CLINICAL AND HEMATOLOGICAL EVALUATION OF LEUKEMIAS, USING CYTOCHEMICAL STAINS AND IMMUNOPHENOTYPING

DISSERTATION

SUBMITTED FOR M.D. BRANCH III

[PATHOLOGY]

APRIL 2017

THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY

CHENNAI – TAMIL NADU

CERTIFICATE

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DECLARATION

I, Dr.A.Abu Arshad, do solemnly declare, that this dissertation **" CLINICAL AND HEMATOLOGICAL EVALUATION OF LEUKEMIAS, USING CYTOCHEMICAL STAINS, AND IMMUNOPHENOTYPING"**, is a bonafide record of work done by me, in the Department of Pathology, Thanjavur Medical College, Thanjavur, under the guidance and supervision of my Professor and Head of Department **Dr.A.L.SANTHI, M.D.,D.G.O,** between July 2014 to June 2016. This dissertation is submitted to the Dr. M.G.R. Medical University, Chennai, in partial fulfilment of the University's regulations, for the award of M.D. Degree (Branch – III) in Pathology, to be held in April 2017.

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submitted by Dr. .....ABU ARSHAD..... **of**

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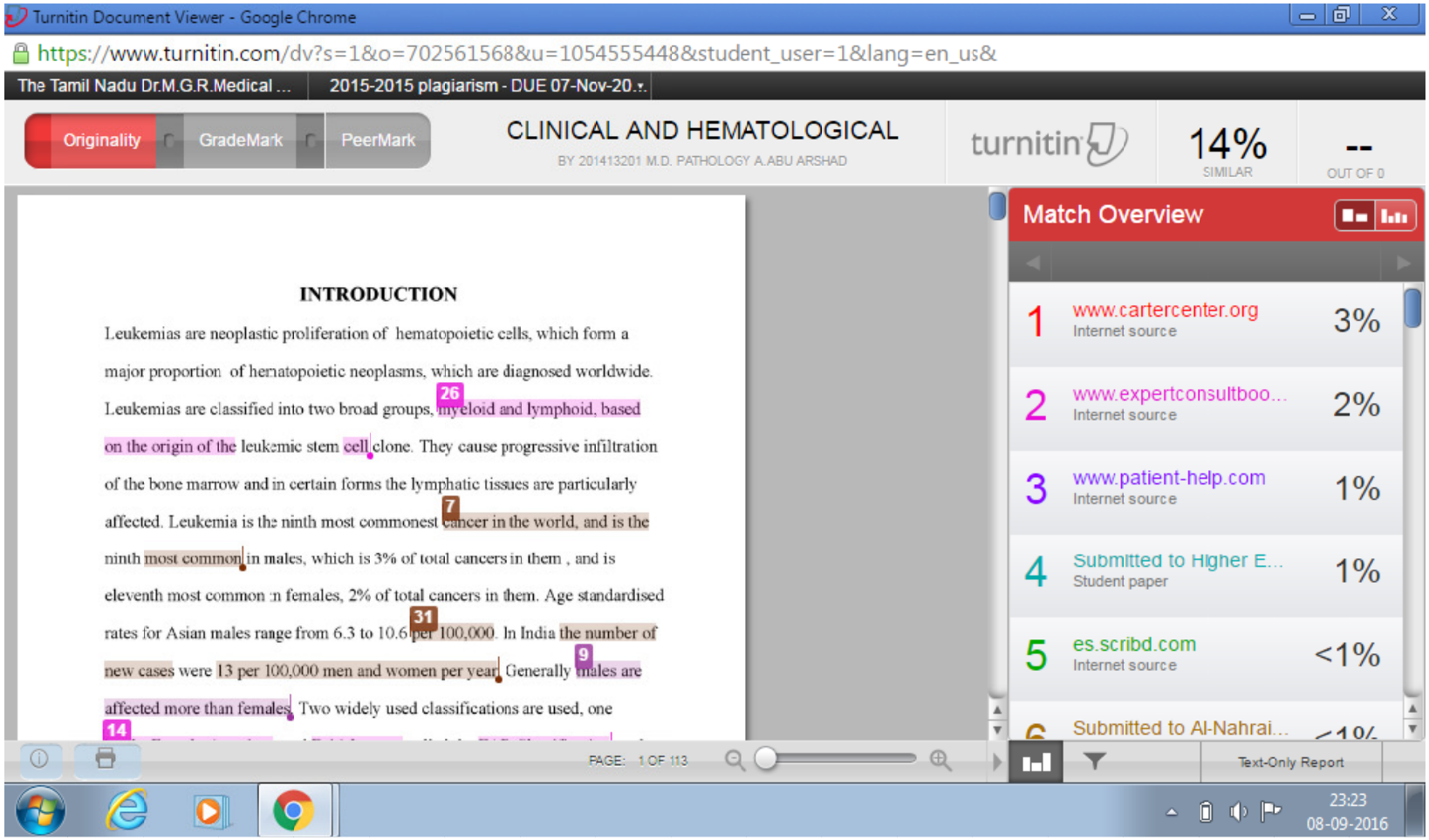
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INTRODUCTION
Leukemias are neoplastic proliferation of hematopoietic cells, which form a major proportion of hematopoietic neoplasms, which are diagnosed worldwide. Leukemias are classified into two broad groups, myeloid and lymphoid, based on the origin of the leukemic stem cell clone. They cause progressive infiltration of the bone marrow and in certain forms the lymphatic tissues are particularly affected. Leukemia is the ninth most commonest cancer in the world, and is the ninth most common in males, which is 3% of total cancers in them , and is eleventh most common :n females, 2% of total cancers in them. Age standardised rates for Asian males range from 6.3 to 10.6 per 100,000. In India the number of new cases were 13 per 100,000 men and women per year. Generally males are affected more than females. Two widely used classifications are used, one
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INTRODUCTION

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blood smear, as well as correlate with the clinical and haematological findings of the cases, with available means in our laboratory. Cytomorphological assessment, based on FAB Classification was made, Immunophenotyping, was done for a select few cases, where there was doubt, or ambiguity, and its concordance with the cytochemical staining patterns and findings were taken into account. Peripheral blood smear, suspected of leukemia,in cases received at the Department of pathology, Thanjavur Medical college, was taken during the period from January 2015 to June 2016, and studied for this purpose. The study also aims to tell that the simple and cost effective method of cytochemical stains, are a valuable tool, in aiding the diagnostic methods in leukemia.

AIMS AND OBJECTIVES

1. To study the demographic data (Age and Sex) distribution in the various types of leukemias.

2. To study the clinical and haematological manifestations of the various types of leukemias.

3. To classify leukemias and subtype acute leukemias with the help of cytochemistry according to the cytomorphology.

MATERIAL AND METHODS

The present study had a sample size of 56 cases of leukemia, diagnosed in the department of pathology, Thanjavur medical college. Those cases referred to the department of pathology, with clinical suspicion , were subjected to peripheral smear study by standard Romanowsky stains first, mainly by Leishman's stain and a presumptive diagnosis was made. Following which peripheral blood smears were subjected to special cytochemical stains using Sudan Black B(SBB) and Periodic acid Schiff's reagent stain(PAS).The percentage of blast cells were enumerated, and the cytomorphology studied based on their positive staining effects on the respective blood smears, were noted. Thereby the types and subtypes of leukemias were classified and reported as per FAB Classification. Relevant clinical history was obtained from each case, with paramaters relating to Splenomegaly, Hepatomegaly, and lymphadenopathy. Hematological investigations were done on a three part automated cell counter, and values pertaining to Haemoglobin, Total count, and Platelet count were also noted. Clinical and Hematological correlation was thus made with the diagnosed cases, and statistical analysis was undertaken. In six cases, immunophenotyping using flow cytometry, and standard panel markers, was undertaken, where in, doubt and ambiguous nature was thought of, and its concordance patterns were also noted.

Inclusion criteria :

Peripheral blood smear from all cases of all age groups who presented with clinical features and abnormal haematological values suggestive of leukemia.

Exclusion criteria :

Other Hematological neoplasms, like Lymphomas and multiple myelomas were excluded in the study

REVIEW OF LITERATURE

Leukemias arise from abnormal proliferation of hematopoietic cells, mainly from the hematopoietic stem cells, the process of differentiation is abnormal and results in immature morphological appearance. They are a heterogenous group of malignancies with different clinical, morphological , immunological and molecular characteristics. Leukemia stands eleventh in the worldwide list of cancers, as per the world cancer research foundation data, with an incidence of 352 per 1000, with 201 per 1000 in men, and 151 per 1000 in women as per the statistical data received by GLOBOCAN 2012, IARC Cancer base 2014, clearly showing a slightly more male preponderance. The SEER fact sheet shows number of new cases of leukemia at 13.5 per 100,000 men and women per year, the Lifetime Risk of developing it standing at approximately 1.5 percent of men and women and the Relative surviving percent at 5 years being 59.7.In children it accounts for almost 30% of all cancers.

AETIOLOGY :-

Leukemia is a neoplastic disorder arising from the hematopoietic cell which has undergone intrinsic change, thereby escaping from the normal restraints imposed on its proliferative activity. Population can consist of one or more different pathways of differentiation. The critical step in its leukemogenesis is the alteration of its DNA in the nucleus of its cell, in which it is initiated.

Chromosomal abnormalities are common in leukemias and occur in all major categories of acute and chronic leukemias.Translocation, addition and deletion do occur in these abnormalities, thereby leading to produce a clone of cells which overtly becomes a leukemic cell clone undergoing blast changes. Translocation is seen, as evidenced in the Philadelphia chromosome, which is caused by the activation of the Abelson cellular proto-oncogene(c-abl) and its transfer to the breakpoint cluster region(BCR).

Evidence that ionizing radiation is leukemogenic and the incidence of leukemia increasing in the cumulative dose received has been noted. Ionising radiation produces chromosomal abnormalities, thereby the process of leukemogenesis. Exposure to high linear energy transfer radiation from alpha emitting radio-isotopes such as thorium dioxide increases the risk of leukemia.

Exposure to chemicals over a period of time have been implicated too. Chemicals like benzene and its products,pesticides and herbicides, alkylating agents as in chemotherapy, such as chlorambucil , melphalan, procarbazine , and nitrosoureas,are noteworthy agents in their causage of chromosomal breakage, thereby producing leukemogenesis.

Viruses like the Human T cell lymphotropic virus type-1 are known to cause oncogenetic process thereby producing T-Lymphocytic leukemia.

Genetic factors have also been implicated, a number of disorders with inherited abnormalities of DNA have been associated with the increased incidence of leukemia. Children with Down's syndrome have been found to have an increased incidence of leukemia with the presence of the additional chromosome 21 in the cells. Inherited disorders like Fanconi's anemia, Bloom syndrome and Ataxia telengeictasia have an increased incidence of chromosomal breakage and leukemogenesis, leading to leukemia. Other conditions like Diamond-Blackfan syndrome, Noonan syndrome, Shwachman syndrome, Dubowitz syndrome, Kostmann syndrome, Poland syndrome, Rothmund-Thomson syndrome, Seckel syndrome, Werner syndrome(progeria), Wolf-Hirschhorn syndrome, MonoMAC and Emberger syndromes(GATA2 Mutation) have been implicated.

A significant association between smoking tobacco and AML has been implicated, with a relative risk of about 1.5 to 2.0.

An endogenous factor that increases increases risk of leukemia is Obesity, studies show AML being associated with it.

Myelodysplastic syndromes(MDS), cause clonal progression, resulting in genomic instability and acquisition of additional mutations, thereby causing transformation into leukemic states.

Other disorders like HIV Infection, Langerhans cell histiocytosis, thyroid disorders and polyendocrine disorders are known to cause leukemic states.

HEMATOPOIETIC LINEAGE:-

Hematopoietic lineage consists of precursor and maturing myeloid cells, erythroid cells, monocytes, megakaryocytes and lymphoid cells. Mast cells and macrophages are both derived directly from the hematopoietic precursors and function as both hematopoietic cells and stromal cells of the bone marrow.

MYELOID SERIES :-

This mainly consists of the granulocytic lineage, which includes neutrophils, eosinophils, basophils and their precursors. It is regulated by the granulocyte colony stimulating factor.

The earliest of the neutrophilic granulocyte is the myeloblast which subsequently differentiates into promyelocyte, myelocyte , metamyelocyte, band cell, and segmented neutrophil. This maturation occurs in a progressive decrease in the nuclear-to-cytoplasmic ratio, loss of nucleoli, condensation of chromatin material, nuclear segmentation and simultaneous accumulation of primary and secondary granules.

MYELOBLAST:-

It measures 10-20µm in diameter. They are characterised by high nucleo- cytoplasmic ratio with a large round , centrally placed nucleus, finely dispersed chromatin, two to five prominent nucleoli, with scant to pale blue cytoplasm. This blast is subdivided into three subtypes, based on the presence of granules. Type 1 has no granules, Type 2 has less than 20 azurophillic granules and Type 3 has more than 20 azurophillic granules

PROMYELOCYTE :-

They are the largest granulocytic cells and measure 10-20µm in diameter. They have a large eccentrically placed round to oval nucleus with a prominent nucleolus and with moderate amount of basophilic cytoplasm containing a few to many purple-red primary azurophilic granules and a pale area called the Golgi zone. Promyelocytes and type 3 myeloblast are almost always indistinguishable and difficult to differentiate between them.

MYELOCYTE :-

This is the last stage capable of division in the lineage, and measures 10-18µm in diameter. These cells have a relatively small eccentrically placed round to oval or slightly indented nucleus with coarse, condensed chromatin and a moderate amount of cytoplasm containing puple-red primary azurophillic granules and light pink secondary granules.

METAMYELOCYTES :-

They measure 10-18µm in size, in this next stage of maturation . They have indented nuclei and abundant acidophilic cytoplasm, with predominant secondary granules.

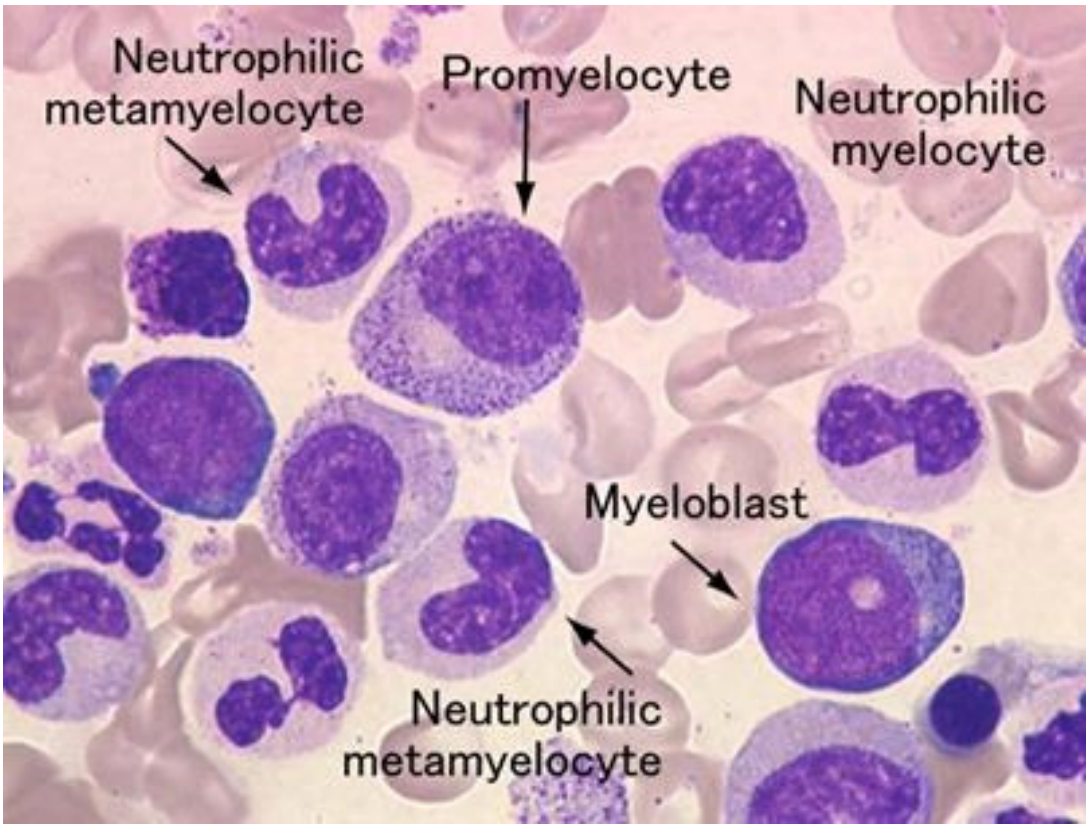
BAND CELLS :-

They measure 10-16µm in diameter and have a horse-shoe or band shape Nucleus (bilobed nucleus with no filaments) and abundant cytoplasm with secondary granules.

SEGMENTED NEUTROPHILS :-

They are the last in the series, measure upto10-16µm in diameter and are characterised as many as five nuclear segmentations connected by each other with filaments.

GRANULOCYTE SERIES IN A SMEAR :-



EOSINOPHILS :-

Eosinophils demonstrate similar stages of proliferation and differentiation as neutrophils. The earliest morphologically identifiable cell is the eosinophil promyelocytes.They have round nuclei with dispersed chromatin and contain two types of granules, one the large, red-orange eosinophilic granules and the other the bluish granules. Eosinophilic myelocytes and metamyelocytes contain only the large red-orange eosinophilic granules.Mature eosinophils are similar or slightly larger in size than the neutrophils , containing bilobed nuclei, and numerous large eosinophilic cytoplasmic granules.

BASOPHILS:-

Basophils, like eosinophils also have similar stages of differentiation and proliferation as well as maturation. The earliest ones present are the basophil promyelocytes. Basophil promyelocytes, myelocytes and metamyelocytes have large, round, deeply basophilic granules. Mature basophils are usually bilobed and have numerous coarse, deeply basophilic granules, that often overlie the nucleus. These granules stain metachromatically with Toluidine blue stain.

MAST CELLS:-

They are derived from multilineage myeloid stem cells and are closely related to basophils. They are of size 12-25µm in diameter. They are round, oval, or spindle shape cells, have a round to oval nuclei,with abundant cytoplasm containing numerous dark purple to red purple granules,they are easily recognised in smears and aspirates stained by Wright-Giemsa stain.

MONOCYTES AND MACROPHAGES :-

They are derived from the same progenitor cells as the granulocytic series. The proliferation and differentiation of these cells are controlled by monocyte/macrophage colony stimulating factor. Gradual nuclear folding and acquisition of cytoplasmic granules characterize the monocytic differentiation and maturation. These stages include monoblast, promonocyte, mature monocyte and macrophage. Monoblasts are similar to myeloblast except that they have slightly lobulated or indented nuclei with scant agranular cytoplasm, and has 1 to 4 nucleoli.Promonocytes are seen earliest, measure 15-20µm in diameter and have large, round, lobulated or folded nucleus with fine chromatin and a moderate amount of cytoplasm containing few azurophillic granules. Mature monocytes have eccentrically placed , oval, lobulated, folded or indented nuclei with fine lacy chromatin, and abundant blue grey, vacuolated cytoplasm, and they measure 15-18µm in diameter. Monocytes are released from bone marrow into the blood circulation , migrating to different body sites, where they transform into tissue macrophages or histiocytes. Macrophages are larger than monocytes and function as phagocytic cells in the bone marrow and measure 20-70µm in diameter, have varying size and shape, cytoplasm is abundant having vacuoles, and may contain cell debris and hemosiderin. The nucleus can also be varying in size with round to oval or indented shape.

LYMPHOID SERIES:-

Lymphoid cells are also derived from multipotential stem cells and are primarily regulated by interleukins.

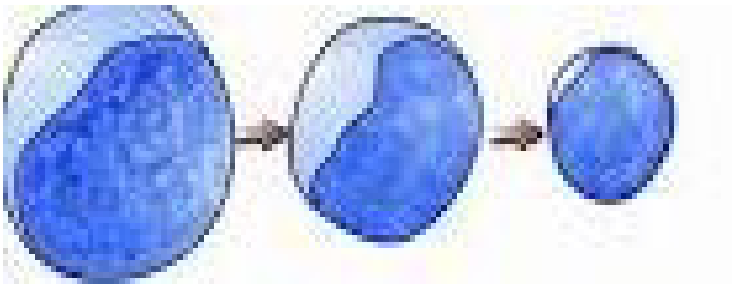
Lymphoblast is the earliest morphologically recognisable cells, 12-20µm in diameter,they are characterized by high nucleo-cytoplasmic ratio, round to oval nuclei with fine coarser chromatin, having one or two nucleoli, and having a small amount of basophilic agranular cytoplasm. They are sometimes difficult to differentiate from other blast cells. The lymphoid maturation results in two group of cells, T and B lymphocytes. B cell maturation occurs in marrow where as T cell maturation is complete in thymus. Prolymphocyte is intermediate between lymphoblast and mature lymphocyte, usually 10-12µm, resembles a mature lymphocyte, with fine blastic chromatin, having a single large nucleolus and pale blue cytoplasm

Large lymphocyte is 10-15µm in diameter, with a centrally or eccentrically placed nucleus, having a clear blue cytoplasm and no nucleoli. Nuclear chromatin is less condensed.

