PATTERN OF IMMUNOPHENOTYPING EXPRESSION IN NEWLY DIAGNOSED ACUTE LEUKAEMIA PATIENTS IN BANGLADESH: A REFERRAL CENTER STUDY

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Date-
Dhaka Cantonment.

Abdullah Al Mamun
Major
AFMI

FORWARDING

This is to certify that BA-101847 Maj Abdullah Al Mamun carried out this research work titled "Pattern of immunophenotyping expression in newly diagnosed acute leukaemia patients in Bangladesh: a referral center study" under my direct supervision. I have found the work and the dissertation satisfactory for partial fulfillment of the requirements of the grading course of the Armed Forces Medical Institute.

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REMARKS

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REMARKS

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LIST OF ABBREVIATIONS

AFIP	Armed Forces Institute of Pathology
AFMI	Armed Forces Medical Institute
AL	Acute Leukaemia
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukaemia
APML	Acute Promyelocytic Leukaemia
AMoL	Acute Monoblastic/Monocytic Leukaemia
BM	Bone Marrow
CD	Cluster Differentiation
cCD3	Cytoplasmic CD3
cCD79a	Cytoplasmic Cd79a
FBC	Full Blood Count
FSC	Forward Scatter
FAB	French American British classification
FITC	Fluorescein isothiocyanate
FLAER	Fluorescent aerolysin
HLA-DR	Human Leukocyte Antigen-DR isotype
MPO	Myeloperoxidase
MRD	Minimal Residual Disease
PNH	Paroxysmal Nocturnal Haemoglobinuria
sm	Surface membrane
SSC	Side Scatter
TdT	Terminal deoxynucleotidyl transferase
WBC	White Blood Cell
WHO	World Health Organization
B-ALL	B-cell acute lymphoblastic leukaemia
T-ALL	T-cell acute lymphoblastic leukaemia

Abstract

Background: Acute leukaemia is a heterogenous group of haematological malignancies characterized by clonal proliferation of immature haematopoietic cells. Immunophenotyping by flow cytometry is essential for accurate lineage assessment, classification and therapeutic planning.

Objectives: To determine the pattern of immunophenotypic expression of newly diagnosed case of acute leukaemia at Armed Forces Institute of Pathology.

Materials and Methods: Study spanned from 01 January 2025 to 31 December 2025.A total 80 newly diagnosed acute leukaemia cases were analyzed by multiparameter flow cytometry. Immunophenotypic markers for B-lineage, T-lineage, and myeloid lineage were assessed. Data were analyzed using SPSS version 26.

Results: Among 80 cases, 53.75% were Acute Myeloid Leukaemia (AML), 40% were Acute Lymphoblastic Leukaemia and Mixed phenotype acute leukaemia were 6.25% cases. B-ALL accounted for 84.3% of ALL cases, T-ALL for 15.6% cases. In AML cases CD13, CD33, MPO were the most frequently expressed markers. Aberrant lymphoid antigen expression was observed in 20.9% cases of AML and aberrant myeloid antigen expression was observed in 44.4% cases of B-ALL. The commonest FAB subtype in AML group was AML-M4/5 (41.8%) which may differ from most published data. It was found that combined use of HLADR and CD34 was much more helpful in distinguishing APL from non-APL AML than either of these antigens alone. It was found that cCD79a and CD19 were the most sensitive marker for B-ALL while cCD3, CD7 and CD5 were the most sensitive antigens for T-ALL.

Conclusion: Immunophenotyping plays a pivotal role in lineage assignment and detection of aberrant antigen expression in acute leukaemia. The majority of cases showed expected lineage specific marker patterns, though a significant minority demonstrated aberrancies, emphasizing the importance of comprehensive panels. Flowcytometry was found to be especially useful in the identification of AML-M0 and differentiation of APL from non-APL AML. Immunophenotyping results and FAB classification of my AL patients were comparable to internationally published studies apart from predominance of AML-M4/5 and more frequent APL.

Keywords: Acute leukemia, Immunophenotyping, Flowcytometry

APPENDICES

APPENDIX-I

Data collection sheet

Title: Pattern of immunophenotyping expression in newly diagnosed Acute Leukaemia Patients in Bangladesh: A Referral Center Study

Sl. No	Question	Response
01.	Age of the patient (in year)	
02.	Sex	1=Male, 2=female
03.	Area of residence	1=Urban, 2=Rural
04.	Type of residence	1=Building, 2= Tin & Wooden, 3= Clay& Bamboo
05.	Occupation	1= Student, 2= Farmer, 3= Housewife, 4= Day labour, 5= Businessman, 6= Service holder, 7= Retired, 8= Infant, 9=children (up to 18 years), 10= Others
06.	Types of Leukaemia	1=AML, 2=ALL
07.	Clinical Symptoms	1= Fever 2= Gum bleeding/ bruise/purpura 3= Generalized weakness 4= Hepatomegaly 5= Splenomegaly 6= Lymphadenopathy 7= Gum hypertrophy 8= Bony Tenderness

08.	Received Blood Transfusion	1=Yes, 2=No
09.	Any exposure history	1=Radiation, 2= Benzene,
		3=Petroleum, 4=Insectiside,5= Tobacco
10.	Comorbidities	1=DM, 2=HTN, 3=IHD, 4=Liver disease, 5=Renal disorder, 6=Respiratory disease
11.	Family history of malignancy	1=No; 2=Yes
12. Invest-igation Profile	Haemoglobin (gm/dl)	1= low, 2 = Normal,3= high
	Total Leukocyte Count($x10^9$)	1= low, 2 = Normal, 3=high
	Total Platelet count($x10^9$)	1= low, 2 = Normal, 3=high
	PBF	1=AML, 2=ALL
	Serum LDH	1= low, 2 = Normal, 3=high
	Bone marrow findings (Blast cell %)	1=AML, 2=ALL
	Immunophenotyping Antibody panel (core markers)	1=CD45, 2=CD34, 3=HLA-DR
	Immunophenotyping Antibody panel (myeloid markers)	1=CD13, 2=CD33, 3=CD117, 4=cMPO
	Immunophenotyping Antibody panel (B-ALL markers)	1=CD19,2=CD10, 3=CD20,4=CD79a
	Immunophenotyping Antibody panel (T-ALL markers)	1=CD3, 2=CD5, 3=CD7
	Lineage interpretation	1= AML, 2= B-ALL, 3= T-ALL, 4=MPAL

(Signature of the investigator)
Abdullah Al Mamun

Major
AFMI

APPENDIX-II

INFORMED WRITTEN CONSENT FORM FOR SUBJECTS

Title: Pattern of immunophenotyping expression in newly diagnosed Acute Leukaemia Patients in Bangladesh: A Referral Center Study

Investigator's name: BA101847 Maj Abdullah Al mamun

Institution: Armed Forces Medical Institute (AFMI), Dhaka Cantonment

Purpose of the study: This study will be done to evaluate the pattern of immunophenotyping expression in newly diagnosed Acute Leukaemia patients. The knowledge generated from this study will be disseminated in the department of Haematology, AFIP. It may contribute to the policy making decisions.

Expectation from and involvement of the participant: You will be asked some questions according to a semi-structured questionnaire that are about your disease. Also, some physical examinations and investigations will be performed. We expect that you will give consent for physical examination, investigation and information given by you will all be correct.

Privacy, anonymity, and confidentiality: All information regarding your identity will be kept confidential. For safeguarding, confidentiality and protecting anonymity each of the patient will be given a special ID no. Research data will be coded with that ID no. and then data will be stored in a locked cabinet. Only research personnel will be allowed to access data. Personal information will not be used during data analysis or publication.

Right to withdraw: Your participation in this study is completely dependent upon your free will and it will not affect your current treatment process. You also reserve the right to withdraw your name anytime during this research procedure.

Incentives:

You will not be provided any incentives to take part in this research. You will be given honorarium conveyance expenditure if you are to come for this research work.

Risks and discomforts:

There is a slight risk that you may share some personal and confidential information by chance or that you may feel uncomfortable about some of the topics. However, we do not wish this to happen. You may refuse to give answer to any question or any portion of it if you need to do so.

Benefits:

You might get direct benefit from this study. You will get appropriate treatment in hospital after your admission. Your participation is likely to help us to acquire more knowledge about this intervention which may be of benefit to other patients of your country.

Procedure of research:

If you agree, we will enroll you as a study participant and will adopt the following procedures for your participation-

i. We will take signature/thumb impression in the attached consent form in duplicate and a copy will be returned to you.
ii. We will ask you some questions to fill in a printed Case Record Form
iii. You will be examined physically for the sake of this study.

If you agree to participate in this study, please sign the attached consent form.

INFORMED CONSENT FORM

Mr/Mrs/Miss/Master……/parent of…… hereby giving informed consent willingly to participate in the study to be done by Dr I agree to participate in the study voluntarily without prejudice. I am fully convinced that during study I will not suffer from any serious physical psychological problems. I am also informed that this study was carried out safely and my participation will bring fruitful result that will be beneficial for most patients in our country. I have right to withdraw myself from this study at any time. I shall not receive any financial benefit. I have understood that my personal information, medical records & laboratory tests will be kept strictly confidential & will be used for research purpose only.

Signature/Thumb impression of Participant

: ……

Date: ……

Name: ……..

Mobile no: …….

Address:

……

……

Signature of Witness: Signature of Researcher:

Name: Name:

Date: Date:

APPENDIX-III

Work Schedule

January 2025 to December 2025

Activities	January 2025 -February 2025	March 2025 – April 2025	May 2025- June 2025	July 2025- August 2025	September 2025- October 2025	November 2025- December 2025
Topic selection						
Literature review						
Protocol submission						
Data collection						
Data entry, edit and analysis						
Report writing and binding						

Submission						

Part-1

Introduction

Part-1

1.1 Introduction:

Immunophenotyping is the process by which the pattern of expression of antigens by a population of cells is determined. The presence of a specific antigen is recognized by its binding to a labelled antibody. Antibodies can be present in a polyclonal antiserum that is raised in an animal but more often they are well characterized monoclonal antibodies produced by hybridoma technology; a hybridoma is a clone of cells created by the fusion of an antibody-producing cell with a mouse myeloma cell. Monoclonal antibodies can be labelled with an enzyme or with a chemical, known as a fluorochrome, that under certain circumstances will fluoresce. Immunophenotyping is carried out primarily by flow cytometry or immunohistochemistry.

Flow cytometric immunophenotyping is applicable to cells in peripheral blood, bone marrow, body fluids (pleural, pericardial, ascitic and cerebrospinal fluids) and fine needle aspirates. Immunohistochemistry of relevance to haematological disease is applied particularly to trephine biopsy and lymph node biopsy specimens, but also to biopsy specimens from any other tissues where infiltration by haemopoietic or lymphoid cells is suspected.[1]

Acute leukaemia represents a heterogeneous group of hematological malignancies characterized by uncontrolled proliferation of immature precursor cells. Accurate diagnosis and classification are essential for effective management. The World Health Organization classification emphasizes integration of morphology, cytogenetics, molecular studies, and immunophenotyping in diagnosis.[2]

Immunophenotyping by flow cytometry plays a central role in identifying lineage and differentiation stage of leukemic blasts. It allows detection of lineage-specific and aberrant antigen expression patterns, which are critical for distinguishing between acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL).[3]

1.2 Rationale of the study:

Acute leukaemia represents a heterogenous group of haematological malignancies characterized by the clonal proliferation of immature precursor cells in the bone marrow, blood and other tissues. Accurate and timely diagnosis is critical because treatment strategies, prognosis and survival outcomes differ significantly between subtypes such as Acute Lymphoblastic Leukemia and Acute Myeloid Leukaemia. In modern haematology, immunophenotyping by flow cytometry has emerged as an essential diagnostic tool that complements morphology and cytogenetics by identifying lineage specific and aberrant antigen expression patterns.

Despite global advancements, there is considerable variation in immunophenotypic expression profile across different populations and geographic regions. These variations may be influenced by genetic, environmental and socioeconomic factors. However, in many developing countries including Bangladesh, there is limited comprehensive data regarding the immunophenotypic patterns of newly diagnosed acute leukaemia patients, particularly in referral centers where

diverse and complex cases are concentrated. This lack of local data may hinder optimal diagnostic classification and risk stratification.
Furthermore, acute leukaemia often presents with overlapping clinical and morphological features, making differentiation between subtypes challenging without immunophenotyping. The identification of lineage- defining markers (e.g., CD markers) and aberrant antigen expression is crucial not only for accurate classification, inappropriate treatment and poorer patient's outcomes.

A referral center plays a pivotal role in managing complicated and atypical cases, thereby providing unique opportunity to study a wide spectrum of immunophenotypic expressions. Investigating the pattern of antigen expression in newly diagnosed cases at such a center can help establish baseline data, identify common and rare marker profiles, and evaluate the frequency of aberrant phenotypes in the local population.

Therefore, this study is justified to:

- Generate region specific data on immunophenotyping patterns of acute leukaemia.
- Improve diagnostic accuracy and sub classification of acute leukaemia.
- Support clinicians in selecting appropriate treatment protocols.
- Contribute to the understanding of disease biology in the local context.
- Provide a foundation for future research, including prognostic and therapeutic studies.

In summary, this study aims to bridge the existing knowledge gap by systematically analyzing immunophenotypic expression patterns in newly diagnosed acute leukaemia cases at a referral center, ultimately contributing to improved patient care and outcomes.

1.3 Research Question:

What is the pattern of Immunophenotyping expression of newly diagnosed acute leukaemia patients at AFIP?

1.4 Objective of the study:

1.4.1 General objective:

- To determine the pattern of immunophenotypic marker expression in 80 diagnosed cases of acute leukaemia using flow cytometry at Armed Forces Institute of Pathology.

1.4.2 Special objective:

- To classify acute leukaemia cases based on immunophenotyping findings into
    - Acute myeloid leukaemia(AML) with FAB subtypes
    - Acute lymphoblastic leukaemia (ALL)
    - Mixed Phenotype Acute Leukaemia (MPAL)
- To determine the frequency of commonly expressed myeloid markers (e.g. CD13, CD33, cMPO, CD117).
- To determine the frequency of B-lymphoid markers (e.g. CD19, CD79a, CD20, CD10).
- To determine the frequency of T-lymphoid marker (e.g. smCD3, cyCD3, CD5, CD7).
- To evaluate aberrant antigen expression in AML and ALL cases.

Part – 2
Literature review

Part-2

2.1 Literature review:

2.1.1 Role of Immunophenotyping:

Flow cytometry enables rapid analysis of multiple antigens, improving diagnostic accuracy. It is used for lineage assignment, detection of aberrant markers, and prognosis.
It is widely used for

a. Lineage assignment
b. Identification of maturation stage
c. Detection of aberrant antigen expression
d. Prognostic stratification

Immunophenotyping has become an essential component of modern leukaemia classification systems. [4][5].

2.1.1.1 Immunophenotypic Pattern in AML:

AML commonly expresses CD13, CD33, CD117, HLA-DR, and CD45. CD13 and CD33 are most frequently expressed markers in AML[8][10] .

2.1.1.2 Subtype variations:

Immunophenotypic expression varies among AMLsubtypes

a. Acute promyelocytic leukaemia (APL): typically HLA-DR negative.
b. Monocytic AML: expresses CD14 and CD11b.
c. Immature AML(M0) : strong CD34 expression.

AML often exhibits aberrant expression of lymphoid markers, such as:

a. CD7 (T-cell marker)
b. CD19 (B-cell marker)

This phenomenon indicating its potential prognostic significance.[10]

2.1.1.3 Immunophenotypic Pattern in ALL:

B-ALL typically expresses CD19, CD10, CD20, CD79a. Studies by Omran et al. demonstrated that CD10 is commonly expressed in precursor B – ALL and plays a role in disease classification.
T-ALL typically expresses CD3, CD5, CD7. These markers are essential for confirming T-lineage differentiation [15][16].

Comparative Patterns
AML shows myeloid markers, while ALL shows lymphoid markers. Combined panels allow accurate differentiation[8][16].

2.1.2 Geographical Variation:

Several studies have highlighted regional differences in antigen expression patterns:

a. Variations in FAB subtype prevalence in Brazil.
b. Differences in immunophenotypic profiles in south Asian populations
c. Geographic heterogeneity between Australian and Japanese populations

These findings suggest that genetic and environmental factors influence the immunophenotypic characteristics of acute leukaemia.[11][12]

2.1.3 Prognostic significance of immunophenotypic markers:

Immunophenotypic markers are important predictors of prognosis:
Expression of CD34 and aberrant markers is often associated with poor outcomes. Aberrant phenotypes are associated with adverse clinical outcomes.[26]

2.1.3.1 Role of flow cytometry panels:

a. Myeloid markers: CD13, CD33, CD117
b. B- cell markers: CD19, CD10
c. T-cell markers: CD3, CD5
d. Stem cel markers: CD34
e. Others: HLA-DR, CD45

Multi-color flow cytometry enhances diagnostic precision and allows detection of mixed phenotype acute leukaemia.[26]

2.1.3.2 Clinical implications:

Immunophenotyping is essential for:

a. Confirming diagnosis.
b. Classify leukaemia subtype.
c. Guiding therapeutic decisions.
d. Monitoring minimal residual disease.[27]

2.1.4 Flow cytometric Immunophenotyping:

This technique determines cell size, structure (to some extent) and antigen expression. Cells in suspension are first exposed to a combination of fluorochrome-labelled monoclonal antibodies (or other lectins or ligands) and then pass in a focused stream through a beam of light generated by a laser. Laser-generated light is coherent (waves of light are parallel) and monochromatic (single wave length/colour). Large multichannel instruments with multiple lasers are used to identify, count, size and otherwise characterize cells that are hydrodynamically focused and pass in a single file through a narrow orifice in a flow cell. The passing of the cell through a light beam leads to both the scattering of light and the excitation of fluorochromes so that they emit a fluorescence signal.[30]

Forward scatter (FSC) of light at a narrow angle is detected and measured and is proportional to cell size. Sideways or side scatter (SSC) of light is detected and measured and is proportional to cell granularity and complexity. Antigens expressed on the surface membrane of cells or with modified techniques, within cells are detected. After 'permeabilisation', both cytoplasmic and nuclear antigens can be detected. For each fluorochrome, a selected laser emits light of a specified wavelength that will be absorbed by the fluorochrome. This leads to excitation of the fluorochrome with subsequent emission of light of lower energy and a longer wavelength as the fluorochrome returns to its basal state; this property is known as fluorescence. [31]

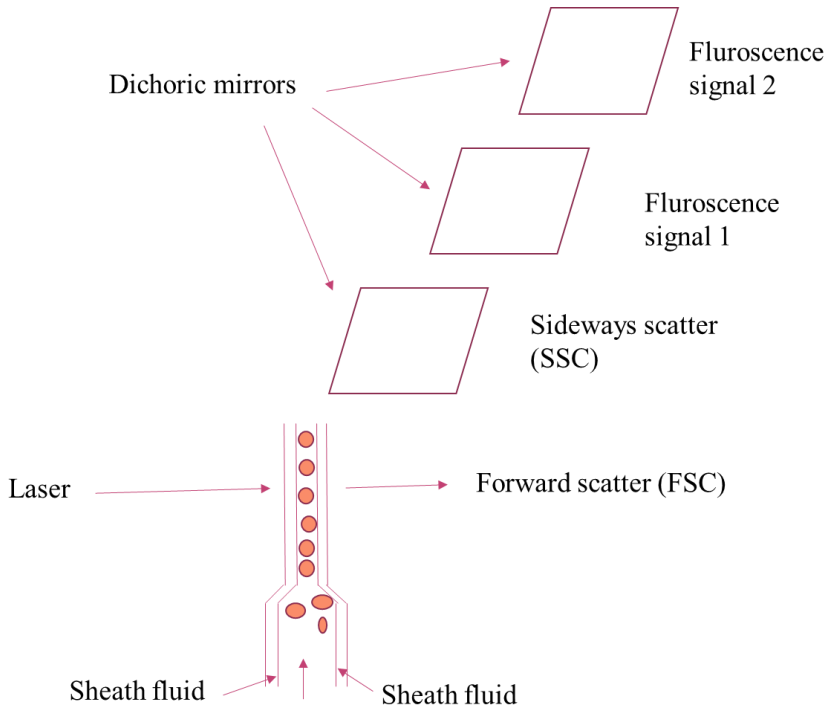


Figure-2.1 Diagrammatic representation of the principles of flow cytometric Immunophenotyping.[31]

The amount of light emitted (the number of photons) is proportional to the amount of fluorochrome bound to the cell. The mean fluorescence intensity of a population indicates the strength of expression of the relevant antigen. The emitted light passes through dichroic mirrors, that is, mirrors that reflect some wavelengths and transmit others, so that it is possible, for example, to reflect SSC for measurement and transmit fluorescence signals to another detector such as a photomultiplier tube [Figure-2.1]. The detector produces an electrical signal that is proportional to the amount of incident light. Some commonly used fluorochromes are shown in Table-2.1.

The cells that are studied must be dispersed. For peripheral blood and bone marrow aspirate specimens, it is necessary to exclude mature and immature red cells. This is most simply done by lysing red cells using an ammonium chloride solution. Otherwise red cells and their precursors will appear in scatter plots and interfere with gating leucocyte populations of interest. If assessment of immunoglobulin expression is required, there must also be a washing step to remove the plasma that contains immunoglobulin, which would neutralize the monoclonal lambda or kappa specific antibody.

