Journal of the Egyptian Society of Haematology & Research, Vol. 3, No. 1, March: 55-67, 2007

Morphological and Immunophenotypic Heterogeneity of Pediatric Acute Megakaryoblastic Leukemia (M7)

AZZA M. KAMEL, M.D.; HEBA S. MOUSSA, M.D.; YASSER H. EL-NAHASS, M.D.; NAHLA EL-SHARKAWY, M.D.; DINA A. YASSIN, M.D.; KHALED M. SHAABAN, M.B.B.C.H. and IMAN SIDHOM, M.D.*

The Departments of Clinical Pathology, Pediatric Oncology, NCI, Cairo University.*

ABSTRACT

Background: M7 was first recognized by the FAB group as a separate subtype of AML in 1985. Since then, variability in its relative frequency has been reported. A major cause of this variability could be attributed to morphological heterogeneity. Some cases may lack the classical morphology; these may not be tested for the specific megakaryocytic markers and will be misdiagnosed as other FAB subtypes of AML.

Aim of the Work: In this work we aimed to investigate the true relative frequency of M7 within pediatric AML diagnosed at NCI, Cairo University. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

Patients and Methods: In the years 2003-2005, 280 cases were diagnosed as pediatric AML (age <18 years). The diagnosis was established according to standard methods including clinical picture, complete blood count, bone marrow aspiration, cytochemistry and immunophe notyping. Bone marrow biopsy was performed when the aspiration was not adequate. All M7 cases were revised for clinical, morphological and immunophenotypic findings. Data on trisomy 21 were obtained from the files. Four M7 cases were tested for t (1; 22) and one was tested for BCR/ABL p 210 chimeric gene.

Results: M7 was encountered in 17/280 (6%) of the cases. Patients included 12 males and 5 females. They showed an age range of 2 days -14 years, median 3 years; they included 3 infants four of them were associated with Down's syndrome including the 2 days old one. Typical M7 morphology with large blasts showing cytoplasmic blebbing was obtained in 12 cases (70.6%); one of them showed many micromegakaryocytes with platelet budding and thrombocytosis of $750x10^9$/L. This latter case expressed BCR/ABL fusion gene p 210 and proved to be acute crisis on top of CML. The other 5 cases (29.4%) showed morphology suggestive of AML in three and of ALL in 2 cases. Bone marrow blasts were <20% in 2 cases and hence bone marrow trephine was performed. Cytochemistry showed a characteristic pattern of acid phosphatase in all cases with strong cytoplasmic positivity distributed in the whole cell. Diagnosis was confirmed in all cases by expression of CD 61 and/or CD 41. CD 41 and CD 61 were both coexpressed in 8/15 (53.3%) of cases tested for both. Four out of 7 remaining cases showed cyt CD 61 only. One case was positive for CD 41 only (CD 61 was 17%) and two cases were positive for CD 61 alone (CD 41 was 4 & 14%). Cyt CD 61 was the tool for diagnosis in one more case. In the remaining case, CD 41 was 90% and CD 61 was not tested. Coexpression of megakaryocytic antigens with other myeloid antigens namely CD 13 and/or CD 33 was encountered in 9 cases (52.9%). CD 7 was expressed in 2 cases, one of them expressing CD 56 as well, and the other was expressing CD 2. CD 4 was encountered in one case. t (1; 22) was encountered in 1/4 cases tested.

Conclusion: M7 showed marked morphological het erogeneity with 29.4% lacking the typical features. These cases could be missed especially if they are expressing myeloid markers or occasionally markers of other lineages. CD 61 and CD 41 should be tested in all cases of AML as well as in cases lacking other lineage markers. Cy tochemistry especially the characteristic pattern of acid phosphatase could be highly suggestive of M7 and directs attention to testing for CD 61 and CD 41 expression.

Key Words: *M7 – Pediatric leukemia – AML – Immunophenotypic.*

INTRODUCTION

Acute Megakaryoblastic Leukemia (M7) comprises a heterogeneous group of disorders that are considered to represent clonal expan sions of megakaryocytic cells, which have un dergone malignant transformation and are ar rested at distinct stages of differentiation **[1]**. Although M7 was first described in 1931 **[2]**, it was not included in the original classification of acute leukemia (AL) of the French-American-British (FAB) leukemia Cooperative Study Group because of the difficulty in recognizing megakaryocytic cell lineage precursors. For this

reason, the disease was considered for many years to be a rare variant of acute myeloid leukemia (AML). In addition, inaspirable bone marrow has contributed to the difficulty in establishing the diagnosis. However, with the development of ultrastructural cytochemistry, immunocytochemistry and monoclonal antibodies (MoAbs) against platelet specific glycoproteins (Gps), it soon became apparent that M7 occurred more frequently than previously suspected **[3,4]**. This fact prompted the FAB group to establish the criteria for the diagnosis and to add this category as a distinct subtype of AML-M7 in 1985 **[5]**.

The incidence of M7 has been estimated to be 4-7% of pediatric AML in large Cooperative Group studies in developed countries **[6,7,8]**. However, higher incidence rates of 10-15% and 29% were reported **[9,10]**. The peak age for M7 in children is 1-3 years in those with and without Down's syndrome **[10,11]**. A special type of M7 is seen in Down's syndrome (DS) that may present in the neonatal period and remits spontaneously **[12]**; in which case the disorder is termed transient myeloproliferative disorder or transient abnormal myelopoiesis (TAM). Around a quarter of these cases recur later on in life into frank M7, but typically respond well to a modified therapeutic regimen for childhood acute myeloid leukaemia. Recent evidence shows that M7 occurring in a patient with a history of TAM may represent clonal evolution of the same disease **[13]**.

Recently t (1; 22) (p 13; q 13) has been described to be associated with M7 **[14]**. It occurs mainly in infants, but is uncommon in cases with Down's syndrome and accounts for one third of other pediatric M7 cases **[6,14,15,16]**. The genes RBM15 on chromosome 1p13 and MLK1 on 22 q 13 have been identified leading to an RBM15/MLK1 fusion gene and a fusion transcript **[17]**. Cases with detectable RBM15/MLK1 fusion transcripts in the absence of t (1; 22) have been reported as well **[14]**. In the small number of children reported, the presence of the t (1; 22) appears to be associated with poor prognosis, though long-term survivors have been noted following intensive therapy **[15]**.