Small lymphocytes are 7-10µm in diameter, slightly larger than a mature RBC, nuclear chromatin is densely coarse, with a thin rim of pale blue cytoplasm, have an inconspicuous nucleoli too, and these are the mature forms. Morphologically T and B lymphocytes are indistinguishable and can only be differentiated by surface antigen detection by immunophenotyping.

Plasma cells measure 10-18µm in diameter and are the last stage of maturation in the B cell lineage. They are present in low numbers in the marrow. They are characterized by eccentrically placed nucleus with coarse chromatin and amply deeply basophilic cytoplasm containing a perinuclear golgi zone.

In adults, lymphocytes constitute less than 20% of the total nucleated cells of the marrow, however in children , younger ones less than 6years of age have as many as 40% of the nucleated cells in the marrow.



Lymphoblast ⇨ prolymphocyte ⇨lymphocyte

With this knowledge of basic myeloid and lymphoid series, we now classify the myeloid and lymphoid cell neoplasms, based on their morphologic characteristics and the clinical presentation as well as with their cytochemical and cytogenetics.

CLASSIFICATION OF LEUKEMIAS

Leukemias are now classified as per the FAB(French American British) classification, as well as the WHO(World Health Organisation) classification.[1,8] The FAB Classification is purely based on the cytomorphological pattern of the presentation seen in it. It relates subtypes to the stages of differentiation and myeloid development and is easy to understand. Has a cut off value for blasts, 30% or more of its presence as the diagnostic point. But the categories do not separate subtypes by prognostic relevance, and hence has largely supplanted by the WHO classification. Revised FAB classification included paramaters which included prognosis, incorporating cytogenetics and immunophenotyping The WHO classification, is based on morphology,history, clinical picture, cytogenetics and flow cytometric analysis with immuno markers. The cut off value of percentage of blasts being 20% or more in the revised FAB Classification also. The significance here lies for prognosis and therapy.

In our study we include FAB classification, incorporating the simple to understand, cytochemical analysis, as well as the morphology, which is easy to classify and corroborate the findings in the context of feasible standards, and with available means in our backyard. We also do immunophenotyping in a select few doubtful cases which corroborated with our findings initially.

WHO CLASSIFICATION.[1] :-

1. Minimal deviation neoplasms (no increase in blast cells(<2%)are evident in the marrow)

A. Underproduction of mature cells is prominent

1. Clonal anemia (refractory sideroblastic or non-sideroblastic)

2. Clonal bi or tricytopenia

3. Paroxysmal nocturnal hemoglobinuria

B. Overproduction of mature cells is prominent

1. Polycythemia vera

2. Essential thrombocythemia

2. Moderate deviation neoplasms(very small proportion of leukemic blast cells present in marrow)

A. Chronic myelogenous leukemia

1. Philadelphia(Ph) chromosome positive BCR Rearrangement positive (~6%)

2. Philadelphia chromosome negative BCR Rearrangement negative (~4%)

B. Primary myelofibrosis (chronic megakaryocytic leukemia)

C. Chronic eosinophilic leukemia

1. PDGFR Rearrangement positive

2. FGFR Rearrangement positive

D. Chronic neutrophilic leukemia

1. CSF3R Rearrangement positive

2. CSF3R and SETBP1 Rearrangement positive

3. $JAK2^{v617}$ Rearrangement positive

E. Chronic basophilic leukemia

F. Systemic mastocytosis (chronic mast cell leukemia)

1. $KITD^{816V}$ mutation positive (~90%)

2. $KITV^{560G}$ mutation positive (rare)

3. Moderately severe deviation of neoplasms(moderate concentration of leukemic blast cells present in marrow)

A. Oligo myelogenous leukemia(refractory anemia with excess blasts)

B.Chronic myelomonocytic leukemia

C.Atypical myeloproliferative disease (atypical chronic myelogenous leukemia)

D.Juvenile myelomonocytic leukemia

4.Severe deviation neoplasms (leukemic blasts or early progenitor cells frequent in the marrow)

A. Phenotypic variants of acute myelogenous leukemia

1.Myeloblastic (granuloblastic)

2.Myelomonocytic (granulomonoblasts)

3.Promyelocyte

4.Erythroid

5.Monocytic

6.Megakaryocytic

7.Eosinophilic

8.Basophilic

9.Mastocytic

10.Histiocytic or dendritic

B. High frequency genotypic variants of acute myelogenous leukemia [t(8;21) , Inv16 or t(16;16), t(15;17) or (11q23)]

C.Myeloid sarcoma

D.Acute biphenotypic (myeloid lymphoid markers) leukemia

E.Acute leukemia with lymphoid markers evolving from a prior clonal disease

2008 WHO CLASSIFICATION OF ACUTE MYELOID LEUKEMIA. [2,4,5]

Acute myeloid leukemia and related neoplasms

1.Acute myeloid leukemia (AML) with recurrent genetic abnormalities

AML with t(8;21)(q22;22) (RUNX1-RUNX1T1)

AML with inv(16)(p13,1q;22) or t(16;16)(p13,1;q22) (CBFB-MYH11)

Acute promyelocytic leukemia with t(15;17)(q22;q12) (PML-RARA)

AML with t(9;11)(p22;q23) (MLLT3-MLL)

AML with t(6;9)(p23;q34) (DEK-NUP214)

AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2) (RPN1-EV11)

AML (Megaryoblastic) with t(1;22)(p13;q13) (RBM15-MKL1)

Provisional entity : AML with mutated NPM1

Provisional entity: AML with mutated CEBPA

2. AML with myelodysplasia related changes

3. Therapy related myeloid neoplasms

4. AML Not otherwise specified

AML with minimal differentiation

AML without maturation

AML with maturation

Acute myelomonocytic leukemia

Acute monoblastic/monocytic leukemia

Acute erythroid leukemia

Pure erythroid leukemia

Erythroleukemia, erythroid/myeloid

Acute megakaryoblastic leukemia

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

5.Myeloid proliferations related to Down syndrome

Transient abnormal myelopoiesis

AML associated with Down syndrome

6.Myeloid sarcoma

7. Blastic plasmacytoid dendritic cell neoplasm

Myeloproliferative Neoplasms (MPNs)

1.Chronic myelogenous leukemia, BCR-ABL1 Positive

2.Chronic neutrophilic leukemia

3.Polycythemia vera

4.Primary myelofibrosis

5.Essential thrombocythemia

6.Chronic eosinophilic leukemia, not otherwise specified

7.Mastocytosis

8.MPN, Unclassifiable

Myeloid and Lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA,PDGFRB or FGFR1

1.Myeloid and lymphoid neoplasms associated with PDGFRA rearrangement

2.Myeloid neoplasms associated with PDGFRB rearrangement

3.Myeloid and lymphoid neoplasms associated with FGFR1 abnormalities

Myelodysplastic/Myeloproliferative neoplasms (MDS/MPNs)

1.Chronic myelomonocytic leukemia

2.Atypical chronic myeloid leukemia, BCR-ABL1 Negative

3.Juvenile myelomonocytic leukemia

4.Myelodysplastic/Myeloprolifertive neoplasm, unclassifiable

5.Provisional entity: Refractory anemia with ring sideroblasts and thrombocytosis

Myelodysplastic syndrome (MDS)

1.Refractive cytopenia with unilineage dysplasia

Refractive anemia

Refractive neutropenia

Refractive thrombocytopenia

2.Refractive anemia with ring sideroblasts

3.Refractive cytopenia with multilineage dysplasia

4.Refractive anemia with excess blasts

5.MDS with isolated del(5q)

6. MDS, unclassifiable

2008 WHO CLASSIFICATION OF THE LYMPHOID NEOPLASMS .[2,4,5]

1**.Precursor lymphoid neoplasms**

B lymphoblastic leukemia/lymphoma

T lymphoblastic leukemia/lymphoma

2.Mature B cell neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma

B cell prolymphocytic leukemia

Splenic B cell marginal zone lymphoma

Hairy cell leukemia

Lymphoplasmacytic lymphoma

Heavy chain disease

Plasma cell neoplasm

Follicular lymphoma

Mantle cell lymphoma

Diffuse large B cell lymphoma

Burkitt lymphoma

3.Mature T and NK cell neoplasms

T cell prolymhocytic leukemia

T cell large granular lymphocytic leukemia

Mycosis fungoides

Sezary syndrome

Peripheral T cell lymphoma, not otherwise specified

Angioimmunoblastic T cell lymphoma

Anaplastic large cell lymphoma

Adult T cell leukemia/lymphoma

Extranodal NK/T cell lymphoma, nasal type

4.Hodgkin lymphoma

Classical Hodgkin lymphoma

Nodular sclerosis

Mixed cellularity

Lymphocytic rich

Lymphocytic depleted

Nodular lymphocytic predominance Hodgkin lymphoma

WHO CLASSIFICATION OF ACUTE MYELOID LEUKEMIA AND RELATED PRECURSOR NEOPLASMS IN CONTEXT TO FAB CLASSIFICATION.[3]

Acute myeloid leukemia and related neoplasms

1.Acute myeloid leukemia (AML) with recurrent genetic abnormalities

AML with t(8;21)(q22;22) (RUNX1-RUNX1T1) **- FAB –M2**

AML with inv(16)(p13,1q;22) or t(16;16)(p13,1;q22) (CBFB-MYH11) **–M4eo**

Acute promyelocytic leukemia with t(15;17)(q22;q12) (PML-RARA)–**M3,M3v**

AML with t(9;11)(p22;q23) (MLLT3-MLL) **– FAB -M4 , M5**

AML with t(6;9)(p23;q34) (DEK-NUP214)

AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2) (RPN1-EV11)

AML (Megaryoblastic) with t(1;22)(p13;q13) (RBM15-MKL1)

Provisional entity : AML with mutated NPM1

Provisional entity: AML with mutated CEBPA **– FAB Variable**

2. AML with myelodysplasia related changes – FAB Variable

3. Therapy related myeloid neoplasms – FAB Variable

4. AML Not otherwise specified

AML with minimal differentiation **– FAB – M0**

AML without maturation **- FAB – M1**

AML with maturation **- FAB – M2**

Acute myelomonocytic leukemia **– FAB – M4**

Acute monoblastic/monocytic leukemia **– FAB – M5a , M5b**

Acute erythroid leukemia **– FAB – M6a ,M6b**

Pure erythroid leukemia

Erythroleukemia, erythroid/myeloid

Acute megakaryoblastic leukemia **– FAB – M7**

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

5.Myeloid proliferations related to Down syndrome

Transient abnormal myelopoiesis

AML associated with Down syndrome

6.Myeloid sarcoma

7. Blastic plasmacytoid dendritic cell neoplasm

8. Acute leukemias of ambiguous lineage, undifferentiated and mixed types

Revised update of the WHO CLASSIFICATION 2016.[23]

There has been a revision of the WHO classification of tumours of hematopoietic and lymphoid tissues, at the time of this study. It incorporates clinical features, morphology, immunophenotyping, cytogenetics and molecular genetics, to define the various entities for clinical importance. Reasons for its revision has been, discovery of new molecular features with regards to diagnostic and prognostic markers, in understanding better the pathology. Secondly, improved standardisation and characterisation of morphological features,aiding in the differentiation of the disorders, such as the BCR-ABL negative myeloproliferative neoplasms,which has increased the reliability of the diagnosis. Thirdly an integrated approach was postulated on the basis of hematologic, morphologic, cytogenetic, and molecular genetics, which was validated. The changes include, removal of Mastocytosis from the MPN list, due to its unique clinical and pathological features and is considered a separate disease category in the classification. Bone marrow karyotyping in the criteria for chronic phase of CML, monitoring of the fusion gene in accelerated phase during therapy, looking for extramedullary accumulation for blasts in blast phase, are changes made now. CALR mutations in MPN, CSF3R mutations in Chronic Neutrophilic Leukemia have been recognised, Bone marrow criteria in polycythemia vera has been incorporated, marrow fibrosis to differentiate pre-fibrotic from primary myelofibrosis and Essential thrombocythemia, and to have a say on its prognosis, and have an accurate histologic diagnosis.

FAB CLASSIFICATION.[3,4,19]

It was devised by a group of French, American , and British haematologists in 1976, based on blood and bone marrow morphological features defined by Romanowsky's stains and cytochemical staining patterns. The FAB classification remains the gold standard against which the WHO classification was developed. It primarily divides leukemias into acute and chronic types. The myeloid ones were Acute myeloid leukemia(AML) and chronic myeloid leukemia(CML). The lymphoid ones were Acute lymphoblastic leukemia(ALL) and chronic lymphocytic leukemia(CLL). The assigned cut of percentage of blasts in peripheral blood and bone marrow smears being >30% in them. It was relatively simple to understand as well as easy to classify according to the cytomorphological pattern observed.

FAB CLASSIFICATION OF ACUTE MYELOID LEUKEMIA.[3,4,19]

AML- M0 Minimally differentiated

AML- M1 Without maturation

AML-M2 With maturation

AML-M3 Promyelocytic

AML-M4 Myelomonocytic

AML-M5

M5a- monoblastic (>80% monoblasts)

M5b- monocytic (<80% monoblasts)

M5c- malignant histiocytic spill over

AML- M6 – Erythroid

M6a- Erythroleukemia

M6b- pure erythroid leukemia

AML-M7 – Megakaryocytic leukemia

FAB CLASSIFICATION OF CHRONIC MYELOID LEUKEMIA.[3,4,19]

Chronic granulocytic leukemia (CGL) Classical CML

Atypical chronic myeloid leukemia (a CML)

Chronic myelomonocytic leukemia (CMML)

FAB CLASSIFICATION OF ACUTE LYMPHOBLASTIC LEUKEMIA.[3,4,19]

Acute lymphoblastic leukemia

L1- Subtype

L2- Subtype

L3- Subtype

FAB CLASSIFICATION OF CHRONIC LYMPHOCYTIC LEUKEMIA.[3.4]

CLL- Prolymphocytes >10%

CLL-PLL – Prolymphocytes 11-55%

PLL- Prolymphocytes more than 55%

CYTOMORPHOLOGY IN FAB CLASSIFICATION.[3,4]

ACUTE MYELOID LEUKEMIA

AML M0 with minimal differentiation

The blasts are usually 15-18µm in diameter, the nucleus occupies most of the cell, the nuclear membrane is fine, the nuclear chromatin is open and fine, with 1-

3 nucleoli. The cytoplasm is pale blue, forming a rim around the nucleus, and does not have any inclusions. Less than 3% of blasts show both myeloperoxidase and Sudan black B(SBB) Positivity. These cells are CD 13, CD33, and CD 117 positive by flow cytometric analysis for immunophenotyping, which show confirmation of AML lineage. CD15, CD36, and CD64 are negative for maturity. Erythroid precurs show dyserythropoiesis and megaloblastic features.

AML M1 without maturation

The blasts are 15-18µm in diameter, the nucleus occupies most of the cell, the nuclear membrane is fine, nuclear chromatin is fine, 2 to 3 nucleoli are also seen. The cytoplasm is pale blue in nature, forming a rim around the nucleus, and may contain thin pink rod like structures called Auer rods. This is formed by the condensation of the primary granules and when present are diagnostic of AML. M1is differentiated from M2, by the presence of more than 90% blasts and less than 10% containing other maturing granulocytic component. M1 occurs in almost 15-20% of cases of AML. Larger nucleocytoplasmic ratio is also seen. More than 3% of blasts are myeloperoxidase and Sudan black B(SBB) positive. Acid phosphatase shows diffuse positivity.

AML M2 with maturation

The blasts are 15-18µm size in diameter, the nucleus occupies most of the cell, nuclear membrane is fine, nuclear chromatin is fine, 2 to 3 nucleoli are seen. The cytoplasm is pale blue, forms a rim around the nucleus, contains early granulation and thin rod like pink structures called Auer rods. More than 30 to 100% of blast

cells are seen, which are positive for myeloperoxidase and Sudan blackB(SBB) stains. Alpha naphthyl chloracetate esterase reaction is also positive in them.More than 10% of myeloid precursors are also present. This is the commonest subtype of AML seen in over 30% of cases. The cytogenetic abnormality noted is t(8;21). Often young individuals are affected.

AML M3 Promyelocytic

The blast is promyelocytic, 18-20µm in diameter size, the nucleus has reticular chromatin, has 1 to 2 nucleoli. The nucleus appears to be folded on itself or may be bilobed. The cytoplasm is moderate in amount and has pink azurophilic granules. Many Auer rods can be seen, and these cells are called Faggot cells. Sometimes these cells have less granulation, so they are called hypogranular variant. The hypergranular variant is seen in 5to10% of AML cases. Dense granules obscuring the nucleus are seen, Auer rods can be seen in bundles and are larger in size, a few of them show Phi body too, paranuclear hof is also present. Myeloperoxidase is strongly positive, as well as Sudan black B. PAS can show cytoplasmic positivity.More than 30% blasts are seen. 90% of the blasts are specific esterase positive.Majority of the hyper granular variant are associated with leucopenia .Hypogranular variant, have sparse finer microgranules, have a bilobed or reniform or convoluted nucleus, and apparently agranular cytoplasm. Few cells have Auer rods, or Phi body. Most often the leucocyte count are higher in hypogranular variety. Cells usually are indistinct from both types, and should be carefully evaluated morphologically as well as by cytochemistry and cytogenetic evaluation, so as to distinguish it from AML M5b type, as

monoblasts like cells are encountered. PML- RARA alteration can be assayed by PCR as well as by FISH. The cytogenetic alteration seen is t(15;17). CD13 and CD33 are positive by flow cytometry,immature markers CD34 ,HLA-DR, CD15 and CD65 are negative.

AML M4 myelomonocytic

This variant has two types, one like the promyelocytic, and the monocytic Component, the other. It occurs in almost of 20% of cases. The myeloblasts are upto 20% of monocytes and monoblasts, more than 30% coexist as blastic components. Increased monocytes are seen in peripheral blood. The blasts are 20-22µm in diameter, nucleus may be round or convoluted as in promyelocytes, has reticular chromatin, and 2 to 3 nucleoli. The cytoplasm is abundant, vacuolated, and may contain few azurophilic granules and sometimes Auer rods. Myeloperoxidase ,Sudan black B and Non specific esterase are positive, especially in the monocytic component. A distinct variant **M4eo** has eosinophilic differentiation and a cytogenetic abnormality of inversion 16. They have more than 5% eosinophilic precursors with basophilic granules too which is diagnostic. Immunophenotyping expresses CD64, CD14, CD11b, CD11c in the monocytic component, and are positive in CD34 and CD117 in the M4eo entity. Variable expression for CD13,CD33,MPO, and CD15 as well as HLA-DR are seen. Double esterase stains, chloracetatase esterase (CAE) for myeloid differentiation and non specific esterase for monocytic differentiation are useful to distinguish M4 from M2 and M5.

AML M5 Monocytic

In this type, the blast is predominantly monocytoid, and restricted to the monocyte series, usually has high cell counts too, and a propensity to infiltrate skin, gums, meninges , lungs and lymph nodes with rapid progression. This accounts for 7 to 10% of AML cases, and is uncommon. Three types of it has been recognised, **M5a**,**M5b**, and **M5c**. **M5a** type has predominant monoblasts , almost more than 80% are seen in them, promonocytes and monocytes are less than 20%. The monoblasts are large cells having pale blue basophilic cytoplasm having vacuolations , with few granulations, nuclei are round to oval, having a lacy fine chromatin , which contain more than 1 nucleoli. Blasts show non specific esterase positivity with sodium fluoride inhibition, weak granular myeloperoxidase positivity, and granular positivity with Sudan black staining in cytochemistry. **M5b** type has predominant promonocytes in the marrow and monoblasts are less than 80%. Promonocytes have abundant cytoplasm with azurophilic granules, the nuclei is lobulated or convoluted with prominent nucleoli. Monocytes have lesser nucleocytoplasmic ratio, with lobulated or reniform nuclei and basophilic cytoplasm which is vacuolated and borders are irregular, the nuclear chromatin is condensed and have variable nucleoli. They are positive to non specific esterase(NSE) staining and are similar to monoblasts in immunophenotyping. Promonocyte enumeration helps in distinguishing M5b from chronic myelomonocytic leukemia. In addition to cytochemistry , serum and urine muramidase levels are raised. **M5c** is rare and show malignant histiocytic spill in the peripheral blood. Variable expression of immunomarkers are seen in AML M5. CD33, CD13, CD 14, CD 11, CD 11b , CD 11c, lysozyme, CD64 and

CD68 are positive. HLA-DR is also positive. Myeloperoxidase can be weakly expressed.