Table-2.1 Commonly used fluorochromes.[32]

Name of the flurorochromes
Fluorescein isothiocyanate (FITC)
Phycoerythrin (PE)
Allophycocyanine (APC)
Peridinin chlorophyll (PerCP)
Cyanine 5 (Cy5), cyanine 5.5 (Cy5.5) and cyanine 7 (Cy7)
Texas red
Pacific blue
Brilliant violet
Krome orange
Alexa Fluor 488 (AF488)
Alexa Fluor 647 (AF647)
Phycoerythrin-Texas Red X (ECD)
Phycoerythrin-cyanine 5 (PE-Cy5)
Phycoerythrin-cyanine 5.5 (PE-Cy5.5)
Phycoerythrin-cyanine 7 (PE-Cy7)

The great majority of monoclonal antibodies used in immunophenotyping have been characterized at a series of international workshops and those with the same specificity have been assigned a cluster of differentiation (CD) number. This number can be used to refer to both the antibody and the antigen it recognizes. There are now more than 350 specificities recognized so that a careful selection of antibodies for diagnostic use is important. In addition to fluorochromes conjugated to monoclonal or polyclonal antibodies, it is also possible to use either fluorochromes that can bind directly to cellular constituents, such as DNA, or labelled modified aerolysins that bind to membrane glycosylphosphatidylinositol glycan A (GPI) (used in the diagnosis of paroxysmal nocturnal haemoglobinuria). Propidium iodide binding can be used to identify non-viable cells and exclude them from analysis. Results of immunophenotyping are

usually shown as a two-dimensional plot in which FSC, SSC and the expression of certain anti-gens are plotted against each other, permitting the recognition of the probable nature of a cell cluster in a particular position. It is thus possible to gate on a cellular population of interest. A gate is an electronic boundary; it can either be predetermined or drawn by the operator. There are four commonly used approaches to gating of target populations: FSC versus SSC, CD45 versus SSC, CD19 versus SSC and CD34 versus SSC. FSC versus SSC is a useful way of screening a specimen to identify normal populations and to highlight abnormal cells as illustrated in Figure-2.2. [32]

Forward scatter is increased in relation to increasing cell size whilst SSC is influenced by cytoplasmic granularity and nuclear complexity. It is a useful means of gating on blasts when CD34 is not expressed, for example in monoblastic leukaemias. Such plots are helpful in identifying large activated lymphocytes, an excess of small lymphocytes or monocytes and even the presence of hairy cells. Granular blasts show increased SSC and this is reflected in a shift to the right in the scatter plot. This can be an early indication of a possible acute promyelocytic leukaemia.[32]

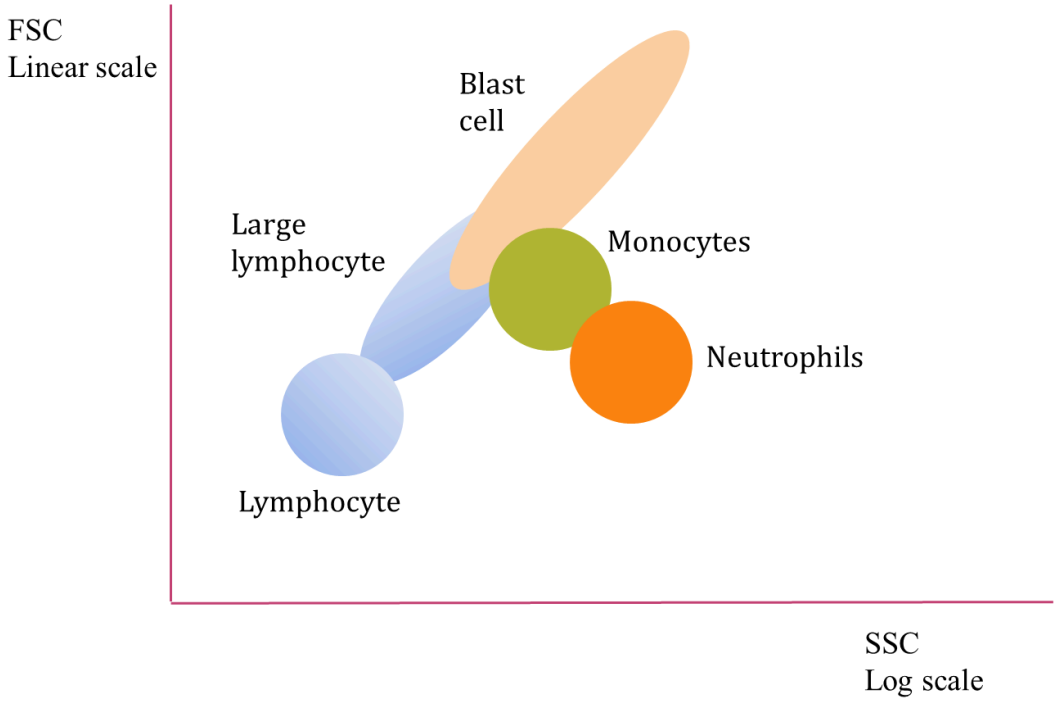


Figure-2.2 Delineation of peripheral blood leucocyte populations using forward scatter (FSC) and side scatter (SSC) characteristics.[33]

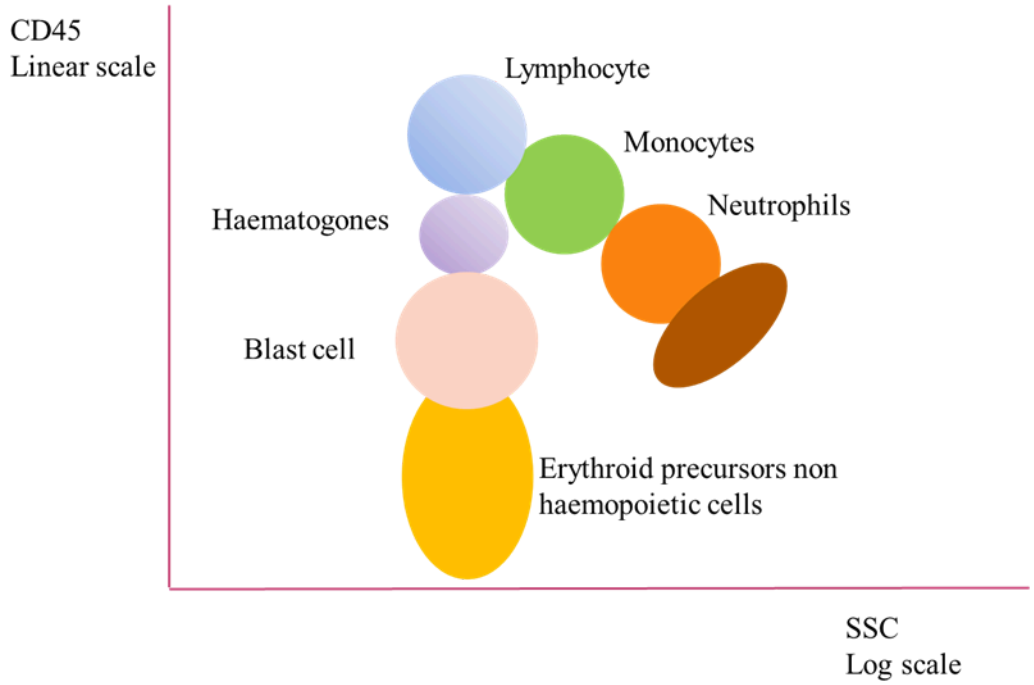


Figure-2.3 Delineation of peripheral blood or bone marrow leucocyte populations using CD45 expression and SSC.[33]

A plot of CD45 expression and SSC is not only useful for separating normal cell populations but also helps identify precursor cell populations, which frequently show only weak CD45 expression [Figure-2.3]. CD19 versus SSC [Figure-2.4] and CD34 versus SSC [Figure-2.5] plots are useful for isolating B cells and blast cells, respectively. Back gating is a process whereby a target population identified in one approach can be tracked in another. For example, CD34+ myeloblasts can be isolated using CD34 versus SSC, then colour tracked into the FSC versus SSC plot to show cell size and granularity. With modern multichannel instruments it is possible to study 6-8 or more antigens in a single tube. If multiple tubes are studied, several core antibody-flurochrome conjugates can be included in each tube analyzed so that cross comparison between the same cells stained with different antibody panels in different tubes is possible.

Flow cytometric Immunophenotyping is used particularly in the investigation of haematological neoplasms, but there are other roles (Table-2.2).[33]

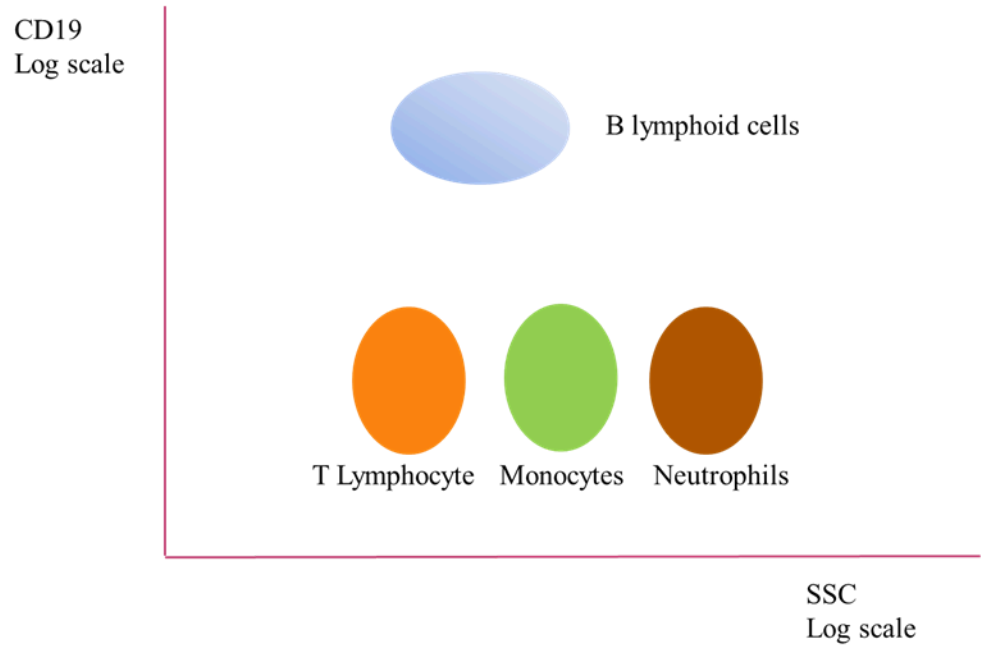


Figure-2.4 Delineation of peripheral blood or bone marrow B-cell populations using CD19 expression and SSC.[33]

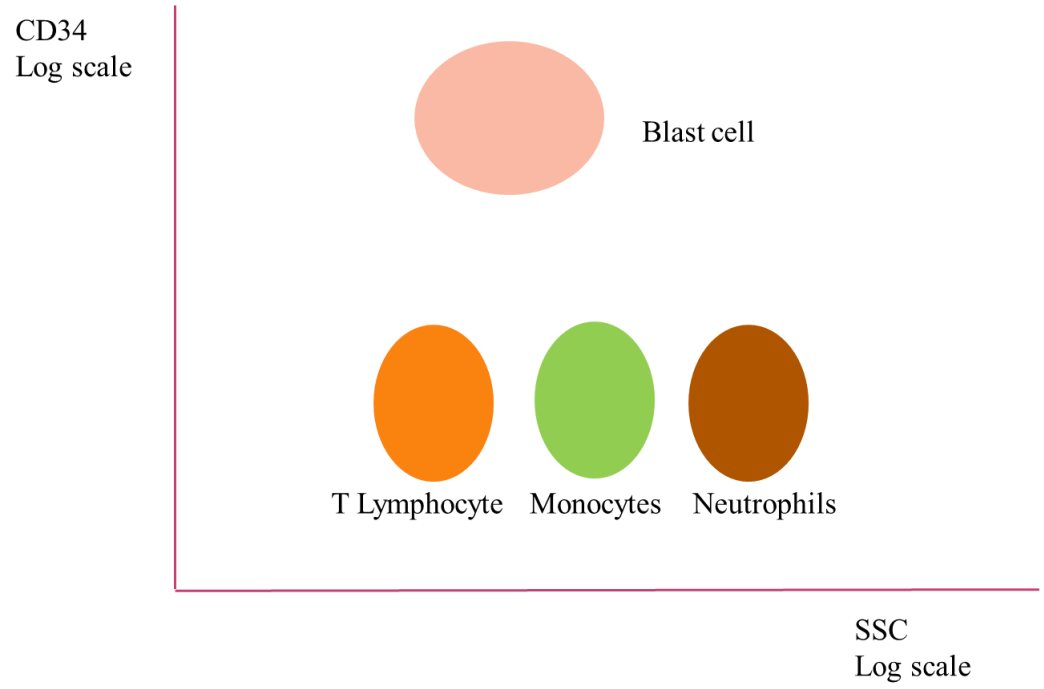


Figure-2.5 Delineation of peripheral blood or bone marrow CD34+ blast populations using CD34 expression and SSC.[33]

Table-2.2 Role of flow cytometric immunophenptyping.[34]

Haematological neoplasms
Diagnosis of haematological neoplasms
Further classification e.g. of AML, B-ALL, T-ALL
Identification of disease spread e.g. to the central nervous system
Identification of a therapeutic target e.g. CD19, CD20, CD30, CD33, CD52
Detection of minimal residual disease (which may include identifying a leukaemia-specific phenotype at diagnosis)
Identification of hypodiploidy and hyperdiploidy in B-ALL, including the detection of masked hypodiploidy when there has been duplication of a small hypodiploid clone
Investigation of erythrocytes and their disorders
Diagnosis of paroxysmal nocturnal haemoglobinuria (CD15, CD16, CD24, CD55, CD59, CD66b, CD157, FLAER on neutrophils; CD14, CD55, CD157 and FLAER on monocytes; CD55, CD59 and FLAER on erythrocytes)
Identification of a PNH clone in aplastic anaemia (predictive of better prognosis and a response to immunosuppressive therapy)
Diagnosis of hereditary spherocytosis (eosin-5-maleimide binding). Binding is also reduced in hereditary pyropoikilocytosis, South-East Asian ovalocytosis and congenital dyserythropoietic anaemia, type II
Diagnosis of hereditary stomatocytosis due to RHAG mutation (reduced expression of CD47, which is part of the Rh protein complex)
Detection and enumeration of fetal red cells in maternal circulation (using anti-RhD when mother is RhD-positive, or using permeabilised erythrocytes and an antibody to haemoglobin F) or using the two techniques in combination
Investigation of platelets and their disorders
Diagnosis of inherited platelet disorders: Glanzmann's thrombasthenia, deficiency of platelet glycoprotein IIb/IIIa (CD41/CD61 absent or reduced in three quarters of patients); Bernard-Soulier syndrome, deficiency of glycoprotein I/V/IX (CD41 and CD42a/CD42b moderately reduced); Scott syndrome (annexin V not expressed on activated platelets); GFI1B mutation (CD34 expressed on platelets); Wiskott-Aldrich syndrome (deficiency of WAS protein, reduced or defective CD43 on T lymphocytes)

Investigation of leucocytes and their disorders including investigation of immune function
Investigation of suspected primary immunodeficiency syndromes
Diagnosis of autoimmune lymphoproliferative syndrome (CD3+TCRαß+CD4-CD8-lymphocytes)
Diagnosis of leucocyte adhesion deficiencies type I (CD18 and CD11a, 11b and 11c deficient) and type II (CD15s deficient); reduced expression of CD11b, CD18 or CD15s by phorbol esterase-stimulated neutrophils is demonstrated
Diagnosis of neutrophil specific granule deficiency (reduced SSC, CD15, CD16, CD66, myeloperoxidase and lactoferrin)
Diagnosis of chronic granulomatous disease using dihydrorhodamine as a marker of H2O2 production after stimulation of neutrophils; carrier detection is also possible
Enumeration of CD4-positive T cells in HIV infection
Investigation for lymphocytic variant of hypereosinophilic syndrome (aberrant phenotypes such as CD3-CD4+CD8-or CD3+CD4-CD8-)
Diagnosis of haemophagocytic lymphohistiocytosis (HLH) (upregulation of HLA-DR on T cells; CD57 and perforin can also be upregulated; testing for deficiency of perforin, SAP, XIAP or CD107a is used to screen for various underlying genetic defects
Diagnosis of persistent polyclonal lymphocytosis
Identification of sepsis by CD64 expression on neutrophils
Other
Enumeration and isolation of haemopoietic stem cells (CD45 weak, CD34+, SSC low)
Differential leucocyte counting; the Beckman Coulter Hematoflow, for example, can distinguish neutrophils, eosinophils, basophils, CD16- and CD16+ monocytes, B cells, CD16+ cytotoxic T cells and NK cells, CD16-T cells, myeloblasts, monoblasts, B lymphoblasts and T lymphoblasts
Enumeration and characterisation of reticulocytes or platelets by the binding of a fluorochrome (e.g. a proprietary mixture of polymethine and oxazine in Sysmex instruments) to RNA or the binding of a fluorescence-labelled CD61 monoclonal antibody to platelets (CellDyn instruments)
AML, acute myeloid leukaemia; B-ALL, B-lineage acute lymphoblastic leukaemia; CD, cluster of differentiation; FLAER, fluorescent aerolysin; HIV, human immunodeficiency virus; HLA-DR, human leucocyte antigen-DR;

PNH, paroxysmal nocturnal haemoglobinuria; RNA, ribonucleic acid; SAP, SLAM-associated protein; T-ALL, T-lineage acute lymphoblastic leukaemia; XIAP, X-linked inhibitor of apoptosis.

Following analysis, the immunophenotyoing laboratory will issue a report detailing the characteristics of any abnormal population identified and offering an interpretation. The strength of expression of any antigen is also relevance. This may be expressed as
i) -, ±, +, ++;
ii) negative, weak, moderate, strong;
iii) negative, dim, moderate, bright;
iv) hi, lo.

It should be noted that ± indicates weak expression whereas +/- indicates that expression may be positive or negative. An immunophenotyping result will often also be subsequently incorporated into an integrated report that includes the results of other types of investigation, for example, morphological assessment and cell counts, and cytogenetic or molecular genetic analysis.[34]

2.1.4.1 Immunohistochemistry:

Immunohistochemistry predominantly employs a primary monoclonal antibody directed at the target antigen, followed by a secondary anti-immunoglobulin antibody that is coupled to an enzyme; the enzyme can subsequently participate in an enzymatic reaction, producing a coloured product that can be visualized. The most frequently used technique is an immune peroxidase reaction. For some purposes, for example, the detection of immunoglobulin components, polyclonal antisera may be preferred. Immunohistochemistry has an advantage over flow cytometry in that antigen expression can be related to cytological and histological features. Co-expression of antigens can be studied by using two different enzymatic reactions (such as peroxidase and alkaline phosphatase) or by identifying the same cell population in serial sections of the tissue. [34]

2.1.5 Interpretation and limitations of flow cytometric Immunophenotyping:

Flow cytometry must not be interpreted in isolation but in the light of the clinical history, findings on physical examination and the results of other investigations. In particular, the blood or bone marrow film should be carefully examined in the light of the clinical and laboratory findings. Specimens are frequently sent for flow studies where the referring clinician does not have a working diagnosis. For example, a patient presenting with pancytopenia could have a number of potential diagnoses including acute leukaemia, myelodysplastic syndrome, a lymphoproliferative disorder or aplastic anaemia.

A morphological review is essential in order that an appropriate panel of antibodies is utilized. Not uncommonly an abnormal cell population in the blood is present at low levels, examples being acute leukaemia, high grade lymphoma and hairy cell leukaemia. The cells identified as being of potential interest morphologically must be correlated with abnormal populations seen in scatter plots so that an appropriate gating strategy is utilized.

2.1.5.1 Problems and pitfalls:

Clearly technical errors can lead to erroneous results of immunophenotyping. Rigorous quality control is required. Inappropriate selection of antibodies and erroneous interpretation can result from inadequate clinical information being provided or from failure to examine a film of the peripheral blood or bone marrow aspirate that is to be tested. Delays in transportation of a sample to the laboratory can lead to cell death and make testing of the sample unwise since results are likely to be misleading.

Errors in interpretation can occur if the results of immunophenotyping are not integrated with clinical, haematological, cytogenetic and genetic information. Not all cases of a specific condition will have a typical immunophenotype and, in some entities, the immunophenotype is not distinctive.

In certain circumstances flow cytometry of a bone marrow aspirate will show no abnormality despite a neoplastic infiltrate being present. This is likely to occur when there is diffuse or focal bone marrow fibrosis, when the aspirate is of low cellularity and when neoplastic cells are infrequent, fragile or dead Findings are typically negative in Hodgkin lymphoma where the disease cells, Hodgkin and Reed-Sternberg cells, are present at a low frequency amongst a reactive environment of lymphocytes, plasma cells and eosinophils with associated reticulin fibrosis. In these circumstances it is trephine biopsy histology and immunohistochemistry that yield the diagnosis. It is therefore important not to exclude a diagnosis completely based solely on the results from one approach, particularly where the specimen quality is poor. No single investigation in isolation is infallible - by correlating the results of several investigations using different modalities, a unifying diagnosis can be achieved.[35]

2.1.6 General aspect of acute leukaemia:

2.1.6.1 Definition of leukaemia:

The leukaemias are a group of disorders characterized by the accumulation of malignant white cells in the bone marrow and blood. These abnormal cells cause symptoms because of bone marrow failure, e.g. anaemia, neutropenia, thrombocytopenia; and, less commonly, (i) infiltration of organs, e.g. liver, spleen, lymph nodes, meninges, brain, skin or testes.

2.1.6.2 Classification of leukaemia:

The main classification is into four types: acute or chronic leukaemias, which are further subdivided into lymphoid or myeloid leukaemias. Acute leukaemias are usually aggressive diseases in which malignant transformation occurs in a haemopoietic stem cell or early progenitor. Acquired genetic damage results in an increased rate of proliferation, reduced apoptosis and a block in cellular differentiation. Together these events cause accumulation in the bone marrow of early haemopoietic cells known as blast cells. The dominant clinical feature of

acute leukaemia is usually bone marrow failure caused by accumulation of blast cells, although organ infiltration also can occur. If untreated, acute leukaemias are usually rapidly fatal, although with modern treatments most young patients are ultimately cured of their disease.[36]

2.1.6.3 Diagnosis of acute leukaemia:

Acute leukaemia is normally defined as the presence of at least 20% of blast cells in the bone marrow or blood at clinical presentation. However, it can be diagnosed with less than 20% blasts if certain leukaemia-specific cytogenetic or molecular genetic abnormalities are present [Table-2.3] and [Table-2.4]. The lineage of the blast cells is defined by microscopic examination (morphology, immunophenotypic [flow cytometry; Figure-2.6], cytogenetic and molecular analysis [Table-2.4]. These assessments define whether the blasts are of myeloid or lymphoid lineage and also localize the stage of cellular differentiation [Table-2.4]. A typical "myeloid" immunophenotype is CD13+, CD33+, CD117+, TdT- [Figure-2.6]. Special antibodies are helpful in the diagnosis of the rare undifferentiated, erythroid or megakaryoblastic subtypes [Table-2.5]. Occasionally, a case of leukaemia will express both myeloid and lymphoid markers. Acute leukaemias of ambiguous lineage (mixed phenotype acute leukaemias) are rare cases that express markers for both myeloid and lymphoid differentiation, either on the same blast cells or on two different cell populations in the same patient.

