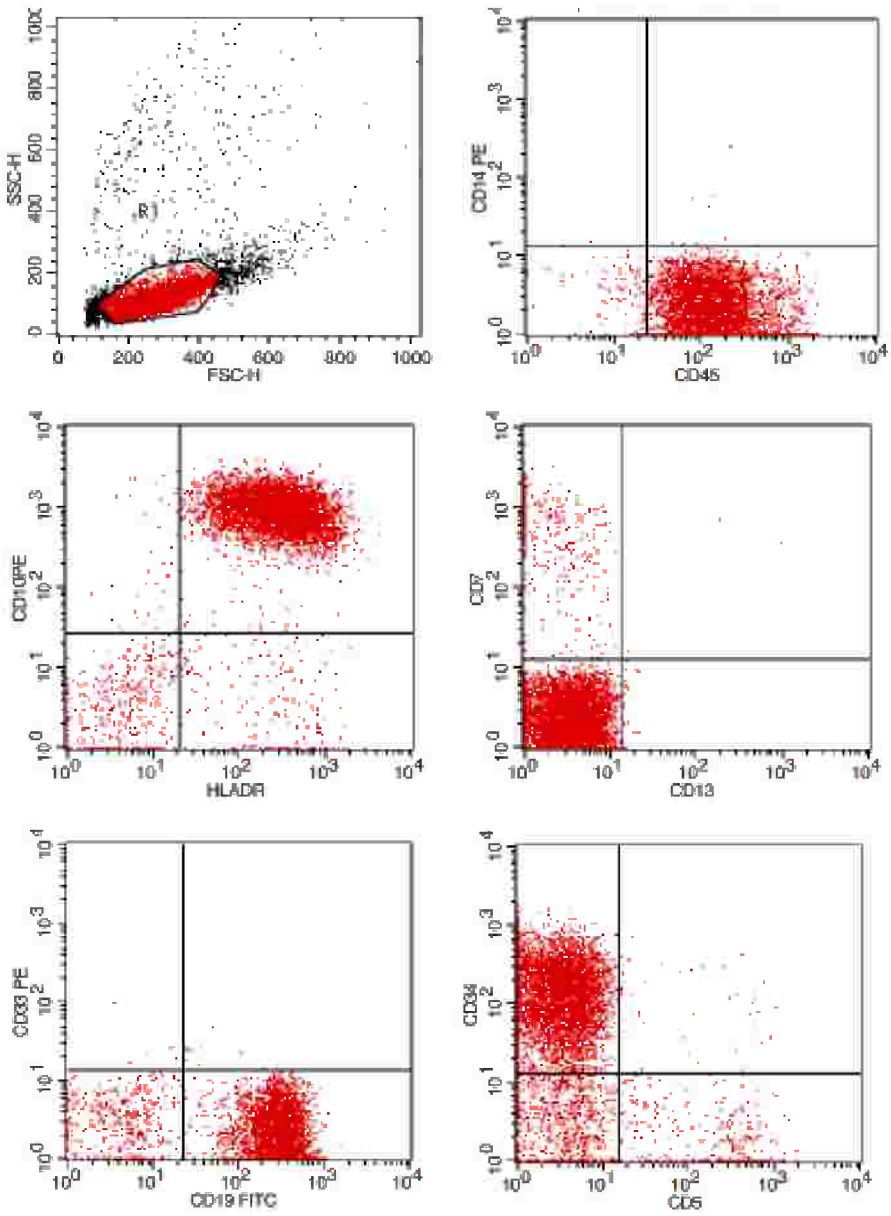


Figure (27): Day 28 follow up bone marrow aspiration smear of a B-ALL case showing increased hematogones

Flow cytometric immunophenotyping:

A) B-ALL cases at diagnosis:

The initial diagnostic BMA specimens from all B-ALL cases were subjected to FCI using the routine laboratory panel for acute leukemia (Figure 28). The results of immunophenotyping are shown in table (13).



Figure(28): Flow cytometric immunophenotyping panel done for the initial diagnostic BMA specimens of all B-ALL cases showing the presence of leukemic blasts of B-ALL

Table (13): Flow cytometric immunophenotyping results of B-ALL cases at diagnosis (n = 32)

Case No.	Immunophenotype							
	CD10	CD19	CD34	HLA-DR	NG2	Cytoplasmic μ	Myeloid markers	T-cell markers
1	Positive	Positive	Positive	Positive	-	Positive	Negative	Negative
2	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
3	Positive	Positive	Negative	Positive	-	Positive	Negative	Negative
4	Positive	Positive	Negative	Positive	-	Negative	Negative	Negative
5	Positive	Positive	Positive	Positive	-	Positive	Negative	Negative
6	Negative	Positive	Positive	Positive	Positive	Negative	Abberrant CD33	Negative
7	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
8	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
9	Positive	Positive	Negative	Positive	-	Positive	Negative	Negative
10	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
11	Positive	Positive	Positive	Positive	-	Positive	Negative	Negative
12	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
13	Positive	Positive	Positive	Positive	-	Positive	Negative	Negative
14	Positive	Positive	Negative	Positive	-	Negative	Negative	Negative
15	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
16	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
17	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
18	Positive	Positive	Positive	Positive	-	Negative	Abberrant CD33	Negative
19	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
20	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
21	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
22	Positive	Positive	Positive	Positive	-	Positive	Negative	Negative
23	Negative	Positive	Positive	Positive	-	Negative	Negative	Negative
24	Negative	Positive	Positive	Positive	Positive	Negative	Negative	Negative
25	Positive	Positive	Negative	Positive	-	Positive	Negative	Negative
26	Positive	Positive	Negative	Positive	-	Positive	Negative	Negative
27	Positive	Positive	Negative	Positive	-	Positive	Negative	Abberrant CD5
28	Negative	Positive	Positive	Positive	-	Negative	Negative	Negative
29	Negative	Positive	Positive	Positive	-	Negative	Negative	Negative
30	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative
31	Negative	Positive	Positive	Positive	-	Negative	Negative	Negative
32	Positive	Positive	Positive	Positive	-	Negative	Negative	Negative

Cutoff for positivity: ≥ 20% and for negativity: < 20%.