The aim of this study was to assess the incidence, clinical features at presentation, hematological, immunophenotypic and when possible molecular characteristics of M7 in pediatric AML cases in Egypt. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

PATIENTS AND METHODS

This study included all cases (17 cases) diagnosed as M7 in the period 2003-2005. All patients presented to the Pediatric Oncology Department at NCI, Cairo University. During the study period, 280 pediatric AML cases were newly diagnosed. Written informed consents were obtained from the patients' guardians to use the data in the records and the protocol was approved by the Institution Research Board. Diagnosis of AML was performed according to standard criteria including clinical, morpholog ical and cytochemical criteria as well as immu nophenotyping. The FAB subtype was deter mined **[5]** and cases with diagnosis of de novo M7 were selected. Details of clinical presenta tion, laboratory findings at the time of diagnosis, therapy received, and outcome were collected from patients' medical records. The diagnosis of M7 was established on the basis of the FAB criteria by studies of morphology (peripheral blood (PB), bone marrow aspirate (BMA), as well as BM biopsy in selected cases), cytochem istry (Sudan Black B (SBB), Acid Phosphatase (AP) and alpha naphthyl acetate esterase (ANAE) **[5]** and was finally confirmed by im munophenotyping (CD 41 & CD 61) **[9]**. Cyto genetics for Down's syndrome was available in patients' files. The 17 M7 cases showed an age range from 2 days to 14 years with a median of 3 years; they included 3 infants they were 12 males and 5 females. Four patients had Down syndrome. They were all males aged 2 days, 1.5 years and two patients 3 years.

Immunophenotypic analysis:

Immunophenotypic analysis was performed using whole blood staining method. Fresh samples were obtained from peripheral blood or bone marrow at the time of diagnosis. A wide panel of monoclonal antibodies (Mo Abs) was used (Table 1). Double and Triple marker labeling was performed, including proper isotype controls. Samples were analyzed on Flow Cytometer (Coulter Epics, XL, Hialeh).

Detection of surface markers by direct staining:

The whole blood staining method was performed as previously described **[18]**. In short,

10µl labeled Mo Ab was added to 100µl whole blood (or BM), incubated in the dark for 20 minutes, hemolyzed (Optilyse® No-wash Lysing solutions, A11894, Beckman Coulter) and washed by PBS then analyzed.

Detection of intracellular markers:

Hundred µl of whole blood was lysed using lysis solution (Becton & Dickinson) for 10 minutes. Cells were washed once and re-suspended in 1mL PBS. A mixture of 500 µl 4% paraformaldehyde as fixative, 500 µl PBS and 5 µl tween 20 as detergent was added to the cells and incubated for 10 min. The cells were washed and 10 µl Mo Ab was added and incubated for 30 min at 4ºC. Cells were washed, resuspended in 500 µl PBS and analyzed **[19]**. Any antigen (Ag) was considered positive when ≥20% of blast cells were stained above the negative control except for CD 34 and CD 10 where ≥10% was considered positive.

DNA index (DI): Was done for 16/17 cases. Bone marrow or blood samples were processed with the DNA-Prep coulter® Reagents Kit. The sample was shaken on an automatic shaker then 100µl of the suspension was taken. 100µl of the DNA-Prep LPR for lysing and permeabi lizing cells was added to the suspension with continuous shaking for 8 to 12 seconds and 2 mL of the DNA-Prep Stain (propidium iodide) was added to the previous suspension with continuous shaking for another 30 seconds **[20]**. The DNA-Prep stains DNA and double stranded RNA with Propidium iodide; ribonuclease is included to digest RNA. Samples were analyzed on the Coulter Epics® XL-MCL flow cytometer.

Molecular characterization:

Molecular detection of t (1; 22) was performed on 4 cases for whom RNA was available.

RNA extraction and reverse transcription:

Total RNA was extracted from patient samples using Total RNA isolation kit (Purescript, Gentra, Minneapolis, USA) according to the manufacturer's instructions. Whole blood and/or BM was collected on EDTA and processed immediately. The RBCs in 300ul blood/BM were lysed by RBCs lysis solution, the cell pellet was lysed by cell lysis solution and protein and DNA precipitated by protein-DNA precipitation solution. The supernatant containing RNA was collected on 100% isopropanol, centrifuged to precipitate RNA which was then washed by ethanol. RNA pellet was left to dry then dissolved in 20ul RNA hydration solution. RNA was stored at -80ºC until use.

One µg RNA was reverse transcribed to cDNA in a final volume of 20 µL containing 2 ul of 10 X reverse transcription-polymerase chain reaction (RT-PCR) buffer, 4ul 25 mM MgCl2, 2ul 10mM dNTPs, 0.5ul RNAsin, 15 U AMV reverse transcriptase enzyme, 1ul random hexamers and nuclease free water to a final volume of 20ul (Promega, Madisson, WI). Cyclic temperatures consisted of 10 min at 25°C, 1hourr at 42°C and 5 min at 95°C.

Detection of t (1; 22):

This part of the work was performed at the Molecular Pathology Department of Saint Jude Children's Research Hospital (SJCRH). PCR reaction mix consisted of 300 ng DNA, 5ul 10x PCR buffer containing 25 mM MgCl2, 3.5 ul of 1.25 mM of each dNTPs, 0.5ul of 75 pmol Forward and Reverse Primers, 2.5 U of Amplitaq Gold DNA polymerase enzyme and 5% DMSO. Nuclease free water was added to a total reaction volume of 50 ul.

Thirty five cycles of amplification were performed in a thermocycler with a step program consisting of an initial denaturation step at 95ºC for 11 min and 30sec, 15 cycles of 94ºC for 30sec, 54ºC for 1min & 72ºC for 1 min followed by 20 cycles of 94ºC for 30sec, 54ºC for 1min & 72ºC for 2min. A final extension step was performed for 5 min at 72ºC. A housekeeping gene, Glyceraldehyde Phosphate Dehydrogenase Enzyme (GAPDH) was run with every PCR reaction to check DNA integrity and exclude any PCR failure. A synthetic positive control was used for confirmation of the translocation. PCR products obtained were separated on a 1.2% ethidium bromide agarose gel for 2 hours at 70 volt **[21]**. Fragments size was determined by running a molecular weight marker of known size and comparing the distance of unknown fragment in relation to the ladder. DNA on gels was denatured, neutralized and transferred by a Southern blot technique **[22]** in a high salt buffer solution by either capillary action or positive pressure from its position on agarose gel to a nitrocellulose or nylon membrane (Nytran; Schleisher & Shuell, Keene, NH, USA).

Denatured single-single stranded DNA was permanently bound to the membrane by UV cross linking (UV stratalinker 2400, Stratagene). Single stranded probes were labeled utilizing a 5' DNA end labeling kit (RPN 1509, Amersham Biosciences, USA). The enzyme T4 polynucleotide kinase was used to specifically transfer the γP32 phosphate from ATP to a 5'OH group of DNA. After hybridization, the membrane was washed and labeled to remove unbound or weakly bound probes and then exposed to an autoradiographic film. Results for fusion gene expression were expressed as positive or negative according to the presence or absence of the specific band on the autoradiographic film.