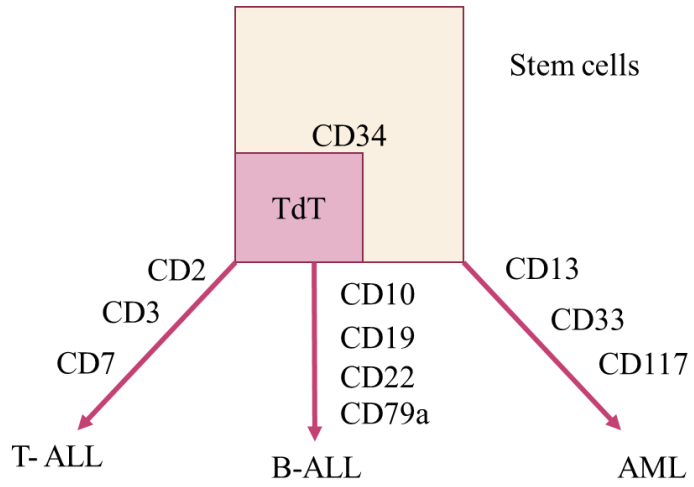


Figure- 2.6 Development of three cell lineages from pluripotential stem cells giving rise to the three main immunological subclasses of acute leukaemia.[36]

Cytogenetic and molecular analysis is essential and is usually performed on marrow cells, although blood may be used if the circulating blast cell is high. Cytochemically can also be useful in determining the blast cell lineage [Figure-2.7] but is no longer performed in centres where the newer and more definitive tests are available.

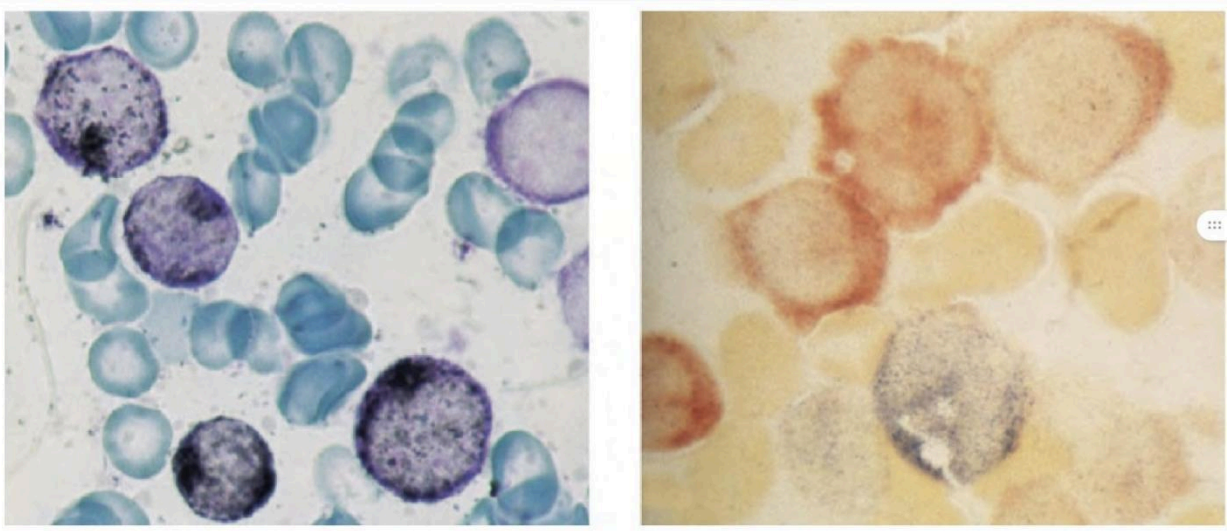


Figure-2.7 Cytochemical staining in acute myeloid leukaemia. (a) Sudan black b shows black staining in the cytoplasm. (b) Myelomonocytic non-specific esterase staining shows orange-staining monoblast cytoplasm and blue-staining (myeloblast) cytoplasm.[37]

2.1.7 Acute myeloid leukaemia:

2.1.7.1 Pathogenesis:

The AML genome contains an average of about 10 mutations within protein-coding genes in each case, among the smallest number of any adult cancer. Many AML 'driver mutations' promoting clonal expansion have been identified, with the most common being within FLT3, NPM1 and DNMT3A. Some other mutations, e.g. of ASXL1 or mutations in splicing associated genes, are frequent in myelodysplastic neoplasia (MDS) and when found in AML suggest that it is secondary to MDS, which may not have been recognized clinically. This has resulted in a list of cytogenetic and somatic mutations which are associated with MDS, and as such, the presence of which suggests a preceding MDS phase [Table- 2.4]. These secondary AML are often associated with chemotherapy resistance and a poor prognosis. The mutations usually occur on only one of the two alleles for the gene and, depending on the gene, may be 'loss of function', 'gain of function' or 'neomorphic', i.e. conferring a novel function.

Some AML cases are characterized by a gene fusion event, which usually arises from translocations, with the most common being PML::RARA, CBF::MYH11 and RUNX1::RUNX1T1, which are found in around 15%, 12% and 8% of cases, respectively. The wide variety of cytogenetic abnormalities and molecular mutations is such that there are hundreds of patterns of mutations. However, molecular cooperativity of mutations result in recurring patterns of mutations, for example NPM1, FLT3-ITD DNMT3A mutations frequently co-occur in the same patient. Mutations of genes DNMT3A, TET2, ASXL1 and less commonly IDHI, IDH2, TP53 or spliceosome genes may be found in the blood cells of healthy subjects especially after age 60 years (age-related clonal haemopoiesis or clonal haemopoiesis of indeterminate potential). Patients with these mutations can develop AML which may present many years later. The likelihood of AML development is higher in those with IDHI, IDH2, TP53

or spliceosome gene mutations, more than one mutation, high mutation allele burden, or an mutations can elevated red cell distribution width.

2.1.7.2 Incidence:

AML is the most common form of acute leukaemia in adults and becomes increasingly common with age, with a median onset of 65 years. It forms only a minor fraction (10-15%) of the leukaemias in childhood. Cytogenetic and molecular abnormalities and response to initial treatment have a major influence on prognosis [Table-2.7][37]

2.1.7.3 Classification:

AML is classified according to the World Health Organization (WHO; 2022) scheme
[Table-2.3]. There is an increasing focus on the genetic abnormalities within the malignant cells, and it is likely that ultimately all AML cases will be classified by specific genetic subtype. Currently this is not yet possible, but many genetic subtypes have been determined. Approximately 60% of ML cases exhibit karyotypic abnormalities on cytogenetic analysis and most cases with a normal karyotype carry mutations in genes such as FLT3, NPM1, CEBPA or DNMT3A, detected only by molecular methods (see below). 5 main groups of AML are recognized:

AML with defining genetic abnormalities. The detection of these abnormalities defines the neoplasm as AML, and so the diagnostic criteria for this subgroup are relaxed in that the bone marrow blast cell count does not need to exceed 20% in order to make a diagnosis (with the exception of AML with BCR::ABL1 fusion and AML with CEBPA mutation).

Table-2.3 Classification of acute myeloid leukaemia (AML) according to World Health Organization (WHO) (2022).[36]

Acute Myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia (APL) with PML::RARα fusion
AML with RUNX1::RUNX1T1 fusion
AML with CBFB::MYH11 fusion
AML with DEK::NUP214 fusion
AML with RBM15::MRTFA fusion
AML with BCR::ABL1 fusion
AML with KMT2A rearrangement
AML with MECOM rearrangement
AML with NUP98 rearrangement

AML with NPM1 mutation
AML with CEBPA mutation (require blast count of ≥20%)
AML, myelodysplasia-related
AML with other defined genetic alterations
Acute myeloid leukaemia, defined by differentiation
AML with minimal differentiation
AML without maturation
AML with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia
Myeloid sarcoma
Secondary AML (myeloid neoplasm)

AML defined by differentiation. These include cases lacking a defining genetic abnormality.

AML, myelodysplasia-related. In this group the AML is defined by the presence of cytogenetic or molecular abnormalities related to myelodysplasia, and/or a history of MDS (or MDS/MPN) [Table-2.4]

Myeloid sarcoma is rare, but refers to a disease that resembles a solid tumour but is composed of clustered myeloid blast cells. This is often called 'extramedullary leukaemia', 'granulocytic sarcoma' or 'chloroma'. Bone marrow involvement often occurs concurrently.

Secondary myeloid neoplasms include those that arise in patients

a. With germline predisposition. Genetic counselling and family history is an important part of the assessment of these patients. This subtype of AML also includes myeloid leukaemia of Down syndrome (ML-DS). This usually occurs before the age of 5 years and may follow an episode of transient abnormal myelopoiesis (TAM). TAM occurs as a multi-step process provoked by trisomy 21 associated abnormal foetal haemopoiesis. There are acquired mutations of GATA1. TAM begins in utero and often presents in

newborns, about 10% of those with Down syndrome, as a self-limiting leukaemic syndrome which resolves spontaneously in the majority of patients. Patients with TAM which proceed to ML-DS have megakaryoblastic features, but often respond to chemotherapy alone.

b. Following exposure to radiation or cytotoxic drugs such as etoposide or alkylating agents. AML secondary to cytotoxic drugs or radiation commonly exhibit mutations in the TP53 or KMT2A (MLL) gene. The clinical response is usually poor.

c. AML arising in patients with myeloproliferative diseases is categorized as MPN while that arising as transformation of MDS is categorized as myelodysplasia related.[38]

Other subtypes of AML

2.1.7.4 Acute leukaemias of mixed or ambiguous lineage:

These acute leukaemias are grouped together in WHO (2022). They are separated into those with defining genetic abnormalities and those defined on immunophenotyping alone. The assignment of lineage by immunophenotyping depends on the strength and pattern antigen expression as well as the coordinated expression of more than one antigen of the same lineage. They usually have a poor prognosis. There is no clear evidence for optimal treatment strategies, but use of ALL style chemotherapy schedules, or if not responsive, to use AML directed regimens are reasonable. In the case of BCR::ABLI mutated leukaemia, use of TKI should be considered. Menin inhibitors are promising treatments for MLL fusion driven acute leukaemias.[40]

Table-2.4 Cytogenetic and molecular abnormalities defining acute myeloid leukaemia, myelodysplasia related (WHO, 2022).[37]

Defining cytogenetic abnormalities
Complex karyotype (>3 abnormalities)
5q deletion or loss
Monosomy 7, 7q deletion, or loss of 7q
11q deletion
12p deletion or loss
Monosomy 13 or 13q deletion
17p deletion or loss
Isochromosome 17q
idic(X)(q13)
Defining somatic mutations
ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2
Idic = Isodicentric, contains mirror-image segments of genetic material.

1.4.4.5 Blastic Plasmacytoid Dendritic Cell Neoplasm (BPDCN):

This is a rare but aggressive neoplasia, characterized by skin and heterogeneous systemic manifestations. These include blood, bone marrow, lymph node and CNS infiltration. It is often placed in the myeloid disorders category (with "myeloid pattern of genetic mutations) though the cell of origin is unclear. It is important to differentiate BPDCN from AML with leukaemia cutis, and BPDCN immunophenotypically expresses CD123, CD4, CD56 and TCL1, but is negative for lineage markers such as MPO. Treatments include intensive AML or ALL protocols including SCT. Novel CD123- targeting treatments have an emerging role.

1.4.4.6 Clinical features:

The clinical features of AML are dominated by the pattern bone marrow failure caused by the accumulation of malignant cells within marrow [Figure-2.8]. Infections are frequent, and anaemia and thrombocytopenia are often profound. A bleeding tendency caused by thrombocytopenia and disseminated intravascular coagulation (DIC) is characteristic of the promyelocytic variant of AML. Tumour cells can infiltrate a variety of tissues. Gum hypertrophy and infiltration [Figure-2.9], skin involvement (leukaemia cutis) and central nervous system (CNS) disease are characteristic of the myelomonocytic and monocytic subtypes.

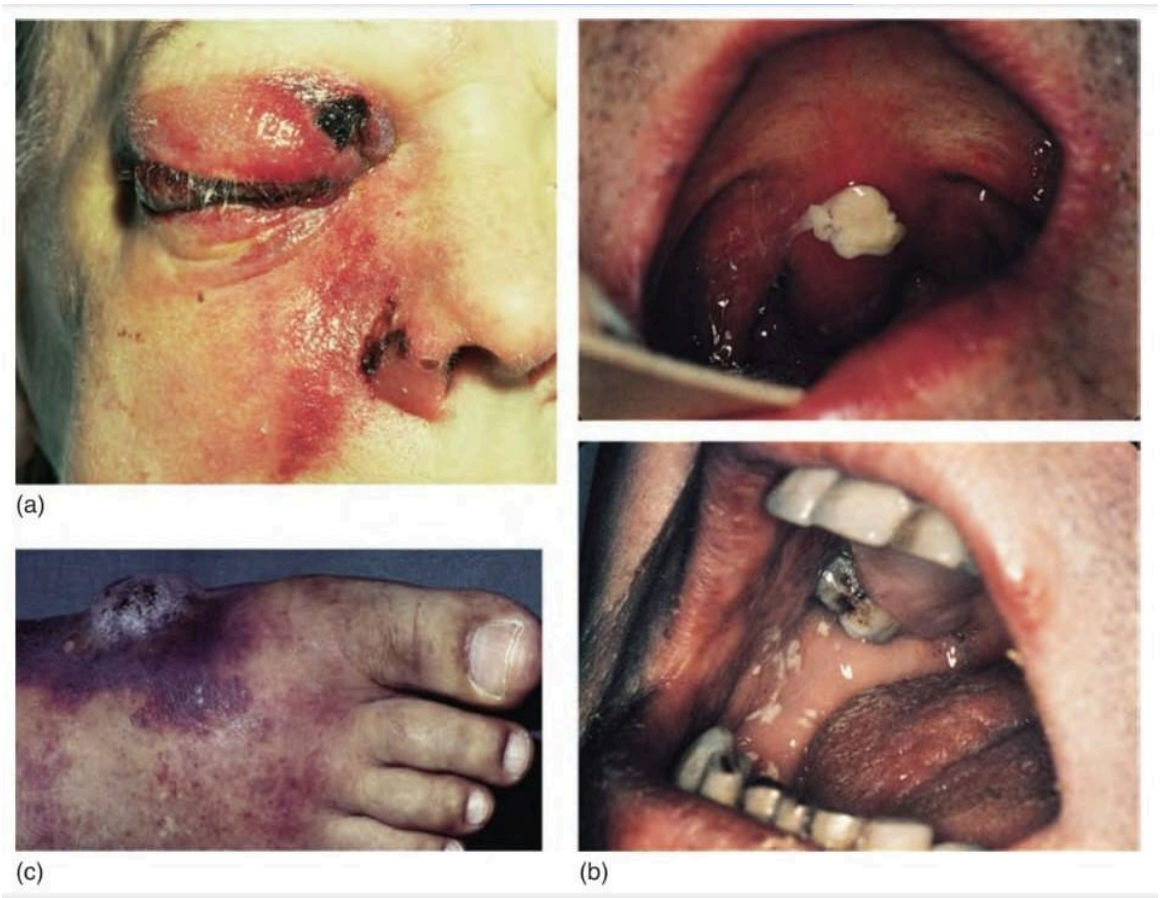


Figure-2.8 (a) An orbital infection in a female patient (aged 68 years) with acute myeloid leukaemia and severe neutropenia. (b) Acute myeloid leukaemia: top: plaque Candida albicans on soft palate; lower: plaque Candida albicans in the mouth, with lesion of herpes simplex on the upper lip. (c) Skin infection (Pseudomonas aeruginosa) in a female patient (aged 33 years) with acute lymphoblastic leukaemia receiving chemotherapy and with severe neutropenia.[38]

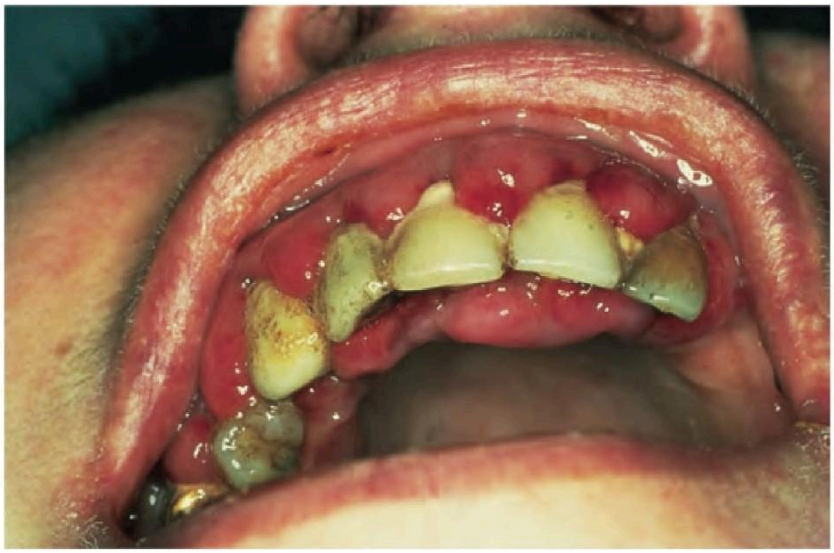


Figure-2.9 Monocytic acute myeloid leukaemia: the gums are swollen and haemorrhagic because of infiltration by leukaemic cells.[38]

2.1.7.5 Investigations:

Table-2.6 lists the initial clinical and laboratory tests to be performed in newly diagnosed cases of AML; similar work-up is needed for all new haematological malignancies. Haematological investigations reveal a normochromic normocytic anaemia with thrombocytopenia in most cases. The total white cell count is usually increased, and blood film examination typically shows a variable numbers of blast cells. The bone marrow is hypercellular and typically contains many leukaemic blasts [Figure-2.10]. Blast cells are characterized by morphology, immunological (flow cytometric) [Table-2.5], cytogenetic and molecular genetic analysis for confirming the diagnosis, determining prognosis and developing a treatment plan [Tables 2.6 and 2.7]. Tests for DIC are often positive in patients with the pro-myelocytic variant of AML, and in some cases with monocytic differentiation (see below). Biochemical tests are performed as a baseline before treatment begins and may reveal raised uric acid and lactate dehydrogenase.[38]

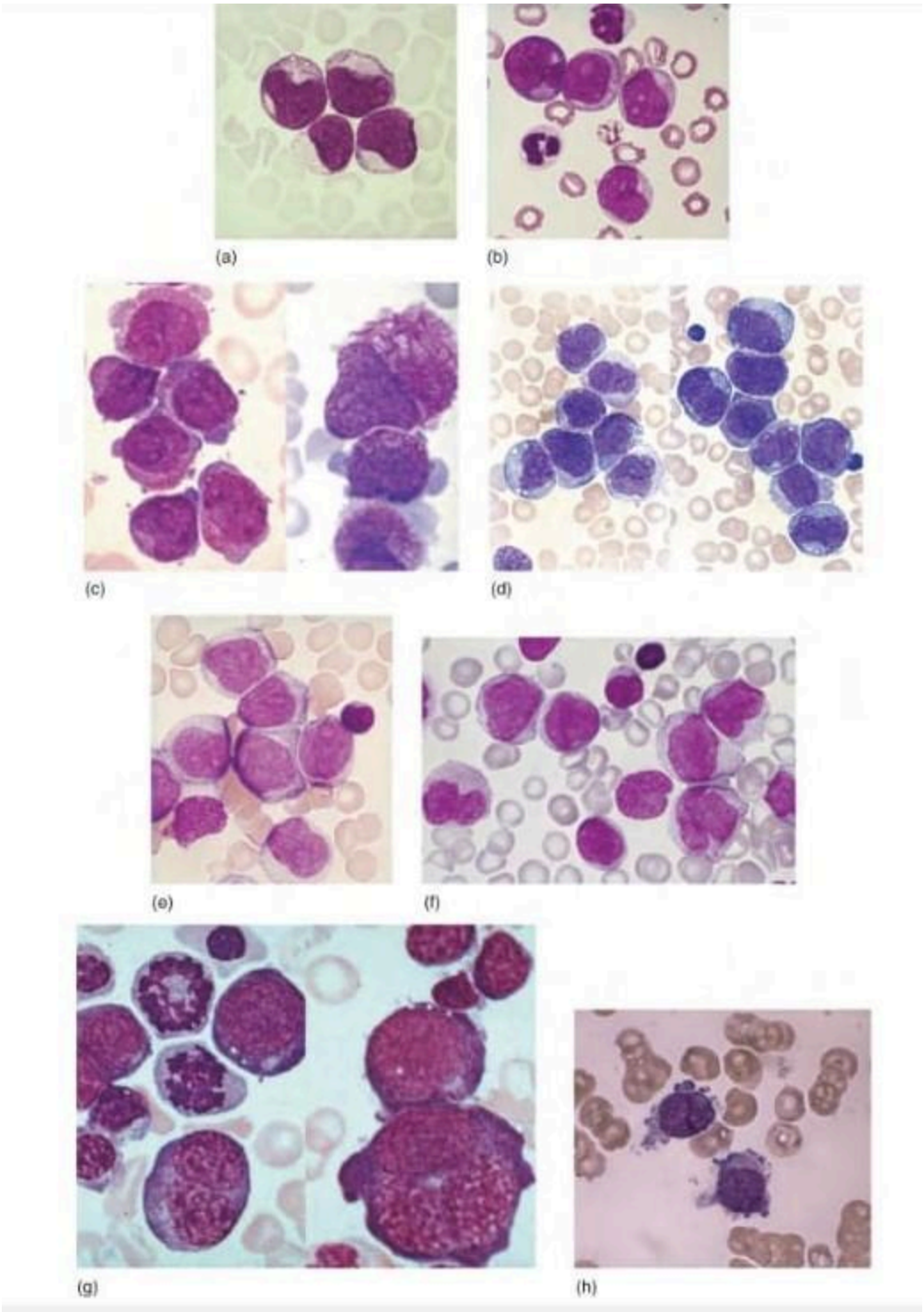


Figure-2.10 Morphological examples of acute myeloid leukaemia. (a) Blast cells without differentiation show few granules but may show Auer rods, as in this case; (b) cells in differentiation show multiple cytoplasmic granules; (c) acute promyelocytic leukaemia blast cells contain prominent granules or multiple Auer rods; (d) myelomonocytic blasts have some monocytoid differentiation; (e) monoblastic leukaemia in which >80% of blasts are monoblasts;

(f) monocytic with <80% of blasts monoblasts; (g) erythroid showing preponderance of erythroblasts; (h) megakaryoblastic showing cytoplasmic blebs on blasts.[39]

1.4.4.8 Cytogenetics and Molecular Genetics:

Cytogenetics and molecular genetics have two roles: initial diagnostic classification [Table-2.1] but following treatment a number of translocations and mutations can be used as markers of measurable/minimal residual disease, e.g. t(8;21) or NPM1 mutations. Two of the most common - t(8;21) and inv(16) – are associated with a good prognosis. Acute promyelocytic leukaemia (APML) is a variant of AML that contains the t(15;17) translocation in which the gene PML on chromosome 15 is fused to the retinoic acid receptor a gene, RARA, on chromosome 17. The resultant PML::RARα fusion protein functions as a transcriptional repressor, whereas normal (wild-type) RAR at is an activator. Normally, the PML protein forms homodimers with itself, whereas the RARα protein forms heterodimers with the retinoid X receptor protein, RXR. The PML::RARα fusion protein binds to PML and RXR, preventing them from linking with their natural partners. This results in the cellular phenotype of arrested differentiation.