B-ALL cases were classified according to their immunophenotypic features into pro-B-ALL subtype (6 cases); {expressing CD19, CD34 and HLA-DR and negative for CD10 and cytoplasmic μ}, common B-ALL subtype (16 cases); {expressing CD10, CD19, HLA-DR, ± CD34 and negative for cytoplasmic μ} and pre-B-ALL subtype (10 cases) {expressing CD10, CD19, HLA-DR, ± CD34 and cytoplasmic μ} (Table 14 and figure 29).

Aberrant expression of myeloid or T-cell markers were found in 3 cases; two cases showed aberrant expression of CD33, and one case had aberrant expression of CD5.

Table (14): Distribution of B-ALL cases at diagnosis according to the immunological classification of ALL (n = 32)

Subtypes of precursor B-ALL	No.	%
Pro-B-ALL	6	18.8
Common B-ALL	16	50.0
Pre-B-ALL	10	31.3

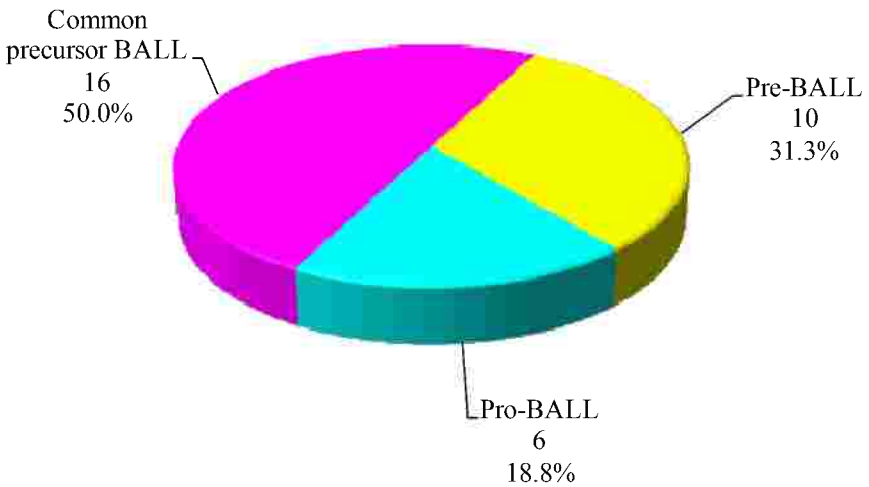


Figure (29): Distribution of B-ALL cases at diagnosis according to the immunological classification of ALL

B) B-ALL cases on day 28 post-induction chemotherapy:

Thirty B-ALL cases on day 28 post-induction chemotherapy were subjected to FCI for MRD detection, using the following panel of monoclonal antibodies: TDT, CD10, CD19, CD34 and CD38 (Figure 30). This combination was shown to be quite pertinent to differentiate residual leukemic blasts from hematogones (normal B-cell precursors), based on the difference in fluorescence intensity displayed by both (as will be explained later). According to immunophenotypic results, all cases were negative for MRD (<0.01%) on day 28 post-induction chemotherapy. Hematogones were identified by flow cytometry in eighteen cases (60%). The remaining two B-ALL cases died during induction chemotherapy and couldn't be followed up (Table 15 and figure 31).