AML M6 with erythroid differentiation

This type also is a rarer form constituting 3 to 4% of AML cases. It is seen with severe anemia. Two types are present M6a and M6b**. M6a** has more than 50% of blasts in marrow and non erythroid cells being more than 20%. Erythroid component is prominently seen in this type, the erythroblast show megaloblastoid or macronormoblastic reaction , with nucleocytoplasmic asynchrony. Blasts can have binucleus , the nucleus is multilobed, few ring sideroblasts are seen. Cytoplasm has vacuoles. Some blasts can have Auer rods and can be dysplastic. Myeloperoxidase stains positive,the erythroid component can demonstrate coarse granules in the cytoplasm which show cytoplasmic granular positivity with Per iodic Schiff"s stain (PAS). Focal non specific esterase is also seen positive.CD71, CD 117 and glycophorin A also are positive with immunophenotyping. CD13 and CD33, which are myeloid markers are dimly positive too in this type.**M6b** is pure erythroid type, the proerythroblastic population is more than 80% of the marrow cells. The blast cells are large with basophilic cytoplasm and has vacuoles, the nuclei are round with fine chromatin and 1 to 2 nucleoli. M6b expresses glycophorin A , haemoglobin A . MPO is absent CD 34, CD 41, CD61, CD64, and HLA-DR are negative in immunophenotyping.

AML M7 megakaryocytic leukemia

In this variant the cell line involved is megakaryoblastic. The blasts superficially resembles lymphoblasts. It is seen very uncommonly, and is present in 8 to 10% of adult cases and 2 to 3% of cases in children. It can also occur in 20% of Philadelphia chromosome positive chronic myeloid leukemia patients too. In this type more than 20% of the blasts are in the bone marrow nucleated cells,and more than 50% are of the megakaryocytic lineage, and flow cytometry shows positivity for CD41 and CD61 which are platelet markers. The megakaryoblasts are large cells and variable in size, having abundant pale blue cytoplasm, cytoplasmic protusions, with blebs, the nuclei are rounded with condensed chromatin, having a single or multiple nucleoli. Micromegakaryocytes and fragments can also be seen. These micromegakaryocytes are small cells with round nuclei having condensed chromatin and mature cytoplasm, these are not included in the blast count too. Bizarre sized platelets are also present. The blasts are negative for Myeloperoxidase and Sudan black B(SBB) cytochemical stains. Periodic acid schiff's stain(PAS) show cytoplasmic granular positivity, some blast may show non specific esterase positive which is fluoride sensitive. Other markers like CD34,CD45 and HLA-DR are negative. Myeloid markers like CD13 and CD33 may be positive.

CHRONIC MYELOID LEUKEMIA

Chronic granulocytic leukemia (CGL) Classical CML

This is the commonest form of CML, and is either philadelphia chromosome positive or negative, but BCR/ABL1 positive, primarily seen in the fifth and sixth

decades of life. Males are affected slightly more than females. Onset is gradual with non specific symptoms, 50% of them are asymptomatic and diagnosed at routine blood exam. There are three different phases of CML. Chronic phase , accelerated phase and blast phase with crisis.

Chronic phase

In the chronic phase , the total count varies from 30 × 10^9/L to 1000×10^9/L, and can even be above it too. The leucocytosis has mainly neutrophils, and all stages of white cells from myeloblasts to mature neutrophils. Dysplasia of myeloid cells are usually not seen. Myelocytes and mature cells are mostly seen with blasts usually being less than 10%, often less than 3%. Myelocyte bulge is the major immature cell present, the neutrophilic precursors are usually normal, later stages can present with Pelger Huet cells and hypersegmented neutrophils. Hypogranular basophils are seen in this phase, mild eosinophilia is also observed with both basophilic and eosinophilic granules. Monocytes can also be present,and when seen is usually associated with BCR ABL 1 and needs to be differentiated from chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia. Most of these cases have normocytic normochromic anemia,with nucleated RBCs also seen with some nuclear budding. Presence of anisopoikulocytosis and tear drop cells are suggestive of marrow fibrosis. Platelets are moderately increased in this phase, can be upto 1000×10^9/L , and vary in size and shape with lack of granules. Some megakaryocytes are also seen with fragments too. Platelet function can also be deranged. Neutrophil alkaline phosphatise score is markedly reduced and can be as much as 0 to 20 , normal being 40 to 100. Marrow is usually hypercellular,containing hyperplastic

hematopoietic cells, and has marked myeloid hyperplasia, with M:E Ratio being as much as 15:1 to 32:1. Proliferation of neutrophilic precursors are seen, with slight increase in basophilic precursors and eosinophilic precursors, myeloblasts are usually less than 5%. Megakaryocytes are smaller in size and usually a dysmegakaryopoietic picture is present. Erythroid series is decreased, morphologically normal, and unevenly distributed in focal areas. Macrophages with linear striations or with wrinkled granular cytoplasm having a sea blue colour, also called as Pseudo Gaucher cells or Sea blue Histiocytes are also seen, which is mainly due to increased lipid formation. The NAP score helps in differentiating CML from leukemoid reaction. Sudan black B(SBB) can be variably positive, showing coarse granules, as well as MPO too. Molecular findings in chronic phase are , to detect Philadelphia chromosome and the presence of BCR-ABL1 fusion gene. The chimeric size of the protein is 210kd,which is the p210 fusion protein.

Accelerated phase

In the accelerated phase, usually more than 5 to 20%, in the range of 10 to 19%. There is persistent basophilia , which is more than 20% in the peripheral blood. The total count values can be more than 10×10^9/L, and will be increasing. Thrombocytopenia is a key feature, where the count gets reduced below 100×10^9/L . Myeloblasts are seen upto 19% in the smears. Marrow shows similar blast range upto 19%, and are seen in clusters, with promyelocytes seen more in the paratrabecular and perivascular areas.increasing anemic picture is also seen. Immunophenotyping with CD34 markers is positive and focal. Additional clonal cytogenetic and chromosomal abnormalities are also seen like

trisomy 8, isochromosome 17q, or duplication of the Philadelphia chromosome. Megakaryocytic series proliferation is associated with reticulin fibrosis and granulocytic dysplasia. Granulocytic dysplasia can be well seen by Sudan black B (SBB) staining as they stain granules black in the cytoplasm. Majority of the cases progress to blast crisis gradually.

Blast crisis (Blastic phase)

The chronic phase gets transformed to terminal phase of blast crisis in almost 70% of the cases. Here the blasts are more than 20%. The clinical picture looks that of acute leukemia. Blastic phase is myeloid in 60 to70% of cases. MPO and SBB staining is weak and variable with subtype being either M1, M2, M4, M5, M6, or M7. M3 transformation is very rare. About 10 to 15% undergo M7 transformation. Basophils are seen, and increase to more than 20%. Thrombocytopenia is also present with bleeding manifestations. Bone marrow fibrosis can also develop, leading to marrow failure. Flow cytometry for immunophenotyping with CD13, CD33, CD41, CD14, CD117, CD34, HLA-DR, is positive for these myeloid blast cells. Aberrant lymphoid antigens can also be expressed. Nearly 15 to 30% of cases undergo lymphoblastic blast crisis, this can be identified with immunophenotyping with CD10, TdT and CD19 markers showing the aberrant nature of the myeloid neoplasm. Some of them have a mixed phenotype of acute leukemia.

Atypical Chronic myeloid leukemia (aCML)

This variant of CML is Philadelphia chromosome negative , there is increase in monocytoid population of cells, and lie in an intermediate position between classical CML and chronic myelomonocytic leukemia (CMML). There is prominent dysplasia of granulocytes , megakaryocytic and and erythroid series too. The total count is lower, usually between 2 to 5 $\times 10^9$/L. Myelocytes are less in number, but monocytoid and atypical neutrophilic cells are more . Thrombocytopenia and anemia is more frequent in them. BCR-ABL1 fusion gene is negative in these cases.

Chronic myelomonocytic leukemia (CMML)

This type of CML is Philadelphia chromosome negative , and now classified under the MDS/MPN category of the 2008 WHO Classification. There is absolute monocytosis with more than 1×10^9/L , which is a prominent feature. Thrombocytopenia is very common unlike classical CML. Normocytic normochromic anemia is present.Total leucocytic count varies from 0.9 to 160 $\times10^9$/L and can rise moderately. Bone marrow shows hypercellularity with granulocytic and monocytic hyperplasia, features of myelodysplasia of the hematopoietic cell lines are evident. Basophils are more than 2%, monocytes 3 to 10%, with severe granulocytic dysplasia, blasts more than 2% and immature granulocytes 10 to 20%. MPO and SBB stain variably. CD45, CD68 and lysozyme are positive for immunostaining. Cytogenetics show they are associated with trisomy 8 in approximately 35% of cases and to a lesser extent monosomy 7 and –Y. Mutations have been associated with K-RAS or N-

RAS, RUNX1, JAK2, TET2, IDH1/2 , SRSF2. PDGFR rearrangements were also present

Juvenile chronic myeloid leukemia (JMML)

Juvenile CMML is rare and found in children less than 14 years of age , 75% occurring in less than 3 years of age, it is a clonal hematopoietic disorder with proliferation of mainly granulocytic and monocytic lineage, total leukocytic count will be more than 10×10^9/L , blasts are more than 20%, with monocytosis more than 100 per cu.mm. Fetal haemoglobin(HbF) is increased in 70% of cases. Philadelphia chromosome is positive, mutations in PTPN1 have been found common. BCR-ABL1 fusion gene is negative. Abnormality in chromosome 7 is present.

Chronic neutrophilic leukemia

It is a rare disorder, having BCR-ABL1 fusion gene negative, commonly seen in the elderly. Blood picture shows marked neutrophilia with total count more than 25×10^9/L , segmented neutrophils and band forms are more than 80% of the white cell population, immature cells like promyelocytes, myelocytes and metamyelocytes are seen less than 10% of the cells, myeloblasts are less than 1%. Mature cell pattern appear normal. Megakaryocytes can also be present. There is no philadelphia chromosome or the BCR-ABL1 fusion gene or any PDGFRA or PDGFRB or FGFR1 rearrangement seen cytogenetically. No evidence of any myelodysplasia or myeloproliferative neoplasm or granular dysplasia. Monocytes are less than 100 per cu.mm, no identifiable cause for neutrophilia or any evidence of infection or inflammation is seen. No other

tumour underlying also is seen. NAP activity is increased. Variable staining patterns are noticed by MPO and SBB.

Chronic eosinophilic leukemia

It is a BCR-ABL fusion gene negative chronic myeloid disease, striking eosinophilia is the hallmark picture. Total count can be high normal or elevated. Platelet counts are normal or decreased. Marrow shows myelocytic and eosinophilic hyperplasia, charcot leyden crystals and increased mast cell population. Megakaryocytes can be dysmorphic. Reticulin fibrosis is common. Cytogenetically translocations are common in chromosome 5 at the PDGFR gene site. MPO and SBB staining patterns are variable.

Chronic basophilic leukemia

It also is a BCR-ABL fusion gene negative disorder. Basophilia is striking in peripheral blood and marrow smears. Marrow is hypercellular with the three major lineages. Dysmorphic megakaryocytes are also seen. The disorder is a clonal myeloid disease and quite rare. Cytogenetics, do show rearrangements in PDGFR gene. Cytochemical stains like MPO and SBB can be variably positive.

ACUTE LYMPHOBLASTIC LEUKEMIA ALL

Acute lymphoblastic leukemia originates in the lymphoid precursors of lymphopoietic stem cells of the bone marrow, lymph nodes and thymus. Almost 75 to 80% are of B cell lineage and 15 to 20% are of T cell lineage.

ALL L 1

This type is seen in 70 to 75% of acute lymphocytic leukemia cases. Mostly pediatric population are affected.The leukemic blast cells are homogenously uniform small cells, ranging from the same size to twice the size of small lymphocytes,with scanty light blue cytoplasm. The nucleus is round or slightly indented and has coarse chromatin or clumped chromatin and contains 1 or 2 nucleoli. Some cases have larger blast cells. The cytoplasm in some cases have coarse granules which are amphophilic and found to be that of mitochondria too in electron microscopy.The blast cells stain positive with Periodic Acid Schiff's stain (PAS), giving a purple magenta colour, as block positivity in the cytoplasm. MPO and SBB are negative in these cases. PAS positive cases are usually CD10 positive for immunomarkers. Other markers are also used to assess the cell lineage , if they are from pre-B, pro-B, or T cell as well as for CALLA positivity. B cell lineage markers used are CD19, CD79a, and CD22, T cell markers used are CD3, CD7,CD34, CD117, TdT, HLA-DR and CD2 mainly.

ALL L2

In this type which is seen in almost 20 to 25% of ALL cases, the lymphoblasts are larger in size as well as heterogenous in nature with larger amount of cytoplasm. The nucleus are also varied in size, and has indentations, with irregular membranes, and finer to coarse chromatin, 1 or 2 nucleoli will also be present. Minimal cytoplasmic vacuolation can also be seen. L2 includes most of the adult cases and T ALL cases seen. MPO and SBB are negative while PAS shows block positivity.

ALL L3

This type is rare and seen in 1 to 2% of all ALL cases. The blast cells are large homogenous and have deep blue basophilic cytoplasm with multiple vacuoles overlying the nucleus. These prominent vacuolations are lipids. Blasts resemble that of cells in Burkitt's lymphoma. The vacuoles stain for fat stains like Oil Red O positive, and are MPO and SBB negative. PAS can also be negative, rarely positive.Most of these cases have c MYC rearrangement.

Morphologic correlation with FAB Sub types of Precursor B, T lymphocytic leukemia and Burkitt's leukemia.[3,4,19]

Precursor B lymphoblastic leukemia/lymphoma have FAB L1/L2 or Mixed morphology. Precursor T lymphoblastic leukemia/lymphoma have FAB L1 or L2 or Mixed morphology and the cells are CD3 positive. Burkitt's leukemia/lymphoma is derived from small cells which are not cleaved and are mature B cell neoplasms. The blasts have FAB L3 morphology

CHRONIC LYMPHOCYTIC LEUKEMIA CLL

This disease is heterogenous in nature and is seen in middle and old age group patients. It is a B Cell lymphoproliferative disorder, where CD5+ and CD23+ mature lymphocytes are seen in blood, bone marrow and lymphoid tissues. The lymphocyte count is increased and is more than 20 to 200×10^9/L , more than 90% are mature lymphocytes in the peripheral blood. The leukemic blasts are uniform, with round nucleus and cytoplasmic rim. The nucleus has coarse chromatin with no nucleoli. Nuclear indentation, clefting and large atypical

lymphocytes will also be seen. These are associated with trisomy 12 cytogenetic abnormalities too. Large smudge cells are also present due to the making of the smear, as the cells are fragile and almost always seen in the smears. Few prolymphocytes are also seen. The prolymphocytes are larger with moderate cytoplasm, and has nucleus with coarse chromatin and a single nucleoli. Based on this, the FAB classification subtypes CLL as follows

a) CLL where prolymphocytes are less than 10%

b) CLL –PLL where prolymphocytes are 11-55%

c) PLL where prolymphocytes are more than 55%

80% of cases are classical CLL, and 20% are CLL/PLL or atypical CLL. Atypical CLL are cases which have mixed type, where there are predominant small lymphocytes and few large cells, which might have nucleoli. Some 15% of the cells show clefted or lobulated nuclei, which can be seen in trisomy 12 and other cytogenetic abnormalities. Red cells are usually normocytic normochromic , haemoglobin can be low, erythroid elements can get diminished in the marrow as the disease progresses. Platelets are generally normal and thrombocytopenia is rare. Bone marrow is hypercellular because of lymphocytic infiltration, which is more than 30% and can be nodular , mixed or diffuse in infiltrating the interstitium. Lymphocytes in the marrow vary from 30 to 95%, there by the erythroid population gets diminished. In cases of associated autoimmune haemolytic anemia, there is erythroid hyperplasia. Megakaryocytes are normal in number. Rarely Red cell aplasia develops which is due to parvo B19 virus infection and giant sized erythroblasts can be seen. PAS stain is variable in nature. Immunomarkers used for B cell lineage are CD19, CD23 and CD5, which

are positive, CD5 being a T cell marker identifies co-expression with B cell lineage. CD10,CD79a and FMC7 is negative in CLL, and CD20 is seen in atypical CLL. Aberrant CD5 expression signifies CLL/SLL types. Small lymphocytic lymphoma(SLL), the blast cell in the lymph nodes, has absent cytopenias in peripheral blood and lymphadenopathy is the common feature.

International workshop CLL guidelines for the diagnosis (2008).[4]

1) Clonal proliferation of abnormal B lymphocytes in peripheral blood for more than 3 months.
2) B lymphocytes should be atleast 5×10^9/L.
3) Atypical / immature blast cells/ prolymphocytes should be less than 55%.
4) More than 30% of lymphoid cells should occupy the bone marrow.
5) Low density of surface immunoglobulin (IgM or IgD) with κ or λ light chains should be present.
6) B cell surface antigens (CD19, $CD20^{dim}$, CD23) is to be present.
7) CD5 surface antigen is present.
8) Lack of pan T cell markers other than CD5 is to be seen.

Hairy cell leukemia

It is an uncommon form of adult chronic B cell leukemia, the characteristic leukemic cells are found in the marrow, blood and spleen. Patients present with infections , fatigue and splenomegaly. Most of them have pancytopenia, due to impaired hematopoiesis in the infiltrated marrow and sequestrated spleen. Anemia , absolute neutropenia are also present.Monocytopenia is also seen. Morphologic presence of hairy cells, with pale blue or grey cytoplasm with

ruffled border, nucleus round to oval or reniform with indistinct nucleoli and spongy chromatin is seen. These cells are TRAP positive. Flow cytometry identifies the profile with immunomarkers CD2O, CD11c, CD103,CD123, and is negative for CD5, CD10, CD27, and CD43. BRAFV600E mutation is present and confirmatory. Marrow fibrosis is very common and characteristic, characteristic mononuclear cells with a "fried egg appearance" is also seen.

CYTOCHEMICAL STAINS IN LEUKEMIA

Despite a lot of advances in defining the diagnosis of leukemias, careful examination of Romanowsky stained peripheral blood smears and bone marrow smears remains the fundamental in the haematological diagnosis.[10] Microscopy and cytology remain the gold standard in elucidating the provisional diagnosis of leukemias. With the advent of cytochemistry in the earlier part of the twentieth century, the understanding of leukemic cell morphology and its staining patterns ushered in new changes and challenges in confronting the disease. FAB Classification which came into existence in 1976, was solely based on the cytomorphological pattern seen in the special stains used, and helped a lot in understanding leukemias in general, and depending on its classification the Acute and Chronic forms of the disease were well documented. Specific cytochemical stains have now been used to differentiate leukemic cell abnormalities in their nucleus and cytoplasm. Deformities in the nucleus such as the acquired Pelger-Huet anomaly of neutrophils, abnormalities of the cytoplasmic granules in the cytoplasm called the Auer rods or by defective granularity in the eosinophils have been demonstrated. Hayhoe and his co-

workers demonstrated the staining patterns of periodic acid schiff's stain (PAS), peroxidase and the Sudan black reaction to classify the leukemic blast cells in lymphoblastic, myeloblastic, monoblastic and erythroblastic cells.[12] This ancillary tool remained the gold standard until immunocytochemistry took over, and still continues be an handy one in diagnosing the disease, and is one among the components in diagnosis apart from immunophenotyping with markers and cytogenetics, thus giving a multifaceted approach to it.

Leukocyte cytochemistry.[2]

This includes techniques used to identify diagnostically useful enzymes or other substances in the cytoplasm of hematopoietic cells. These techniques are particularly useful in the identification of immature blast cells in AML and identification of maturation abnormalities in the myelodysplastic syndromes and myeloproliferative neoplasms. The use of it in characterizing lymphoproliferative neoplasms have been superseded by immunological techniques and therefore should be interpreted with Romanowsky's stains and immunophenotyping. Various techniques have been employed, and recommended by an Expert Panel of the International Council for Standardization of Hematology, which gives the theoretical and practical aspects of cytochemistry.