Treatment:

All patients received the same treatment protocol: Induction therapy consisting of one course of ADE (Cytarabine 100 mg/m^2/d 24 hours infusion d1-2; 100 mg/m^2 IV q12 hours days 3-8, Doxorubicin 25 mg/m^2/d IV days 3-5, Etoposide 100 mg/m^2/d one hour infusion days 6-8) followed by another course as consolidation, then four courses of MIDAC as continuation therapy (Mitoxantrone 10 mg/m^2/d 6 hours infusion days 1-3 and intermediate dose Cytarabine 1g/m^2/d two hours infusion q12 hours days 1-3).

Clinical evaluation and follow-up:

Bone marrow aspirate was performed to evaluate response to chemotherapy (status post induction) following first course of ADE in AML. Evaluable patients were followed up for a period ranging from 14-24 months.

Complete remission (CR) was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements.

RESULTS

During the study period, 2003-2005, 280 consecutive pediatric AML cases presented to the Pediatric Oncology department at NCI, Cairo University.

FAB subtype was available for 242 cases. The 38 cases with missing FAB were not M6 or M7 as confirmed by immunophenotyping. Thus M7 constituted 17/280 (6%) of all cases. Other FAB subtypes included 85/242 M1 cases (35.1%), 66/242 M2 (27.3%), 30/242 M3 (12.4%), 19/242 M4 (7.9%) and 24/242 M5 cases (9.9%). M6 was encountered in one case only (0.3%).

The laboratory features of the 17 M7 patients at the time of diagnosis are presented in Table (2). Twelve patients 12/17 (70.6%) were males and 5/17 (29.4%) were females. Their median age was 3 years with a range from 2 days to 14 years, but most cases 10/17 (58.8%) were children < 36 months and 3/17 were infants. The 4 Down's syndrome cases were all males aged 2 days, 1.5 years and two patients 3 years.

Clinical and hematological features: Clinical presentation was similar to those observed in patients with other types of AML including pallor, fever, easy fatigability, headache, hyporexia, bleeding and bone pain. Hepatomegaly was encountered in 11/15 (73.3%), splenomegaly in 10/15 (66.6%) and lymphadenopathy in 9/12 (75%), (Table 3).

Anemia was present in 16/17 cases (94.1%) with haemoglobin (Hb) ranging from 3-15.4 g/dl. White blood cell count ranged from 1.8-190x10^9/L with 5/17 patients only having leukocytosis. Fifteen patients 15/17 (88.2%) had thrombocytopenia.

Morphology: Morphological presentation showed a remarkable degree of pleomorphism. BM cellularity was either normal or decreased with marked reduction in megakaryocytes and marrow platelets in most cases (12/15, 80%). The number of blast cells in the bone marrow aspirates ranged from 3-90%. Diagnosis was based, in all cases, on immunophenotyping by Flow Cytometry. An open gate strategy was used to determine the true percentage of malignant cells that could not be detected by morphology. In 12/17 (70.6%), variable percentage of blasts showed typical morphologic features of megakaryoblasts which differ from other classical myeloblasts by relatively hyperchromatic nuclei and abundant cytoplasm with blebbing and sometimes cytoplasmic granulations (Figs. 1-A, 2). In other cases, blasts could not be distinguished from myeloblasts (Fig. 3). In others, blasts resembled lymphoblasts being small with a high nucleocytoplasmic ratio and chromatin condensation (Fig. 4). One case out of the 17 showed normal platelet count and another one showed thrombocytosis of 750 x

10^9/L. This latter was the one with micromegakaryocytes and proved to be M7 on top of CML (Fig. 5). The peripheral blood of almost all cases (15/16 93.8%) even those with leucopenia showed blast cells. In cases with < 20% blasts in the BM (2 cases), trephine biopsy was performed. Extensive fibrosis was encountered with fibrous tissue entangling blast cells (Fig. 6).

Cytochemical findings: Cytochemistry was performed on PB and/or BM for all 17 cases where they showed myeloperoxidase (MPO) and SBB negativity. Acid Phosphatase showed characteristic pattern in all cases with strong cytoplasmic positivity distributed in the whole cell while ANAE showed moderate positivity partially inhibited by sodium fluoride. Figs. (1-B, 3-B) represent the characteristic pattern of positivity of AP in typical and atypical M7 morphology.

Immunophenotypic features (Table 4): In 7/17 cases (41.1%) blast cells expressed platelet specific markers only while in the remaining 10 cases (58.%) blast cells simultaneously co-expressed megakaryocytic as well as markers of other cell lineages. One case (Nº16) was biphenotypic expressing T, myeloid/ megakaryocytic markers. The coexpression of megakaryocytic, lymphoid and myeloid markers by single cells was confirmed by standard 2 or 3 color flow cytometric analysis using appropriate monoclonal antibodies (MoAbs). CD41 and CD61 were both coexpressed in 8/15 (53.3%) of cases tested for both. Four out of 7 remaining cases showed cyt CD 61 only. One case was positive for CD 41 only (CD 61 was 17%) and two cases were positive for CD 61 alone (CD 41 was 4 & 14%). Cyt CD 61 was the tool for diagnosis in one more case. In the remaining case (nº6), CD41 was 90% and CD61 was not tested. There was no reactivity with platelet specific markers in the remaining cases of AML (M1-M6).

CD 13 and/or CD 33 were expressed in 10/17 (58.8%), both in 3/17(17.7%) and 7/17 (41.1%) had either. As for HLA DR expression, 7/17 (41.1%) cases were positive. CD 33 and HLA-DR were simultaneously expressed on 4/17 (23.5%) of cases. Simultaneous expression of CD 61, CD 41, HLA-DR and CD 33 was detected in 1/17 (5.8%) of cases.

DNA index (DI): Most cases were diploid (13/16 cases). One case was hyperdiploid (DI 1.3) and 2 cases were hypodiploid (DI 0.86, 0.89).

t (1; 22): Molecular detection of t (1; 22) was performed for 4/17. One case showed the gene expression (a 2 years old female).

Two cases deserve elaboration:

The first (Nº4) was a 5 years old girl. PB and BM blast morphology was typical and acid phosphatase showed classical positivity (Figs. 1-A, b). Initial immunophenotyping showed CD 61 and CD 41 expression on 8.7% and 5.5% respectively; the case expressed as well CD 45 and CD 34 and was negative for all other lineage markers. Due to failure to achieve the 20% threshold for the markers together with classical morphology and cytochemistry, immunophenotyping was repeated 5 days later. CD 61 and CD 41 were then expressed on 25% and 26% respectively. The case was documented as M7.