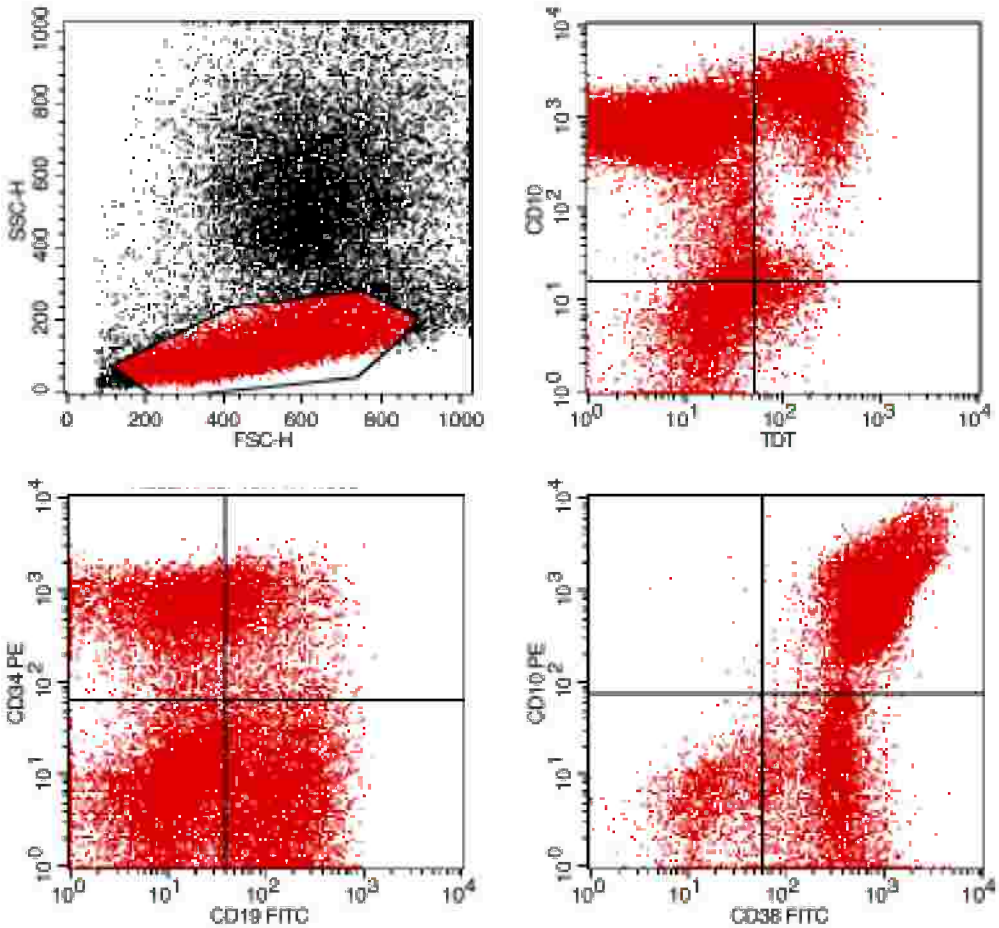


Figure (30): Flow cytometric immunophenotyping on BMA specimens of B-ALL cases on day 28 post-induction chemotherapy for MRD detection showing absence of leukemic blasts and presence of hematogones

Table (15): Outcome of induction chemotherapy of B-ALL cases

	No.	%
Complete remission	30	93.8
Induction death	2	6.25

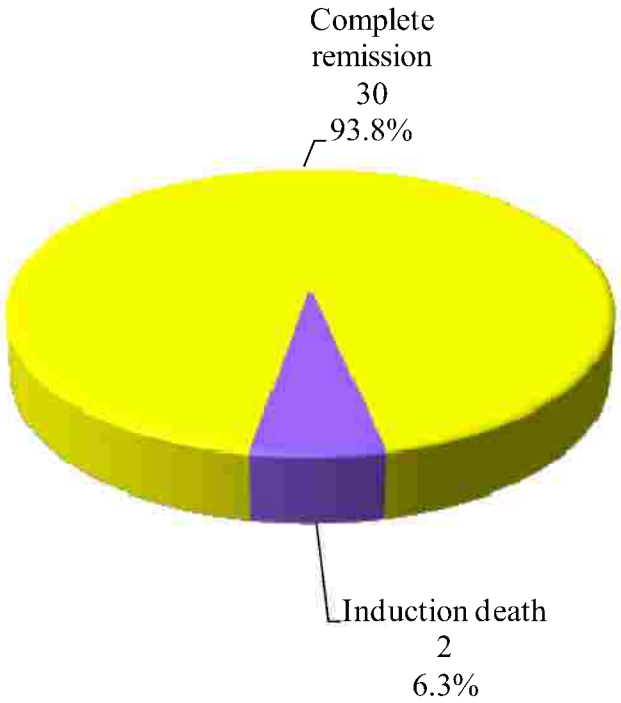


Figure (31): Outcome of induction chemotherapy of B-ALL cases

C) Control group:

Bone marrow aspirate specimens from all controls were subjected to FCI using a combination of TdT, CD10, CD19, CD34 and CD38. On FCI, the BM hematogones population of the control group exhibited a typical continuous spectrum of antigen expression that defines the normal B-cell maturation (Figure 32). The pattern of sequence and intensity of antigen expression was virtually identical in all cases. The earliest recognizable B-lineage precursors expressed the progenitor cell markers; TdT and CD34 in combination with CD38, CD19 and bright CD10. These progressed to the next stages by down-regulating TdT, and CD34 completely and CD10 partially. Finally, CD10 was down-regulated completely and CD38 partially producing the mature stage of B-cell development. Asynchronous expression of antigens and aberrant over- or under-expression of antigens was not observed in hematogone populations.

This pattern of antigen expression exhibited by hematogones allowed their differentiation from B-ALL blasts that exhibit incomplete maturation and immunophenotypic asynchrony and aberrancy from the normal, continuous and complete B-lineage maturation spectrum observed for hematogones. In addition, neoplastic lymphoblasts commonly exhibited aberrant antigen expression.