Principal uses of cytochemistry[2]

1.To characterize the blast cells in acute myeloid leukemia, leading to its diagnosis, unless there is evidence of lymphoid differentiation.

2.To demonstrate the myeloperoxidase or non-specific esterase activity, thus

contributing to a mixed phenotype acute leukemia, according to the 2008 WHO classification of tumours of Hematopoietic and Lymphoid tissues.

3. To identify granulocytic and monocytic components of AML.

4. To identify unusual lineages occasionally involved in clonal myeloid disorders (e.g basophils and mast cells)

5. To detect cytoplasmic abnormalities and enzyme deficiencies in myeloid disorders (e.g myeloperoxidase deficient neutrophils in myelodysplasia or acute leukemia, neutrophil alkaline phosphatase deficient neutrophils in CML.

6.To identify Auer rods in MDS and thus classify a case as refractory anemia with excess of blasts II in the WHO Classification

7. To confirm the diagnosis of Hairy cell leukemia

List of cytochemical stains used

1. Myeloperoxidase (MPO).

2. Sudan Black B (SBB).

3. Neutrophil Alkaline Phosphatase.

4. Acid Phosphatase Reaction including

Tartrate Resistant Acid Phosphatase (TRAP).

5.Periodic Acid-schiff Reaction (PAS).

6. Esterases

Naphthol AS-D Chloroacetate Esterase (CAE)

α – Naphthyl Butyrate Esterase (ANBE)

α - Naphthyl Acetate Esterase (ANAE)

Double Esterase method.

7. Toluidine Blue.

Myeloperoxidase (MPO)

Myeloperoxidase is seen in the primary and secondary granules of neutrophils and their precursors, also in the eosinophils and in the azurophilic granules of monocytes. The MPO in eosinophil granules is cyanide resistant , where as that in the neutrophils and monocytes is sensitive. MPO splits H_2O_2 and in the presence of chromogenic electron donor and forms an insoluble product. DAB (3,3$^{/}$ - diaminobenzidine) is the preferred chromogen used here. The reaction product is stable, insoluble and non-diffusable too. MPO is not inhibited by heparin, oxalate or EDTA anticoagulants. Staining is done in room temperature, which remains satisfactorily for a week. DAB should be in frozen temperature storage, kept at -20^{o}C . Optimum results can be obtained with good DAB buffer mixture and adjustment of counterstaining time with hematoxylin to give good nuclear details.

Results and interpretation

The results of the staining product, show a brown and granular Red cells, and erythroid precursors show diffuse brown cytoplasmic staining. The most early primitive myeloblasts are negative, but granular positive appears as they progress to the promyelocytic stage. Positivity is seen in the Golgi region. Promyelocytes and myelocytes are strongly positive, as the positive primary granules are present in the cytoplasm. Metamyelocytes and neutrophils have lesser secondary granules and are progressively fewer positive. Eosinophils granules stain strongly and the larger granules are easily distinguished from the neutrophil granules, as the peroxidase is distinctly different both biochemically and immunologically in it. Monoblasts and monocytes can be positive or negative, and when seen

positive, they lie scattered in the cytoplasm of the cells. MPO activity is seen basophil granules but not in mature basophils. Individuals with congenital deficiency of neutrophil MPO show negative in the neutrophilic lineage, but eosinophils stain normally. Dysplastic neutrophils may show negative staining pattern. Auer rods stain positive and are seen well with DAB, than with the Romanowsky's stains. 3% of blasts in the stained smears is the cut off point.[7]

Sudan Black B (SBB)

Sudan Black B is a lipohilic dye that binds irreversibly to the granular component in the granulocytes, eosinophils and some monocytes. This gives almost the same information as that of the MPO staining pattern. Here the fixative used is formalin vapour for the smears. Alternative fixative used is formal acetone. The working staining solution is to be replaced after four weeks. Marrow smears which have lipid soluble SBB, can be rinsed for 5 seconds in xylene prior to counterstaining with May-Grunwald Giemsa or Leishman stains. Thereby the cytological detail is seen well with it.

Results and interpretation

The reaction product is black and granular. The results are similar to MPO staining, both in leukemic cells and normal cells. MPO negative neutrophils are also negative for SBB. Eosinophils give a clear core in their granules when stained with SBB, which is not seen in MPO. Rare cases of ALL can show non-granular smudgy positive reactions which is not seen in MPO staining. Basophils show bright red or purple metachromatic granules. More than 3% of positive blasts is the cut off point for the diagnosis.[7]

Neutrophil Alkaline Phosphatase (NAP)

Neutrophils contain Alkaline phosphatise activity. It can also be seen in metamyelocytes. It is demonstrated as a granular product in the cytoplasm, the enzyme activity is associated with intracytoplasmic membranous component, distinct from primary and secondary granules. Some cases of lymphoid malignancies do show positive activity. Bone marrow macrophages also show positivity. Azo dye techniques, using substituted naphthols as the substrate, and its ensuing action in liberating naphthol , to combine with the azo dye, a fast blue, gives an insoluble precipitate at the site of enzyme activity, which is read as positive. Formal methanol fixatives are used for smears, Naphthol AS is the substrate used and Fast blue the azo dye mixed in the working solution. Counterstain used is neutral red. Glass tubes are used for substrate, as plastics can get dissolved with formamide. Blood smears should be done as early as possible within 30 minutes of collection, as EDTA anticoagulant can reduce NAP activity, and should be stained within 6 hours. Control films also are to be used.

Results and interpretation

The positive smears show blue and granular. Intensity is noted from negative to positive in the reaction product of neutrophils, with coarse granules filling the cytoplasm and overlying the nucleus. Score is obtained by assessing the intensity in 100 neutrophils consecutively, with each scored on a scale of 1 to 4.

0 – negative or no granules

1 – occasional granules scattered in cytoplasm

2 – moderate number of granules

3 – numerous granules

4 – heavy positivity with numerous coarse granules in the cytoplasm and overlying the nucleus.

Score ranges from 0 to 400 in 100 assessed neutrophils.Each laboratory has its established scoring done.Normal individuals usually do not have a score of 3 and 4 . New born babies, pregnant women and children have high scores. Perimenopausal women do have a score one third higher than men. Significant low score is seen in CML, which is diagnostic of use, especially in the chronic phase, where it is usually zero. In accelerated phase, and in myeloid transformation, the score rises. Moderate rise is seen in intercurrent infection. Low scores are seen in paroxysmal nocturnal hemoglobinuria (PNH), and hereditary hypophosphatasia. Raised scores are seen in neutrophilia of infection, leukemoid reaction, and Hodgkin's lymphoma. In aplastic anemia, the score is high and can get low once PNH sets in.

Acid phosphatase reaction including Tartrate Resistant Acid Phosphatase Reaction (TRAP)

The main use of this cytochemical stain is in the diagnosis of T- cell ALL and Hairy cell leukemia. The pararosaniline method is commonly employed , and is used for the demonstration of the positivity in the T-cells. Fast Garnet as a coupler in the reaction demonstrates the tartrate resistant acid phosphatase activity. Methanol, acetone and citric acid are used as fixatives, before staining. Naphthol AS-BI phosphate is used as the substrate, dissolved in N,N-dimethylformamide. The coupler agent used is the pararosaniline. The working solution is made up of Michaeli's veronal buffer, with naphthol substrate, and

hexazotized pararosaniline and tartaric acid. Smears are stained by incubating in the working staining solution for 1 hour and counterstained with methyl green or hematoxylin.

Results and interpretation

The Acid phosphatase enzyme present in the cell, will be inhibited by the tartrate, and no colour is seen, where as in hairy cell leukemic cells, they are not inhibited, and a precipitate red colour is seen as positivity in the cytoplasmic granules. They can be diffuse positivity too. Almost all T-cell lineage show strong positivity. In T-cell ALL, the positivity is highly polar. Granulocytes also show positivity, so do macrophages, plasma cells and megakaryocytes in the bone marrow. In hairy cell leukemia, almost all leukemic cells, are positive in the absence or presence of tartrate . Though immunophenotyping methods are more reliable, the TRAP stain method is more useful, when they are not available.

Periodic Acid–Schiff Reaction (PAS)

It is one of the main stains used for identifying Acute lymphoblastic leukemias, the stain indicates the presence of glycoproteins, mucoproteins, and high molecular weight carbohydrates in blood cells. Periodic acid oxidizes the glycol groups to produce aldehydes. When these are exposed to schiff's reagent, which is leuco basic fuchsin, they produce a magenta red colour in the granules or cytoplasm. The reaction occurs positively with glycogen, monosaccharides, polysaccharides, glycoproteins, mucoproteins, phosphorylated sugars, inositol derivatives, and cerebrosides. Glycogen is sensitive to diastase digestion and this distinguishes it from other positively reacting substances. In blood cells the

main source of positive reaction is glycogen. The fixative used is methanol,the periodic acid and schiff's reagent are used for staining, and the counterstain used is hematoxylin. Other fixatives used are formal vapour and formal acetone. Previously fixed, iron stained or Romanowsky's stained films can be overstained with PAS reaction well. Romanowsky's stained smears can be dipped in methanol before the staining process to partly decolourize the smear. The intensity of the colouration depends on the quality of the schiff's reagent. Normal neutrophils stain red, deterioration in the Schiff reagent can be detected by seeing control smears. Sodium metabisulphite can be used, with schiff's reagent, but does not require often.

Results and Interpretation

The stain produces a magenta red colour, with intensity ranging from pink to bright red. Cytoplasmic positivity is either diffuse of granular. The granulocyte precursors show diffuse weak positivity, neutrophils show strong granular positivity. Eosinophils show granular negativity, but diffuse cytoplasmic positivity. Basophils may be negative, but often show block positivity, not related to the granules. Monocytes and their precursors show variable diffuse staining positivity, seen at the periphery of the cytoplasm. Normal erythroid cells and its precursors stain negative. Megakaryocytes and platelets show variable intense diffuse positivity, with the granules being superimposed. Granular positivity with cytoplasmic negativity is seen in 10 to 40% of peripheral lymphocytes. lymphoblasts show block positive cytoplasmic granules, and can be variable too. Block positive lymphoblasts clearly differentiates it from myeloblasts, which are

negative. Cytoplasmic blush is seen in acute promyelocytic leukemia, and in M7 of acute myeloid leukemia, it shows granular positivity in the megakaryocytic type. M6 of AML can also show granular block positive. Abnormal erythroblasts give coarse granular positivity and dysplastic megakaryocytes in MDS can give diffuse cytoplasmic positivity with coarse granules. More than 5% of stained blasts in the smears is the cut off point for positivity, less than it is considered negative.[9,21]

Esterases

Naphthol AS-D Chloroacetate esterase (CAE)

Neutrophils have the specific esterase, and they are isoenzymes in the group band 1,2,7,8,and 9 and stain specifically with CAE. Fixatives used is the buffered formal acetone with Naphthol AS-D chloracetate as the working solution, coupling agent is the hexazotized new fuchsin and sodium nitrate, with the counterstain used being hematoxylin. Fast blue is also used instead of fuchsin, Here incubation of slides is important, as more the time if given, scattered granular staining occurs, hydrolysis is rapid and the staining time is usually over within 3 to 5 minutes.

Results and interpretation

Staining causes a bright red colouration, confined to the cells of the neutrophilic series and the mast cells. Cytoplasmic positivity is seen in promyelocytes amd myelocytes. Hence it is useful in staining smears of Acute promyelocytic leukemia. Auer rods take up the stain positively.

α – Naphthyl Butyrate esterase (ANBE) – Non specific esterase

Bands 3,4,5, and 6, of the isoenzymes of the esterase correspond to this enzyme. Fixative used is the formal acetone, buffer used is the Sorenson's phosphate buffer, substrate is the alpha naphthyl butyrate, coupling agent used is fast garnet and the counterstain employed is hematoxylin. Here the slides are mounted in gum arabic mountant or glycerine, as the reactant product is soluble in immersion oil and synthetic mounting media. Staining is complete when dark brown colour appears, which is checked at incubation period. Pararosaniline is another coupling agent which can be used, and is insoluble , so a synthetic mounting media can be used.

Results and interpretations

The positive reaction is a brown colour seen in majority of monocytes, over 80% of them stain positively. Neutrophils, basophils, eosinophils and platelets are negative. B lymphocytes are negative, where as T lymphocytes stain variably. In the marrow monocyte and its precursors as well as macrophages stain positively. ANBE is more specific for identifying the monocytic component in AML.

α – **Naphthyl Acetate Esterase (ANAE)**

This also is a non specific esterase, the fixative used is buffered formal acetone, substrate is alpha naphthyl acetate, phosphate buffer is used and the coupling agent used is the hexazotized pararosaniline with sodium nitrate,and counterstain being hematoxylin. Fast blue can also be used as a coupling agent. The substrate solution is soluble in mounting media, glycerine hence ,is used for mounting.

Results and interpretation

The staining reaction is diffuse red to brown in colour. Monocytes stain strongly as well as the leukemic cell. Granulocytes stain negative, but in MDS or AML can give positive reactions with variable staining. Megakaryocytes and its leukemic cells stain strong diffuse positive, and also focal too. T lymphocytes and lymphoblasts show focal dot like positivity. Leukemic erythroblasts also show focal positivity.

Combined esterase stain (ANAE and CAE)

The staining protocol is similar as in ANAE , Fast blue is used as the coupling agent here, naphthol AS-D chloroacetate is mixed in the working medium for incubating the slides, and hematoxylin is used as the counterstain.

Results and interpretation

The staining product is a brown colour and the CAE gives a bright blue colour to the granules. This can be used in acute myelomonocytic leukemias, MDS and in AML with dysplastic granules.

Double esterase method

Both ANAE and ANBE substrates are used here, the coupling agent is the Fast blue, phosphate buffer is used, and formal acetone is the fixative for the smears. Timing of staining can be extended upto 30 minutes in order to have maximal staining with ANBE.

Results and interpretation

The CAE stains bright blue in the granulocytes, and the ANBE stains dark green to brown in monocytes. ANBE does not stain megakaryocytes or T cells as much as the CAE stain. It is useful for identifying monocytic and granulocytic cell components.

Toluidine Blue

This stain is useful for basophil and mast cell identification and for its enumeration. It binds strongly to the granules and is helpful in AML as well as CML and other myeloproliferative neoplasms. 1% toluidine blue in methanol is the staining substrate used.

Results and interpretations

Basophil and mast cell granules are stained bright red to purple and are very much distinct and discrete. Nuclei stains blue in colour and cells having abundant RNA can show a bluish tint in the cytoplasm. If staining period is kept for more than 10 minutes, the granules of the promyelocytes also take up the colour. These granules are smaller compared to those of the basophils and mast cells.

Cytochemical reactions in MDS

MDS shows a variety of morphological abnormalities, the marrow is hypercellular, and has peripheral cytopenias can be identified. Perl's reaction identifies ring sideroblasts, other cytochemical evidence of dysplasia are identified by the double staining of cells with ANAE and chloroacetate,

neutrophils show SBB or MPO negativity, but Auer rods can be identified with them. ANAE and ANBE are useful in identifying the monocytic cells in AML, as well as demonstrating Auer rods and dysplasia.

Differences between myeloblast and lymphoblast based on morphology and cytochemistry.[4]

	Lymphoblast	**Myeloblast**
Size	2 to 3times the size of lymphocyte	3 to 5 times the size of lymphocyte
Cytoplasmic characteristics		
Amount	Scanty, lesser than myeloblast	Scanty to moderate, more cytoplasm than lymphoblast
Colour	Blue	Gray
Cytoplasmic granules	Agranular	May contain granules
Auer rod	Negative	Positive
Nuclear characters		
Nuclear chromatin	Uniform, coarse	Uniform, fine
Nucleoli	Inconspicuous or 1 to 2	3 to 5, prominent
N:C Ratio	High	High
Accompanying cells	Lymphocytes	Promyelocytes, myelocytes, metamyelocytes, band forms and neutrophils
Cytochemistry		
Myeloperoxidase	Negative	Positive
Sudan Black B	Negative	Positive
PAS	Block positive	Negative
Non-specific esterase	Negative	Positive in M4 and M5

CYTOCHEMICAL STAINS USEFUL IN TYPING THE BLAST CELLS

STAIN	Precursor B-ALL	Precursor T-ALL	AML M1-M3	AML M4-M5	AML M6-M7
Myeloperoxidase	Negative	Negative	Positive	Positive	Negative
Sudan Black B	Negative	Negative	Positive	Positive	Negative
PAS	Positive (coarse)	Negative	Negative	Positive	Positive (fine)
Non specific esterase	Negative	Negative	Negative	Positive	Positive (focal)
Acid phosphatase	Negative	Positive (focal)	Negative	Positive (diffuse)	Positive (focal)

Immunophenotyping.[1,2,3]

Immunophenotyping have revolutionised the methods of diagnosing leukemias, and today is the gold standard in its diagnosis and management . A multifaceted approach has evolved, including basic cytology, cytochemical study pattern, along with immunophenotyping and cytogenetics in the current setting of therapy and prognosis.[19] The WHO classification emphasizes the importance of immunophenotyping, and defined the antigenic nature of the neoplastic cells in myeloid and lymphoid malignancies.[11] This technique uses the identification of antigens on hematopoietic cells, which are lineage specific and are restricted to particular level of differentiation in it. Detection of cytoplasmic and nuclear antigens, usage of multicolour immunostaining with labelled monoclonal antibodies, quantification of the number of molecules of an antigen as well as analysis of selected population of cells by the estimation of gating methods, are the main technical derivatives of immunophenotyping. Flow cytometry is the technique used for immunophenotyping. A lot of improvements in the instrumentation have been done and Multicolour flow cytometry, with multiparametric immunophenotyping have facilitated accurate identification of normal and abnormal cell populations. This has provided a comprehensive knowledge of the data obtained and interpreted. Assignment of lineage detecting panels, like the lymphoid panel and the myeloid panel of markers is important, and used in most laboratories. There is an abundance of monoclonal and polyclonal antibodies, to assess the myeloid and lymphoid lineage by flow cytometry. The blood and bone marrow samples are commonly employed, and the natural fluid suspension helps a great deal in its assessment. Flow cytometers

use the principle of light scatter and flouresence with flourochromes to define the cellular population, by tagging with antibodies. The cell suspension is passed on the laminar flow, which passes through a laser beam. The scattered light and fluorescent emitted light data are collected after appropriate filtration by the photomultiplier tubes. Here the light scatter information is collected at 90 degree angle, which correlates with cytoplasmic granularity and nuclear complexity. This helps in differentiating between myeloid progenitors, monocytes, and mature granulocytes from lymphoid cells and blasts. The primary requirement here is that the cells should be viable in a suspension prior to staining. Most of the Flow cytometric methods, use a four to six colour analysis, and even three, to detect the side and forward light scatter for routine panels. Following washing and lysis of red cells, direct addition of monoclonal antibodies is done to analyse the cell surface proteins. Assessment of intracytoplasmic and nuclear associated proteins is accomplished after staining for surface markers, and fixing the cells, and adding relevant antibodies to the cell suspension with a membrane permeable agent. Gating is important to have the relevant cell population under study, especially in heterogenous samples like bone marrow, where the studied population is less in overall. Here the cells at the front end selection of the flow cytometry use a CD45antigen and 90 degree light scatter to distinguish the blast cells from the normal cells in the population. The back end has the fluorescent markers. The blast gate helps to identify the cells with specific markers. Care is taken for unusal light scatters, to make sure abnormal cells are not hiding.

Indications for flow cytometry.[3]

Indications include mainly the evaluation of haematological malignancies.

1.Diagnosis and classification of hematopoietic neoplasms.

2. Used for the prognostic significance of the neoplasm.

3. To identify potential therapeutic targets in the haematological neoplasms.

4. To monitor response to therapy, by looking for minimal residual disease.

5. To evaluate the adequacy of blood stem cell collection.

Data analysis and reporting

Analysis of the data received for the equipment uses a sophisticated computer and programme devised to perform compensation, two dimensional histograms for two parameter dot plots or scatter plots, to identify the abnormal cell population and enumerate the various cell populations for study. After the gating strategies, to exclude all non-viable cells to delineate the population for study, the antigenic abnormalities are observed. The histogram plots forward light scattering to side light scattering in a diagrammatic graphic colour representation, which helps in identifying the phenotype, the proportion of abnormal cells to the total viable cells is described in a free text format.