The second (Nº1) was a 6 years old male who presented with huge splenomegaly, anemia (7gm/dl) leukocytosis ($50X10^9$/L) and thrombocytosis ($750X10^9$/L). PB and BM showed a lot of micromegakaryocytes with plentiful platelet production (Fig. 5-A). Immunophenotyping showed expression of DR: 19%, CD 41: 71% and CD 61: 63%. Molecular characterization documented the presence of BCR/ABL p 210 fusion gene (Fig. 5-B) denoting acute crisis on top of CML.

Clinical outcome: In this study, 9/17 cases were non evaluable; either they died early in the course or before the start of treatment, 1/17 lost follow-up after one month of therapy, 1/17 became refractory, 3/17 relapsed and 3 were in CR till end of study (for 12,14 & 24 months).

DISCUSSION

M7 was first recognized as a separate subtype of AML in 1985 **[18]**. Since then, variability in its relative frequency has been reported **[1,6-10,23]**. A major cause of this variability could be attributed to morphological heterogeneity. Some cases may lack the classical morphology would not be tested for the specific megakaryocytic markers, and hence misdiagnosed as other FAB subtypes of AML.

Table (1): Panel of monoclonal antibodies (MoAbs).

Monoclonal Ab	Clone	Source
Myeloid markers:		
CD13	My7-PE	Coulter Hialeah, FL
CD14	RmO52 PE	Coulter Hialeah, FL
CD33	M9-PE	Coulter Hialeah, FL
CD41	P2-PE	Coulter Hialeah, FL
CD61	SZ21-FITC	Coulter Hialeah, FL
Glycophorin A	11E4B.7.6 (KC16)	Coulter Hialeah, FL
Myeloperoxidase	MPO7 FITC	DAKO
Lymphatic markers B Lineage:		
CD19	BL6-FITC	Immunotech Marseille, France
CD22	Sd10 PE	Immunotech Marseille, France
T Lineage:		
CD1	BL6	Coulter Hialeah, FL
CD2	39C1.5 FITC	Coulter Hialeah, FL
CD3	UCHT1 FITC	Coulter Hialeah, FL
CD4	13B8.2-FITC	Immunotech Marseille, France
CD5	BL1A-PE	Coulter Hialeah, FL
CD7	3A FITC	Coulter Hialeah, FL
CD8	B9.11-PE	Immunotech Marseille, France
NK:*		
CD16	3G8 FITC	Coulter Hialeah, FL
CD56	N901 (NKH-1) PE	Coulter Hialeah, FL
Others:		
CD45	Immu19.2-FITC	Coulter Hialeah, FL
HLA-DR	B8.12.2 FITC	Immunotech Marseille, France
CD10	d5 FITC	Coulter Hialeah, FL
CD34	581	Immunotech Marseille, France
Isotypic controls:		
IgG1 (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG1 (Mouse)	PE	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	PE	DAKO/COULTER/DIACLONE

*NK: Natural killer.

Table (2): Laboratory features of 17 pediatric M7 cases.

No	FAB	TLC	HB	PLT	Blasts	Initial BM blasts %	DS	Molecular testing
1	M7	50	7.4	750	70	HC, 43%	No	Philadelphia+
2	M7	9	7.1	70	10	HC, 3%	No	NA
3	M7	8.2	3	13	0	NC,16%	Yes	NA
4	M7	7.1	7.1	8	11	DILUTED	No	NEG t (1; 22)
5	M2	83	15.4	124	55	NC, 30%	Yes	NA
6	M7	48	8.4	64	90	HC, 70%	No	NA
7	M7	3	8	150	50	DILUTED	No	NA
8	M7	13	3.5	15	70	NC,63%	Yes	NA
9	M7	190	4	9	80	NC, 90%	No	NA
10	M7	7	4.2	19	17	HC, 70%	Yes	NEG t (1; 22)
11	L2	9.5	7.8	40	NA	NA	No	NA
12	M7	74	5.9	35	28	NOT DONE	No	NA
13	M7	12	5.8	12	6	HC, 22%	No	POSITIVE t (1, 22)
14	L1	1.8	6	22	15	HC, 20%	No	NA
15	M2	2.9	4.4	22	20	NC, 58%	No	NEG t (1; 22)
16	M1	5	10	100	10	HC, 20%	No	NA
17	M7	4	10	100	3	DILUTED	No	NA

HB: Hemoglobin. PLT: Platelets. NA: Not available. HC: Hypocellular. NC: Normocellular.

Table (3): Clinical features of 17 pediatric M7 cases.

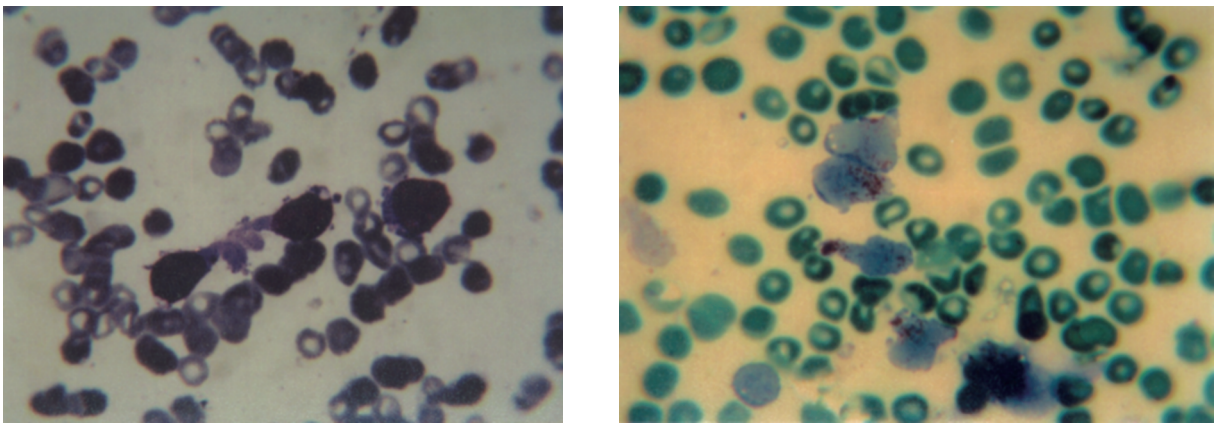
No	AGE (years)	SEX	CXR	CSF	LIVER (in cm)	SPLEEN (in cm)	LNS
1	6	M	free	free	3	huge	–
2	0.25	M	NA	free	huge	huge	–
3	3	M	free	free	3	2	–
4	5	F	NA	NA	no	no	yes
5	2 days	M	NA	NA	yes	yes	no
6	0.9	M	free	free	yes	yes	yes
7	13	M	free	NA	yes	yes	yes
8	1.5	M	free	free	palp	yes	yes
9	2	F	free	free	yes	no	yes
10	3	M	NA	NA	yes	yes	no
11	3	F	NA	free	no	no	yes
12	2	M	NA	NA	yes	yes	yes
13	2	F	NA	free	huge	huge	yes
14	6	M	NA	NA	no	no	no
15	14	F	free	abcess	no	no	yes
16	11	M	NA	NA	NA	NA	NA
17	14	M	NA	NA	NA	NA	NA