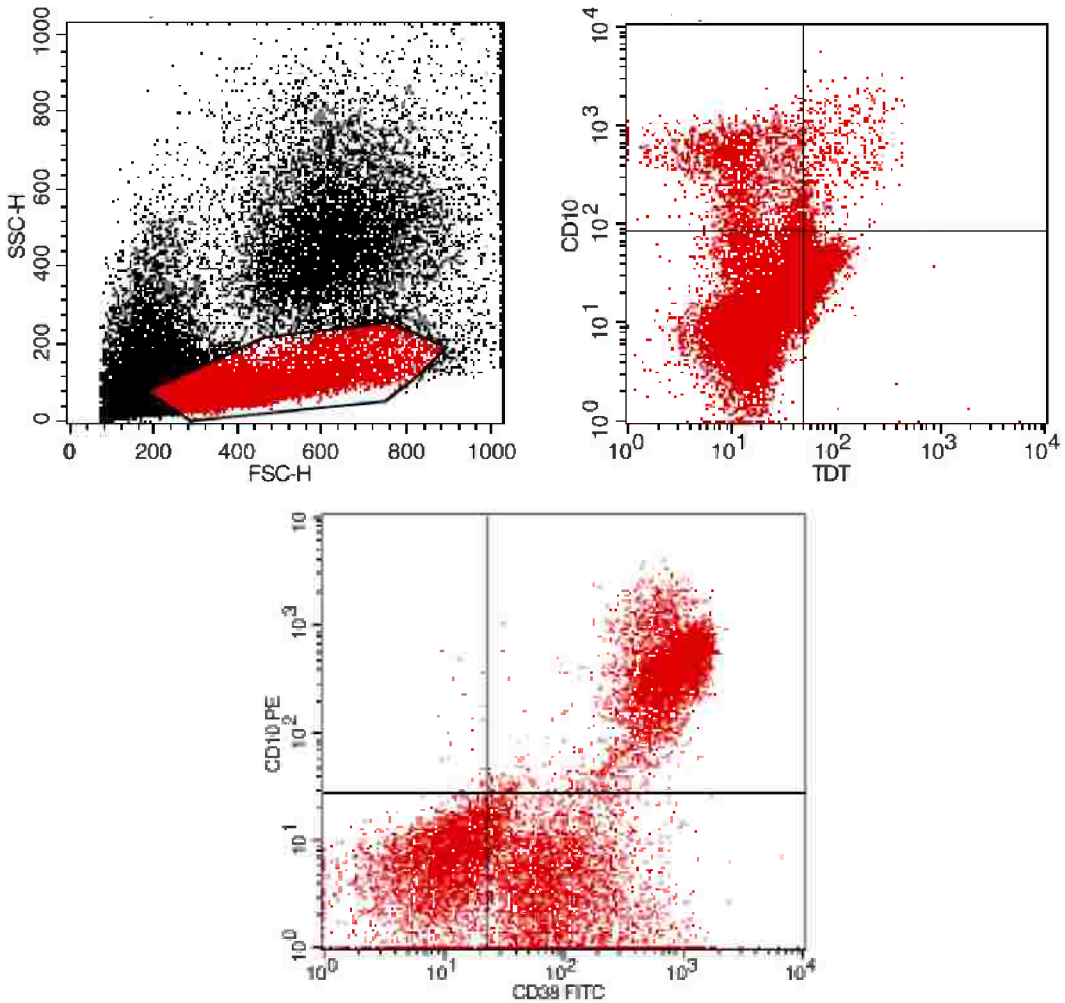


Figure (32): Flow cytometric immunophenotyping on BMA smears of controls showing presence of hematogones.

D) Analysis of CD81 expression:

- **Analysis of CD81 on normal BM cells:**

The pattern of CD81 expression was assessed on normal BM cells. Hematogones showed the brightest CD81 expression. Lymphocytes, monocytes and myeloid blasts showed moderate to weak expression, while the granulocytes showed the weakest expression level of CD81 (Figure 33).

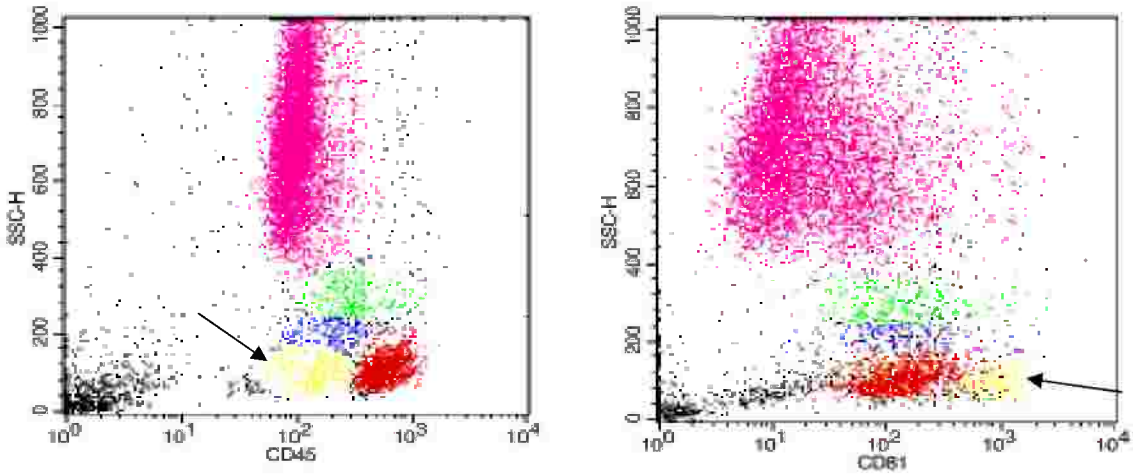


Figure (33): CD81 expression pattern on normal BM cells; hematogones (yellow); showed the brightest expression. Lymphocytes (red), myeloid blasts (blue) and monocytes (green); showed moderate to weak expression. Granulocytes (pink); showed the weakest expression.

- **Analysis of CD81 expression on B-ALL blasts and hematogones in cases and controls:**

The pattern of CD81 expression on leukemic blasts (in the initial diagnostic specimens of B-ALL cases) and BM hematogones (in the control specimens and in the follow up BMA specimens of B-ALL cases), was analyzed using FCI (Figure 34). Leukemic blasts showed a dim CD81expression (low CD81 mean fluorescence intensity (MFI)), with a mean of 304.49 ± 202.18. Hematogones from both controls and day 28 follow up specimens showed bright CD81 expression (high CD81 MFI) with a mean of 2684 ± 502.1 and 2434.11±309.16 respectively. The differences between CD81 MFI in leukemic blast cells and immature hematogones of both controls and follow up B-ALL specimens were statistically significant (P <0.001) (Tables 16 and 17) (Figures 35 and 36).