Table-2.5 Specialized tests for acute myeloid leukaemia (AML**)**.[39]

Immunological markers (flow cytometry)	**Indicates**
CD13, CDS3, CD34, CD117	Usually positive in AML
CD11c, CD14, CD64	Monocytic differentiation
Glycophorin (CD235a), CD36	Erythroid differentiation
CD41, CD61	Megakaryoblastic differentiation
Myeloperoxidase, CD65	Granulocytic differentiation
Chromosome and genetic analysis [see Tables 2(a) and 2(d)]	
Cytochemistry	
Myeloperoxidase	Myeloid differentiation (usually bright in Auer rods)
Sudan black	Myeloid differentiation (usually bright in Auer rods)
Non-specific esterase	Monocytic differentiation

Table-2.6 The initial evaluation of a new patient with suspected acute myeloid leukaemia.[39]

Assessment of medical history, examination and performance status; analysis for co-morbidities
Full blood count and differential with blood film
Bone marrow aspirate and trephine biopsy
Immunophenotyping of bone marrow (and/or blood if blast cells present)
Cytogenetic analysis by karyotype
Mutation
Cytochemical analysis
Coagulation - PT, APTT, Fibrinogen, D dimer
Pregnancy test
Information on oocyte or sperm storage
Early tissue typing and donor search
CMV serology, Hepatitis B, C and HIV test
CXR with ECG and ECHO
CXR, chest X-ray; ECG, electrocardiography; ECHO, echocardiography; HIV, human immunodeficiency virus; LDH, lactate dehydrogenase.

Point mutations affecting the genes FLT3, NPM1, DNMT3A, IDH1, IDH2 TET2, RUNX1, TP53 and others are frequent in AML, especially in those cases without a cytogenetic abnormality. They may be used to subclassify the disease [Table-2.1] and have prognostic significance. Some of these genes are involved in DNA methylation or histone methylation or acetylation and are also mutated in cases of myelodysplasia and myeloproliferative neoplasms. The presence in de novo AML of an MDS-associated mutation, c.g. ASXL1 or SF3B1, is unfavourable.[39]

Table-2.7 Examples of prognostic factors in acute myeloid leukaemia.[40]

Points	**Favourable**	**Intermediate**	**Unfavourable**
Cytogenetics	t(15;17) t(8,21)	Normal t(9;11) Other changes neither unfavourable or favourable	Deletions of chromosome 5 or 7 or 17p, Inv(3) or t(3.3) t(6:9). t(v;11q23); KTM2A rearranged Complex rearrangements (>3 unrelated abnormalities) t(9;22) BCR::ABL1
Molecular genetics	NPM1 mutation CEBPA mutation	Wild type/Mutated NPM1 and FLT3-ITD	Mutations of TP53, RUNX1, ASXL1 and spliceosome Mutations:
Bone marrow response to remission induction	<5% blasts after first course		>20% blasts after first course
Age	Child	<60 years	>60 years
Performance status	Good		Bad
Co-morbidities	Absent		Present
White cell count	$<10x10^9/L$		$>100x10^9/L$
Post-cytotoxic therapy (based on medical history) or transformation of MDS or MPN			unfavourable
Minimal residual disease in remission	Absent	Absent	Present

2.1.8 Acute lymphoblastic leukaemia:

Acute lymphoblastic leukaemia (ALL) is caused by an accumulation of lymphoblasts in the bone marrow and is the most common malignancy of childhood, though it can occur at any age. The definition of acute leukaemia and distinguishing ALL.

2.1.8.1 Incidence and pathogenesis

The incidence of ALL is highest at 3-7 years, with 75% of cases occurring before the age of 6. There is a secondary rise in incidence after the of 40 age years. B-cell lineage represents 85% of cases and these have an equal sex incidence; there is a male predominance for the 15% of T-cell ALL (T-ALL).

The pathogenesis is varied. A proportion of cases of childhood ALL are initiated by genetic mutations that occur during development in utero. Studies in identical twins have shown that both may be born with the same chromosomal abnormality, e.g. the t(12;21), ETV6-RUNX1 translocation. This has presumably arisen spontaneously in a haemopoietic progenitor cell that has passed from one twin to the other as a result of shared placental circulation. Massive expansion of the lymphoid system in the fetus predisposes to somatic mutations. Also, the process of VDJ recombination to generate antigen receptor diversity is prone to generation of 'off target genomic abnormalities. Environmental exposure during pregnancy may be important for this first event. One twin may develop ALL early, e.g. at age 5 because of a second transforming event affecting the copy numbers of several genes, including those in B-cell development (see below).

The other may remain well or develop ALL later, perhaps as a result of a different transforming event. The ETV6-RUNX1 translocation is present in the blood of approximately 10% of newborn infants, but only 1 in 100 of these go on to develop ALL at a later date. The mechanism of the 'second genetic hit' within the neoplastic cell is unclear, but an abnormal response of the immune system to infection is suggested by epidemiological studies. In other cases, the disease seems to arise as a postnatal mutation in an early lymphoid progenitor cell.[41]

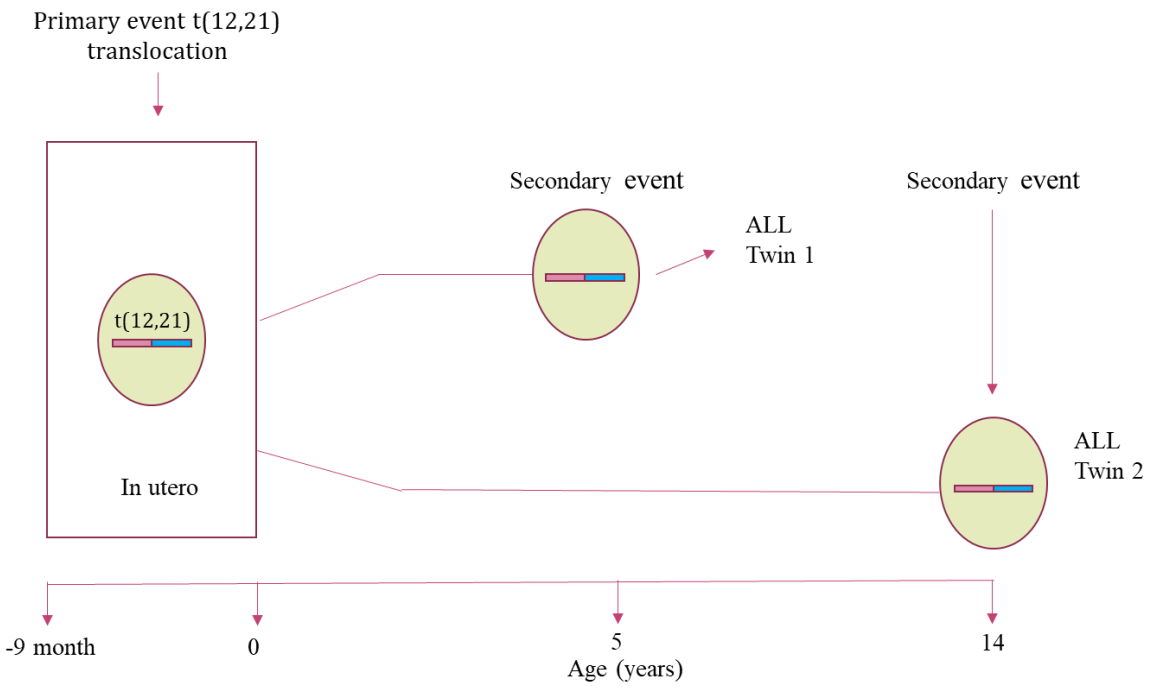


Figure-2.11 Prenatal origin of acute lymphoblastic leukaemia (ALL) in a pair of identical twins. Both tumors had an identical t(12,21) translocation. ALL was diagnosed in the first twin at age 5 years and in the second at the age 14 years, indicating probable origin of the leukaemic clone in utero and dissemination to both twins via a shared placental blood supply.[41]

Certain germline polymorphisms in a group of genes mainly involved in B-cell development (e.g. IKZFI) appear to predispose to ALL, since they are more frequent in children with B-cell ALL (B-ALL) than controls. IKZF1 is also deleted in the leukaemic cells in 30% of high-risk B-ALL and 95% of ALL BCR::ABL1 positive cases. Children with constitutional trisomy 21 (Down syndrome) have a remarkably increased risk of B-ALL and AML compared with other children. There is a 33-fold increase in B-ALL and 150-fold increase in AML in young children with Down syndrome.

In general, the genomic landscape in ALL is characterized by primary chromosomal abnormalities and a wide range of secondary deletions and mutations involving key pathways implicated in leukaemogenesis. These are described in more detail below. For childhood ALL, an average of 11 somatically acquired structural variations are present. [Figure-2.11].

2.1.8.2 Classification

Acute lymphoblastic leukaemia, B cell or T cell, is sub-classified by the World Health Organization (WHO 2016) according to the underlying genetic defect [Table-2.8]. Within B-ALL there are several specific genetic subtypes, such as those with the t(9;22) [BCR::ABL1] or t(12;21) [ETV6-RUNX1] translocations, rearrangements of the KMT2A(MLL) gene or alteration in chromosome number [aneuploidy, Table-2.8]. The subtype in both B-ALL and T-ALL is an important guide to the optimal treatment protocol and to prognosis.[42]

Among BCR::ABLI (Philadelphia chromosome) negative cases, some patients have a gene expression signature similar to BCR-ABL1 positive cases. These 'BCR::ABL1-like cases comprise about 15% of children with ALL (more common in Down syndrome), 20-25% of adolescents and young adults, and at least 10-15% of older adults. About 60% of patients with BCR::ABLI-like ALL have overexpression of CRLF2 and 85% of these cases have JAK-STAT pathway mutations,
including of JAK2. In BCR::ABL1-like ALL without CRLF2 overexpression, fusions involving JAK2, ABL1, ABL2 and other tyrosine kinases are common.

Table-2.8 World Health Organization (2022) classification of acute lymphoblastic leukaemia (ALL)/lymphoblastic lymphoma. The corresponding chromosomal translocations are included in the Table.[42]

B-cell lymphoblastic leukaemias/lymphomas
B-lymphoblastic leukaemia/lymphoma NOS
B-lymphoblastic leukaemia/lymphoma with high hyperdiploidy (>50 chromosomes)
B-lymphoblastic leukaemia /lymphoma with hypodiploidy (<45 chromosomes)
B-lymphoblastic leukaemia/lymphoma with iAMP21
B-lymphoblastic leukaemia/lymphoma with BCR::ABL1 fusion, t(9;22)
B-lymphoblastic leukaemia/lymphoma with BCR-ABL1-like features
B-lymphoblastic leukaemialymphoma with KMT2A rearrangement
B-lymphoblastic leukaemia/lymphoma with ETV6::RUNX1 fusion, t(12;21)
B-lymphoblastic leukaemia/lymphoma with ETV::RUNX1-like features
B-lymphoblasic leukaemia/lymphoma with TCF3::PBX1 fusion, t(17;19)
B-lymphoblastic leukaemia/lymphoma with IGH::IL3 fusion, t(5;14)
B-lymphoblasic leukameia/lymphoma with other defined genetic abnormalities
T-cell lymphoblastic leukaemias/lymphomas
T-lymphoblastic leukaemia/lymphoma NOS
Early T-precursor lymphoblastic leukaemia/lymphoma

In T-ALL an abnormal karyotype is found in 50-70% of cases and the NOTCH signaling pathway is activated in most cases. Early T-precursor (ETP) ALL leukaemia has a unique immunophenotype. Blasts in ETP ALL express the T-cell marker CD7, but lack CD1a and CD8 that characterize mature T cells. They also express at least one myeloid/stem more cell-associated marker. The genetic profile of ETP ALL is also distinct. Common T-cell-associated gene alterations such as of NOTCH1 or CDKN1/2 are rare in this subtype, but myeloid gene mutations are common. The prognosis is worse associated than for other patients with T-ALL.[42]

2.1.8.3 clinical features

Clinical features are a result of the following;

Bone marrow failure

- Anaemia (pallor, lethargy and dyspnoea).
- Neutropenia (fever, malaise, features of mouth, throat, skin, respiratory, perianal or other infections).
- Thrombocytopenia (spontaneous bruises, purpura, bleeding, gums and menorrhagia).

Organ infiltration

This can cause tender bones, lymphadenopathy, moderate splenomegaly, hepatomegaly and meningeal syndrome (headache, nausea and vomiting, blurring of vision and dipolpia). Fundal examination may reveal papilloedema and sometimes retinal haemorrhage. Many patients have a fever at presentation, which usually resolves include testicular swelling or signs of mediastinal compression, after starting chemotherapy. Less common manifestations which is more common in T-ALL. If lymph node or solid extranodal masses predominate with <20% blasts in the marrow, the disease is classified as lymphoblastic lymphoma, but is treated as ALL.[42]

2.1.8.4 Investigations:

Haematological investigations reveal a normochromic normocytic anaemia with thrombocytopenia in most cases. The total white cell count may be decreased, normal or increased,
sometimes to 200 × 10^9/L or more. The blood film typically shows a variable number of blast cells. The bone marrow is hypercellular with >20% leukaemic blasts. The blast cells are characterized by morphology [Figure-2.12] immunological tests, cytogenetic and molecular genetic analysis [Table-2.9]. Identification of the immunoglobulin or T-cell receptor (TCR) clonal gene rearrangement, (aberrant) immunophenotype and molecular genetics of the neoplastic cells is important to determine treatment and to detect minimal residual disease (MRD) during follow-up.[43]

Lumbar puncture for cerebrospinal fluid (CSF) examination is important in disease staging, but should be performed only by experienced physicians, as a traumatic procedure may promote the spread of neoplastic cells from blood to the central nervous system (CNS). Initial assessment of the CSF should always be combined with the concurrent administration of intrathecal chemotherapy. Biochemical tests may reveal a raised serum uric acid, serum lactate dehydrogenase or, less commonly, hypercalcaemia. Liver and renal function tests are performed as a baseline before treatment begins. Radiography may reveal lytic bone lesions and/or mediastinal lymph nodes, which is characteristic of T-ALL.

The differential diagnosis includes acute myeloid leukaemia (AML), aplastic anaemia, marrow infiltration by other malignancies, e.g. rhabdomyosarcoma, neuroblastoma and Ewing sarcoma, infections such as infectious mononucleosis and pertussis, juvenile rheumatoid arthritis and immune thrombocytopenia.

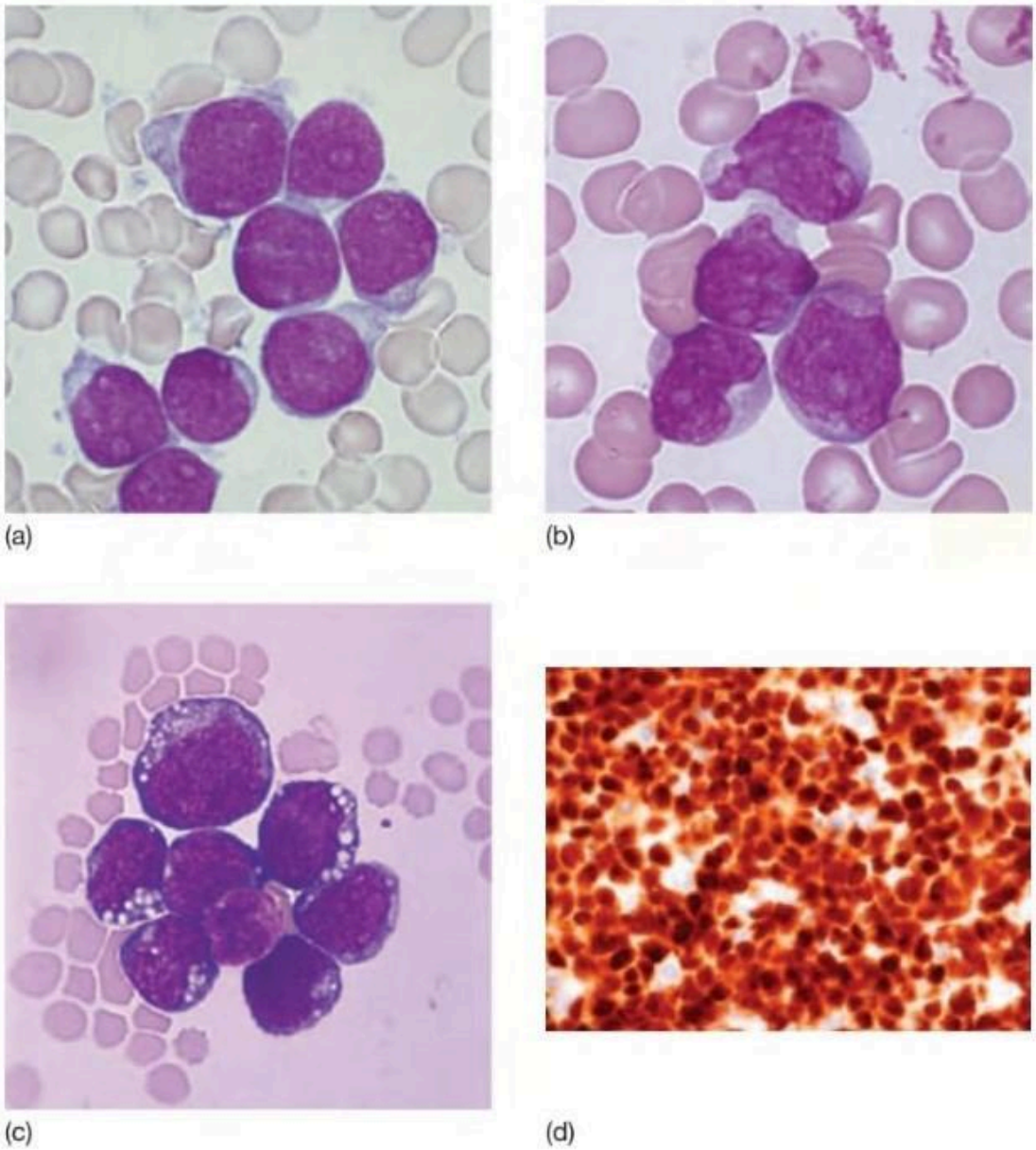


Figure-2.12 Morphology and immunophenotyping of acute lymphoblastic leukaemia. (a) Lymphoblasts show scanty cytoplasm without granules. (b) Lymphoblasts are large and heterogeneous with abundant cytoplasm. (c) Lymphoblasts are deeply basophilic with cytoplasmic vacuolation. (d) Acute lymphoblastic leukaemia: bone marrow cells staining positive for terminal deoxytidyl transferase (TdT) by Immunoperoxidase.[43]

Table- 2.9 Immunological markers for classification of acute lymphoblastic leukaemia.[43]

Marker	ALL	
	B	T
B lineage-associated		
CD19	+	-
CCD22	+	-
CCD79a	+	-
CD10	+ or –	-
clg	+ (pre -B)	-
sig	-	-
TdT	+	+
T lineage-associated		
CD7	-	+
CCD3	-	+
CD2	-	+
TdT	+	+
CD1a	-	+
CD4, CD8	-	+
Myeloid or stem cell lineage-associated CD34, CD117, HLADR, CD13, CD33, CD11b, or CD65	Negative except in biphenotypic acute leukaemia	Negative except in early T-cell precursor subtype or biphenotypic acute leukaemia
C, cytoplasmic; s, surface, TdT, terminal deoxynucleotidyl transferase		

2.1.8.5 Cytogenetics and molecular genetics:

Cytogenetic analysis shows differing frequencies of abnormalities in infants, children and adults, which partly explains the different prognoses of these groups. Cases are stratified according to the number of chromosomes in the neoplastic cell (ploidy) or by specific molecular abnormalities. The two parameters define good and poor-prognosis disease.

Hyperdiploid cells have more than 50 chromosomes and cases with this abnormality generally have a good prognosis, whereas hypodiploid cases (less than 45 chromosomes) carry a poor prognosis. The most common specific chromosome abnormality in childhood B-ALL is the t(12;21)(p13;q22) ETV6::RUNX1 translocation. The RUNX1 protein plays an important part in transcriptional control of haemopoiesis and is repressed by the ETV6::RUNX1 fusion protein. The frequency of the Ph translocation t(9;22) increases with age and carried an unfavourable

prognosis, though outcomes have improved significantly with the addition of BCR-ABL1 tyrosine kinase inhibitors (TKIs) to treatment regimens. ALL with BCR::ABL1-like features also has an unfavourable prognosis and the role of kinase inhibitors is being tested in trials. Translocations of chromosome 11q23 involve the KMT2A (MLL) gene and are seen particularly in cases of infant leukaemia.

Using more sensitive molecular genetic tests, as well as fluorescence in situ hybridization (FISH) analysis, some cases that have normal results from conventional cytogenetic testing are found to have fusion genes, e.g. BCR-ABL1. These molecular genetic changes may carry prognostic significance.

T-ALL accounts for 15% of childhood and 25% of adult ALL. The clinical presentation is often with a very high white cell count, mediastinal mass or pleural effusion. TCR genes (and in 20% the IGH gene) show clonal rearrangement and cytogenetic changes often involve the TCR loci with different partner genes. The majority of cases have acquired genetic abnormalities that lead to constitutive activation of the NOTCH signaling pathway and drugs that target those abnormalities are being developed. *KMT2A* fusion, ABL class fusions and CDKN2A/B deletions may also be present as in B-ALL.[43]

2.1.9 Immunophenotyping in the diagnosis and monitoring of acute leukaemia:

2.1.9.1 Normal peripheral blood and bone marrow cells, lineage and stem cell markers:

Markers that are often studied and that are expressed by normal cells in the peripheral blood and bone marrow shows at Table-2.10. When relevant, the strength of expression is shown as: - negative, ± weak, + moderate, ++ strong. [Figures-2.13-2.17] show the alteration in expression of various markers with maturation within a lineage. The nature of the B-cell precursors known as haematogones and their distinction from neoplastic B lymphoblasts is discussed later.