Cell type and antigens detected by monoclonal antibodies

Cell type	**Antigen designation**
T cell	CD1, CD3, CD4, CD5, CD8
B cell	CD10, CD19, CD20, CD21, CD23, CD79a
Monocyte or Macrophage	CD11c, CD13, CD14, CD15, CD33, CD64
Stem cell and progenitor cell	CD34
All leukocytes	CD45 Leukocyte common antigen
NK cell	CD16, CD56

Immunological markers used in the panels for diagnosis.[1,2,3]

AML panels

First line panel used are CD13, CD33, CD117, anti-MPO.

Second line panel used CD41, CD 42, CD62p, anti glycophorin A (CD235a).

Optional markers like CD14, antilysozyme, CD36 and CD64 are used for

Identifying monocytic component.

Phenotypes and markers positive for AML

Myeloblastic	CD11b, CD13, CD15, CD33, CD117,HLA-DR
Myelomonocytic	CD11b,CD13, CD14, CD15, CD32, CD33, HLA-DR
Erythroid	Glycophorin,spectrin, ABH antigens, carbonic anhydrase I, HLA-DR, CD71
Promyelocytic	CD13, CD33
Monocytic	CD11b, CD11c, CD13, CD14, CD33, CD65, HLA-DR
Megakaryoblastic	CD34, CD41,CD42, CD61, anti- von Willebrand factor
Basophilic	CD11b, CD13, CD33, CD123, CD203c
Mast cell	CD13, CD33, CD117

ALL panels

B Cell lineage

First line panels used are CD19, CD22, CD79a, CD10[a], CD20.

Second line panels used are cμ and SmIg.

CD11c, CD25, CD103, CD123, CD38, CD138, cIg are also used.

CD10 and cμ are useful in pediatric cases to identify common –ALL (CALLA),

pro-B-ALL, and pre-B-ALL.

T Cell lineage

First line panels used are CD7, CD2, cCD3, TdT, HLA-DR, CD34.

Second line panels used are CD1a, CD5, CD4, CD8, anti-TCR.

CLL panels

B Cell lineage

First line panels used are SmIg(kappa/lambda), CD19, CD23, FMC7, SmCD79b, SmCD22, CD5[a],CD20[b].

Second line panels used are CD11c, CD25, CD103, CD123, CD38, CD138, cIg.

T Cell lineage

First line panels used are CD2, CD5[a].

Second line panels used are CD3, CD4, CD7, CD8, CD57.

CML panels

Panels used here included CD3, CD10, CD11c,CD13, CD19, CD33, CD41a, and CD45. CD7 expression is seen myeloid and undifferentiated blast crisis, and CD34 is seen in lymphoid blast crisis and myeloid blast crisis. CD203c, gives an accurate assessment of the Basophils and its frequency. CD56 detects NK cells, they are very much reduced in CML. CD45 and CD45RO is expressed in CML. CD45RA is not expressed, and helps to differentiate from AML. The fusion gene identification of BCR-ABL1 by FISH technique, and the identification of the Philadelphia chromosome(22q-), is the hallmark for the diagnosis and constituting therapy.

Clinical features of leukemia.[1,4,5]

AML

Predominantly seen in younger age individuals more than 25 years of age, the general symptoms and signs encountered usually are Pallor, fatigue, weakness, palpitations, and dyspnea. Bleeding tendencies due to thrombocytopenia can also manifest as, easy bruisability, petechiae, epistaxis, gingival bleeding, and bleeding from GI tract, genitourinary tract, pulmonary or CNS bleeding. Pustules and minor skin infections can also occur, major infections of the lung, CNS, and pneumonia, as well as pyelonephritis are uncommon. Post chemotherapy can lead to severe infections due to neutropenia. Anorexia and weight loss can also be present. Splenomegaly and hepatomegaly does occur in a quarter of cases. Lymph node enlargement is also uncommon.Meningeal involvement is common in the monocytic type. Myeloid sarcomas are associated, and seen as an extramedullary mass, in areas of skin, orbit,sinuses, bone,chest wall, breast, spleen, lymph nodes, urinary tract, gastrointestinal tract, respiratory tract, and CNS. Marrow expansion causing bone pain also can be seen. Leukostasis is common as the blasts are larger in size, and expresses adhesive proteins. This can lead to cerebral leukostasis causing headache,confusion and visual disturbances. Pulmonary leukostasis can cause dyspnea at rest. Gingival hypertrophy is common in M4 and M5 cases, as cellular extravasation is seen. Disseminated intravascular coagulation and primary fibrinolysis is commonly seen in M3 cases. Tumour lysis syndrome can occur leading to variety of metabolic derangements.

CML

It is seen more, above the ages of 50 and 60, they have a gradual onset, most of them are diagnosed incidentally, pallor due to anemia and hypermetabolism due to rapid turn over of cells is seen, fatigue, weight loss, anorexia is seen. Massive splenomegaly is common, which is due to extramedullary hematopoiesis, causing a fullness in abdomen. Sternal tenderness, bone pain are also seen. Lymphadenopathy if present, indicates the progression from accelerated phase to blast crisis. Bleeding tendencies are rarely seen, if present can have retinal haemorrhages, causing visual disturbances.Venous thrombosis is rare, leukostasis causing symptoms like dyspnea, drowsiness, confusion, priapism are rare and is seen in accelerated and blast phases. Gouty arthritis due to high turn over of leukemic cells can also be present.

ALL

It is predominantly seen in children between 1 to 5 years of age, and also be seen in adolescent and adults too, also commonly seen in older patients above 65 years of age. Symptoms can present insidiously or can be acute in presentation. Fever is a common symptom, caused by the presence of neutropenia, producing infections, and the leukemic cells producing a variety of cytokines. Fatigue and lethargy are also seen. Anemia and pallor can be seen in older individuals, presenting as dyspnea. Bone pain and arthralgia are common in children, presenting with limping walk, due to the leukemic infiltration into the periosteum, bone and joints, as well as due to the marrow expansion. Some can have marrow necrosis. Less common symptoms include, vomiting, headache,

altered sensorium, oliguria and anuria. Occasionally , hepatosplenomegaly can be seen, especially more in children. Leukemic meningitis can also present. Gingival hypertrophy, coagulopathy are not seen here. Leukostasis is rare. Lymph nodes can be enlarged also. Rare cases of intra cranial bleed have been reported. Leukemic infiltration in skin, causing petechiae, and ecchymosis, are present. Extramedullary involvement in the liver, spleen, lymph nodes, testes, mediastinum, orbit, iris, retina,cornea, conjunctiva, subcutaneous tissues, salivary glands,and cranial nerves are also seen. Priapism due to leukostasis of the corpora can also be a feature. Epidural spinal cord compression is rare. Tonsils, adenoids, appendix, and mesenteric nodes can get infiltrated, especially in children.

CLL

CLL is seen in older age group, above the age of 65. Most of them present as asymptomatic ones, on diagnosis. Mild fatigue, with limitations in daily activity can be seen. Night sweats, fever, weight loss, pallor related to anemia, lymphadenopathy, splenomegaly are also seen. Hypersplenism and thrombocytopenia can be found too.Hepatomegaly, because of liver infiltration is unusual. Advanced disease can result in multiple organ involvement. Pulmonary infiltration is also seen due to infiltration in the interstitium. Chylous pleural effusion can occur. Gastrointestinal mucosal infiltration causing iron deficiency anemia, chronic diarrhoea, and malabsorption can be present. Involvement of the CNS is rare, and can present with meningeal signs as well as cranial palsies. Moreover these patients can have opportunistic infections, in the central nervous system due deficient immune system. Insect bite hypersensitivity is also present

with painful eruptions and erythematous lesions, which is due to skin infiltration of the leukemic cells. Tumour lysis syndrome also can occur.

Laboratory haematological features. [1,3]

AML

Anemia is a common feature and hemoglobin values fall drastically in the course of the disease. Values are seen below 8gm/dl commonly. Reticulocyte count is between 0.5 to 2.0%. Red cell morphology can varied to dimorphic in picture, with poikulocytosis. Nucleated red cells can also be seen. Thrombocytopenia is common and values of platelet count are below 50×10^9/L. Giant platelets and granulated platelets can also been. The total leucocyte count is initially less than 5×10^9/L , and gradually raises to above 50×10^9/L. The absolute neutrophil count is initially lower than 1×10^9/L, gradually becoming normal or more. Neutrophils can be hypo or hypersegmented and hypogranular. Peripheral smears show myeloblasts with mixture of immature blasts, promyelocytes and with Auer rods in the cytoplasm. Faggot cells having bundles of Auer rods can also be present. Marrow shows upto 95% leukemic blasts, more than 20% of blast is the WHO cut off value. Leukemic blasts give positive reaction to MPO, SBB,and Chloroacetate esterase stains. Flow cytometric analysis using the myeloid panel of markers, can be diagnosed as per the criteria. Cytogenetics determine the aneuploidy and pseudoploidy of the cases.

CML

Hemoglobin values are markedly reduced. Red cells show variations in size and shape,with elliptocytes, and a few nucleated cells. The reticulocyte count is normal or slightly raised. The total leucocytic count, is markedly raised, and is mostly seen above 25×10^9/L. All stages of development of the granulocytes are seen in the smears.In the chronic phase, there is mild anemia,the platelet count is usually normal or elevated, can go upto 1000×10^9/L, The serum Lactic acid dehydrogenase levels as well as serum uric acid levels are raised. Marrow shows hypercellularity, with blasts less than 5%, with classical morphology. Accelerated phase shows, increased basophils, more than 20%, peripheral blood blasts show more than 10% upto 19% blast cells, increasing total count, and the platelet count is low, falling below 100×10^9/L, or can be high, going as high as upto 1000×10^9/L. Basophils are more than 20%. In the blast phase, the blasts are more than 20%, extramedullary proliferation of blasts are seen, marrow show aggregates of myeloid blasts, but in 20 to 30% cases lymphoid blasts are also seen, mixed lineage can be found. Cytogenetic analysis to identify the Philadelphia chromosome,by FISH techniques, as well as to identify the BCR-ABL fusion gene is also done.

ALL

Cytomorphology of the blasts can be identified by Romanowsky's stained smears, and the special staining with PAS, gives the block positivity feature in the smears. Anemia is a common feature, with falling Hemoglobin values, less than 8gm/dl. Neutropenia, can be seen, as also thrombocytopenia, in newly

diagnosed cases. Platelet counts, can be less than 50×10^9/L, and in some can be more than 100×10^9/L. The total leucocyte count can vary, as much as from 0.1 to 1500×10^9/L. Hyperleucocytosis can be seen in most of T-cell ALL cases, and values can be more than 100×10^9/L. Marked neutropenia is seen, in almost 40% of cases, and is associated with risk of infections, values can be low and less than 0.5×10^9/L. Pancytopenia, followed by a recovering state, can be seen preceding the diagnosis of ALL. Serum lactic dehydrogenase levels can be raised. An hypereosinophilic picture can also be present in cases preceding the diagnosis. Serum uric acid levels also can be raised due to large leukemic cell burden and purine catabolism. Infiltration of the kidneys, can present with raised creatinine levels too. Disseminated intravascular coagulation, hypercalcemia, and liver dysfunction, can be seen in adolescent age group of cases of B-cell ALL, mainly the t(17;19)(q22;p13.3) with E2A-HLF fusion abnormality. Examination of Cerebro-spinal fluid , can reveal blasts in it, and is commonly seen, with at least 5 blasts per/μL, and reveals Central nervous system involvement. ALL panels identify the gated blasts and are confirmatory in the evaluation of the disease, using flow cytometry. They help in identifying the lineage and the precursors. Cytogenetics determine the ploidy status, hyperdiploidy is mostly seen in children and hypodiploidy in adults, FISH, RT-PCR, and flow cytometric methods are used. Micro-array based genomic analysis of gene expression, identifies specific genetic alterations.

CLL

Smears show the typical blast picture with smudge cells in Romanowsky's stains. Anemia is a feature, with fall in haemoglobin values, the red cells are normocytic and normochromic, platelets can be normal or can have an associated thrombocytopenic picture. Lymphocytosis is sustained, and the values can be more than 5×10^9/L. Atypical immature lymphocytes are seen as more than 55% can be present. Marrow can show more than 30% of the lymphoid cells, decrease in red cell precursors and megakaryocytes. Panel of CLL markers in flow cytometry, establishes the lineage. Karyotypic analysis, using FISH, evaluates the abnormalities in the study, and evolution of their clonal extent. Cases with macrocytic anemia, should be evaluated for Coomb's test, haptoglobin test, and reticulocytic count, to rule out auto-immune haemolytic anemia. Hypogammaglobinemia is a feature, as there is reduced immunoglobulins. paraproteinemia can also be seen, identified by light chain electrophoresis. Heavy chain disease, can also result in cases of CLL.

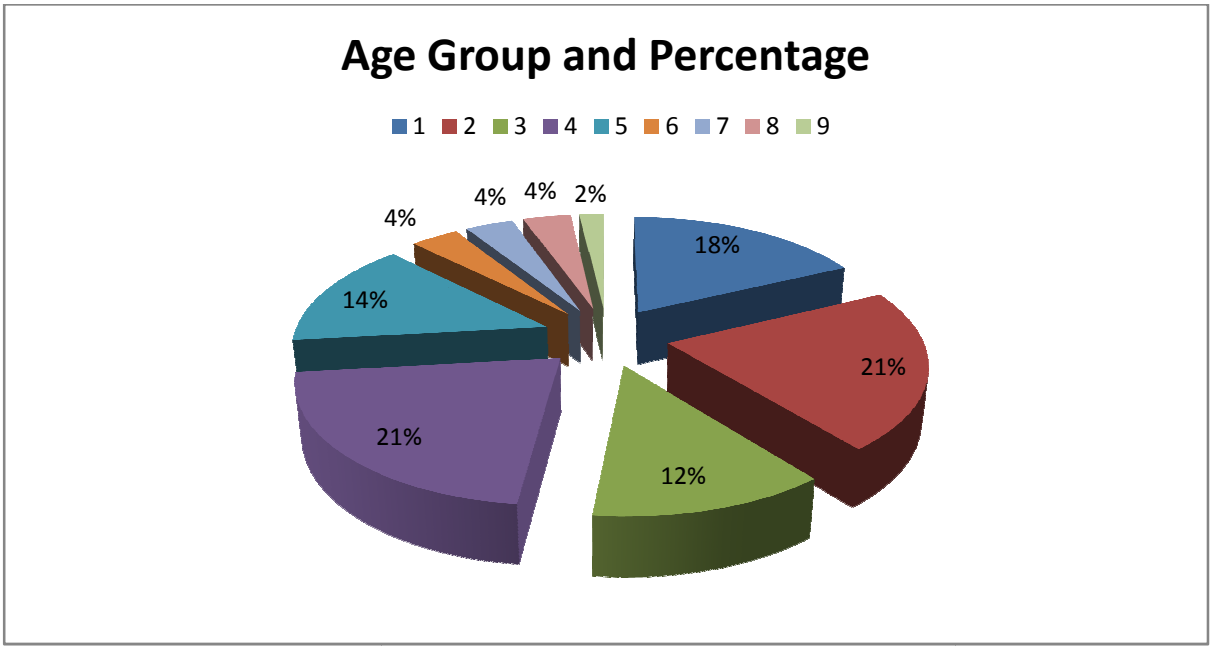
OBSERVATION AND RESULTS

The present study comprised of 56 cases of suspected Leukemia, taken from the department of pathology, Thanjavur Medical college, between January 2015 and June 2016. The observations and the results deduced were as follows.

Table 1

Age Distribution of patients

AGE IN YEARS	No. of Cases	%
≤ 10	10	18
11-20.	12	21
21-30	7	12
31-40	12	21
41-50	8	14
51-60	2	4
61-70	2	4
71-80	2	4
> 81	1	2
TOTAL	**56**	



The cases were in the range of 1 to 93 years. Table1, shows the age distribution, with the majority of the percentage lying in the age group 11 to 20 years, and 31 to 40 years, each having 12 cases in the category, which was found to be 21%. This was followed by the age group less than 10 years, with 10 cases, which was 18%, and 41 to 50 years group with 8 cases having 14%, closely followed by the 21 to 30 years group,with 7 cases, amounting to 12%.

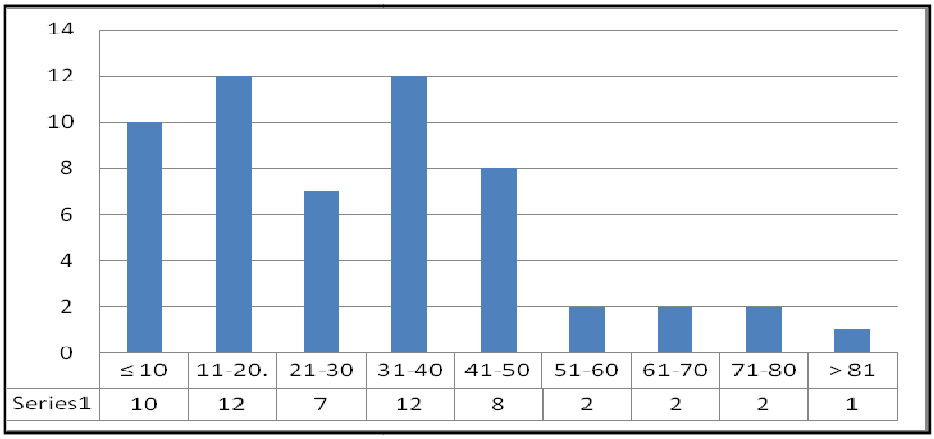


Table 2

Age Distribution of leukemia cases

Age in Years	NO. OF PATIENTS							
	ALL		AML		CML		CLL	
	Nos.	%	Nos.	%	Nos.	%	Nos.	%
1-20.	11	92%	10	42%	0	0%	0	0
21-40	1	8%	8	33%	10	62%	1	34%
41-60	0	0%	5	21%	4	25%	1	33%
61-80	0	0%	0	0%	2	13%	1	33%
>80	0	0%	1	4%	0	0%	0	0%

Table 2 shows, the age distribution for the diagnosed cases, where ALL is common in the age group of 1 to 20, with a percentage of 92. Followed by AML, in the same age group with 42%, and as much as 33% in the 21 to 40 age group. CML, was common in the age group 21 to 40, with 62%, and 25% presenting in the 41 to 60 age group. CLL, had an equal percentage of presentation in both 41 to 60 and 61 to 80 groups, and had almost 34%, in the 21 to 40 group.