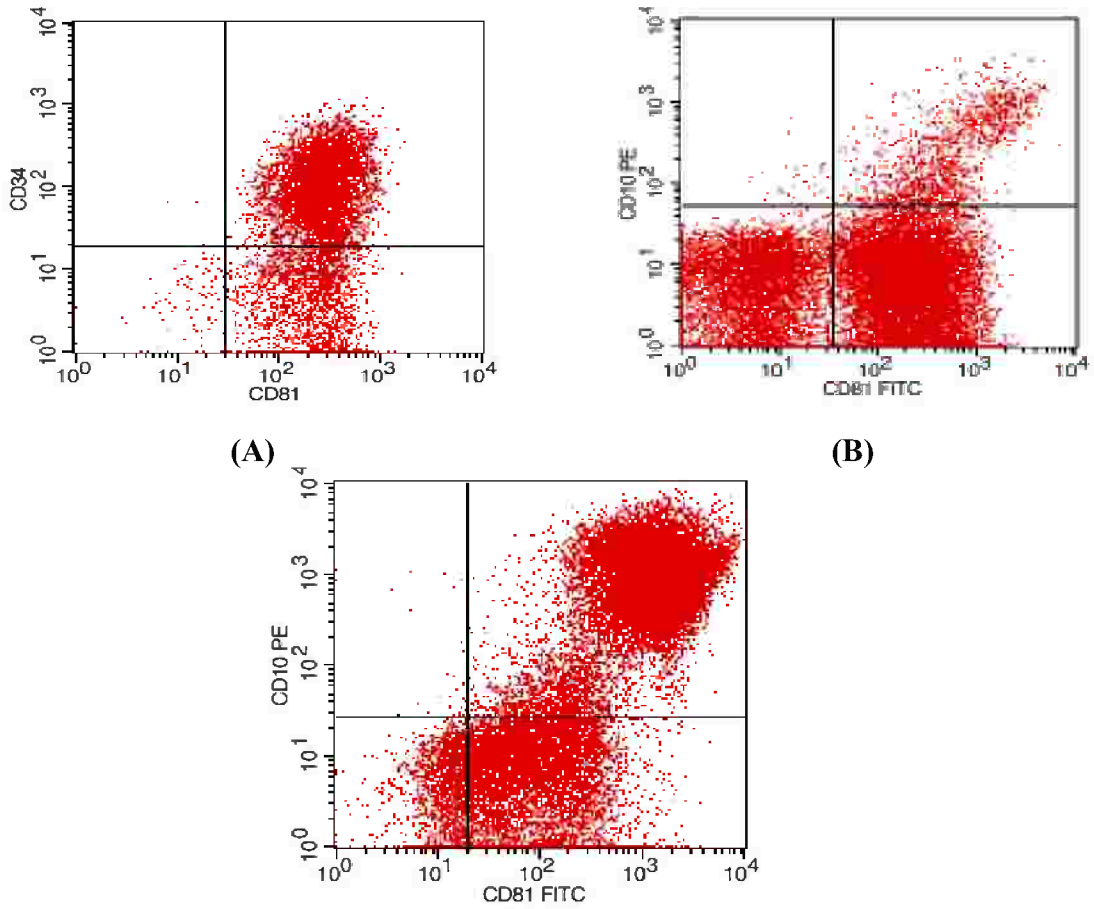


Figure (34): CD81 expression patterns: A) Dim CD81 expression on leukemic blasts of B-ALL case. B) Bright CD81 expression on CD10+ve hematogones of an ITP case (control). C) Bright CD81 expression on CD10+ve hematogones of a day 28 bone marrow aspirate specimen (from a B-ALL case)

Table (16): Comparison between CD81 MFI on leukemic blasts in the initial diagnostic specimens of B-ALL cases and hematogones of control specimens

CD81 MFI	B-ALL blasts (n = 32)	Hematogones of controls (n = 32)	T	P
Min. – Max.	88.0 – 975.0	1293.0 - 3657.0	24.868*	<0.001*
Mean ± SD.	304.49 ± 202.18	2684 ± 502.1		
Median	269.50	2648.5		

t: Student t-test
*: Statistically significant at $p \leq 0.05$

Table (17): Comparison between CD81 MFI on leukemic blasts in the initial diagnostic specimens and hematogones in follow-up BM specimens of B- ALL cases

B-ALL cases CD81 MFI	At diagnosis (n = 32)	Follow up day28 (n = 30)#	T	P
Min. – Max.	88.0 – 975.0	2100 - 2920.0	26.6812*	<0.001*
Mean ± SD.	304.49 ± 202.18	2434.11±309.16		
Median	269.50	2312.0		

t: Student t-test
*: Statistically significant at $p \leq 0.05$
#: Two cases died during induction chemotherapy