2.1.9.2 Acute myeloid leukaemia:

The major role of immunophenotyping in acute myeloid leukaemia (AML) is the recognition, as myeloid, of cases of acute leukaemia lacking cytological evidence of their lineage; this includes the recognition of monoblasts, megakaryoblasts and primitive erythroid cells as well as early myeloblasts that lack cytoplasmic granules or Auer rods. Other important roles are: (i) making a distinction from mixed phenotype acute leukaemia (MPAL); (ii) the identification of an immunophenotype that suggests a specific genetic subtype; and (iii) the identification of a leukaemia-associated immunophenotype that can be used for monitoring for minimal residual disease (MRD). Table-2.11 shows antigens that are expressed in AML, including those that are associated with a specific genetic subtype. [44]

2.1.9.3 Immunophenotype of Cells of Specific Myeloid Lineages:

Myeloblasts typically express CD34, CD117, CD13, CD33, CD38, CD45 and CD133 and usually also myeloperoxidase (MPO) and human leucocyte antigen (HLA)-DR. Monoblasts express CD36, CD45, HLA-DR and CD64; they are MPO negative. Maturing cells of monocyte lineage express MPO, CD4, CD11a, CD11b, CD11c and CD14; there can also be expression of CD2, CD56, CD71 and CD123. Megakaryoblasts express CD41, CD42b and CD61; by immunohistochemistry, CD42b has been found to be most sensitive, followed by CD61, then von Willebrand antigen with immunohistochemistry for von Willebrand antigen yielding no further cases if CD42b andCD61 had been tested for while CD36 is expressed but is not specific. Erythroblasts express glycophorin A (CD235a), which is lineage specific, together with CD36 and CD71,which are not lineage specific. E-cadherin can be detected by immunohistochemistry, permitting detection of earlier cells than those expressing glycophorin A and is erythroid specific within haemopoietic lineages.

2.1.9.4 Correlation of Immunophenotype with Genotype:

Specific immunophenotypic features can provide a clue to the underlying genetic abnormality. This is particularly important in the identification of acute promyelocytic leukaemia, since rapid diagnosis and treatment can be crucial.

Acute promyelocytic leukaemia with t(15;17)(q24.1;q21.2); PML-RARA shows high side scatter (SCC), as a result of the granular cytoplasm, and expression of myeloid markers such as CD13 (heterogeneous), CD33 (strong), MPO and usually CD117; HLA-DR and CD34, which are usually expressed in AML, are generally negative although CD34 may be expressed in the microgranular variant; CD11b, CD11c, CD15, CD18 and CD16 are negative or weak; CD64 is often expressed; sometimes there is aberrant expression of CD2 and CD56 (about 10% of cases). CD9 is expressed in 95% of cases; CD9+CD11b- HLA-DR- has been found to have 85% sensitivity and 95% specificity for this diagnosis.[44]

Table 2.10 Lineage and stem cell markers. [44]

Cell type	Commonly used markers	Other markers	Markers that are generally negative
Neutrophil	CD11b++, CD11c+, CD13++, CD15++, CD16++, CD33+, CD38, CD45+, CD65+	CD10++, CD24++; CD64 is + or - but upregulated during infection	CD34, CD117, HLA-DR
Eosinophil	CD11b++, CD11c+, CD13+, CD15+, CD33+, CD45++, high SSC	CD38+	CD34, CD117, HLA-DR, CD4,CD10,CD16

Basophil	CD9+, CD13+, CD33+, CD22+/+, CD123++, CD203c, CD45+	CD25+/+, CD36+, CD38++	CD34, CD117, HLA-DR, CD64
Monocyte	CD11b++, CD11c++, CD13++, CD14++, CD15++, CD33++, CD45++, CD64++	HLA-DR variable, CD4+, CD36++, CD38++, CD300e on mature monocytes	CD34, CD117,CD16
T lymphocyte	CD2+, SmCD3+, CD5+, CD7+, CD4+ or CD8+ (cytotoxic T cells are CD8+), CD43+, CD45++	CD56+ (minor population), CD57+ (minor population representing cytotoxic T cells), TCRaß+ (majority of T cells), TCRyō+ (minority of T cells)	CD25, HLA-DR (unless activated)
B lymphocyte	CD19+, CD20+, CD22+, CD79a+, CD79b+, surface membrane kappa or lambda+, HLA-DR+	Cytoplasmic immunoglobulin	CD5 (+ in mantle zone lymphocytes), CD23, CD38
NK cells	CD2+, CD16, CD56, CD57+ or -, CD45+	CD158a+, CD158b+, CD158+e	HLA-DR, CD3
Myeloid dendritic cell	CD45+, CD1c+, CD11c++, HLA-DR+, CD16 variable		
Plasmacytoid dendritic cell	CD45+, CD1c-, CD11c-, CD123++, HLA-DR+		
Mast cell	CD117++, CD9+, CD11c+, CD29+. CD33+, CD44+, CD45+, CD49d+. CD49e+, CD51+, CD54+, CD71+, CD38-, CD138-		
Plasma cell	CD19+, CD20-, CD22-, CD38++, CD138+/++, CD45		CD56,CD117

	variable (-/+), cytoplasmic Ekappa or lambda+, Smig-, CD56-, CD117-		
Erythroblast	CD235a+/++, CD36+, CD71+, E-cadherin+		CD45
Haemopoietic stem cell	CD45+, CD34+, CD38+/-	CD49f+,CD90+	

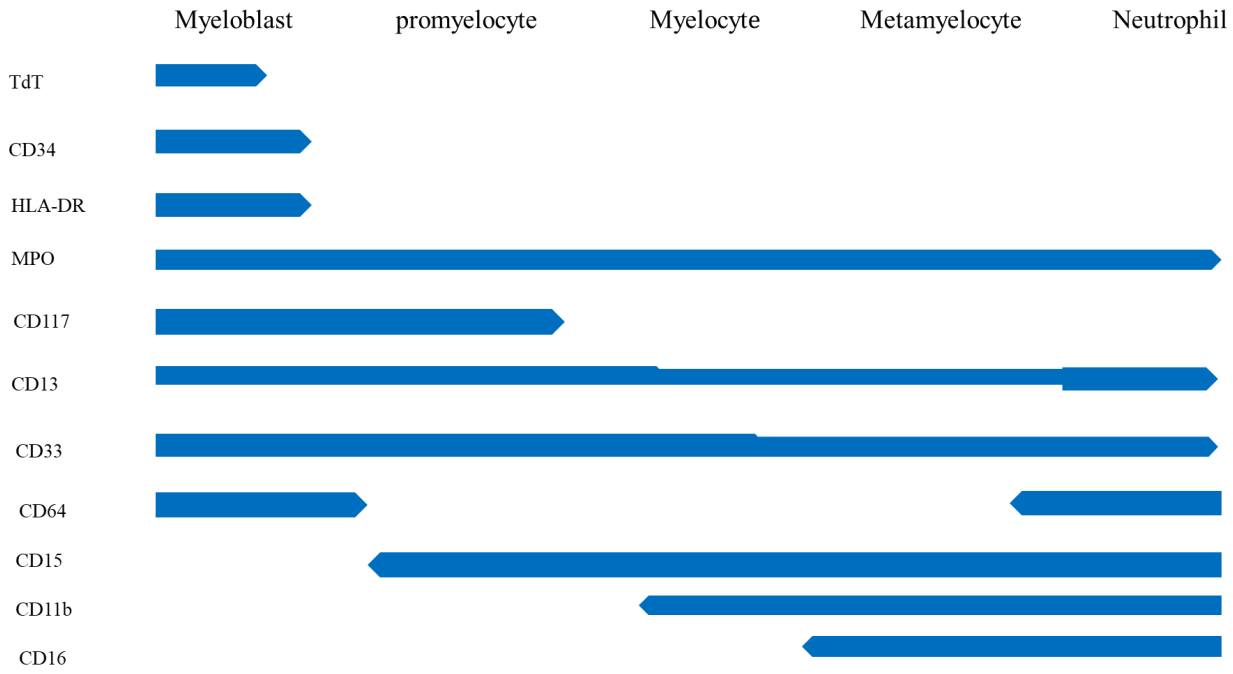


Figure-2.13 Antigen expression during maturation of the neutrophil lineage within the bone marrow. In addition, CD56 is expressed from the promyelocyte stage onwards and CD10 and CD24 on mature neutrophils. MPO, myeloperoxidase; Tdt, terminal nucleotidyl transferase.[45]

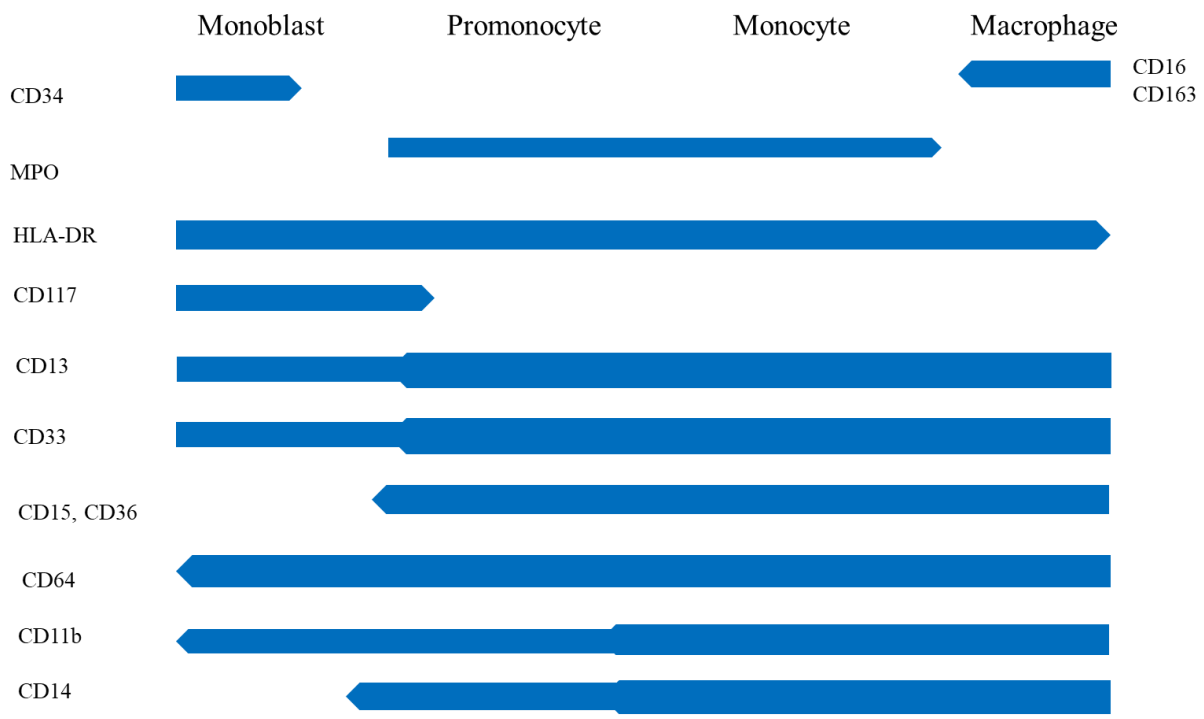


Figure-2.14 Antigen expression during maturation of the monocyte lineage in the bone marrow and in tissues, to macrophages. In addition, CD34 is expressed at all stages of maturation.[45]

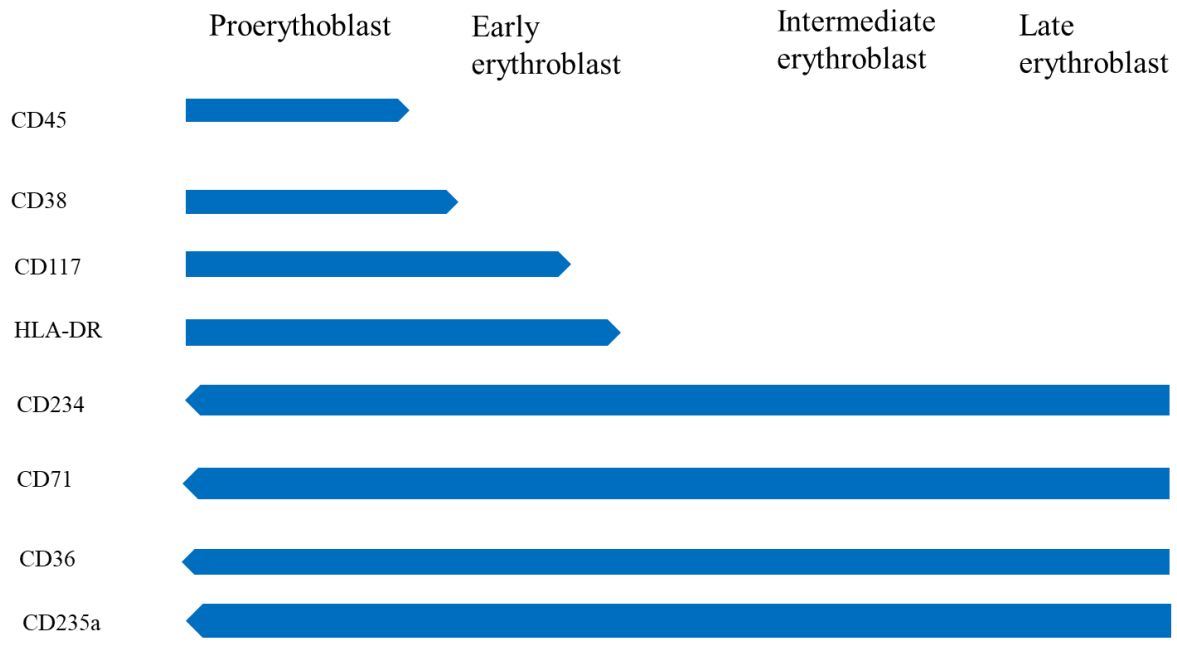


Figure-2.15 Antigen expression during maturation of the erythroid lineage in the bone marrow. CD34 is not expressed by proerythroblasts.[45]

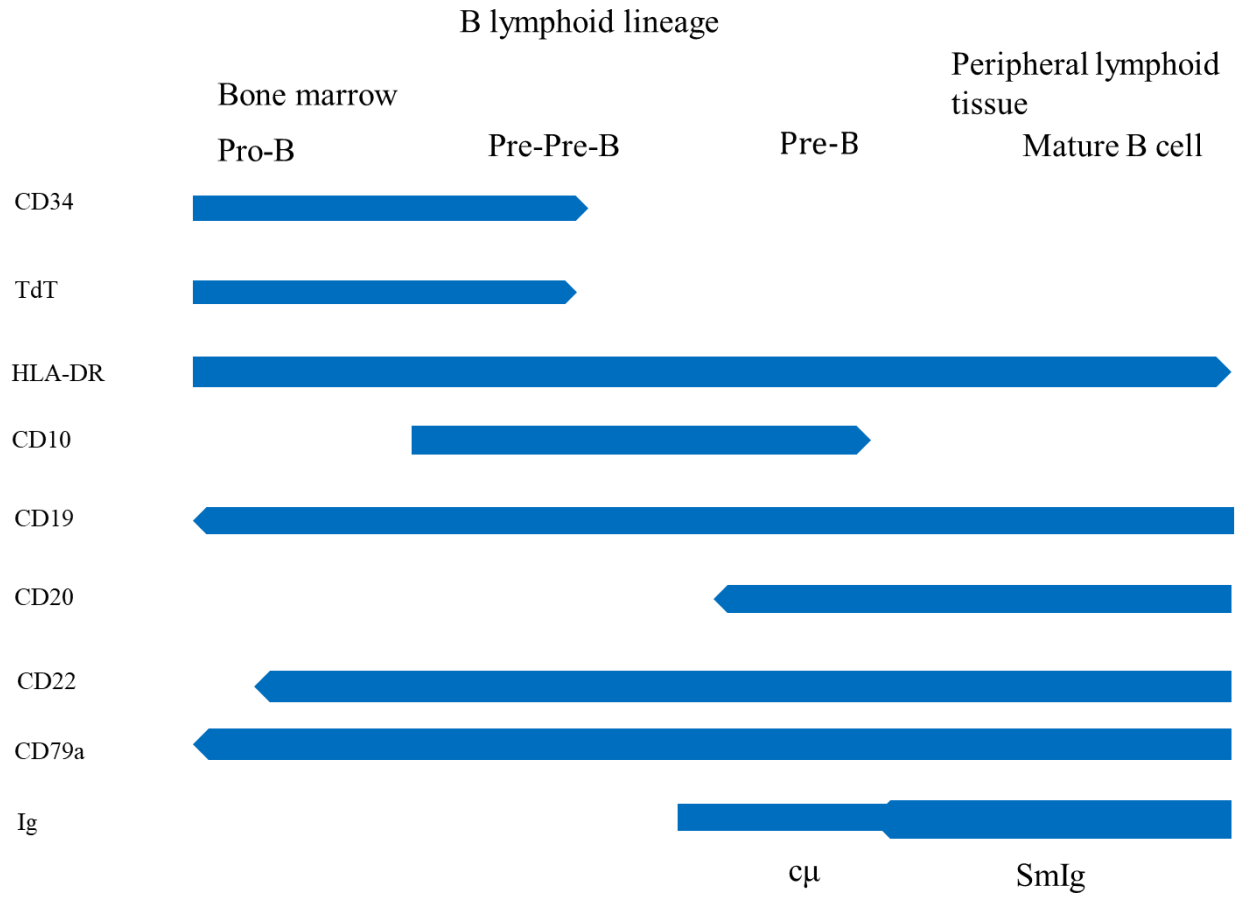


Figure-2.16 Antigen expression during maturation of the B lymphocyte lineage in the bone marrow and in peripheral lymphoid tissues. For CD79a, it is a cytoplasmic epitope that is detected by flow cytometry. cμ, cytoplasmic μ; Ig, immunoglobulin; SmIg, surface immunoglobulin.[45]

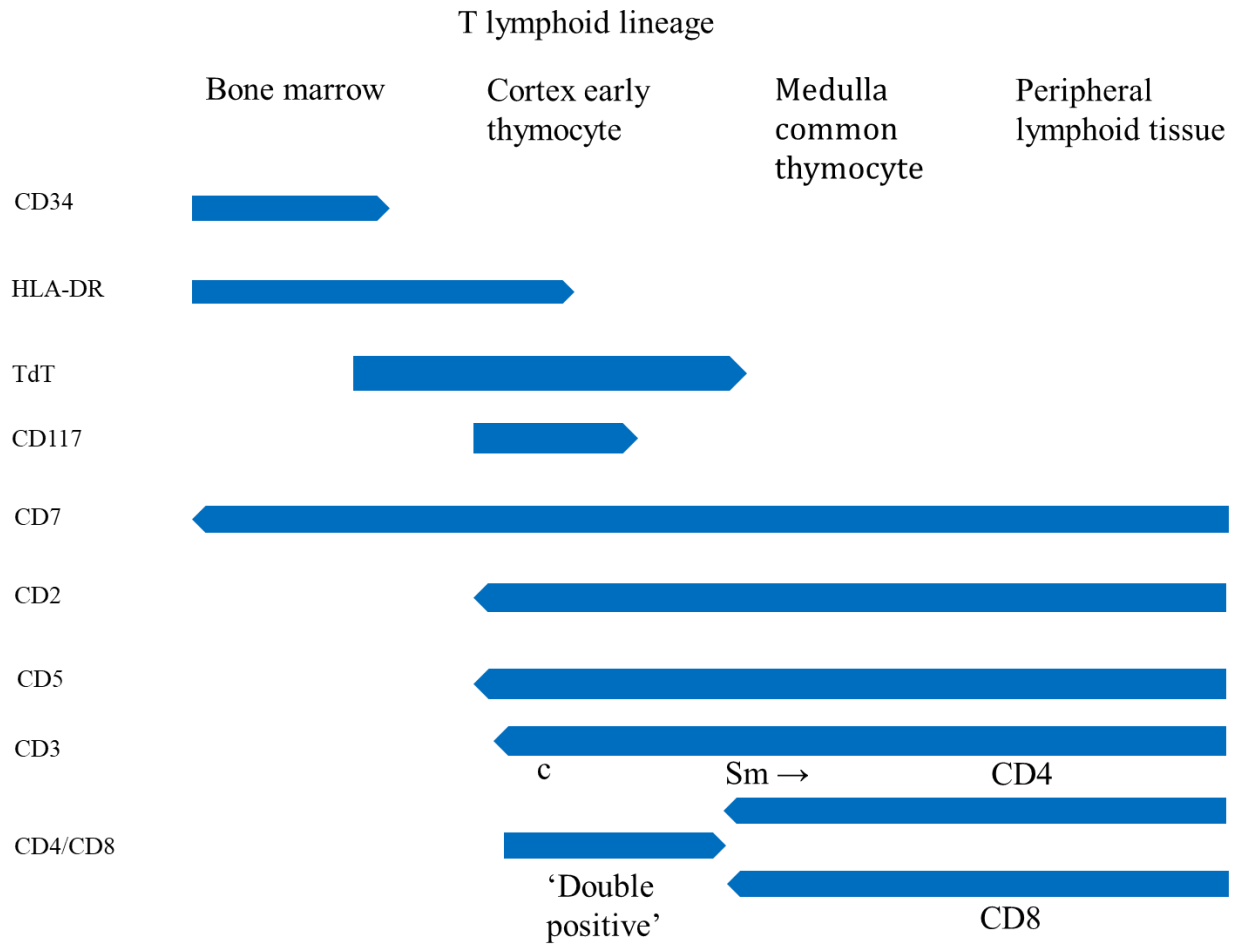


Figure-2.17 Antigen expression during maturation T lymphocyte lineage in the bone marrow, thymus and peripheral lymphoid tissues. In addition, CD10 is expressed by the earliest recognized T cell precursors. Early thymocytes are CD3 negative and it can thus be deduced that the bone marrow precursor of the T lineage is also CD3 negative. A leukaemia derived from such a precursor would be classified as undifferentiated since expression of CD3 is necessary to define T lineage. c, cytoplasmic.[46]

CD56 is expressed in ≥20% of leukaemic promyelocytes in 10% of cases and is prognostically adverse, correlating with more induction deaths, a higher rate of relapse and lower over. AML with t(8;21)(q22;q22.1); RUNX1 RUNX1T1 shows expression of CD34, HLA-DR and myeloid markers. Maturation can lead to expression of CD15 and CD65. Aberrant expression of CD19 (weaker than in B-lineage acute lymphoblastic leukaemia), CD79a, PAX5 and sometimes CD56 can be present, such So expression being useful in the detection of MRD.

AML with inv(16)(p13.1q22) or t(16;16) (p13.1;q22); CBFB-MYH11 usually shows expression of monocytic markers such as CD4, CD14, CD36 and CD38, sometimes markers of the neutrophil lineage such as CD15 and CD65, and often aberrant expression of CD2.