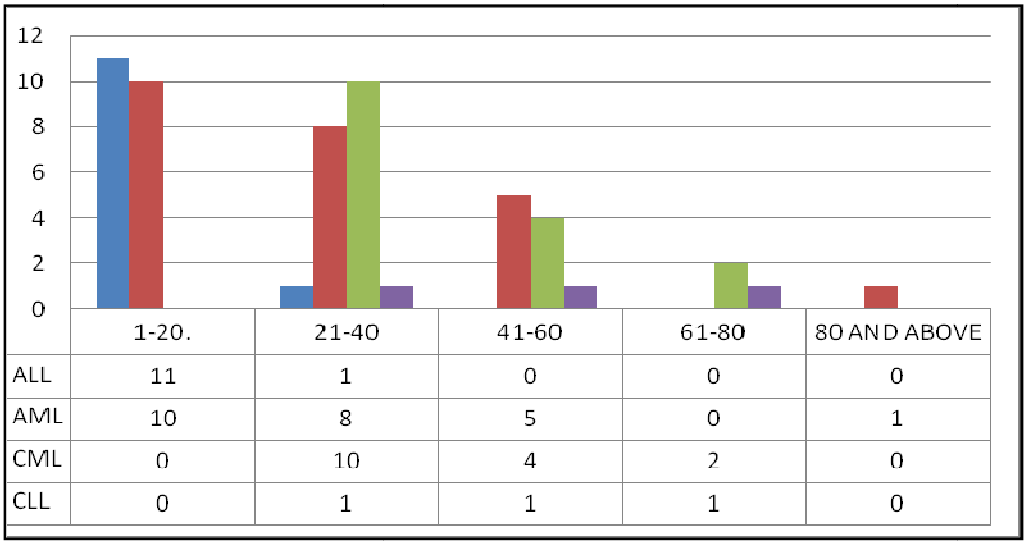
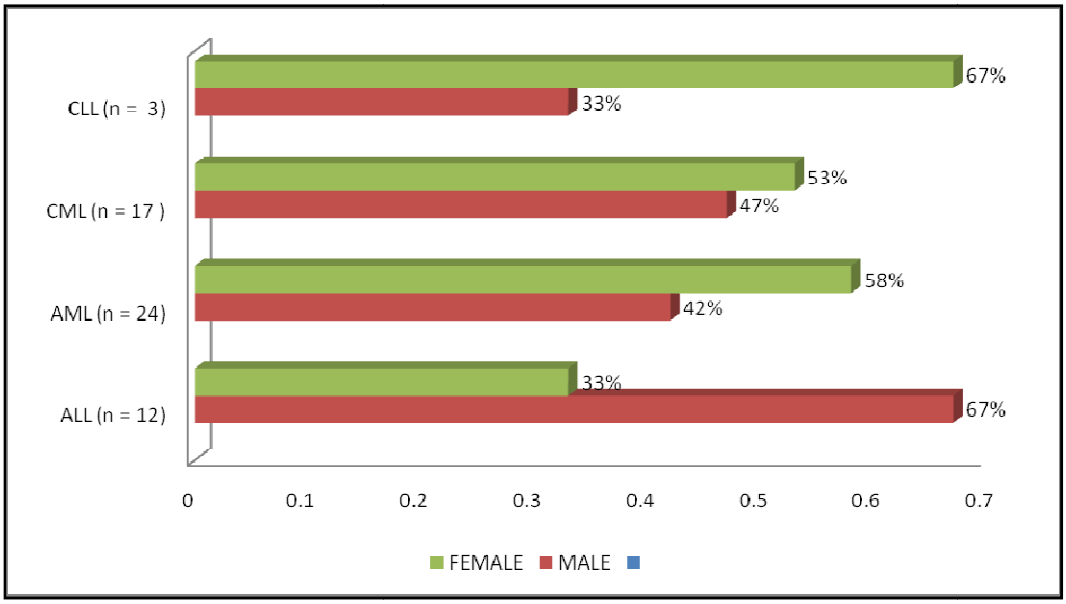


Table 3

Sex Distribution of Cases

SEX	ALL		AML		CML		CLL	
	Nos.	**%**	**Nos.**	**%**	**Nos.**	**%**	**Nos.**	**%**
MALE	8	67%	10	42%	8	47%	1	33%
FEMALE	4	33%	14	58%	9	53%	2	67%



TOTAL MALE CASES – 27 – 48%

TOTAL FEMALE CASES – 29 – 52%

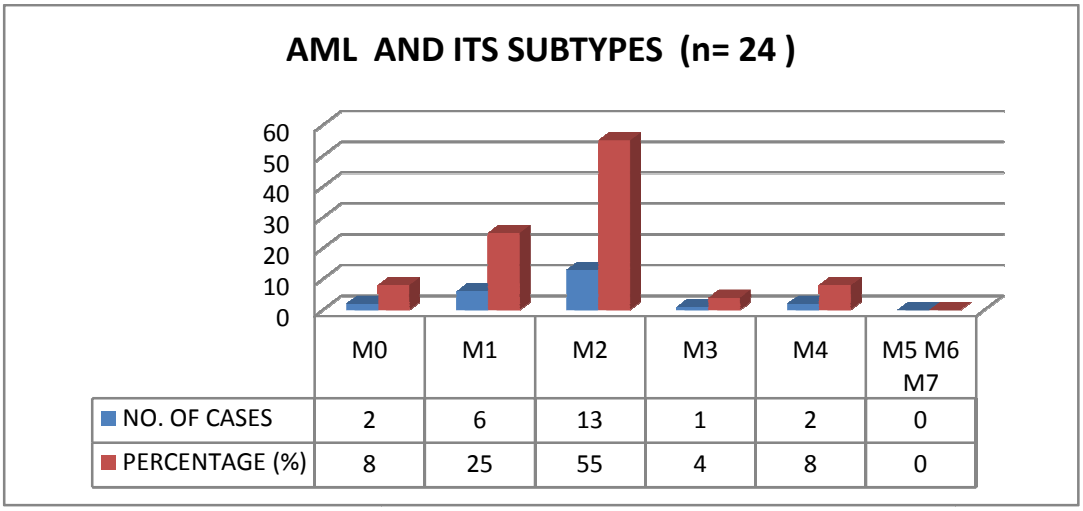
The total Male cases in AML, was 10 in number, and Females were 14, accounting to 42% and 58% respectively. The total Male cases in ALL, were 8, and females were 4, with a 67% and 33% respectively.

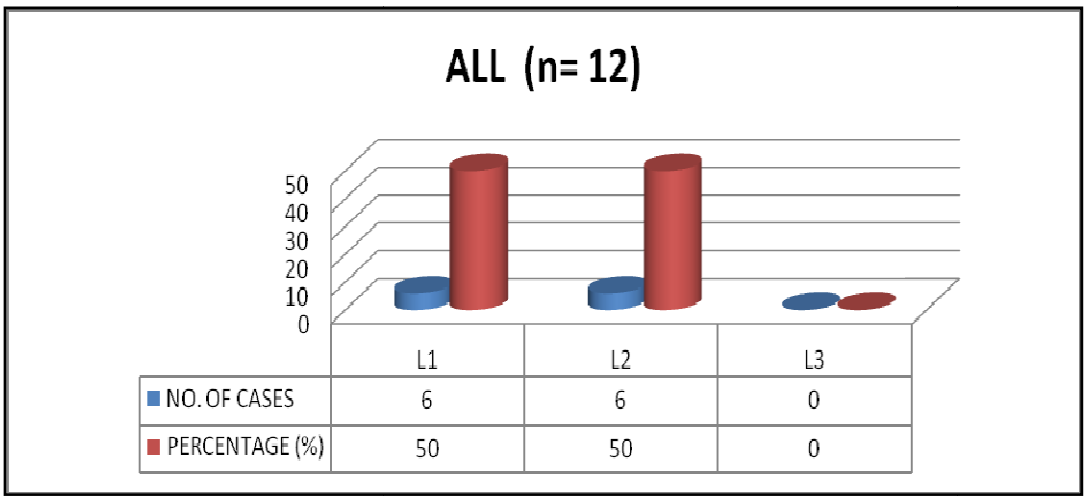
Table 4

Distribution of patients according to types and sub types of leukemia

TYPES AND SUBTYPES OF LEUKEMIA	NO. OF CASES AND PERCENTAGE (%)	
AML (n=24)	**NO. OF CASES**	**PERCENTAGE (%)**
M0	2	8
M1	6	25
M2	13	55
M3	1	4
M4	2	8
M5 M6 M7	0	0
ALL (n= 12)		
L1	6	50
L2	6	50
L3	0	0
CML (n=17)		
Chronic phase	9	52
Accelerated phase	3	18
Blast crisis	3	18
Chronic Neutrophilic leukemia(CNL)	1	6
Juvenile CML	1	6
CLL (n=3)	3	100

This table depicts the distributive nature of the different types and subtypes of leukemia. Of the total 56 cases, AML, with 24 cases(43%), was the highest, followed by CML with 17 cases(30%), ALL with 12 cases(22%) and CLL with 3 cases(5%), was the least. In AML, 13 cases were of M2(55%), followed by M1, which had 6 cases(25%). In ALL, 6 cases were seen, each in L1 and L2, 50% equally distributed. CML, had 9 cases(52%) in the chronic phase, which was highest, with 3 each in accelerated phase(18%) and blast crisis(18%). One case of Juvenile CML, and another case of CNL, was also seen.


AML AND ITS SUBTYPES (n= 24)
60
50
40
30
20
10
0
M0
M1
M2
M3
M4
M5 M6 M7
NO. OF CASES
2
6
13
1
2
0
PERCENTAGE (%)
8
25
55
4
8
0


ALL (n= 12)
50
40
30
20
10
0
L1
L2
L3
NO. OF CASES
6
6
0
PERCENTAGE (%)
50
50
0

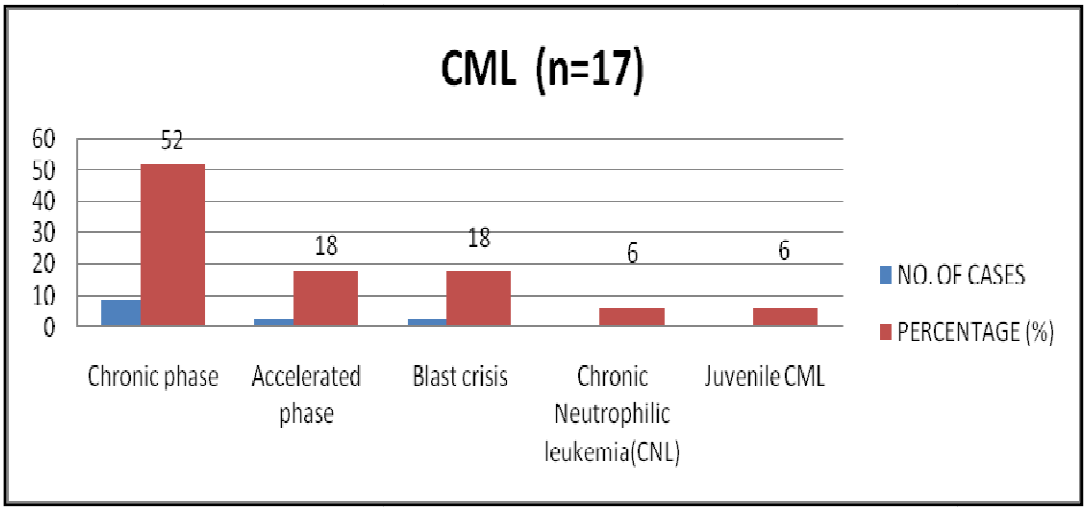
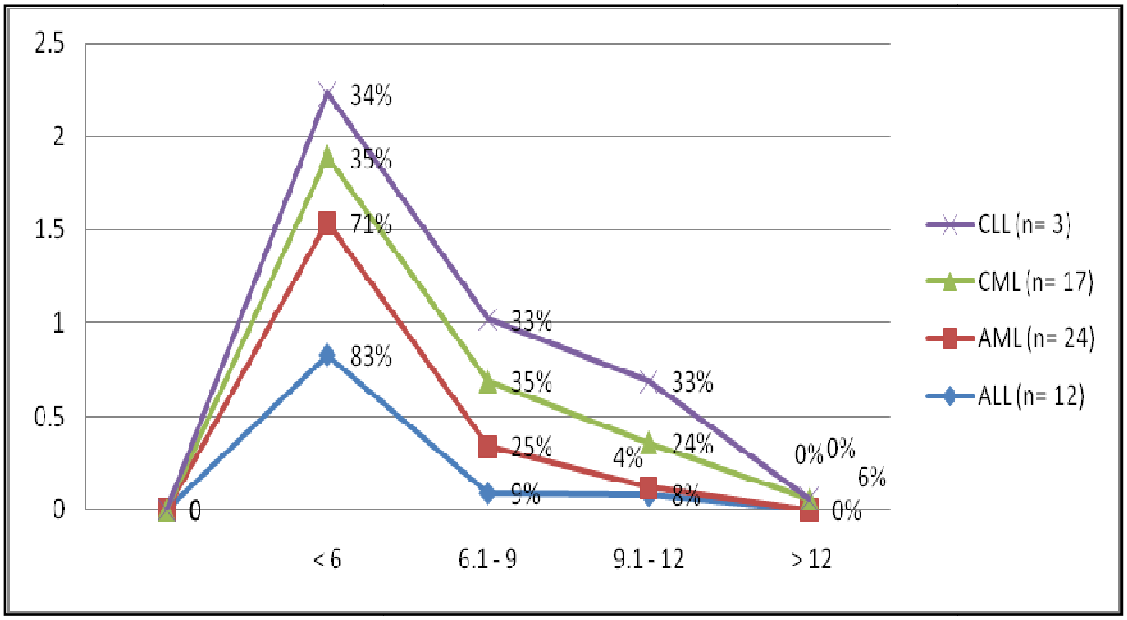

CML (n=17)
60
50
40
30
20
10
0
52
18
18
6
6
Chronic phase
Accelerated phase
Blast crisis
Chronic Neutrophilic leukemia(CNL)
Juvenile CML
NO. OF CASES
PERCENTAGE (%)

Table 5

Hemoglobin distribution among the cases

Hb in Gram %	ALL		AML		CML		CLL	
	Nos.	%	Nos.	%	Nos.	%	Nos.	%
< 6	10	83%	17	71%	6	35%	1	34%
6.1 - 9	1	9%	6	25%	6	35%	1	33%
9.1 - 12	1	8%	1	4%	4	24%	1	33%
12 and above	0	0%	0	0%	1	6%	0	0%

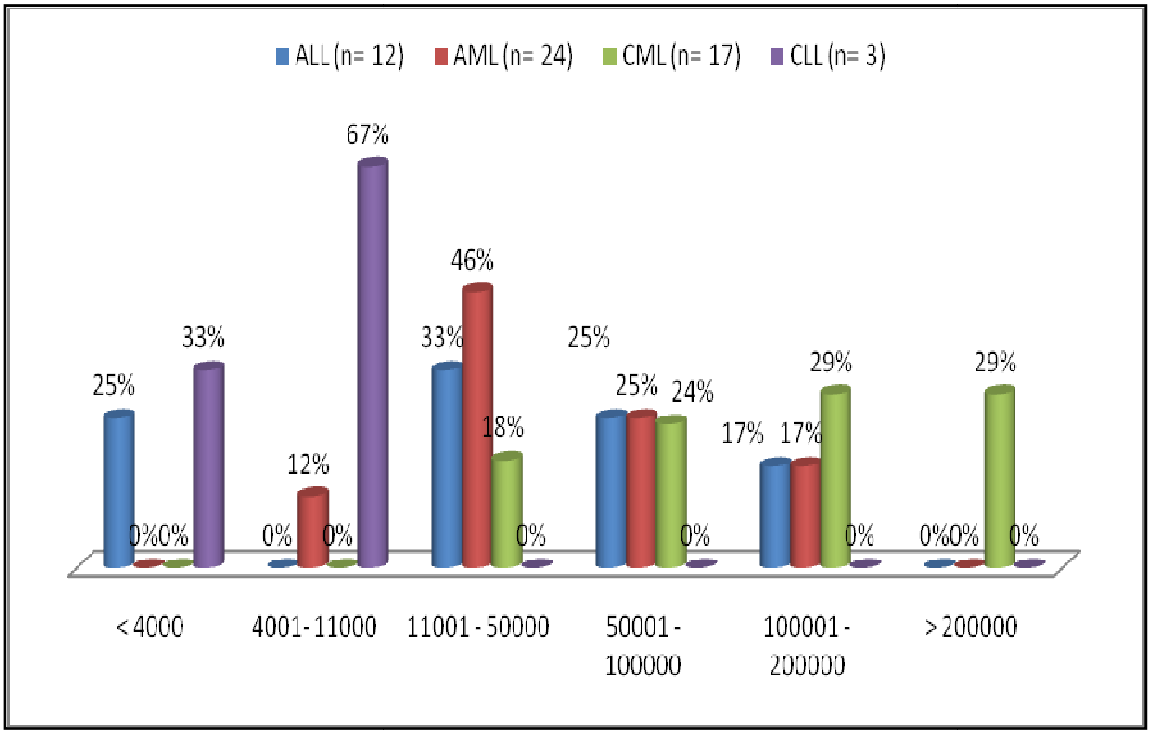


Haemoglobin was seen to be less, and anemia more prevalent in acute leukemias than the chronic ones . ALL had as high as 83% cases with below 6gm/dl values, followed by AML which had 71% cases . CML and CLL had 35% and 34% respectively.

Table 6

Total leukocyte count in cases

TLC	ALL (n= 12)		AML (n= 24)		CML (n= 17)		CLL (n= 3)	
	Nos.	%	Nos.	%	Nos.	%	Nos.	%
< 4000	3	25%	0	0%	0	0%	1	33%
4001- 11000	0	0%	3	12%	0	0%	2	67%
11001 - 50000	4	33%	11	46%	3	18%	0	0%
50001 - 100000	3	25%	6	25%	4	24%	0	0%
100001 - 200000	2	17%	4	17%	5	29%	0	0%
> 200000	0	0%	0	0%	5	29%	0	0%



The total leukocyte count showed higher values of upto 50,000/µl, in 46% of AML cases, and upto 100,000/µl in 25% of them. ALL showed 33% and 25% in the same value range. Both AML and ALL, had 33% cases with a total count value upto 200,000/µl. CML, had 29% cases of total count values, upto and with more than 200,000/µl. CLL, by and large had a normal range of total count value between 4000 to 11,000/µl in 67% of the cases. 33% of CLL, and 25% of ALL, had a total count value less than 4000/µl.

Table 7

Platelet count in cases

Platelet Count / cu mm	**ALL (n= 12)**		**AML (n= 24)**		**CML (n= 17)**		**CLL (n= 3)**	
	Nos.	**%**	**Nos.**	**%**	**Nos.**	**%**	**Nos.**	**%**
< 50000	9	75%	17	71%	4	24%	0	0%
50000 - 1 lac	1	8%	2	8%	0	0%	1	34%
1 Lac - 1.5 Lacs	0	0%	1	4%	0	0%	1	33%
> 1.5 Lacs	2	17%	4	17%	13	76%	1	33%

Platelet counts were seen less than 50,000/cu.mm, in 75% of AML, and in 71% of ALL cases. CML, had 76% cases, with more than 100,000/cu.mm, where as 24% had less than 50,000/cu.mm. CLL had 34% cases, upto the value of 100.000/cu.mm, and more than 150,000/cu.mm in 33% cases.

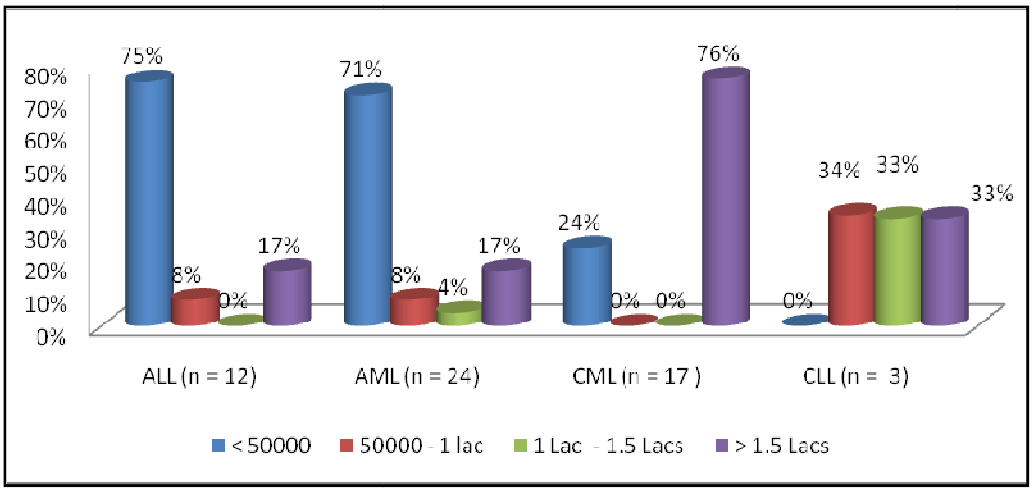


Table 8

Different Hematological parameters according to the types of leukemia

Parameters	**AML (n = 24)**	**ALL (n = 12)**	**CML (n = 17)**	**CLL (n = 3)**
Mean SD : Hb (gm/dl)	5.533 ± 2.77	4.975 ± 1.24	6.724 ± 1.68	8.601 ± 2.150
Mean SD : TLC (per µl)	56166.667 ± 52432.25	72343.166 ± 70265.325	106258.824 ± 82563.265	31900 ± 28564.325
Mean SD : Platelet Count (/cmm)	55625.012 ± 58268.635	68916 .011 ± 34865.854	226647.059 ± 123562	162666. 66 ± 143652.21

Table 5, provides the different statistics for the haematological parameters according the types of leukemia. The maximum mean haemoglobin level was observed in CLL, followed by CML, AML and ALL, the least. The mean Total count, was maximum in CML, followed by ALL, AML and CLL. The mean platelet count ,was high in CML, followed by CLL, ALL, and least in AML.

Using One way Anova , by SPSS, the significance analysis of the differences in the group means, resulted into a *p* value of < 0.0001, and indicated , it was highly significant across the leukemia types.