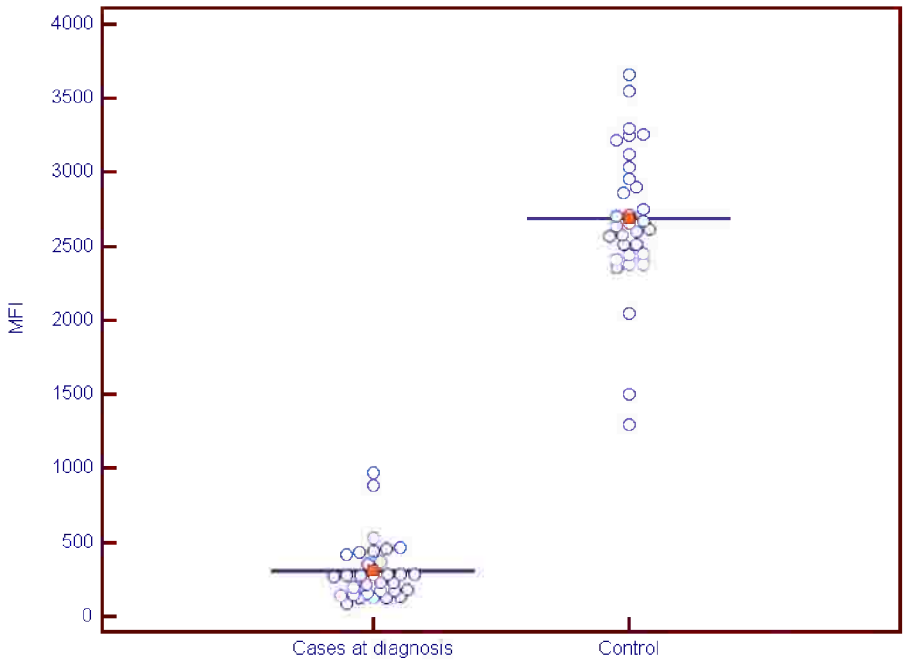


Figure (35): CD81 MFI on leukemic blasts in the initial diagnostic specimens of B-ALL cases versus hematogones of control specimens. The CD81 MFI was significantly lower for leukemic blasts (304.49) than for hematogones (2684) (P<0.001)

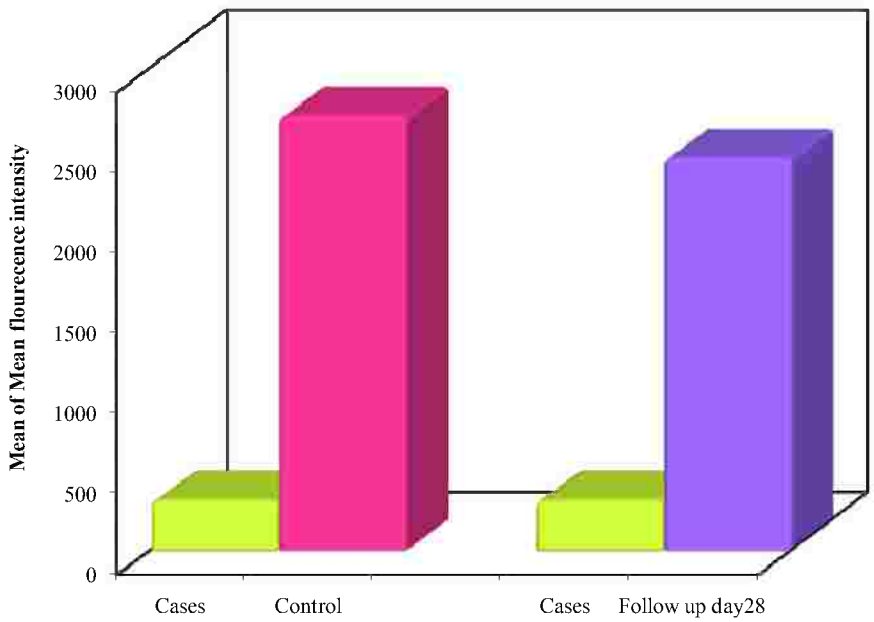


Figure (36): Comparison between CD81 MFI on leukemic blasts in the initial diagnostic specimens of B-ALL cases and hematogones of controls and follow up BMA specimens of B-ALL cases

Two of our 32 B-ALL cases who were further followed up after induction phase presented with relapse after the consolidation phase. This relapse was confirmed by the conventional MRD panel. CD81 expression was studied on the leukemic blasts of the BM specimens of the relapsed cases to assess its stability through time and following chemotherapy.

CD81 MFI remained aberrantly dim on the leukemic blasts of the relapsed cases (CD81 MFI = 120 and 157 respectively) as compared to their MFIs in the initial diagnostic specimens (CD81 MFI = 88 and 162 respectively). This might reflect the stability of CD81 expression. Interestingly, one patient was pro-B-ALL expressing NG2 (reflecting MLL rearrangement) which confers poor prognosis, while the other patient was pre-B-ALL (Figure 37).

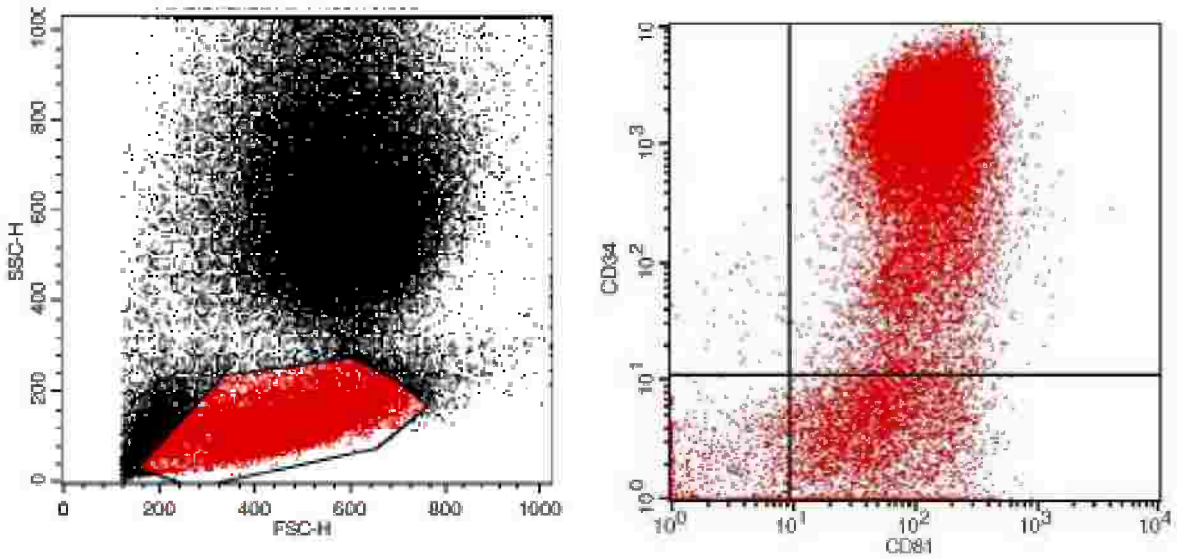


Figure (37): Flow cytometric immunophenotyping of BMA specimen of a relapsed case of precursor B-ALL after consolidation showing dim CD81 expression on leukemic blast cells