AML with t(9;11)(p21.3;q23.3); KMT2A-MLLT3 usually shows expression of CD4, CD9, CD13 (weak), CD33, CD38, CD65, CD123, HLA-DR and NG2 (detected by the 7.1 antibody); CD15 and terminal deoxynucleotidyl transferase (TdT) are often positive; CD11b, CD11c, CD14, CD36 and CD64 may be expressed. AML with t(6;9)(p23;q34.1); DEK-NUP214 usually shows expression of CD9, CD13, CD15, CD33, CD34, CD38, CD117, CD123 and HLA-DR. AML with inv(3)(q21.3q26.2) or t(3;3) (q21.3;q26.2); GATA2, MECOM can express CD7 and CD56 in addition to CD34, HLA-DR, CD13, CD33, CD65 and CD117. When there is megakaryocytic differentiation, there can be expression of CD41, CD42 and CD61. CD7 can be aberrantly expressed. [46]

Table-2.11 Immunophenotyping of acute myeloid leukaemia and blastic plasmacytoid dendritic cell.[47]

Marker	**Expression**
CD34	Usually positive on blast cells except in AMOL, some cases of AML with NPM1 mutation some pure erythroid leukaemia and most acute megakaryoblastic leukaemias; usually negative in APL
HLA-DR	Usually positive except in APL, AML with NPM1 mutation, some pure erythroid leukaemia and some acute megakaryoblastic leukaemia
CD45	Common leucocyte antigen; useful for gating on blast cells as expression is often weaker than on lymphocytes; often more strongly expressed by monoblasts than myeloblasts; megakaryoblasts are often negative; a generally negative in pure erythroid leukaemia
Myeloperoxidase	Positive except in AML with minimal evidence of differentiation, acute megakaryoblastic leukaemia and pure erythroid leukaemia
CD117	Positive; may be negative in AMOL and weak in acute megakaryoblastic Leukaemia
CD13	Positive
CD33	Positive; expression is relevant to monoclonal antibody treatment

CD11a	May be positive in AML, particularly with monocytic differentiation but not in APL; may be positive in acute megakaryoblastic leukaemia but in cases with Down's syndrome or in transient abnormal myelopoiesis
CD11b,CD11c	Strongly expressed by normal monocytes; positive in AML when there is monocytic differentiation with maturing cells; can be expressed, more weakly, when there is granulocytic differentiation
CD14	Strongly expressed by normal monocytes; positive in AML when there is monocytic differentiation with maturing cells; variably positive on promonocytes but often negative on monoblasts
CD15	Positive when there is granulocytic or monocytic differentiation; more weakly expressed on neutrophils than monocytes
CD16	Positive on mature cells when there is granulocytic differentiation
CD64	Strongly expressed by normal monocytes; positive in AML when there is monocytic differentiation; often weakly positive in APL, both classical and variant, with heterogeneous distribution; may be weakly positive in acute megakaryoblastic leukaemia
CD65	Positive when there is granulocytic differentiation and sometimes when there is monocytic differentiation
CD36	Positive when there is monocytic differentiation and in pure erythroid leukaemia and acute megakaryoblastic leukaemia
CD38	Often positive; positive on leukaemic stem cells ; usually positive in acute megakaryoblastic leukaemia
CD2	Positive in a minority of cases of classical APL; usually positive in variant APL; may be positive in AMI with inv(16)
CD4	Expressed on maturing monocytes; positive in AML with monocyte differentiation and in a minority of cases of classical APL and somewhat more often in variant APL; expressed in blastic plasmacytoid dendritic cell neoplasm
CD10	Expressed by neutrophils

CD41, CD42,CD61	Positive in megakaryoblasts
CD235a	Glycophorin A, expressed in pure erythroid leukaemia
CD7	Aberrantly expressed in some cases; expressed in two-thirds of blastic oplasmacytoid dendritic cell neoplasm
CD56	May be aberrantly expressed; often positive in AML with t(8;21) and when there is monocytic differentiation; expressed in a minority of cases ofAPL, both variant and classical; may be expressed by megakaryoblasts
CD19	Can be aberrantly expressed in AML with t(8;21)
CD79a	Can be aberrantly expressed in AML with t(8;21)
CD71	Sometimes positive, particularly in pure erythroid leukaemia, when expression is characteristically strong, and acute megakaryoblastic leukaemia, when expression is moderate
CD25	Expressed in a minority of cases of AML and is prognostically adverse
CD123	Positive on leukaemic stem cells, sometimes positive on myeloblasts and monoblasts; positive in blastic plasmacytoid dendritic cell neoplasm
CD133	Positive on leukaemic stem cells, on myeloblasts and in APL; monoblasts are usually negative
CD200	Expression is prognostically adverse, including in cytogenetically normal Cases
CD43	Often positive in pure erythroid leukaemia
Terminal deoxynucleotidyl transferase	Positive in a minority of cases
E-cadherin	Positive in pure erythroid leukaemia
PAX5	Pan-B marker; can be aberrantly expressed in AML with t(8;21)
Epithelial membrane antigen	Often positive in pure erythroid leukaemia

Acute megakaryoblastic leukaemia with t(1;22)(p13.3;q13.1); RBM15-MKL1 characteristically occurs in infants. Immunophenotyping is important is permitting its rapid diagnosis. There is expression of megakaryocytic markers such as CD41, CD42 and CD61 and, by immunohistochemistry, von Willebrand factor. CD13, CD33 and CD36 may be expressed while CD34 and HLA-DR are often negative. AML with NPM1 mutated can have an 'APL-like' immunophenotype, with CD34, HLA-DR or both being negative, CD33 being strong and CD13 often being weak; CD133 is also usually negative while CD110 and CD123 are often positive; some cases express monocytic markers - CD14, CD36 and CD64. CD19 can be aberrantly

expressed. On immunohistochemistry, NPM1 is inappropriately expressed in the cytoplasm rather than the nucleus. A monoclonal antibody that recognizes mutant NPM1 is available and can be used for monitoring MRD.

AML with biallelic CEBPA mutation usually shows expression of CD34, CD117, CD15, CD64, HLA-DR and strong MPO with asynchronous expression of CD15 and CD65 and aberrant expression of CD7 and CD56 being common. CD14 is generally not expressed. CD64 can be asynchronously expressed by neutrophils.

AML with mutated RUNXI usually shows expression of CD13, CD34, HLA-DR and CD13 and often expression of CD33 with variable expression of MPO and monocytic markers, expression of CD15, CD19 and CD56 is less common than in other categories of AML. AML with myelodysplasia-related changes and therapy-related AML have variable immunophenotypic features.

AML, not otherwise specified has a variable immunophenotype, depending on differentiation. Pure erythroid leukaemia shows expression of CD36, CD71, CD117, CD235a and E-cadherin with HLA-DR, CD34 and CD45 usually being negative. Acute megakaryoblastic leukaemia shows expression of platelet glycoproteins and CD36 with CD34, CD45 and HLA-DR often being negative; CD7 can be aberrantly expressed. Acute basophilic leukaemia usually shows expression of CD9, CD11b,CD13,CD33,CD123 and CD203c but not CD117.Transient abnormal myelopoiesis of Down's syndrome; GATAI mutated shows variable co-expression of stem cell and early myeloid markers (CD34 and CD117), myeloid markers (CD13 and CD33) and megakaryocyte markers (CD41, CD42 and CD61) with often aberrant expression of CD7 or CD56. CD13 and CD11b are often not expressed. There is also usually expression of CD4 (weak), CD36, CD71, CD110 (the thrombopoietin receptor) and HLA-DR.

AML associated with Down's syndrome has a similar immunophenotype to that of transient abnormal myelopoiesis except that CD34 is negative in about half of cases and CD13 and CD11b are often expressed.[47]

2.1.9.5 Acute lymphoblastic leukaemia, mixed phenotype acute leukaemia and undifferentiated acute leukaemia:

Immunophenotyping is crucial in confirmation of the diagnosis of acute lymphoblastic leukaemia (ALL), in distinguishing between B-lineage and T-lineage cases, and in making a distinction from MPAL. The distinction of early T-cell precursor ALL from other T-ALL is also important. Immunophenotyping is applicable to MRD monitoring. Table-2.12 shows antigens

that are expressed by normal T and B lymphocytes and those that can be applied to the diagnosis and further categorization of ALL.

2.1.9.6 B lineage Acute Lymphoblastic Leukaemia:

Cases of B-ALL usually express CD19, CD79a (cytoplasmic epitope detected), of and often CD45 (can be weak or TdT, HLA-DR and PAX5; they often express CD10 and CD22 (initially cytoplasmic) and sometimes CD20 or cytoplasmic μ chain.CD200 expressed in about two-thirds patients and CD56 in about 10%. Four stages of maturation are recognized, these showing some correlation with genetic subtypes. A mature B immunophenotype is very rare [Table-2.13]. Expression of myeloid antigens, such as CD13 and CD33, is common, and is applicable to MRD monitoring. On immunohistochemistry, PAX5 and CD79a are most often used for lineage assignment (but PAX5 can also be expressed in AML with t(8;21) and CD79a can be expressed in T-ALL). CD19 expression can be lost after CD19-targeted therapy.

B-ALL with high hyperdiploidy characteristically has the immunophenotype of common ALL. Approaching two-thirds of cases express CD66c. CD123 is often strongly expressed.

B-ALL with t(12;21)(p13.2;q22.1); ETV6 RUNX1 characteristically has the immunophenotype of common ALL. CD27 is often expressed. CD13 is often strongly expressed. There is rarely expression of CD9, CD20 or CD66c.

B-ALL with t(4:11)(q21.3;q23.3); KMT2A with t(4;11) AFF1 often has a primitive, pro-B, immunophenotype with no expression of the common ALL antigen (CD10). There is characteristically expression of NG2, CD9 and often myeloid antigens, CD15, CD33, CD65 and CD123. Unlike most B-ALL, CD24 is not expressed.

B-ALL with t(9;22)(q34.1;q11.2); BCR-ABL1 typically has a common ALL immunophenotype, expresses myeloid antigens, such as CD13 and CD33, and often expresses CD9, CD25 and CD123. Expression of CD66c is very common (about 80% of cases), expression of this antigen also being seen in about 60% of cases of high hyperdiploid ALL, in comparison with about 25% of other cases of B-ALL. Expression of CD66c and aberrant myeloid antigens are applicable to MRD monitoring.

B-ALL with t(1;19)(q23;p13.3); TCF3-PBX1 often has a pre-B immunophenotype (expression of cytoplasmic μ chain, and usually also CD10). Strong expression of CD9 is typical and CD34 is often negative.

BCR-ABL1-like ALL, which was initially defined by its gene expression profile, usually has a common ALL Immunophenotyping can be useful in identifying those cases resulting from a Translocation involving CRLF2, since there is increased expression of the protein.

Table-2.12 Immunophenotyping of normal mature T and B cells and in acute lymphoblastic leukaemia.[48]

Marker	Normal expression and expression in ALL
SmCD3	Mature T cells and some T-ALL
cCD3	Mature and immature T cells and T-ALL
CD1a	Common thymocytes and about a third of cases of T-ALL
CD2	Mature T cells and most T-ALL
CD5	Mature T cells and most T-ALL
CD7	Normal mature T cells and T-ALL, aberrantly expressed in 15-20% of cases of AML
CD4 and CD8	Normal mature T cells express CD4 or CD8; T-ALL can be CD4-CD8-(about half of cases), CD4+CD8+ (about a third of cases) or, least often, CD4+ or CD8+
CD10	Germinal centre B cells, a proportion of cases of B-ALL ('common ALL'); more weakly expressed in about a third of cases of T-ALL
CD13	Not expressed by normal lymphocytes, can be aberrantly expressed in B-ALL and T-ALL
CD15	Not expressed by normal lymphocytes, can be aberrantly expressed in B-ALL, particularly with KMT2A rearrangement
CD19	Normal B cells and B-ALL
CD20	Normal B cells; positive in some B-ALL with a more mature immunophenotype
CD22	Normal B cells; positive in the cytoplasm in B-ALL and in cases with a more mature Immunophenotype also on the Sm
CD24	B cells and their precursors; most B-ALL but not those with KMT2A rearrangement; can be expressed in AML with monocytic differentiation
CD33	Not expressed by normal lymphocytes, can be aberrantly expressed in B-ALL (particularly with KMT2A rearrangement) and T-ALL
CD34	Normal haemopoietic and lymphoid stem cells; usually positive in B-ALL (about 70% of cases) and AML, often positive in T-ALL
CD38	Haemopoietic stem cells, T-ALL including early precursor T-ALL, some B-ALL
CD45	Normal B and T cells and their precursors; often weak or even negative in B-ALL; generally more strongly expressed in T-ALL than in B-ALL but expression is weaker than that of mature T cells; weaker expression by lymphoblasts than by lymphocytes makes CD45 useful for gating on blast cells
CD56	Not expressed by normal B cells; expressed by NK cells and subsets of CD4-positive and CD8-positive T cells; expressed in a minority of cases of T-ALL
CD65	Can be aberrantly expressed in B-ALL, particularly with KMT2A rearrangement
CD71	Expressed by a minority of cases of B-ALL; more often expressed in T-ALL

CD79a	Expressed by normal and neoplastic B cells and their precursors; expressed in B-ALL; can be weakly expressed by T lymphoblasts
CD117	Not expressed by normal lymphocytes; can be aberrantly expressed in T-ALL
CD123	Often positive in B-ALL; can be positive in T-ALL
CD200	Positive in B cells, a subset of T cells and in B-ALL
cμ	Positive in a subset of B-ALL ('pre-B ALL')
SmIg	Expressed by normal mature B cells; generally negative in B-ALL
Tdt	Positive in B- and T-cell precursors; usually positive in B-ALL and T-ALL
HLA-DR	Positive in immature and mature B cells and B-ALL; not expressed by mature T cells; usually negative in T-ALL with the exception of early T-cell precursor ALL
CRLF2	Upregulated in some BCR-ABL1-like B-ALL
PAX5	Pan-B marker; can also be expressed in AML with t(8;21)

Table-2.13 Maturation stages of Blineage acute lymphoblastic leukaemia.[48]

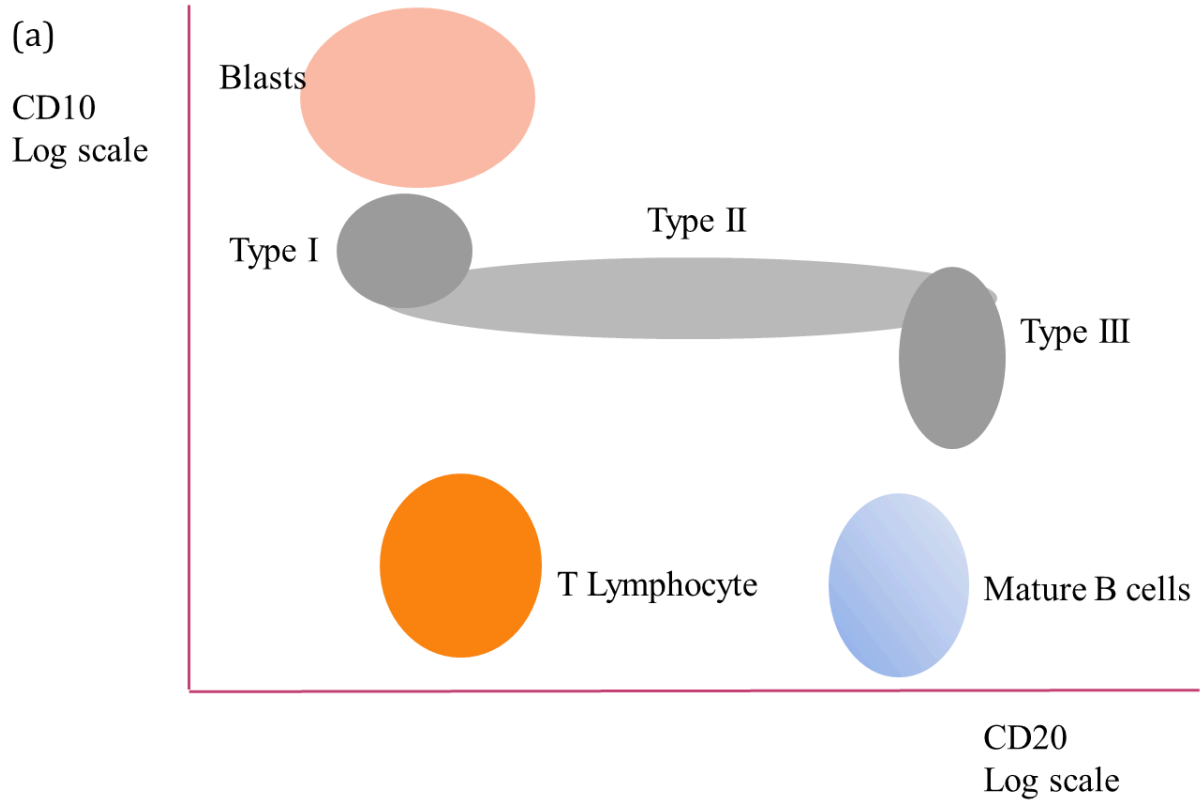
Maturation stage	**Immunophenotype characteristics**	
Pro-B	CD19,cCD22,CD79a and HLA-DR almost always positive. TdT usually positive. CD45 may be weakly expressed or negative	CD10-
Common ALL		CD10+
Pre-B		Cytoplasmic μ+,CD10+ or -
Mature B		SmIg+

2.1.9.7 Haematogones and Their Distinction from B Lymphoblasts:

Haematogones are normal B-cell precursor They are most prominent in the bone marrow all of infants and children, on recovery from chemotherapy and following allogeneic bone marrow transplantation. As they have a precursor phenotype, it is very important to differentiate them from B lymphoblasts, particularly in patients following treatment for common ALL (CD19+, CD10+, CD20+/-. Haematogones are most prominent in healthy and regenerating marrows and tend to be markedly depleted in patients with primary bone marrow diseases such as the myelodysplastic syndromes, AML and aplastic anaemia. Morphologically, they have features intermediate between lymphoblasts and mature B cells, being of medium size with variably mature chromatin and sometimes nucleoli or nuclear clefts.

Regenerating marrows can show very prominent populations of haematogones (up to 20% of nucleated cells in some cases), so they can be easily confused with residual or relapsing disease. There are three stages of maturation of haematogones, designated, depending on the degree of maturation within this precursor population, types I, II and III. Type I haematogones are the least mature often expressing CD34 and nuclear TdT together with CD19 and CD10. As they transition to type II cells, which normally make up the majority of the haematogone population, they lose CD34 and TdT, lose intensity of CD10 expression and gain CD20. It is important to note that type II haematogones often show a spectrum of CD20 expression. Type III cells start to

lose CD10 and show more uniform CD20 expression. Mature B cells lose CD10 completely, show uniform CD20 positivity and gain surface immunoglobulin expression. By plotting marrow cells in a CD10 versus CD20 expression profile, it is usually possible to discriminate between common B-ALL blasts, the three types of haematogone populations can also be identified using CD45 and mature B cells. Haematogone versus SSC characteristics as CD45 expression is also intermediate between that of normal B cells and B lymphoblasts/myeloblasts [Figure-2.18]. By selectively gating on the haematogone zone and analysing the phenotype in relation to CD34, TdT, CD19, CD79a, CD10 and CD20, haematogones of various maturational stages can be accurately identified and separated from neoplastic precursor populations. Figure-2.19 shows the pattern of prominent haematogones in a marrow aspirate following allogeneic stem cell transplantation. The distribution of each subtype according to CD10 versus CD20 expression is illustrated in {Figure-2.18(b)]. Note the spectrum of CD20 expression in the type II cells and that in this case the small type III haematogone population is merging with mature B cells. As haematogones always express CD10, it is particularly important to identify them as such in patients treated for common ALL. It is recommended that at diagnosis, the CD10 versus CD20 expression characteristics are recorded for future reference. Such a plot is illustrated in Figure-2.20 for a diagnostic specimen where the immunophenotype was CD19+, CD34+, CD79a+, TdT+, CD10++ and CD20-. [48]



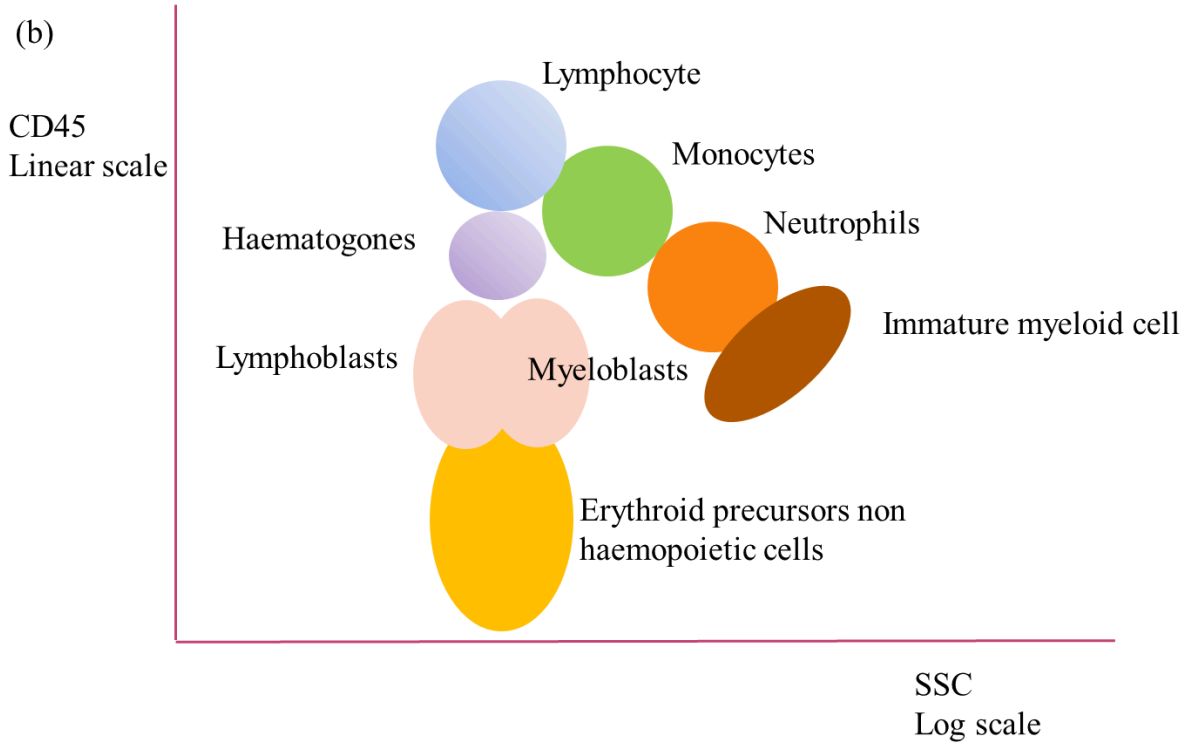


Figure-2.18 Flow cytometric Immunophenotyping: (a) CD10 versus CD20 plot demonstrating the position of B lymphoblasts, haematogone subtypes and mature B cells; (b) utilization of CD45 versus SSC to gate and help identify haematogones.[49]

Typically, common ALL cells express strong CD10 and, regardless of the degree of CD20 expression, this helps to confirm clearance of such cells and separates them from haematogones in follow-up bone marrow aspirates. If patients are transferred between centres for allogeneic transplantation and this diagnostic data is not available, the assessment of post-transplant samples can prove to be substantially more difficult. Since pro-B-ALL does not express CD10, the discrimination of residual blasts from haematogones in this disease should be straightforward. Pre-B-ALL often does not show CD34 and TdT expression so discrimination from type II haematogones is important. The assessment of any given patient relies on a multitude of factors including morphology, flow cytometry and cytogenetic and molecular MRD data. The key immunophenotypic elements used in the identification of haematogones and their discrimination from common B-ALL blasts are summarised in Table 3.5.