Table 9

Comparison of clinical features with reference to haematological values

Signs	**Hb (gm/dl)**	**TLC (/ul)**	**Platelet Count (Lacs /cmm)**
Lymphadenopathy (n = 7)	6.36 ± 0.43	69246.799 ± 33672.368	130200.565 ± 8349.952
Hepatomegaly (n = 16)	6.03 ± 1.23	63226.735 ± 30276.269	108775.755 ± 51687.363
Splenomegaly (n = 21)	4.88 ± 2.14	47874.394 ± 2163.351	103181.282 ± 32269.779
p -Value	**0.52**	**0.54**	**0.51**

With regards to haemoglobin, the highest mean levels were seen with cases having lymphadenopathy, and the lowest in cases with splenomegaly. The difference in mean Hb levels by one way Anova, resulted in a *p* value of 0.52. In the case of total count, the highest value was observed in patients with splenomegaly, and the least was seen in cases with hepatomegaly, the *p* value obtained, was 0.54. And with platelet count, the highest mean values obtained were in patients with lymphadenopathy, followed by hepatomegaly and the least in splenomegaly. The *p* value obtained here was 0.51.There was no statistically significant differences, between the group means , and the clinical features, as determined by one way ANOVA.

Immunophenotyping Results

6 cases underwent flow cytometry,using peripheral blood samples, with 2 cases using regular AML panel and 3 cases with ALL panel, and with one CML case with its panel, for cases suspected and doubtful. Though they showed cytochemical stain positive, the results were obtained , with the plots on the side scatter graph. 4 cases, were concordant with the cytochemical stained smear diagnosis. But 2 cases of ALL, were partially concordant, and were requiring bone marrow aspirate samples for flow cytometry. Flow concordance percentage was 67%, and the remaining 33% was not in concordance, for peripheral blood sample.

1) AML M2 showed CD 13, CD,33,CD,117 positivity

2) AML M1 showed CD13, CD33 positivity

3) ALL L1 showed CALLA positivity with CD10 gated blasts.

4) ALL L2 showed mild blasts, but required a bone marrow aspirate analysis.

5) CML showed CD117, CD13,CD33,CD34 positivity in the gated blasts.

6) ALL L1 showed lymphocytosis, required a bone marrow aspirate analysis.

DISCUSSION

Leukemias, as such are now common, and affects all ages and genders. It requires a multi parameter approach for its diagnosis, which includes cytomorphology study, with phenotypic and genotypic studies. In the present study 56 cases, were evaluated, by studying their morphology using cytochemical stains, as well as clinical features, and with 6 cases for concordant Immunophenotyping, using lineage specific panels with markers. Of the 56 cases, 36 cases were acute leukemias(64%), of which 24 were AML(43%), and 12 were ALL(22%). The rest 20 cases were chronic cases(36%), of which 17 were CML(30%),which include one case of Chronic neutrophilic leukemia, and 3 were CLL cases(5%). This clearly shows Acute leukemias, being more common than chronic ones. In the acute group, AML was more common than ALL, and in the chronic group CML was commoner than CLL. After subtyping of acute leukemias with cytochemistry, the study showed in AML, 2 cases of M0(8%), 6 cases of M1(25%), 13 cases of M2(55%), 1 case of M3(4%), 2 cases of M4(8%). No cases of M5,M6 and M7 were detected. This showed that AML M2 was the commonest, in the FAB classification, and was supported by studies too, e.g Kulshreshtha et al.[6] and also by other comparative studies. Comparing with other studies, population bias could be the reason, why in this study, AML showed higher predominance than ALL or CML.

Comparing the frequency of various leukemias in India (in percentage).[7]

Reference	Region (PERIOD OF STUDY)	No. of cases	ALL	AML	CML	CLL
Chatterjee et al	Calcutta (1949–1961)	544	22.5	32.5	35.9	5.9
Advani et al	Mumbai (1960–1975)	1126	30	13	40	9
Prakash et al	Pondicherry (1970–1979)	278	35	29.5	30.8	3.2
Rani et al	Delhi (1970–1979)	490	15.5	30.8	45.3	5.7
Verghese et al	Kerala (1980–1983)	1016	39.2	19.6	16.4	1.9
Kushawaha et al	Lucknow (1971–1984)	970	9.3	38.7	48	2.6
Shome et al	Chandigarh (1975–1983)	820	24	29.3	36.7	8.8
Dicosta et al	Mumbai (1975–1984)	242	36	22	38	2
Rathee et al	Haryana (2008-2012)	650	17.2	33.8	39	10
Present study	Thanjavur Tamil Nadu (2015-2016)	56	22	43	30	5

The above comparison , clearly shows a higher freguency of percentage in AML cases, in the present study, correlating with All India statistics(20 to

25%).[17] However, CML cases were found to be second commonest, this could be due to population bias, and its limitations in the study.

Comparison of AML subtypes with previous studies in the distribution.[7]

FAB type	Sultanet al (250) 1981	Miguel et al (120) 1986	Chessells et al (112) 1986	Alvi et al (26) 1990	Chaudhry et al (54) 1993	Khalid Hassan (81) 1994	Present Study
M0							8
M1	21	13	10	15.3	13	18.5	25
M2	32	14	26	35	44.4	23.9	54
M3	16	14	6	15.3	11.1	11.1	5
M4	16	22	22	19.2	24	29.6	8
M5	12	21	24	11.5	3.7	11.1	0
M6	3	7	9	3.7	3.7	1.2	0
M7	0	0	0	0	0	1.2	0

The subclassification of AML M2 in FAB subtypes of AML was the predominant type, and was found to be correlating with the various above studies, except in Khalid Hassan 1994 study, where M4 was found to be higher. Two special stains were used in making the diagnosis, SBB and PAS, and on staining, myeloblasts of AML and CML showed coarse brown black granules, with SBB stain, with more than 3% of blasts. PAS was negative in AML. The lymphoblasts of ALL and CLL showed block positive with PAS stain, where as the myeloblasts of AML and CML were negative.

Distribution of acute cases of leukemia as per cytochemistry.

Out of 56 cases in total, 36 cases were acute, AML was 24,which was 67%, and ALL was 12, at 33%, for the cytochemical staining with both SBB, and PAS.

cytochemistry	AML	ALL
SBB+, PAS-	**24 (67%)**	**0**
SBB - , PAS +	**0**	**12(33%)**
TOTAL	**24**	**12**

Gender distribution showed a female preponderance of leukemias as a whole in the study of the 56 cases. Males were of 48% and females 52%. This again reflects the nature of swing in the population, though generally it is commoner in males. AML had a higher female preponderance of 58%, against 42% in males, and it showed the same pattern in CML and CLL too, where as ALL had more males showing 67% and females 33%. But studies show major differences in incidences of AML in males when compared to females,[17] as seen here, where males are 10 and females 14 in number.

Age distribution clearly shows ALL, predominantly in the children and early adolescence group, and ranges to about 92% of the cases, followed by AML with 42%. CML was seen more common, in the 21 to 40 age group, and CLL had 33% in the above 40 age group.

Comparison with other studies.[7]

Reference	**Study**
Neglia, jp, and Robison.10 1988	In children, acute lymphoblastic leukemia (80%) is more common than acute myeloid leukemia
Ribera JM, Oriol A.11 2009	Acute lymphoblastic leukemia (ALL) is the most frequently diagnosed malignancy in children,representing nearly one third of all pediatric cancers
Present study	Acute Lymphoblastic Leukemia is more common in age group of 0 to 20 years (92).

In acute myeloid leukemia , an adult predominance was found ,and it correlated with previous studies too.[7]

Table showing the comparative study.[7]

Reference	Study
Boros, L. and Bennett, J. M (1984)	AML occurs twice as often as ALL, the vast majority of cases occurring in adults
Khalid Hassan Nadeem Ikram, Sajid Hussain Shah13 (1994)	AML was observed more commonly in adults (79%) as compared to children (21%), whereas ALL was commoner in children (72%) as Compared to in 28% amongst adults.
Greer John P, Baer Maria. R, Kinney Marsha. C (2009)	AML accounts for less than 15% of cases of leukemia in children below 10years, 25-30% between 10-15 years, and in adults, it accounts for 80-90% of cases of acute leukemias.
Present study	Acute Myeloblastic Leukemias has an adult predominance (54%)

Laboratory haematological values

With correlation of laboratory values, Hemoglobin values, were less than 6gm% in 83% of ALL cases, and 71% in AML cases. 35% of CML cases, and 34% of CLL cases, had less than 6gm%, this clearly indicates,severe anemia as a co existing disorder, in cases of acute leukemia.[1,7] The main cause being inadequate production of red cells and shortened life span.[1] Abnormalities of size and shape can also occur.

Total count values, were seen in high ranges in CML, with more than 100,000/µl, in 58% of cases, and as much as 29% of them had more than 200,000/µl, followed by AML, which had 46% of cases upto 50,000/µl, and 25% more than that. ALL had 33% upto 50,000/µl, and 25% in more than that. CLL had 67% of cases upto 11,000/µl, and 33% less than 4000/µl. This signifies that elevated counts will have less mature cells, and more of the

immature variety. Leucopenia is an uncommon feature.[1,2]

Platelet counts in general are lower in Acute leukemias, and normal to higher or rarely lower in Chronic leukemias. This was interpreted in the study, and ALL had 75% cases with lesser values below 50,000/cu.mm, and AML had 71% cases, below that level. CML had normal to higher values of more than 150,000/cu.mm in 76% of their cases.CLL had more than 66% of their cases with more than 100,000/cu.mm. This signifies that acute leukemias, end up with less production of platelets,with decreased survival, causing thrombocytopenia.[1] CML, usally has normal ranges in the chronic phase, but in the blast phase, they fall below the normal range just like in the acute cases. A thrombocytopenic picture in CML, always signals that an accelerated phase has started its process, leading to blast crisis.[1]

Anova analysis showed the *p* value to be statistically significant across the group of means of the laboratory values, in the haematological parameters. A clear picture of the haematological values in assessing the Total count, platelet count and haemoglobin is essential in the diagnostic aid of leukemias.

Clinical parameters

Various clinical presentations are seen in leukemias, assessment of splenomegaly, hepatomegaly and lymphadenopathy, were taken into account in this study. Splenomegaly was the commonest feature seen in 21 cases, followed by hepatomegaly in 16 cases, and 7 cases had lymphadenopathy. One particular feature seen here was a lesser platelet count , a lower haemoglobin value, and a total count less than 50.000/µl, in cases which had splenomegaly. There was no statistical significant differences, between the haematological value group

means and the clinical features, as obtained by one way Anova. The varying nature of the clinical features, were seen. CML invariably had splenomegaly in all their cases, and hepatomegaly was mixedly seen in AML , as well as CML and ALL. CLL cases, all had lymphadenopathy. One case of AML M3 had disseminated intravascular coagulopathy, and one other case of CML in chronic phase, had visual disturbances.

Immunophenotyping

It is important to note that, in this study, the resources were limited, and cost effective measures had to be undertaken for the diagnosis. In a country like ours, where sophisticated laboratories having flow cytometric analysis for immunophenotyping, are limited in number, are a costly affair.[7] Hence, here a select few cases, were taken to undergo Flow cytometric analysis. Six cases were taken into account. The main purpose was to see their concordance, with cytochemistry. 4 cases were concordant with the flow cytometric analysis, one ALL, one CML, and two AML cases were found to be concordant, with cytochemical findings, the other two ALL cases required a bone marrow aspirate, and secondary markers for the lineage identity,which as per our study was out of our limitations. 67% were concordant, and effective in diagnosing the cases in our study.This correlates with the study by Belurkar S et.al 2013, which showed 58% concordance with flow analysis.[11] Flow analysis uses a viable single cell suspension for the method, this consideration is important to note that hemodiluting effects can alter the analytical process, and can result in differences in results. Quality control and maintenance of equipment is highly

essential, which should be scrupulously followed.[1] By and large, a practical method, in our setting would be to do a basic peripheral blood smear with routine stains, and with cytochemistry and morphology, a diagnosis can be made. In case of ambiguity or lineage specific identification, for the follow up of therapy and prognosis, flow cytometric analysis and cytogenetics would be of immense help.

CONCLUSION

Leukemias are a heterogenous group of disorders, having varied clinical, morphological, molecular cytogenetic characteristics, and has prognostic and therapeutic implications on identifying them correctly.

In our study, predominace of myeloid neoplasms was found more than that of the lymphoid ones, and AML being the commonest type noted, as per the statistical data, arrived at.

In the age group category, ALL was seen more commonly in the childhood category, and AML was seen more common in the adult age group,closely followed by CML.

The gender distribution showed a slight raise in female preponderance as a whole, but Male predominance was seen in ALL type, with AML and CML having a slight female preponderance.

Anemia, as determined by Hemoglobin values, was seen more severe in Acute leukemias, especially in ALL and AML, where as it was mild to moderate in the chronic types.

Leucocytosis, was seen more in CML, and AML, as well as in ALL, than in CLL, depicting the clonal proliferation of cells.

Thrombocytopenia was seen more in Acute leukemias, than in the chronic types, affirming the criteria for evaluation.

Clinical features like splenomegaly were commonly present in CML, and hepatomegaly was evenly distributed, and lymphadenopathy in a few cases, was seen mostly in CLL.

Cytochemical stains used were profoundly positive, in almost all cases, except in one case of Chronic neutrophilic leukemia. Acute leukemias were well studied, with the stains, SBB and PAS, and accurately classified as per FAB classification.

With resource limitations and considering the fact, that at present, Flow cytometry is either not available, or beyond the capacity of poor patients in our country, this study was carried out, with the aim that cytochemistry would be a cost effective method, in diagnosing leukemias.

Flow cytometry was done in a select few cases, to see the concordance with cytochemistry, and it did show good concordance rate as seen in our study.

A leukemia diagnosis protocol, is need of the hour, considering the economics Involved. So a simple and effective means is to be in place for routine morphology, cytochemistry , so as to give a correct diagnosis, and to make it easily performable, in centres devoid of flow cytometric analysis. This could be an alternative,and an ancilliary tool, till flow cytometry and cytogenetics are made available.

Current therapeutics and prognostic factors, rely more on flow cytometry and cytogenetics, and the WHO has classified leukemias on its basis. This has prompted a new beginning in identification of the types and morphology. Hence a simpler adaptive format, with cost effective means can be devised in countries, where there are least or no facilities, and access to them is made available.

MASTER CHART

S.NO	AGE IN YRS	SEX	DIAGNOSIS	SBB	PAS	IMMUNO PHENOTYPING	Hb% GMS	TOTAL LEUCOCYTIC COUNT	PLATELET COUNT	SPLENOMEGALY	HEPATOMEGALY	LYMPH ADENOPATHY	OTHERS
1	16	F	AML M2	+VE	-VE		4	5000	1	-	-	-	-
2	10	F	AML M1	+VE	-VE		7	33,000	1.2	-	-	-	-
3	25	M	AML M2	+VE	-VE		8.6	41,000	1.9	-	-	-	-
4	38	M	CML chronic	+VE	-VE		11.7	1,20,000	2.02	+	-	-	-
5	2	M	ALL L2	-VE	+VE		3	1,12,000	2	-	+	+	-
6	80	M	CML chronic	+VE	-VE		7.4	92,900	3.41	+	-	-	-
7	14	F	ALL L1	-VE	+VE		2	3,300	2.46	-	+	-	-
8	6	M	AML M2	+VE	-VE		3.4	33,900	0.65	-	-	-	-
9	1	M	ALL L2	-VE	+VE		9.2	98,000	0.38	-	+	-	-
10	32	M	AML M2	+VE	-VE		7.5	40,000	0.46	-	-	-	-
11	9	F	CML juvenile	+VE	-VE		7	53,000	1.7	+	-	-	-
12	30	M	AML M2	+VE	-VE		3	1,40,300	0.24	-	+	-	-
13	12	M	ALL L1	-VE	+VE		6	3,300	0.28	-	-	+	-
14	60	F	AML M1	+VE	-VE		3.8	78,000	0.5	-	+	-	-
15	36	F	AML M2	+VE	-VE	CD13,CD33 +	6.2	66,000	1	-	-	-	-
16	28	F	CML acc phase	+VE	-VE		9.1	1,54,000	6.61	+	-	-	-
17	30	F	ALL L2	-VE	+VE		4.9	40,200	1	+	-	-	-
18	15	M	ALL L2	-VE	+VE		5.6	1,78,000	0.24	+	+	-	-
19	14	M	AML M1	+VE	-VE		11.5	1,46,000	0.13	-	-	-	-
20	47	M	CML chronic	+VE	-VE		4.8	11,300	0.26	+	-	-	-

MASTER CHART													
S.NO	AGE IN YRS	SEX	DIAGNOSIS	SBB	PAS	IMMUNO PHENOTYPING	Hb% GMS	TOTAL LEUCOCYTIC COUNT	PLATELET COUNT	SPLENOMEGALY	HEPATOMEGALY	LYMPH ADENOPATHY	OTHERS
21	2	M	ALL L1	-VE	+VE		5.9	1,06,000	0.46	-	+	-	-
22	70	F	CLL	-VE	+VE		7.1	36,000	0.51	-	+	+	-
23	18	F	AML M2	+VE	-VE		2.7	41,500	0	-	-	-	-
24	25	M	CML acc phase	+VE	-VE		9.5	2,51,700	1.53	+	-	-	HAZY VISION
25	46	F	CML chronic	+VE	-VE		5	2,08,000	2.51	+	+	-	-
26	14	M	ALL L1	-VE	+VE		3.7	56,000	0.32	-	-	-	-
27	4	M	AML M4	+VE	-VE		4.2	10,300	0.28	+	+	-	-
28	40	F	AML M1	+VE	-VE		4.7	37,000	2.01	-	-	-	-
29	22	F	CML chronic	+VE	-VE		6.2	1,21,500	0.1	+	+	-	-
30	12	M	ALL L2	-VE	+VE		2.8	38,000	0.4	-	-	-	-
31	40	F	CML chronic	+VE	-VE		4	80,000	0.46	+	-	-	-
32	40	F	CNL	-VE	-VE		18.7	30,100	1.27	-	-	-	-
33	35	F	AML M2	+VE	-VE		4.2	21,900	0.02	-	-	-	-
34	93	M	AML M4	+VE	-VE		6	64,000	0.34	-	-	-	-
35	38	F	CML chronic	+VE	-VE		5.5	1,02,000	1.78	+	-	-	-
36	2	M	ALL L1	-VE	+VE	CALLA +VE	4.2	37,000	0.19	-	+	+	-
37	17	F	AML M1	+VE	-VE	+VE	6.2	14,600	0.28	-	-	-	-
38	10	F	ALL L1	-VE	+VE	ADVICED BM FCM	5.4	3300	0.18	-	+	+	-

MASTER CHART													
S.NO	AGE IN YRS	SEX	DIAGNOSIS	SBB	PAS	IMMUNO PHENOTYPING	Hb% GMS	TOTAL LEUCOCYTIC COUNT	PLATELET COUNT	SPLENOMEGALY	HEPATOMEGALY	LYMPH ADENOPATHY	OTHERS
39	75	M	CML chronic	+VE	-VE	+VE CD45,CD117	7.1	2,50,000	2.79	+	-	-	-
40	34	M	CML blast	+VE	-VE		10.6	2,00,000	4.22	+	+	-	-
41	35	M	AML M1	+VE	-VE		6	31,000	0.24	-	-	-	-
42	13	F	AML M0	+VE	-VE		5	52,000	0.47	-	-	-	-
43	18	F	AML M2	+VE	-VE		6	37,000	0.12	-	-	-	-
44	60	M	AML M2	+VE	-VE		6.4	68,000	0.45	-	-	-	-
45	1	F	ALL L2	-VE	+VE	MILD ↑ BLASTS ADVICED BM FCM	7	41,000	0.36	+	+	-	-
46	65	F	CLL	-VE	+VE		10.1	29,600	2.64	+	-	+	-
47	46	M	CLL	-VE	+VE		5.4	9800	0.97	-	-	+	-
48	44	F	AML M0	+VE	-VE		7.4	43,800	0.13	-	-	-	-
49	45	F	AML M2	+VE	-VE		5.3	1,43,900	0.42	-	-	-	-
50	23	F	CML acc phase	+VE	-VE		5.8	2,18,000	3.71	+	-	-	-
51	19	M	AML M2	+VE	-VE		3.5	53,600	0.17	-	-	-	-
52	35	F	AML M3	+VE	-VE		4.4	1,37,000	0.08	-	-	-	DIC
53	50	M	CML blast crisis	+VE	-VE		7.8	2,44,500	1.6	+	-	-	-
54	42	F	AML M2	+VE	-VE		5.8	9200	1.26	-	-	-	-
55	40	F	CML chronic	+VE	-VE		7.8	84,300	5.37	+	-	-	-
56	45	**M**	CML BLAST CRISIS	+VE	-VE		5	46,400	0.46	+	+	-	-

ABBREVIATIONS

FAB - French American British Classification

WHO – World Health Organisation

SEER – Suveillance Epidemiology, and End Results Program

GLOBOCAN – Global Cancer project 2012

IARC – International Agency for Cancer Research

AML – Acute Myeloid Leukemia

ALL – Acute Lymphoblastic Leukemia

CML – Chronic Myeloid Leukemia

CLL – Chronic Lymphoid Leukemia

MDS – Myelodysplastic Syndrome

MPN – Myeloproliferative Neoplasms

SBB – Sudan Black B

PAS – Periodic acid Schiff's reagent

CD – Cluster of Differentiation

FISH – Florescence insitu Hybridisation technique

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

BCR – Break point Cluster Region

ABL – Abelson murine Leukemia viral oncogene homologue 1

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APPENDIX - 1

SUDAN BLACK B STAIN

Preparation:

1) Reagent stain SBB 0.3g powder is dissolved in 100ml of absolute ethanol, to make a solution.