- **Pattern of CD81 expression among different B-ALL subtypes:**

The pattern of CD81 expression among different B-ALL subtypes was also analyzed. CD81 MFI was highest in the pro-B-ALL subtype followed by the pre-B-ALL subtype and it was lowest in the common B-ALL subtype, with a mean of 534.0 ± 335.17, 263.90 ± 104.78 and 243.80 ± 119.77 respectively. The difference in CD81 MFI between pro-B-ALL and common B-ALL was statistically significant (p=0.002) and between pro-B-ALL and pre-B-ALL was also statistically significant (p = 0.005), however there was no statistically significant difference between common and pre-B-ALL subtypes (Table 18 and figure 38).

Table (18): Comparison of CD81 MFI on leukemic blasts among the different immunologic subtypes of B-ALL cases

CD81 MFI	Subtypes of precursor B-ALL			F	p
	Pro-B ALL (n = 6)	Common-B ALL (n = 16)	Pre-B ALL (n = 10)		
Min. – Max.	88.0 – 975.0	127.0 - 530.0	128.0 - 442.0	6.482*	0.005*
Mean ± SD.	534.0 ± 335.17	243.80 ± 119.77	263.90 ± 104.78		
Median	449.50	206.50	272.50		
Sig.bet.Grps	$P_1 = 0.002^*$,	$p_2 = 0.005^*$,	$p_3 = 0.776$		

F: F test (ANOVA)
Sig. bet. grps was done using Post Hoc test (LSD)
p_1: p value for comparing between Pro-B ALL and Common-B ALL
p_2: p value for comparing between Pro-B ALL and Pre-B ALL
p_3: p value for comparing between Common-B ALL and Pre-B ALL
*: Statistically significant at $p \leq 0.05$

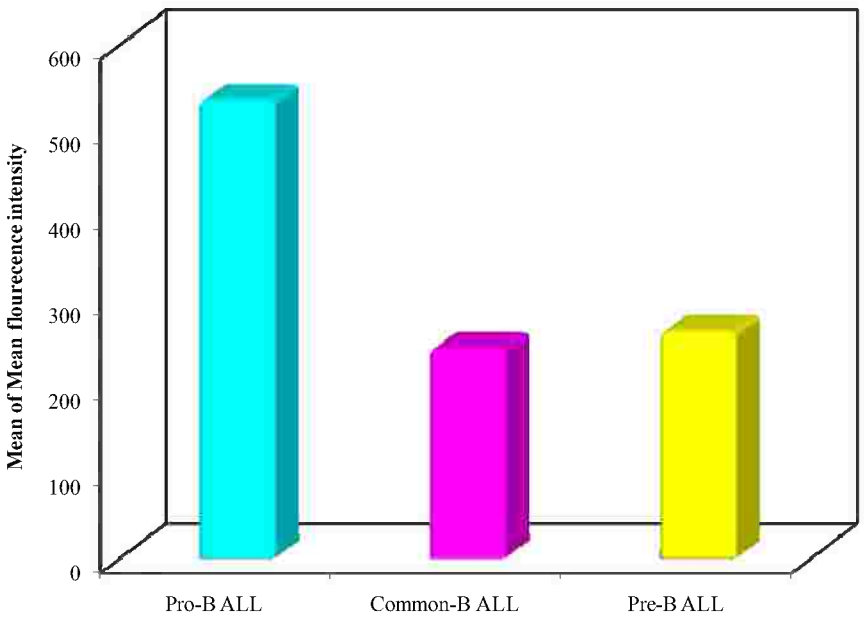


Figure (38): Comparison of CD81 MFI on leukemic blasts among the different immunologic subtypes of B-ALL cases

- **Relation between CD81 and other immunophenotypic markers:**

Assessment of the relation between the level of CD81 expression on leukemic blasts and some immunophenotypic markers, namely CD10 and CD34 revealed that CD10 positive cases had lower CD81 MFI in comparison 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 had slightly higher CD81 MFI than CD34 negative cases, with a mean of 307.36 ± 220.02 and 294.26 ± 131.93 but the difference between them was also not statistically significant (Table 19 and figure 39).

Table (19): Relation between CD81 MFI on blast cells and other immunophenotypic parameters

B-ALL cases at diagnosis (n =32)	N	CD81 MFI			T	p
		Min. – Max.	Mean ± SD	Median		
CD 10						
-ve	**6**	88.0 – 975.0	534.0 ± 335.17	449.50	2.038	0.094
+ve	**26**	127.0 - 530.0	251.53 ± 112.51	228.50		
CD 34						
-ve	**7**	145.0 - 458.0	294.26 ± 131.93	265.0	0.149	0.882
+ve	**25**	88.0 – 975.0	307.36 ± 220.02	274.0		