Figure-2.19 Flow cytometric Immunophenotyping showing haematogones (a) gating on haematogones type I and II according to CD45 expression; (b) haematogone subtype distribution in relation to CD10 and CD20 expression.[49]

Figure-2.20 Flow cytometric Immunophenotyping showing typical CD10 and CD20 expression at diagnosis in a patient with common ALL.[49]

Finally, as noted above, it is important to appreciate that type II haematogones normally form the majority of the total haematogone population. In particular, if there appears to be an excess of type I haematogones, a careful scrutiny of the exact phenotype at diagnosis and comparison with all other response assessment data is absolutely essential.

2.1.9.8 T-lineage Acute Lymphoblastic Leukaemia:

T-ALL usually shows expression of CD2, cytoplasmic (c) CD3, CD5, CD7, TdT and CD34, and sometimes of CD1a, CD10 (weak) and surface membrane (Sm) CD3; CD4-CD8- is most often observed followed by CD4+CD8+ and least often positivity for either CD4 or CD8 alone. TdT and CD99 are expressed. Four maturation stages are recognized [Table-2.15]. In addition to weak CD10, markers that can be aberrantly expressed include CD79a, CD13 and CD33.

Among cases of T-ALL, early T-cell precursor ALL (ETP-ALL) must be distinguished due to its prognostic significance. It has been described by the WHO specialist group and others: there is expression of CD3 (cytoplasmic and rarely membrane) and usually of CD2 and CD7; CD1a, CD4 and CD8 are not usually expressed, and there is expression of one or more of CD34, CD117, HLA-DR, CD13, CD33, CD11b, CD15 and CD65; CD5 is usually weak or negative. The WHO definition requires negativity for CD1a and CD8 and expression of one or more of CD34, CD117, CD11b, CD13, CD33, CD65 and HLA-DR. CD2 and TdT are less likely to be expressed than in other T-ALL and CD10 is much less likely to be expressed. CD45 is usually negative or weak. A scoring system based on 11 immunophenotypic markers [Table-2.16] has been found to give reliable identification of ETP-ALL with all cases scoring at least 8 and other T-ALL having a a score of less than 7. In the case of a mediastinal tumour it may be necessary to distinguish between T-ALL and a thymoma, an epithelial tumour which, particularly in children,

can be rich in immature reactive lymphoid cells, which can express TdT. Misdiagnosis is possible. The presence of CD4-positive, CD8-positive and double positive lymphoid cell populations is seen in thymoma but not T-ALL, which generally has a single homogeneous population (rarely T-ALL has a subset of cells with a somewhat different immunophenotype from the dominant population). Weak or negative CD45 with an abnormal phenotype, such as CD4+ CD8+ CD3-CD10+ with aberrant myeloid markers identifies T-ALL. Immunohistochemical demonstration of cytokeratin and E-cadherin expression is useful to demonstrate sparse thymic epithelial cells.

Table-2.14 Typical immunophenotyic characteristics of common ALL cells compared with those of haematogones.[49]

Antigen	**Common-ALL lymphoblasts**	**Haematogones**
CD10	Strong	Moderate/strong, type I Moderate, type II Weak, type III
CD20	Negative or variable	Negative type I Variable type II Positive type III
CD34	Often positive	Positive type I only
TdT	Often positive	Positive type I only
CD45	Weak or negative	Weak type I Intermediate type II Positive type III

Table-2.15 Maturation stages of T-lineage acute lymphoblastic leukaemia.[49]

Maturation stage	**Immunophenotypic characteristics**	
Pro T	CD7 is usually positive and is the earliest surface marker expressed; cCD3+, TdT usually positive (expression can be lost in later stages)	CD1a-, CD2-, CD4-, CD8-
Pre T		CD1a-, CD2+, CD5+, CD4-, CD8-
Cortical T		CD4 and CD8+, CD1a+
Medullary T		CD4 or CD8+, CD1a-

2.1.9.10 Acute mixed phenotype leukaemia and acute undifferentiated leukaemia:

Immunophenotyping is essential for the diagnosis of MPAL. Table-2.17 shows markers that are required for this diagnosis, as defined in the 2016 WHO classification. It should be noted that although expression of CD13 or CD33 is not sufficient for the identification of myeloid differentiation in suspected MPAL, such expression can be considered sufficient to define a very early myeloid leukaemia when no specific lymphoid markers are expressed.

Acute undifferentiated leukaemia is lineage-specific markers; markers that may be expressed are CD7, CD34, CD38, CD45, HLA-DR and TdT.

Table-2.16 A scoring system for the identification of early T-cell precursor acute lymphoblastic leukaemia.[50]

Marker	**Expressed**	**Not expressed**
CD1a	-2	2
SmCD3	-2	
CD5	-2	2
CD8		2
CD10		1
CD13	1	
CD33	1	
CD34	1	
CD117	1	
TdT		1
MPO	-1	

Table-2.17 Markers that required for the definition of mixed phenotype acute leukaemia.[50]

MPO or CD11c, CD14, CD64, lysozyme	Defines myeloid, particularly granulocytic, lineage OR Expression of at least two of these defines monocytic lineage	Myeloid
cCD3(or SmCD3)	Defines T lineage	T-cell
CD19	If strong, together with strong expression of at least one of CD10, cCD22, CD79a. If weak, together with strong expression of at least two of CD10, cCD22, CD79a.	B-cell

Part-3

Methodology

Part-3

3.1 Materials and methods:

3.1 Study design:

A descriptive cross sectional study.

3.2 Place of study:

Study conducted in department of haematology of Armed Forces Institute of Pathology, Dhaka cantt.

3.3 Study period:

01 January 2025 to 31 December 2025.

3.4 Study population:

Study population will comprise all patients diagnosed with acute leukaemia by bone marrow morphology who underwent flow cytometric Immunophenotyping at the Department of Haematology, Armed Forces Institute of Pathology, during the study period from January 2025 to December 2025.

3.5 Sampling method:

The present study used non probability consecutive sampling technique.

3.6 Sample size:

To conduct the study, the sample size is calculated by using the following statistical formula-

Here,

$$n = \frac{z^2 pq}{d^2}$$

Where, n = sample size,

d= 5% allowable error = 0.05

Z = z- value of SND at a 95% Confidence level

= 1.96

p= 0.02 “acute leukaemia accounts for approximately 3.5-4% of all cancers worldwide.”(Bray et al., CA cancer J Clin, 2021: GLOBOCAN 2020 data)

q=1-p = 1-0.04= 0.96

$$\text{So } n = \frac{(1.96)^2 \times 0.04 \times 0.96}{(0.05)^2}$$

n = 0.147/ 0.0025 = 59.00

A total 80 patients were enrolled in the study.

3.7 Sample selection criteria:

The inclusion and exclusion criteria clearly define the highly specific subset of acute leukemia patients appropriate for this study, ensuring a homogeneous group suitable for advanced diagnostic analysis.

3.7.1 Inclusion criteria:

- All newly bone marrow morpholgically diagnosed cases of acute leukaemia.
- Cases with complete flow cytometry records.
- Cases diagnosed during January – December 2025.

3.7.2 Exclusion criteria:

Following patients were excluded from the study

- Relapse acute leukaemia cases.
- Incomplete Immunophenotyping data.

3.8 Ethical measures:

All data was collected with the permission of the patient. Participations in this research were fully voluntary. The respondents remained entirely free to withdraw their participation at any stage or any time of the study. Written informed consent was taken from each patient. Prior to consent they were explained in detail the aim and objectives of this study. Confidentiality was assured and anonymity was maintained.

3.9 Study variables:

3.9.1 Demographic variables:

These describe the baseline characteristics of the study population.

1. Age
2. Sex

3.9.2 Immunophenotypic Variables:

1. Lineage Markers
    a. Myeloid markers:CD13, CD33, CD117, MPO
    b. B-cell markers: CD19, CD10, CD20, CD79a
    c. T-cell markers: cyCD3, smCD3 CD5, CD7
2. Immaturity Markers:
    a. CD34
    b. Tdt
3. Other markers
    a. HLA-DR
    b. CD45

3.9.3 Pattern based variables (Derived Variables):

These are constructed from immunophenotyping results.

1. Type of leukaemia
    a. AML
    b. B-ALL
    c. T-ALL
    d. Mixed phenotype acute leukaemia (MPAL)
2. Aberrant antigen expression
    a. CD7+ in AML, CD13+ in ALL

3.10 Data collection tools:

1. Structured data collection proforma.
2. Laboratory investigations report.
3. Flow cytometry (immunophenotyping) panel.
4. Patient clinical records.

3.11 Methods of statistical analysis:

Data were analyzed by computer based program SPSS (Statistical package for social Science). Presentation of the result was done by tables and graphs where applicable. Descriptive statistics (frequencies, percentages) summarised socio-demographic and laboratory variables. Chi square

test evaluated associations between categorical variables. A p-value <0.05 was considered statistically significant.

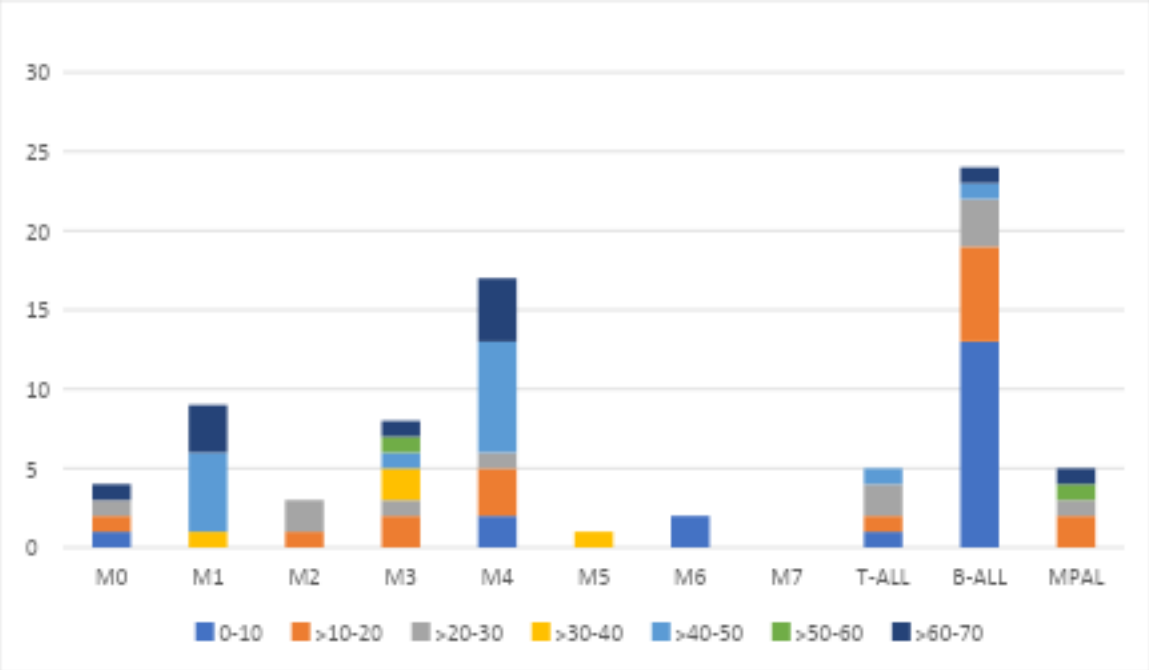
Part-4
Results

Part-4

4.1 Observation and results:

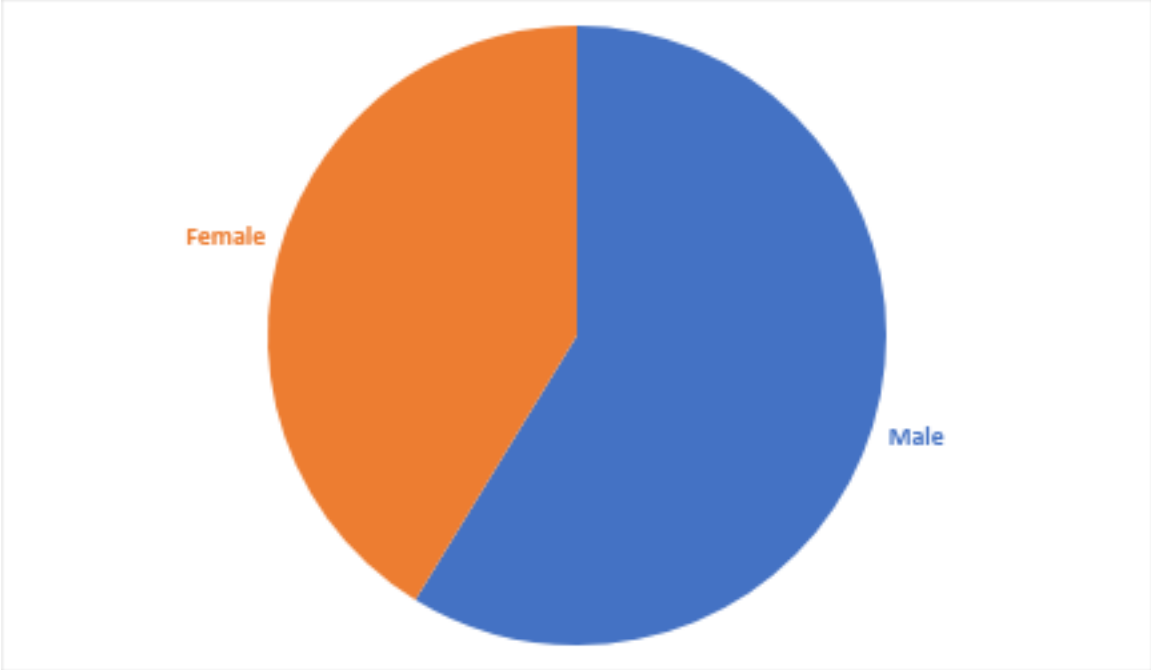
A total 80 cases of newly diagnosed acute leukaemia were included in this study conducted at Armed Forces Institute of Pathology. The observations and results of this study were tabulated and analyzed as below:

Figure-4.1: Age distribution of AL patients group (n=80)



The age distribution of acute leukaemia patients (n=80) showed that the majority were in the younger age groups, particularly 0-10 and 10-20 years. A gradual decline in frequency was observed with increasing age. Very few cases were reported above 50 years. Overall, acute leukaemia was more prevalent among children and young adults in the study.

Figure-4.2: Pie chart Showing percentage of male-female ratio of AL patient groups.



The pie chart illustrates the gender distribution of the study population. It shows that the majority of cases were male, accounting approximately two thirds of the total sample, while female participants constituted roughly one-third.

Table-4.1: Immunophenotypic profile of 43 de novo AML patients.

CD Marker	AML (n=43)							
	M0 (n=4)	M1 (n=9)	M2 (n=2)	M3 (n=8)	M4 (n=17)	M5 (n=1)	M6 (n=2)	M7 (n=0)
CD34+	4(100%)	8(88.9%)	1(50%)	2(25%)	10(58.8%)	0(0%)	1(50%)	0(0%)
HLA-DR+	2(50%)	9(100%)	2(100%)	0(0%)	17(100%)	1(100%)	2(100%)	0(0%)
CD34+HLA-DR+	2(50%)	8(88.9%)	1(50%)	0(0%)	10(58.8%)	0(0%)	1(50%)	0(0%)
CD34-HLA-DR-	0(0%)	0(0%)	0(0%)	6(75%)	0(0%)	0(0%)	0(0%)	0(0%)
CD13+	4(100%)	8(88.9%)	2(100%)	8(100%)	17(100%)	1(100%)	2(100%)	0(0%)
CD33+	4(100%)	9(100%)	2(100%)	8(100%)	17(100%)	1(100%)	2(100%)	0(0%)
CD117+	4(100%)	9(100%)	2(100%)	7(87.5%)	15(88.2%)	0(0%)	2(100%)	0(0%)
CD14+	0(0%)	0(0%)	0(0%)	0(0%)	7(41.2%)	1(100%)	0(0%)	0(0%)
CD64+	0(0%)	0(0%)	0(0%)	3(37.5%)	14(82.3%)	1(100%)	0(0%)	0(0%)
CD11c+	1(25%)	0(0%)	1(50%)	0(0%)	13(76.4%)	1(100%)	0(0%)	0(0%)
CD15+	0(0%)	0(0%)	2(100%)	3(37.5%)	7(41.2%)	1(100%)	0(0%)	0(0%)
cMPO+	0(0%)	9(100%)	2(100%)	7(87.5%)	17(100%)	0(0%)	0(0%)	0(0%)
CD36	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(100%)	0(0%)
CD71	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(100%)	0(0%)
CD235a	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(100%)	0(0%)
CD41	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD61	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
nuTdT+	0(0%)	1(11.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
smCD3+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
cyCD3+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD4+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD5+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD7+	0(0%)	1(11.1%)	1(50%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD19+	2(50%)	0(0%)	0(0%)	0(0%)	1(9.09%)	0(0%)	0(0%)	0(0%)
CD79a+	0(0%)	0(0%)	0(0%)	0(0%)	1(9.09%)	0(0%)	0(0%)	0(0%)
CD20+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
CD10+	0(0%)	0(0%)	0(0%)	1(14.2%)	2(11.8%)	0(0%)	0(0%)	0(0%)

The immunophenotypic profile of 43 de novo AML patients summarized in table 4 (a). The majority of cases belonged to the M4 subtype (n=17), followed by M1 (n=9) and M3 (n=8), while M5 and M6 were least common and no cases of M7 were observed. Myeloid markers CD13 and CD33 were uniformly expressed (100%) across almost all AML subtypes, confirming myeloid lineage. CD117 was also highly expressed in most cases, particularly in M1, M2 and M4 subtypes.

CD34 expression was observed predominantly in immature subtypes (M0 and M1), while it was less frequent in M3 and absent in M5. HLA-DR showed high expression in most subtypes but was negative in M3, consistent with acute promyelocytic leukaemia.

Monocytic markers such as CD14, CD64, and CD11c were mainly expressed in M4 and M5 subtypes, indicating monocytic differentiation. Cytoplasmic MPO (cMPO) positivity was noted in the majority of AML cases, especially in M1, M2 and M4.

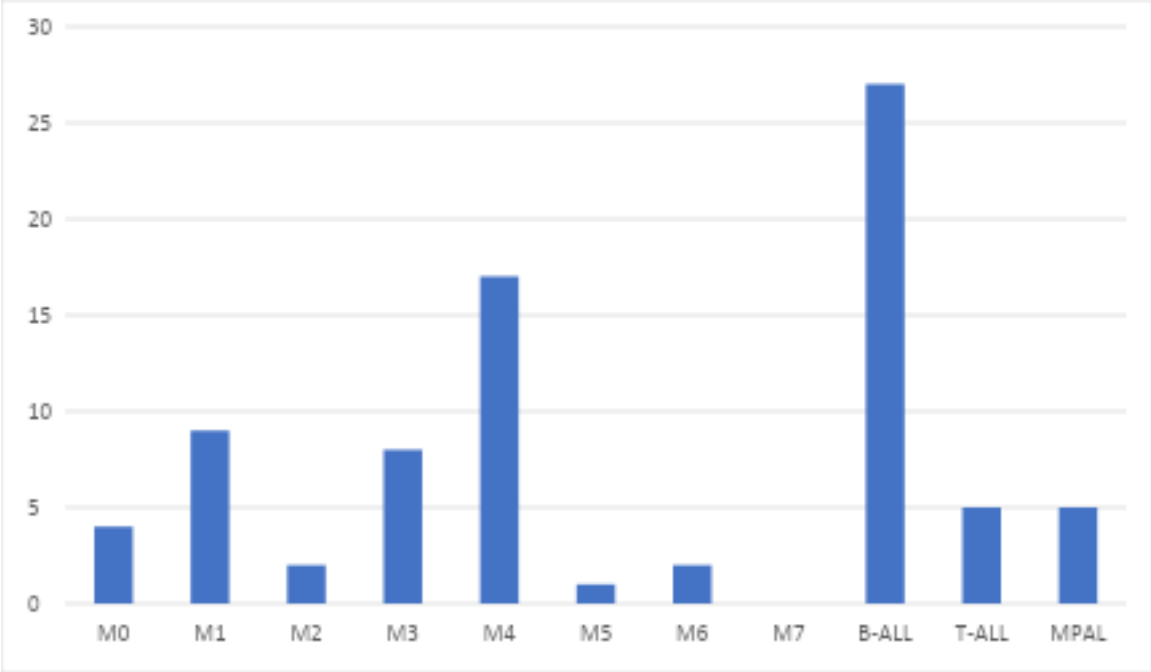
Aberrant expression of lymphoid markers was minimal with occasional positivity for CD7 and CD19 in small number of cases. Other lymphoid markers (CD3, CD5, CD20) were largely negative.

Table-4.2: Immunophenotypic profile of 37 de novo ALL and MPAL patients.