CXR : Chest X ray.
LNS : Lymph nodes.
– : No comment on LNS in patient's records.
NA : Not Available.
NE : Non Evaluable.
R: : Relapse.
CR : Complete Remission.
LFU : Lost follow-up.
Palp : palpable.

Table (4): Immunophenotyping details of 17 M7 patients.

No	CD 13	cCD 13	CD 33	CD 41	cCD 61	CD 61	CD 34	DR	MPO	Others	DI
1	2.27	4.8	0.6	71	nd	63	nd	19	1.5	–	0.86
2	2	2	19	67	nd	32	2	10	1	–	0.89
3	7.2	83	5.3	70	nd	40	0	11	23	–	1
4	5	6	8	26	nd	25	37	-ve	-ve	–	1
5	15	23	48	8	47	12	35	4	12	7 (49%) 56 (21%)	1
6	0.7	1	0.5	90	nd	nd	0.5	5	1	–	1
7	0.4	1	1.5	55	nd	67	1.1	4.9	1.2	–	1
8	19	16	30	27	nd	17	0	43	1.5	CD7 (18%)	1
9	3	88	2	65	nd	67	0.2	5.6	8	–	1.13
10	19	19	0	14	57	9	nd	17	1.5	CD7 (19%)	nd
11	1	–	6	4	nd	54	6	29	2	–	1
12	27	32	30	14	nd	28	20	35	8	–	1
13	2	–	76	54	nd	39	41	0	2	–	1
14	75	nd	10	10	70	10	nd	40	5	CD4 (50%)	1
15	7	72	47	2	58	12	nd	31	7	–	1
16	81	nd	0.3	0.0	79	nd	71	56	0.5	CD7 (80%), CD2 (97%)	1
17	2.5	2.5	40	25	nd	76	1	20	9	–	1

c: cytoplasmic. DI: DNA index. nd: not done.



(A) **(B)**

Fig. (1): BM picture of an M7 patient showing (a) typical M7 morphology (b) characteristic acid phosphatase positivity.

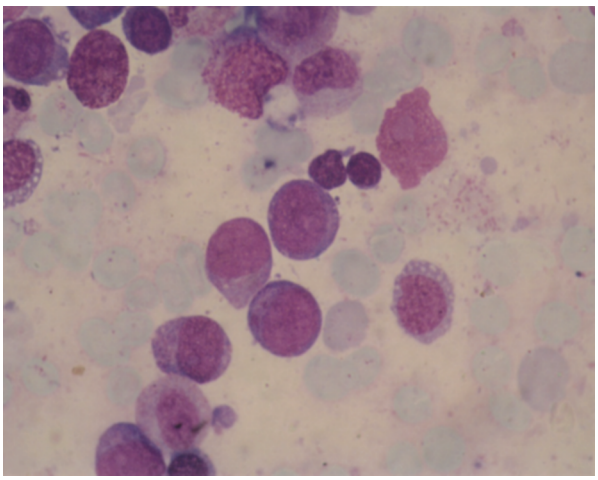
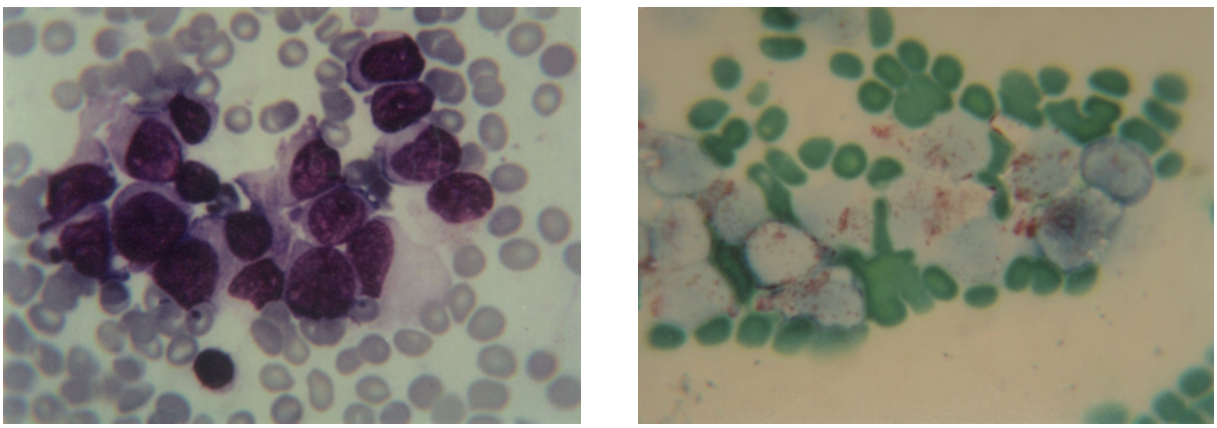
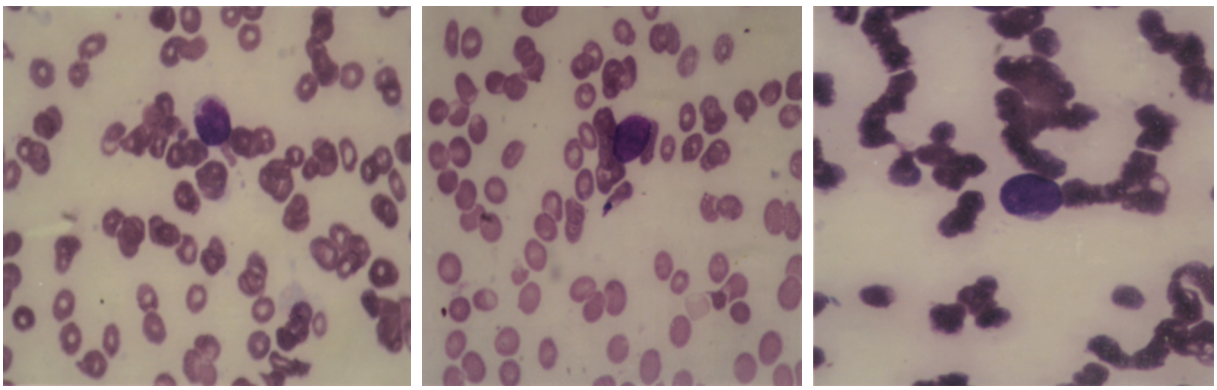


Fig. (2): BM picture of typical M7 patient showing megakaryoblasts with cytoplasmic granulations.