t: Student t-test

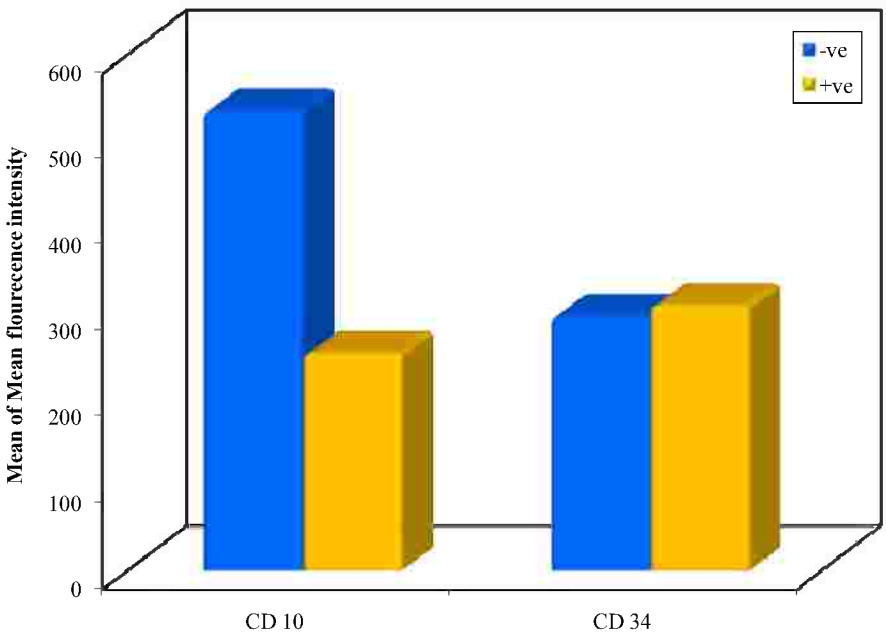


Figure (39): Relation between CD81 MFI on blast cells and CD10 and CD34

The correlation between MFI of CD81 on blast cells and different studied hematological parameters as WBCs counts at diagnosis, blast counts in the peripheral blood and in the BM revealed no statistically significant correlation with any of them (Table 20).

Table (20): Correlation between mean fluorescence intensity of CD81 on blast cells and different studied hematological parameters

B-ALL cases at diagnosis (n = 32)	CD81 MFI	
	r_s	P
WBCs ($\times 10^9$/L)	-0.011	0.955
Peripheral blood blasts (%)	-0.056	0.759
Bone Marrow Blasts (%)	-0.217	0.232

r_s: Spearman coefficient

ROC curve analysis and establishment of a cutoff value for CD81 MFI to distinguish between B-ALL blasts and hematogones

ROC curve analysis was done to establish a lower threshold for normal CD81 expression that would help in the differentiation between normal bone marrow hematogones and leukemic B-ALL blasts.

A value of 1134 was found to provide the greatest separation between leukemic blasts and hematogones with a specificity and sensitivity of 100% (Figure 40). Values greater than 1134 were considered normal (associated with hematogones) and values less than or equal to 1134 were considered low (associated with leukemic blast cells).

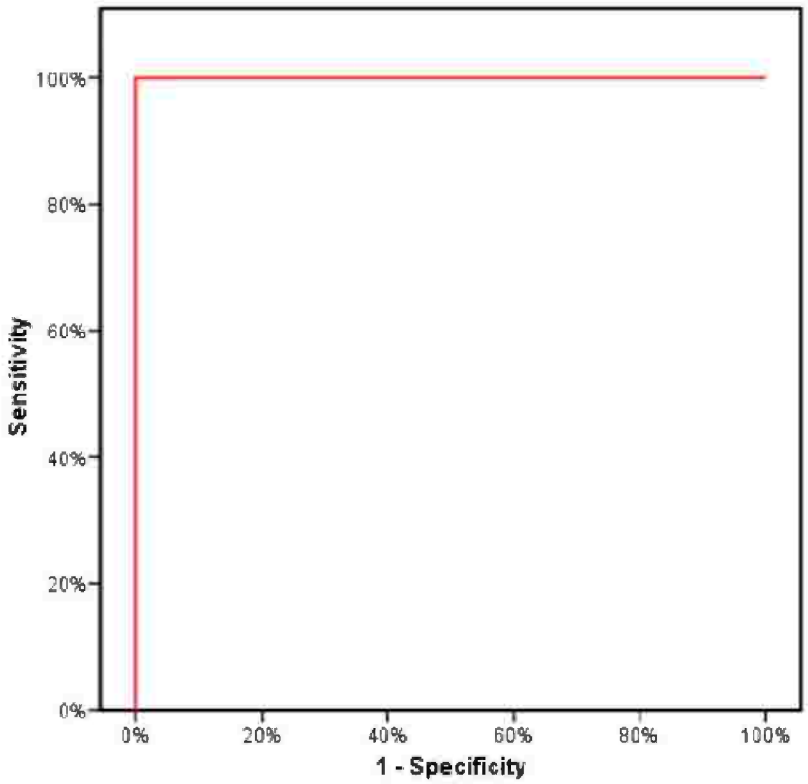


Figure (40): ROC curve for CD81 MFI for differentiating blasts from hematogones

One B-ALL female patient unexpectedly was found to have high CD81 MFI at diagnosis (MFI = 1304), it was thus decided not to include her in the analysis in order not to disturb the homogeneity of our sample, but she was thoroughly investigated. This case was ten years old, presented with fever, pallor and bone aches. On examination no organomegaly or lymphadenopathy were found. Her peripheral blood counts were (WBC count: 1.3×10^9/L, hemoglobin concentration: 5 g/dl and platelet count: 100×10^9/L). Three percent blasts were found in the peripheral blood and 92% were found in the BM. Flow cytometric immunophenotyping on BMA specimen classified her as common B-ALL with the following immunophenotype (CD10: positive, CD34: negative, CD19: positive, HLA-DR: positive, cytoplasmic μ: negative and an aberrant expression of CD5), in addition to high CD81 MFI (1304). Biochemical tests were (uric acid = 12 gm/dl and LDH = 500 IU/L). This case died before ending her induction chemotherapy so we weren't capable of following her up on day 28 post-induction chemotherapy.