CD Marker	**ALL (n=32)**		**MPAL (n=5)**
	B-ALL (n=27)	**T-ALL (n=5)**	
CD34+	19(70%)	1(20%)	5(100%)
HLA-DR+	26(06%)	1(20%)	5(100%)
CD34+HLA-DR+	19(70%)	0(0%)	5(100%)
CD34- HLA-DR-	0(0%)	0(0%)	0(0%)
CD13+	6(22.2%)	0(0%)	5(100%)
CD33+	6(22.2%)	0(0%)	5(100%)
CD117+	0(0%)	1(20%)	0(0%)
CD14+	0(0%)	0(0%)	0(0%)
CD64+	0(0%)	0(0%)	0(0%)
CD11c+	0(0%)	0(0%)	0(0%)
CD15+	3(11.1%)	0(0%)	0(0%)
cMPO+	0(0%)	0(0%)	1(20%)
CD36	0(0%)	0(0%)	0(0%)
CD71	0(0%)	0(0%)	0(0%)
CD235a	0(0%)	0(0%)	0(0%)
CD41	0(0%)	0(0%)	0(0%)
CD61	0(0%)	0(0%)	0(0%)
nuTdT+	22(81.5%)	5(100%)	3(60%)
smCD3+	0(0%)	5(100%)	1(20%)
cyCD3+	0(0%)	5(100%)	1(20%)
CD4+	0(0%)	5(100%)	0(0%)
CD5+	0(0%)	5(100%)	1(20%)
CD7+	0(0%)	5(100%)	1(20%)
CD19+	27(100%)	0(0%)	4(80%)
CD79a+	27(100%)	1(20%)	4(80%)
CD20+	11(40.7%)	0(0%)	0(0%)
CD10+	25(92.6%)	1(20%)	4(80%)

The immunophenotypic profile of 37 de novo ALL and MPAL patients shown in Table 4(b). Among ALL cases, B-ALL (n=27) was more common than T-ALL(n=5), while MPAL accounted for 5 cases.
In B-ALL, there was high expression of CD19(100%), and CD10(92.6%) and CD79a(100%), confirming B lineage differentiation. Tdt positivity (81.5%) and HLA-DR expression were also frequently observed. A minority of B-ALL cases showed aberrant expression of myeloid markers such as CD13 and CD33 in 22.2% respectively.
T-ALL cases demonstrated uniform expression of T-lineage markers, including cyCD3, CD3, CD5 and CD7(100%), along with Tdt positivity (100%), indicating immature T-cell phenotype.
MPAL cases showed co- expression of both Myeloid and Lymphoid markers with CD13 and CD33(100%) along with CD19(80%) confirming mixed lineage characteristics.

Figure-4.3: FAB distribution of AL patients group (n=80)



Out of 80 cases of AL diagnosed in our laboratory over two-year period, there were 43 cases (53.75%) of AML, 32 cases (40%) of ALL and MPAL 05 cases (6.25%). Acute promyelocytic leukemia (APL) accounted for 18.6%, while non-APL AML accounted for 81.4% of all AML cases. The commonest FAB subtype in AML group in our series was AML-M4/5 (41.8%) followed by AML M1/2 (25.6%). AML-M0 accounted only for 9.3% of all AML cases, while AML-M6 and AML-M7 represented 4.6% and 0.0% respectively. As regard ALL, there were 27 cases (84.3%) with B-ALL and 5 cases (15.6%) with T-ALL (Figure 4.1)

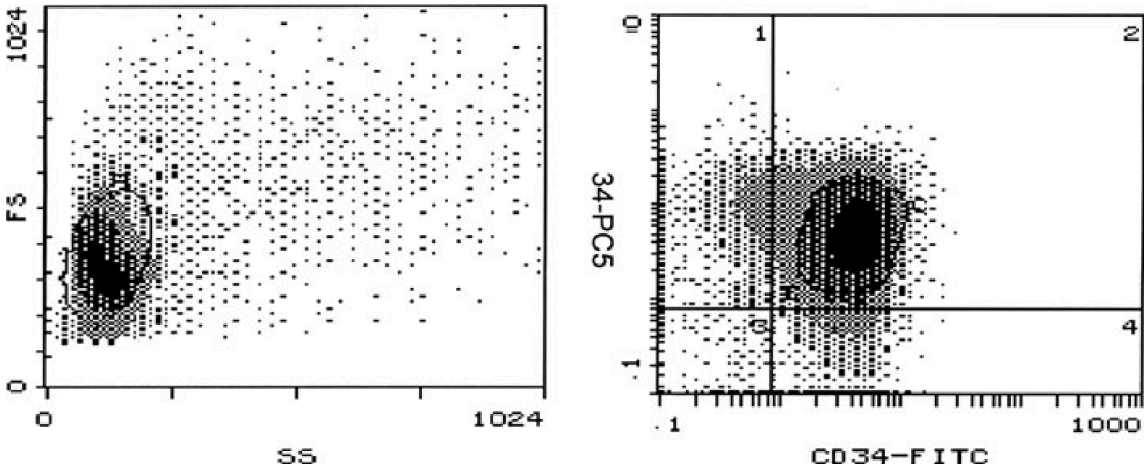


Figure-4.4 Example of HLADR+/ CD34+ expression in AML (this phenotype is not found in any APL case)

Figure-4.5: Column graph representing the expression of CD34, HLADR in AL patients. Notice non of AML-M3 express both antigens simultaneously (n=80)

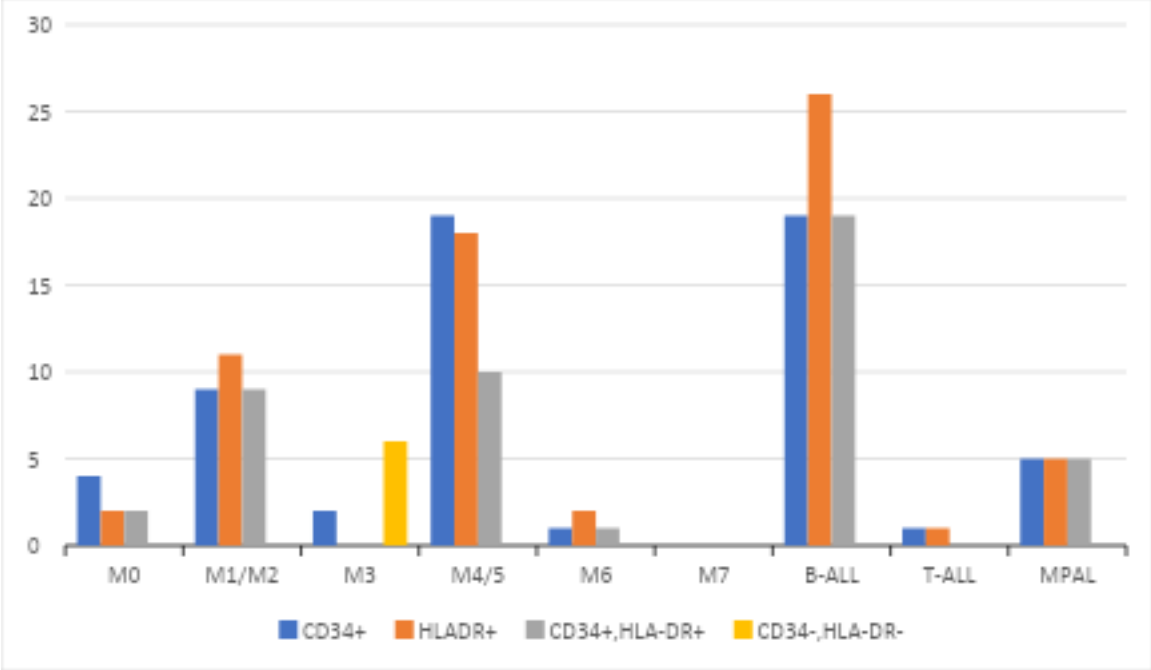


Figure-4.6: Column graph representing the expression of myeloid antigens in AL patients (n=75)

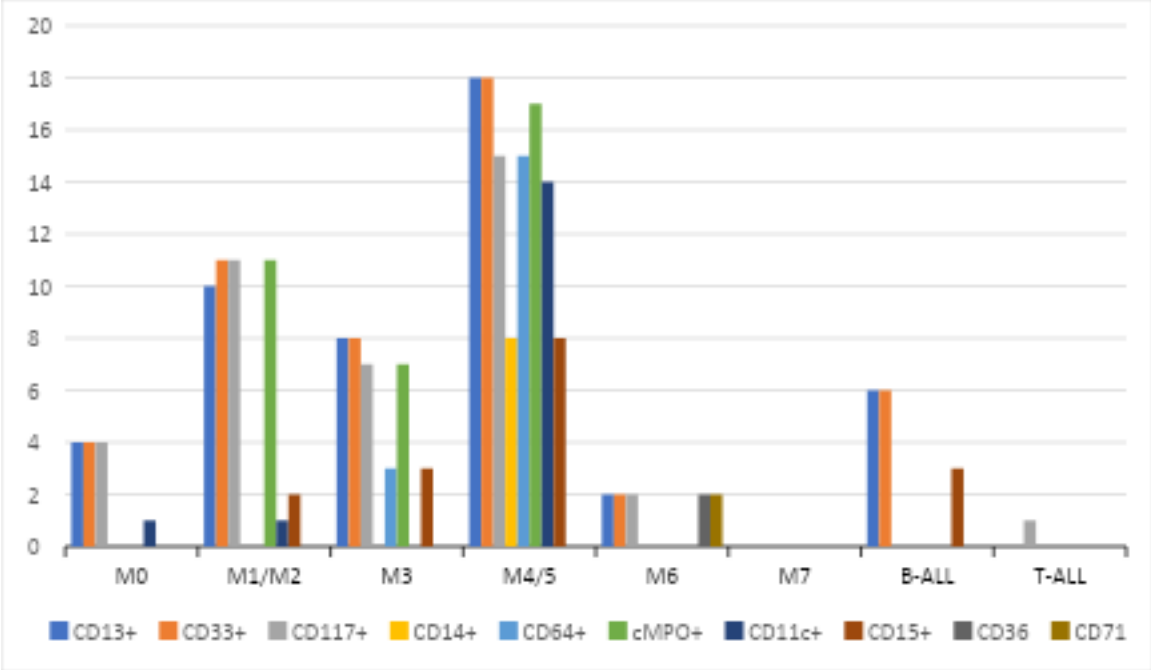
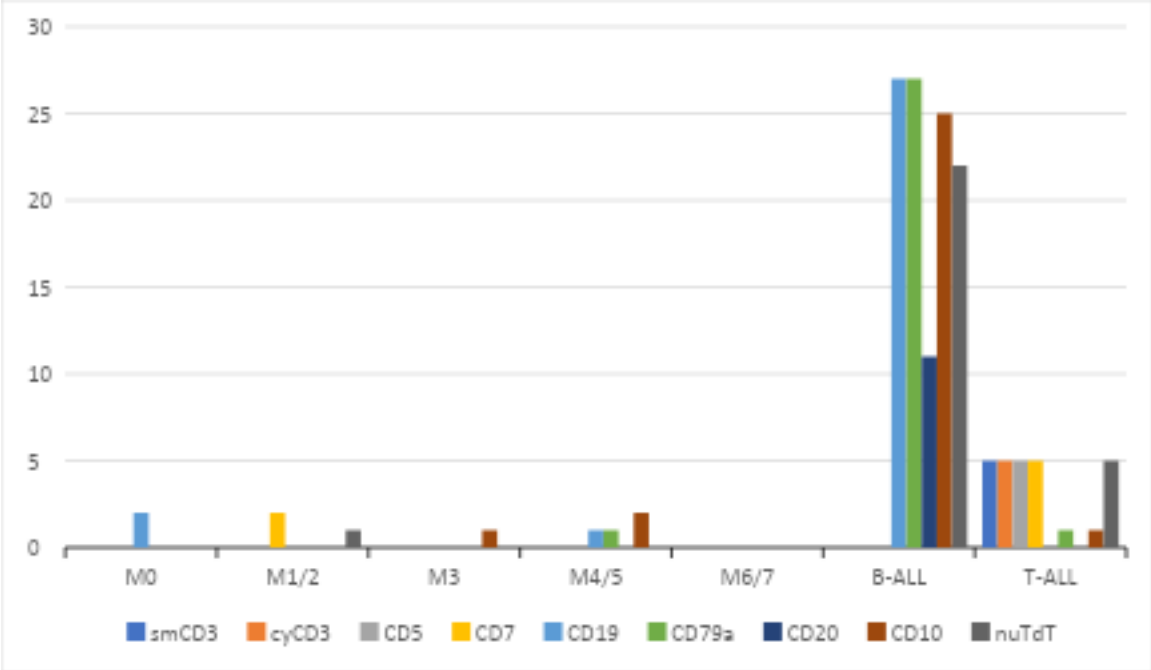


Figure-4.7: Column graph representing the expression of lymphoid antigens in AL patients (n=75)



The leukemic cells in all cases of M0 through M7 commonly express various combinations of CD34, HLADR, CD13, CD33 and CD117 (Figures:4.5-4.7). CD33 was the myeloid marker that most commonly present in all AML subtypes, it's percent was 100%. CD13 was the next most commonly expressed antigen showing 97.6% positivity in all AML categories, while expression of CD117 was seen in 90.7% of AML. CD14 and CD11c positivity were more commonly associated with the monocytic leukemias (83.3 and 77.8%, respectively). CD14 expression was seen with the highest percentage in M4 subtype (82.3%). CD11c expression was highest in M4 subtype (76.4%). Regarding CD71 and CD235a, its expression was restricted to AML-M6 cases. In our study, aberrant expression of lymphoid antigens in AML were seen in of cases. CD19 and CD10 was the most commonly expressed lymphoid antigen (6.97%) respectively and CD7 and CD79a expression was the least often seen in 4.6% and 2.3 % cases respectively. All cases of B-ALL expressed cCD79a and CD19 while CD20, CD10 and TdT were expressed in 40.7, 92.6 and 81.5%, respectively. Few cases of B-ALL expressed CD33 (22.2%) and CD13 (22.2%) without the expression of MPO or CD117 and they were classified as B-ALL with myeloid antigen aberrant expression. None of B-ALL cases expressed T-cell associated antigens (CD3, CD5, CD7).
All cases of T-ALL were positive for cCD3,smCD3,CD5,CD7 and TdT. Only 1 of 5 cases of T-ALL showed CD117 expression and none expressed CD13 and CD33. It was observed that 20% of T-ALL cases showed CD10 and CD79a expression respectively, but none expressed CD19 or CD20.

Table-4.3: HLA DR and CD34 expression in Acute leukemia (n=80)

CD Marker	Non APL-AML (n=35)	APL (n=08)	B-ALL (n=27)	T-ALL (n=05)	MPAL (n=05)	p-value*
CD34+	24(68.6%)	2(25%)	19(70.4%)	1(20%)	5(100%)	~0.0011
HLA DR+	33(94.2%)	0(0%)	26(96.3%)	1(20%)	5(100%)	<0.0001
CD34+ HLA DR+	22(62.8%)	0(0%)	19(70.4%)	0(0%)	5(100%)	<0.0001
CD34- HLA DR-	0(0%)	6(75%)	0(0%)	0(0%)	0(0%)	<0.0001

p-value was determined by *Chi-square test.

Flow cytometric pattern of antigen expression for determination of maturation stage as CD34 and HLA-DR were shown in Table-4.3 and Figure-4.4. Expression of HLADR was seen in 32/35 (94.2%) of patients with non-APL AML, most of them were M4/M5 and 0/8 (0.0%) of patients with APL. On the other hand, expression of CD34 was seen in 24/35 (68.6%) of non-APL AML cases and 2/8 (25%) of APL cases. The combined use of HLA-DR and CD34 was much more helpful in distinguishing cases of non-APL AML from APL cases, as none of 8 cases of APL were positive for both CD34 and HLA-DR in contrast to 62.8% of non-APL AML cases that were positive for both markers.. On the other hand, negativity of both antigens was not seen in any of non-APL AML cases and seen in 75% of APL cases. So, negativity of both antigens does not always suggests the diagnosis of APL. As regard ALL cases, B-ALL showed HLADR and

CD34 expression in 26/27 (96.3%) and 19/27 (70.4%) of cases respectively. T-ALL showed HLADR and CD34 expression in 1/5 (20%) and 1/5 (20%) of cases respectively.

Table-4.4: Lineage demarcation markers expression in acute leukemia (n=75)

CD Marker	M0 (n=4)	M1/M2 (n=11)	M3 (n=8)	M4/5 (n=18)	M6 (n=2)	M7 (n=0)	B-ALL (n=27)	T-ALL (n=5)	P value*
cyCD3+	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	5(100%)	<0.0001
cyCD79a+	0(0%)	0(0%)	0(0%)	1(5.6%)	0(0%)	0(0%)	27(100%)	0(0%)	<0.0001
cMPO+	0(0%)	11(100%)	7(87.5%)	17(94.4%)	0(0%)	0(0%)	0(0%)	0(0%)	<0.0001

p-value was determined by *Chi-square test.

As regard the expression pattern of lineage demarcation markers (MPO, cCD79a and cCD3), MPO was expressed in about 87.5% APL cases and expressed with some variation in non-APL AML cases. The highest percentage of MPO positivity in non-APL AML was seen in AML M1/M2 (100%), followed by AMLM4/M5 (94.4%). MPO was not seen in any of our M0,M5, M6 or M7 cases. As regard ALL, there was no detection of MPO in either B-ALL or T-ALL cases. On the other hand, cyCD3 were negative in all AML cases in contrast to positivity of it in all T-ALL cases.

Part-5
Discussion

Part-5

5.1 Discussion:

Flow cytometric immunophenotyping has become an important and sensitive diagnostic tool in establishing the diagnosis and classification of AL. It is also useful in the early detection of minimal residual disease. Therefore, it has great diagnostic, prognostic and therapeutic implications. Its ability to measure multiple parameters on individual cells in a suspension at high speed is ideal for the study of leukemic cells.

In AL cases, 53.75% were classified as AML while 40% classified as ALL and MPAL as 6.25%. The high percentage of AML may be due to the large number of adults involved in my study (57.5%). The FAB distribution of AML has been extensively studied by many researchers' all over the world [6–13]. In my study, APL accounted for 18.6%, while non-APL AML accounted for 81.4% of all AML cases. Most of the previous studies reported lower APL percentages ranging from 5 to 14% of all AML cases [9–13] while fewer investigators stated nearly similar percentage to my results (24%) [6, 7]. Rego et al. [8] found completely different percentages of APL in two different cities within Brazil (7.8 and 21% of AML). Most published data indicated the predominance of M1-2 as the most common AML subtypes [7, 11–13]. In the current study, the commonest FAB AML subtype was AML-M4/5 (41.8%) and this was in concordance with other published studies who reported marked predominance of M4/5 subtypes varying between 42.2 and 73% of AML cases [6, 10]. Further studies on larger number of cases may be needed to confirm this finding and its cause.

As regard ALL, there were 27 cases (84.3%) with B-ALL and 05 cases (15.6%) with T-ALL. Common ALL (CD10 positive) accounted for 92.6% of B-ALL cases which is concomitant with Rego et al. and Gujral et al. [8, 15].

About 68.6% of our non-APL AML cases were CD34 positive with the highest positivity seen in AML-M0 followed by AML-M1/M2 subtypes. In most reports CD34 positivity in non-APL AML has varied between 55.8 and 69.1% [15–17]. We have found that HLA-DR is the single best marker for distinguishing APL from other AML subtypes. The precision of this distinction is further enhanced if expression pattern of CD34 is also taken into account. HLADR and CD34 double negativity in APL was observed in 75% of cases, this incidence was near to that reported by Wang et al. [18]. On the other hand, none of APL cases expressed both antigens simultaneously, thus the expression of both markers in AML can effectively exclude a diagnosis of APL. There was a strong association between HLA-DR positivity and AML-M4/M5 subtypes and Callea et al. reported similar results [19].

MPO was expressed in 87.5% APL cases and expressed with some variation in non-APL AML with the highest positivity seen in AML-M1/M2 (100%). Expression of CD117 was seen in 91.4% of non-APL AML cases and in 87.5% of APL cases. Similar findings were reported by previous studies [20–22]. So, MPO and CD117 are not reliable markers for differentiation between APL and non- APL AML.

Lymphoid antigens expression in AML seen in 20.9% cases with the highest positivity seen in expression of CD7, CD10, CD19. Some published studies reported that lymphoid antigen positivity in AML between 16 and 22% and CD7 appeared to be the most commonly expressed marker [23–25].

Cytoplasmic CD3 and CD5 expression were seen in 100% of our T-ALL cases but in none of AML-M0 tested cases, so they are the best markers to distinguish AML M0 from T-ALL and this is in agreement with Kaleem et al. [26]. So, the lack of both cCD3 and CD5 dictates the diagnosis of AML-M0 more often than expression of MPO.

All B-ALL cases express CD19 while TdT was expressed in 81.5% and this found to be similar to Bachir et al. [26]. In B-ALL, myeloid aberrant phenotype has been reported in 55.6% of cases [25, 28, 29]. In my study, expression of CD13 and CD33 in B-ALL were 22.2% and 22.2%, respectively.

T-ALL cases in this study were negative for HLADR 80% cases and this incidence is much higher than reported elsewhere [15, 27] which may be explained by low number of cases in our study. Regarding aberrant B-cell marker expression on T-ALL, it was reported that CD10 and CD79a are expressed in 20% of cases respectively [15, 21]. About twenty percent of our T-ALL cases showed CD10 expression while none expressed CD19 or CD20. CD13 was not expressed at any of our T-ALL cases while CD117 observed in 20% cases.

In summary, Beside the routine role of the flowcytometric immunophenotyping in identification and enumeration of blasts in the clinical specimen, it could be uniquely useful in the diagnosis of AML-M0 and in differentiation of APL from non APL-AML. Immunophenotypic criteria of our AL patients were comparable to the internationally published data. On the other hand, AML FAB subtypes showed some geographical variation from most of the previous reports in the form of predominance of M4/5 and more frequent APL.

Part-6
Conclusion, Limitations and Recommendations

Part-6

6.1 Conclusion

This study highlights the significant role of flow cytometric immunophenotyping in the accurate diagnosis and classification of acute leukaemia. The findings demonstrated that acute myeloid leukaemia (AML) cases predominantly expressed myeloid markers such as CD13, CD33 and CD117 with variation across FAB subtypes. Acute lymphoblastic leukaemia (ALL) showed distinct lineage specific expression with B-ALL cases expressing CD19, CD10 and CD79a while T-ALL cases characterized by CD3, CD5 and CD7 positivity. The study also identified aberrant antigen expression in a subset of cases, particularly the expression of lymphoid markers in AML and myeloid markers in ALL, which has diagnostic and prognostic relevance. Mixed phenotype acute leukaemia (MPAL) cases demonstrated co expression of both myeloid and lymphoid markers, confirming the importance of comprehensive immunophenotyping panels. Overall immunophenotyping proved to be an essential and reliable tool for lineage assignment, sub classification and guiding clinical management of acute leukaemia.

6.2 Limitations:

Despite the valuable findings, the study had certain limitations:

- The sample size was modest and drawn from a single center, potentially limiting generalizability.
- Limited antibody panel in some cases may have restricted detection of all aberrant markers.
- Lack of cytogenetic and molecular correlation which are important for comprehensive classification and prognosis.
- Follow up data and survival analysis were not included, limiting prognostic evaluation.

6.3 Recommendations:

Based on the findings of the study, the following recommendations are suggested:

- Future studies should include a larger sample size and multicenter data for better generalization.
- Incorporation of advanced flow cytometry panels with additional markers for improved diagnostic accuracy.
- Integration of cytogenetic and molecular studies with immunophenotyping for comprehensive classification.
- Implementation of minimal residual disease (MRD) monitoring using flow cytometry.
- Long term follow up studies to correlate immunophenotype patterns with treatment outcomes and prognosis.

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