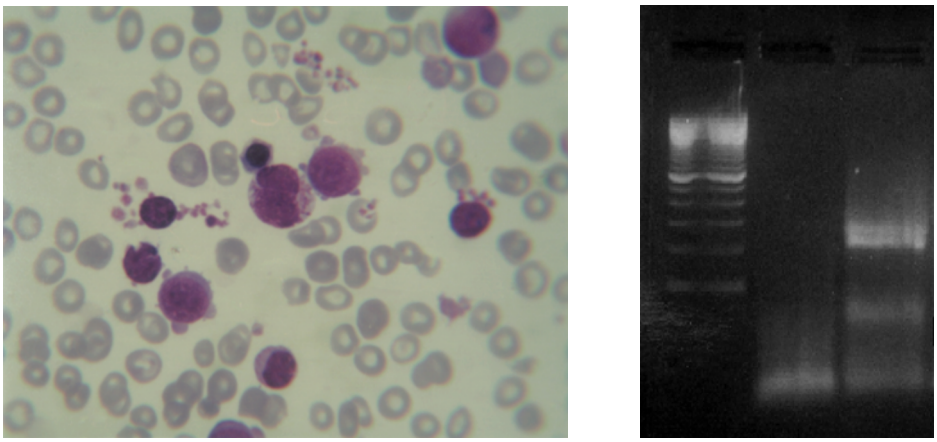
(A) **(B)**

Fig. (3): BM picture of an M7 patient showing (a) blasts resembling myeloblasts (b) characteristic acid phosphatase positivity.



(A) **(B)** **(C)**

Fig. (4): BM aspirate showing megakaryoblasts morpholically similar to lymphoblasts.



(A) **(B)**

Lane 1: 100 bp marker Lane 2: -ve control Lane 3: p210 (patient)

Fig. (5): (a) BM showing micromegakaryocytes in an M7 on top of CML (b) positive bcr/abl amplification band of the same case.

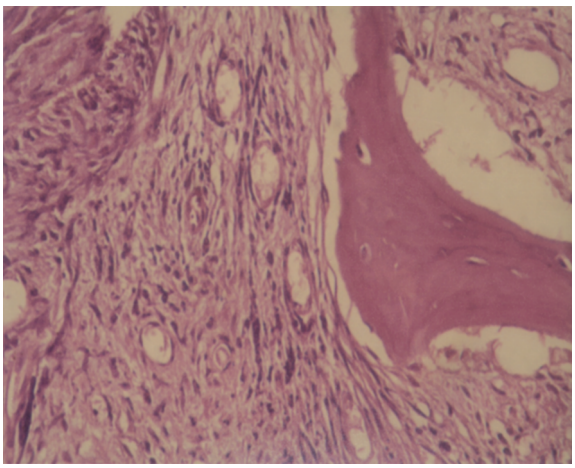


Fig. (6): BM trephine picture of an M7 patient showing marked fibrosis and ectatic sinusoids.

In this work, we aimed to investigate the true relative frequency of M7 within pediatric AML diagnosed at NCI, Cairo University. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

In the present study, 17/280 (6%) of AML cases were M7 including 4 cases with DS. This is comparable to the 4-7% incidence reported in large Cooperative Group studies in developed countries **[6-10]**. The male to female ratio was 2.4:1. Others reported ratios between 1.6:1 and 1.16:1 **[1,10]**. The age range of our cases was from 2 days to 14 years with a median of 36 months; 10/16 (62.5%) of our cases were <3

years including all cases with DS (4/10, 40%) and 7/16 (43.75%) of cases were <2 years.

The median age in our series was slightly higher than that recorded by the St Jude's study (23.9 months) **[10]**. Our data differ from others **[9]** who reported that 79.2% of the patients in their series were <3 years with 58.3% <2 years and also differ from the Mexican group who reported 24% of the cases to be ≤2 years old **[1]**. In our ≤2 years group, 2/16 cases (12.5%) were DS which is an incidence higher than that of the Mexican group who reported 3.45% of their cases with DS **[1]**.

M7 is the most common form of AML in children with DS with a 400-500 fold increased risk **[24-27]**. Several collaborative pediatric group studies have found that approximately half of all pediatric cases of M7 occur in patients with DS **[28,29]** while others reported only 17% of their patients with de novo M7 to have DS. This disparity may reflect a variable rate of referral of patients with DS to different institutions **[10]**.

In our study, 23.5% of cases were DS. Their median age was 26 months which is comparable to the median age recorded (25.8 months) by St Jude's study **[10]** but higher than that reported by others to be <2 years **[30]**. Only 2 of our 4 M7 cases with DS lied in this latter age group (2 days and 1.5 years). All our DS cases were boys in contrast to the reported higher female to male ratio published by other groups **[14]**.

In this work, clinical presentation of M7 was similar in many respects to those observed in patients with other types of acute leukemias.

A study done at St Jude's Hospital proposed a combination of 2 findings to be highly suggestive of M7 **[10]**; typical morphologic features of leukemic cells isolated from the BM together with multifocal punctate cytoplasmic alpha naphthyl acetate esterase cytochemical staining that is incompletely inhibited by sodium fluoride. In our experience, as shown in the current study, characteristic positivity of Acid Phosphatase is highly suggestive of M7, even if the percentage positivity of CD 61 and/or CD 41 did not reach the 20% cutoff value. The diagnosis should always be confirmed by immunophenotyping or immunohistochemistry. In this study, 70.6% of our pediatric patients showed the typical M7 morphology. The other cases (29.4%), displayed heterogeneity in blast morphology at presentation that was very difficult to distinguish from myeloblasts or lymphoblasts and the diagnosis was only possible by immunophenotyping.

In the St Jude's experience, the percentage of blast cells in an aspirated BM specimen was not reliable as a single diagnostic indicator of acute leukemia since 20% of their cases had less than 30% leukemic cells in their aspirates (N.B. the study was performed in the era that required 30% blasts to diagnose acute leukemia). In our study, the blast percent in the bone marrow aspirates was over 20% in 10/12 (83.3%) cases while 2/12 (16.6%) cases had 3 and 16% BM blasts, as judged by morphology; however, the diagnosis was favored by BM trephine findings and the megakaryocytic origin of the blasts was established by immunophenotyping.

In this study the MoAb to platelet GpIIIa (CD 61) was detected in 15/16 cases (93.8%) with 10/15 surface & 5/5 cytoplasmic expression. CD 41 was detected in 10/17 (58.8%) cases and showed a coexpression with CD 61 in 8/15 cases (53.3%). This is in concordance with the observations that GpIIIa is the earliest Gp to be expressed during megakaryocytic maturation **[31]**. Previous studies indicated that the MoAb against GpIIIa should be the marker of choice for the diagnosis of M7 **[32]** however, one case (1/17) expressed CD 41 only. HLA-DR is not normally expressed on circulating platelets or normal megakaryocytes possibly because this Ag appears early in the differentiation of this lineage and is subsequently lost **[33]**. Several studies reported that the blast cells reacted with the anti-HLA-DR MoAb in the majority of their cases **[31,33]** however, only 7/17 (41.1%) of our cases expressed HLA-DR. Some studies viewed HLA-DR expression as an intermediate stage of megakaryocytic differentiation **[34]** but no particular comment was made on its significance. As regards CD 33, it has been reported to appear on megakaryoblasts already expressing CD 41 when HLA-DR Ags were lost **[1]**. In our cases, 5/7 HLA-DR positive cases coexpressed CD 33 and/or CD 13. Coexpression of CD 61, CD 41, HLA-DR and CD 33 was detected in 1/17 (5.8%) of cases. M7 seems to arise from various stages of megakaryocytic differentiation. The expression of HLA-

DR together with CD 13 and/or CD 33 might lead to erroneous diagnosis of AML especially in the absence of the characteristic morphological features. Such a situation is not infrequent and the diagnosis of M7 could be missed. Accordingly MoAb panel should be extended to include CD 61 and CD 41 with both surface and cytoplasmic staining in all AML cases as well as in cases lacking all lineage markers. This will guard against missing an M7 case for a case of AML or undifferentiated leukemia.

In agreement with a previous study **[1]**, we reported a subset of patients (3/17, 29.4%) showing a simultaneous expression of surface membrane Ags of multiple hematopoietic lineages, namely CD 2, CD 4, CD 7 and CD 56. The presence of lymphoid markers especially in the absence of typical M7 morphology may also lead to missing the diagnosis.

In this study, one out of 4 cases tested showed t (1; 22) in a 2 years old female. The close association of 2 specific chromosome aberrations, trisomy 21 and t (1; 22) (p13; q13) with M7, both characterized by early onset of the disease, could account for the younger age at diagnosis reported in several series **[6,9,35]**. This strong association of infant M7 with p13 and q13 breakpoints in chromosomes 1 and 22 suggests that prenatal genetic factors are involved in leukemogenesis and that alteration in genes (N-ras or platelet derived growth factor PDGF-B) at or near these sites participate in malignant transformation and proliferation of megakaryoblasts **[1]**.

The prognosis of children who have de novo M7 in the absence of DS has been difficult to determine. Findings increasingly suggest that these patients have a poorer prognosis than do patients with other FAB subtypes of AML. Studies show that, patients with de novo M7 have significantly lower 5-year survival estimates than do patients treated on similar protocols for other FAB subtypes of de novo AML. **[26]**. Apparently, standard therapies used for AML are not always optimal for patients with M7 **[26,36,37,38]**. The Berlin-Frankfurt-Münster (BFM) group recently reported that M7 independently predicts poor prognosis in children with AML **[38]**. Appropriate treatment for patients with DS, who have a good prognosis and a relatively low tolerance for high-dose chemotherapy, has been described in the literature **[28,39]**. In our series the small number of patients makes it difficult to draw any conclusions with regards to treatment outcome. The high incidence of early deaths might indicate that the standard doses of chemotherapy were not well tolerated. This might necessitate reevaluation of the treatment protocol and drug dosage.

Conclusion:

M7 constitutes 6% of Egyptian pediatric AML as encountered at the NCI, Cairo University. Marked morphological heterogeneity was encountered in 29.4% of the cases lacking the typical morphology and could have been missed for other types of AML or even ALL.

Cytochemistry could be a helpful tool in suspecting the diagnosis of M7 especially Acid Phosphatase. CD 61 and CD 41 membrane and/or cytoplasmic expression are the whole mark for diagnosis and should be tested in all AML cases as well as in cases lacking all other lineage markers. Even if the percentage positivity at diagnosis does not reach the cutoff threshold, repetition is recommended; the diagnosis is still very likely to be M7 especially in the presence of the characteristic Acid Phosphatase positivity.

REFERENCES

1- Paredes-Aguilera R, Romero-Guzman L, Lopez Santiago N, Arana Trejo R. Biology, Clinical and hematologic features of Acute Megakaroblastic Leukemia in Children. American Journal of Hematology. 2003, 73: 71-80.

2- Von Boros J, Korenyi A. Uber einen Fall Akuter Megakaryoblastenleukamie zugleich einige Bemerkungen zum Problem der acuter Leukamie. Z Klin Med. 1931, 118: 697-718.

3- Breton-Gorius J, Vanhaeke D, Pryzwansky KB, et al. Simultaneous detection of membrane markers with monoclonal antibodies and peroxidatic activities in leukemia: Ultrastructural analysis using a new method of fixation preserving the platelet peroxidase. Br J Haematol. 1984, 58: 447-458.

4- Koike T. Megakaryoblastic leukemia: The characterization and identification of megakaryoblasts. Blood. 1984, 64: 683-692.

5- Bennett JM, Catovsky D, Daniel MT, et al. Criteria for diagnosis of acute leukemia of megakaryocyte lineage (M7). A report of the French-American-Britich Cooperative Group. Ann Intern Med. 1985, 103: 460-462.

6- Carroll A, Civin C, Schneider N, et al. The t (1; 22) (p13; q13) is nonrandom and restricted to infants with

acute megakaryoblastic leukemia: A Pediatric Oncology Group Study. Blood. 1991, 78 (3): 748-52.

7- Lion T, Haas OA, Harbott J, et al. The translocation t (1; 22) (p13; q13) is a non andom marker specifically associated with acute megakaryoblastic leukemia in young children. Blood. 1992, 79: 3325-3330.

8- Creutzig U, Harbott J, Sperling C, et al. Clinical significance of surface antigen expression in children with acute myeloid leukemia: Results of study AML-BFM 87. Blood. 1995, 86: 3097-3108.

9- Ribeiro RC, Oliveira MS, Fairclough D, et al. Acute megakaryoblastic leukemia in children and adolescents: A retrospective analysis of 24 cases. Leuk Lymphoma. 1993, 10: 299-306.

10- Uma H Athale, Bassem I Razzouk, Susana C Raimondi, Xin Tong, Frederick G Behm, David R Head, Deo K Srivastava, Jeffrey E Rubnitz, Laura Bowman, Ching-Hon Pui, Raul C Ribeiro. Biology and outcome of childhood acute megakaryoblastic leukemia: a single institution's experience. Blood. 2001, 97: 3727-3732.

11- Gassmann W, Loffler H. Acute Megakaryoblastic Leukemia. Leuk Lymphoma. 1995, 18: 69-73.

12- Massey GV, Zipursky A, Chang MN, Doyle JJ, Nasim S, Taub JW, Ravindranath Y, et al: A prospective study of the natural history of transient leukemia (TL) in neonates with Down Syndrome (DS): Children's Oncology Group (COG) study POG-9481. Blood. 2006, 107 (12): 4606-4613.

13- Ma SK, Wan TS, Chan GC, Ha SY, Fung LF, Chan LC. Relationship between transient abnormal myelopoiesis and acute megakaryoblastic leukaemia in Down's syndrome. Br J Haematol. 2001, 112: 824-825.

14- Duchayne E, Fenneteau O, Pages MP, et al. Acute megakaryoblastic leukaemia: A national clinical and biological study of 53 adult and childhood cases by the Groupe Français d'Hématologie Cellulaire (GFHC). Leuk Lymphoma. 2003, 44: 49-58.

15- Bernstein J, Dastugue N, Haas OA, et al. Nineteen cases of the t (1; 22) (p13; q13) acute megakaryblastic leukaemia of infants/children and a review of 39 cases: Report from a t (1; 22) study group. Leukemia. 2000, 14: 216-218.

16- Lion T, Haas OA. Acute megakaryocytic leukemia with the t (1; 22) (p13; q13). Leuk Lymphoma. 1993, 11 (1-2): 15-20.

17- Ma Z, Morris S, Valentine V, Li M, Herbrick JA, Cui X, et al. Fusion of two novel genes, RBM15 and MLK1, in the t (1; 22) (p13; q13) 0f acute megakaryoblastic leukemia. Nature Genetics. 2001, 28: 220-221.

18- Kamel A, Elsharkawy N, Moussa H, Yassin D, Abdelhamid T, Shaaban K, Mossalam G, ElNahass Y. MPO antigen Negative HLA DR Negative Acute Myeloid Leukemia: Is it a separate clinical entity? J. Egyptian Society of Hematology & Research. 2005, 1: 31- 41.

19- Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. Immunol Method. 1993, 159-197.

20- Hedley DW, Shankey TV, Wheeless LL. DNA Cytometry Consensus Conference. Cytometry. 1993, 14: 471-500.

21- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning; A laboratory manual (second edition), Cold spring harbor laboratory press. 1989, 2: 9.34-9.46.

22- Davis LG, Dibner MD, Battey JF. Basic methods in molecular biology, Elsevier Science Publishing Co, Inc, New York. 1986, p58-61.

23- Behm FG. Diagnosis of childhood acute myeloid leukemia. Clin Lab Med. 1999, 19: 187-237.

24- Bhatia S, Neglia JP. Epidemiology of childhood acute myelogenous leukemia. J Pediatr Hematol Oncol. 1995, 17: 94-100.

25- Zippursky A Editorial. The treatment of children with acute megakaryoblastic leukemia who have Down syndrome. J Pediatr Hematol Oncol. 1996, 18: 10-12.

26- Lange BJ, Kobrinsky N, Barnard DR, et al. Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. Blood. 1998, 91: 608-6.

27- Hitzler J, Zipursky A. GATA 1 mutations as clonal markers of minimal residual disease in acute megakaryoblastic leukemia of Down syndrome a new tool with significant potential applications. Leuk Res. 2005, 29 (11): 1239-40.

28- Ravindranath Y, Abella E, Krischer JP, et al. Acute myeloid leukemia (AML) in Down's syndrome is highly responsive to chemotherapy: Experience on Pediatric Oncology Group AML Study 8498. Blood. 1992, 80: 2210-2214.

29- Zubizarreta P, Felice MS, Alfaro E, et al. Acute myelogenous leukemia in Down's syndrome: Report of a single pediatric institution using a BFM treatment strategy. Leuk Res. 1998, 22: 465- 472.

30- Windebank KP, Tefferi A, Smithson WA, et al. Acute megakaryocytic leucemia (M7) in children. Mayo Clin Proc. 1989, 64: 1339-1351.

31- Rever WN, Breton-Gorius J, Villeval JL, et al. Detection of cells of megakaryocyte lineage in hematological malignancies by immunoalkaline phosphatase-labelling cell smears with a panel of monoclonal antibodies. Bt J Haematol. 1987, 65: 87-94.

32- Helleberg C, Knudssen H, Hansen PB, et al. CD 34+ megakaryoblastic leukemic cells are CD 38- but CD 61+ and glycophorin A+: improved criteria for diagnosis of M7? Leukemia. 1997, 11: 830-834.

33- Pombo de Oliveira MS, Gregory C, Matutes E et al. Cytochemical profile of megakaryoblastic leucemia: A study with cytochemical methods, monoclonal antibodies and ultrastructural cytochemistry. J Clin Pathol. 1987, 40: 663-669.

34- Vinci G, Tabillo A, Deschamps JF, et al. Immunological

study of invitro maturation of human megakaryocytes. Br J Haematol. 1984, 56: 589-605.

35- Lu G, Altman AJ, Benn PA. Review of the cytogenetic changes in acute megakaryoblastic leucemia: One disease or several? Cancer Genet Cytogenet. 1993, 67: 81-89.

36- Ravindranath Y, Yeager AM, Chang MN, et al. Autologous Bone Marrow transplantation intensive consolidation chemotherapy for acute myeloid leukemia in childhood. Pediatric Oncology Group. N Engl J Med. 1996, 334: 1428-1434.

37- Stevens RF, Hann IM, Wheatly K, et al. Marked improvement in outcome with chemotherapy alone in paediatric acute myeloid leucemia: Results of the United Kingdom Medical Research Council 10th AML trial. MRC childhood leucemia working party. Br J Haematol. 1998, 101: 130-140.

38- Creutzig U, Zimmermann M, Ritter J, et al. Definition of a standard risk group in children with AML. Br J Haematol. 1999, 104: 630-639.

39- Ravindranath Y, Taub JW. Down Syndrome and acute myeloid leukemia: Lessons learned from experience with high dose Ara C containing regimens. Adv Exp Med Biol. 1999, 457: 409-414.