2) Phenol buffer, is prepared by dissolving 16g og crystalline phenol in 30ml of absolute ethanol. This is added with 100ml of distilled water, in which 0.3g of

3) Disodium phosphate is dissolved.

4) Working stain solution is made by adding 40 ml of phenol buffer with 60 ml of Sudan Black prepared solution.

Staining method:

1) Fix the air dried blood smears with formalin vapour in a Coplin jar, after this, air wash the slides for 15 minutes.
2) Immerse the smear slide in the working SBB stain solution for one hour in a Coplin jar, closed with a lid.
3) After one hour, place the slide on a staining rack, and flood the slides with70% alcohol, after 30 seconds, tip off the alcohol, and flood again with 70% alcohol for 30 seconds. Repeat the flooding three times.
4) Rinse the smear with running tap water and air dry.
5) Counterstain the slide with leishman stain or May-Grunwald-Giemsa stain.

APPENDIX – 2

PERIODIC ACID SCHIFF'S REAGENT STAIN

Preparation:

1) 1% periodic acid, is made up as a solution as 10g/l in distilled water.
2) Schiff's reagent is made by dissolving 5gm basic fuchsin in 500ml 0f distilled water. It is filtered, and the filtrate is saturated with sulphur dioxide gas by bubbling for 1 to 12 hrs in a fume cup board, 2g charcoal is added for 1 minute and shaken vigourously in a conical flask in the fume cupboard, and is filtered immediately in a dark bottle. This reagent is stable for 6 months at room temperature stored in the dark.

Staining method:

1) Fix the smear in methanol for 15 minutes, or in formalin vapour in a Coplin jar or 5 minutes, or in formal acetone for 45 seconds.
2) Flood the slides with 1% periodic acid for 10 minutes, in a staining rack.
3) Rinse in running tap water for 10 minutes, and air dry it.
4) Immerse the slides in a Coplin jar containing the Schiff's reagent, keeping it Covered with a lid.
5) Rinse in running tap water for 10 minutes and air dry it.
6) Counterstain in aqueous hematoxylin for 5 to 10 minutes, or use May-Grunwald-Giemsa stain.

APPENDIX – 3

Fig. 1 AML M1 SBB Positivity

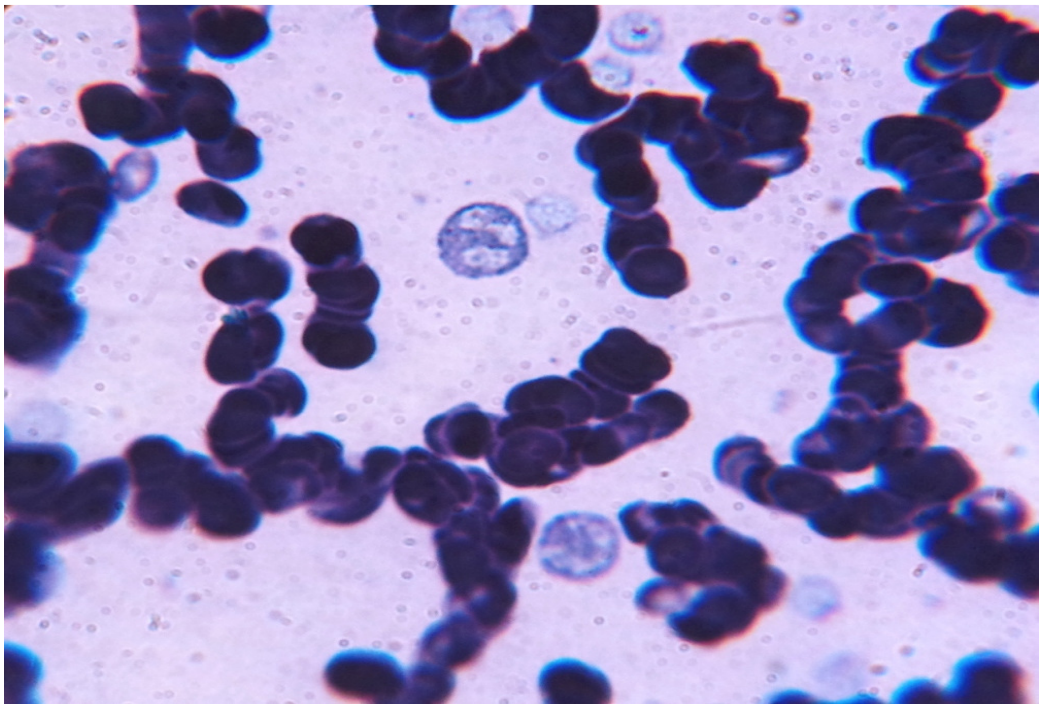


Fig. 2 AML M2 Leishman's stain

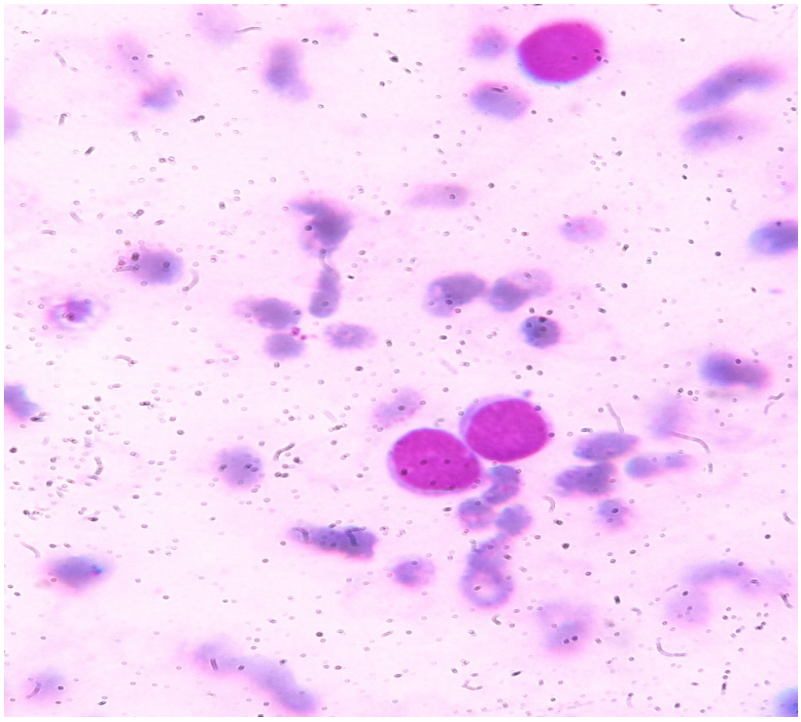


Fig. 3 AML M2 SBB Positivity

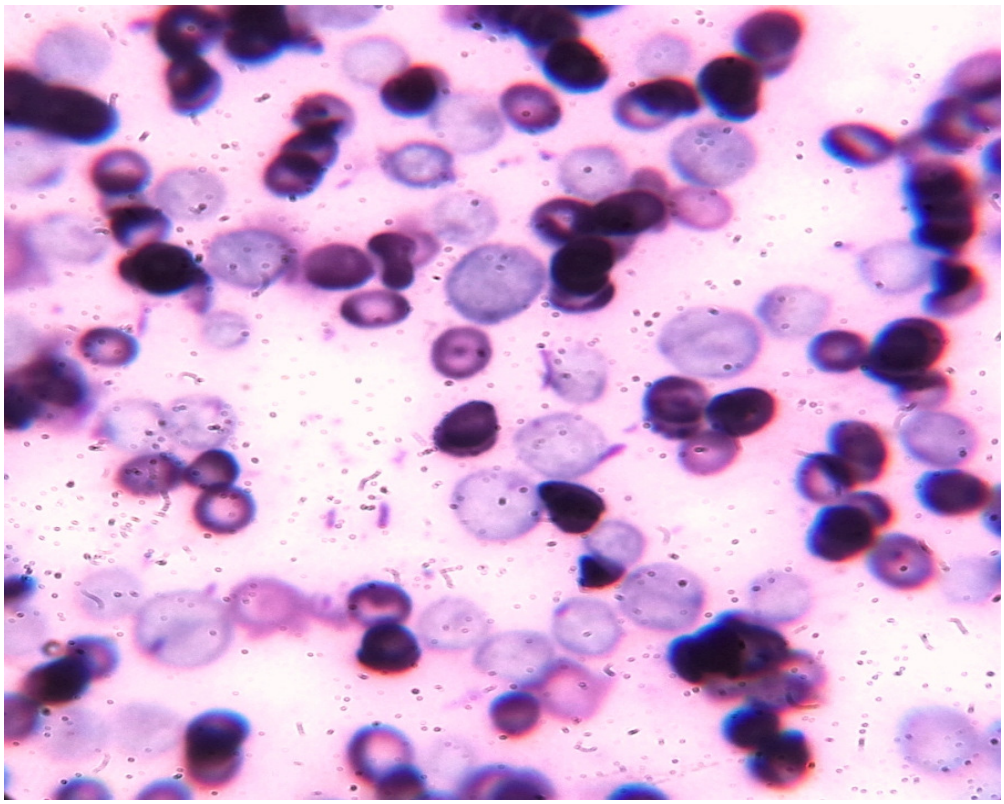


Fig. 4 CML Leishman stain

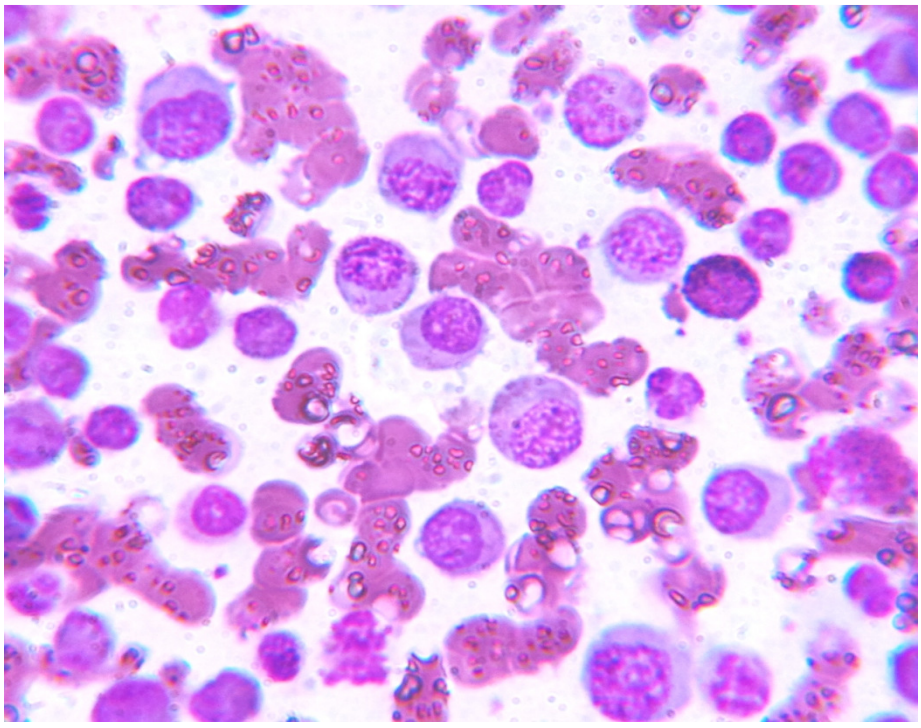


Fig.5 CML SBB coarse positivity

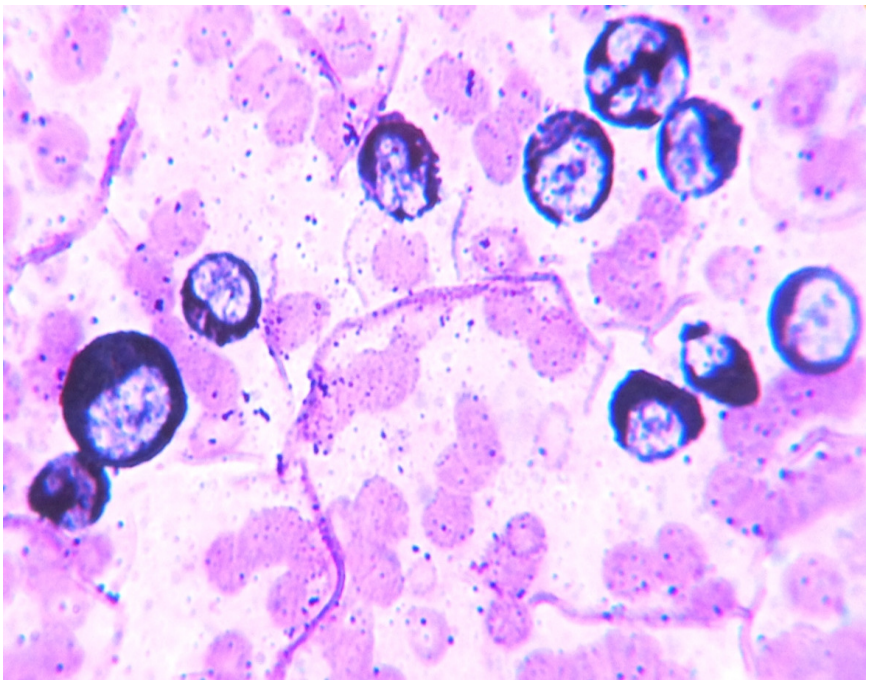


Fig.6 ALL L1 PAS Block Positivity

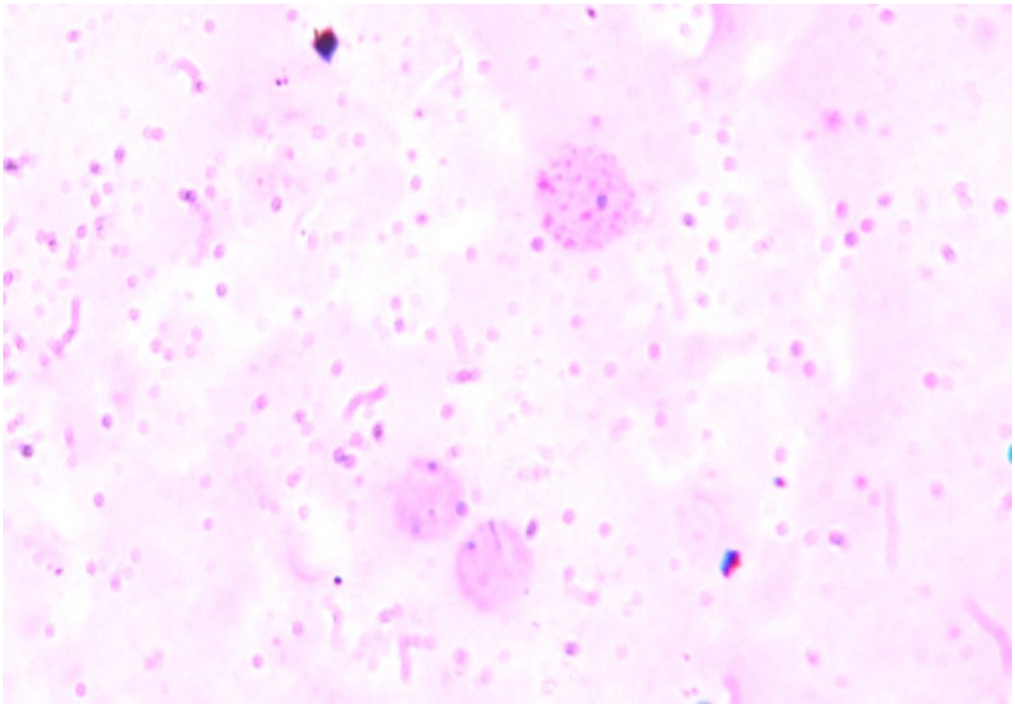


Fig. 7 CLL PAS Cytoplasmic diffuse positivity

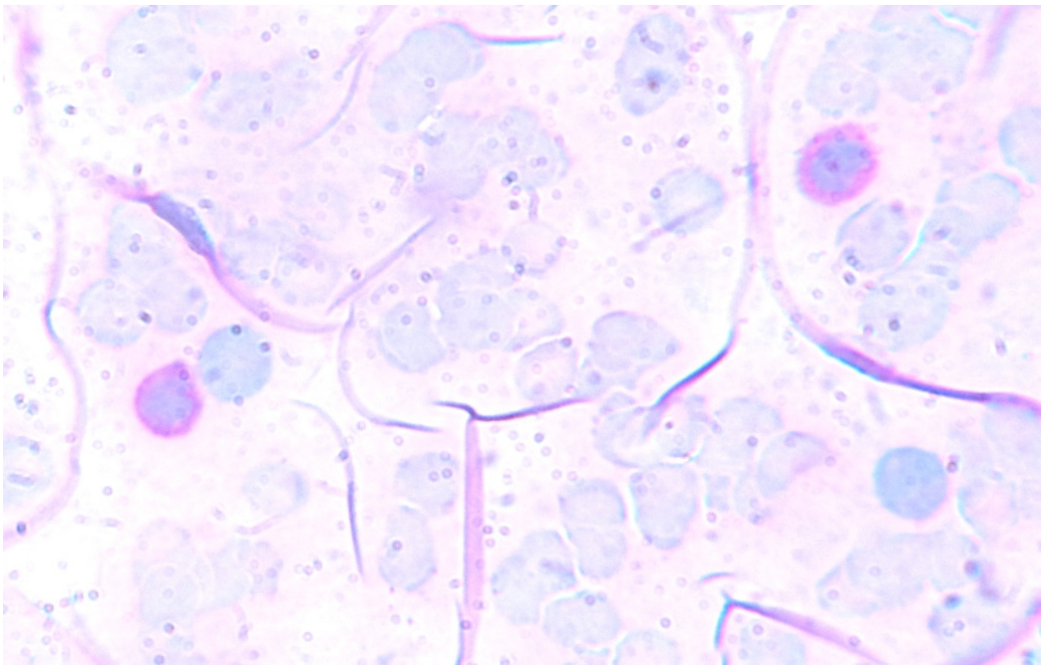
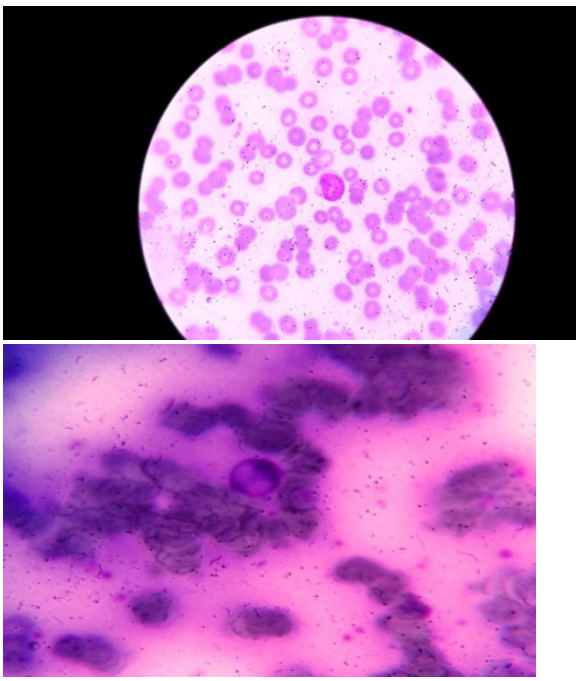


Fig. 8 AML MO Leishman stain and SBB Positivity



Flow cytometric analysis in a case of AML

Flow Cytometric Analysis Rep

Acute Myeloid Leukemia Panel

Sample : Peripheral blood

Gating Type : CD 45 VS Side Scatter (Atypical Cells-33.2 %)

Blast Markers:

CD34 : 86.3%
HLA-DR : 75.4%

Myeloid Markers:

CD33 : 37.2%
CD15 : 25.8%
CD117 : 99.9%
CD13 : 97.8%
cMPO : 98.4%

Monocytic Markers:

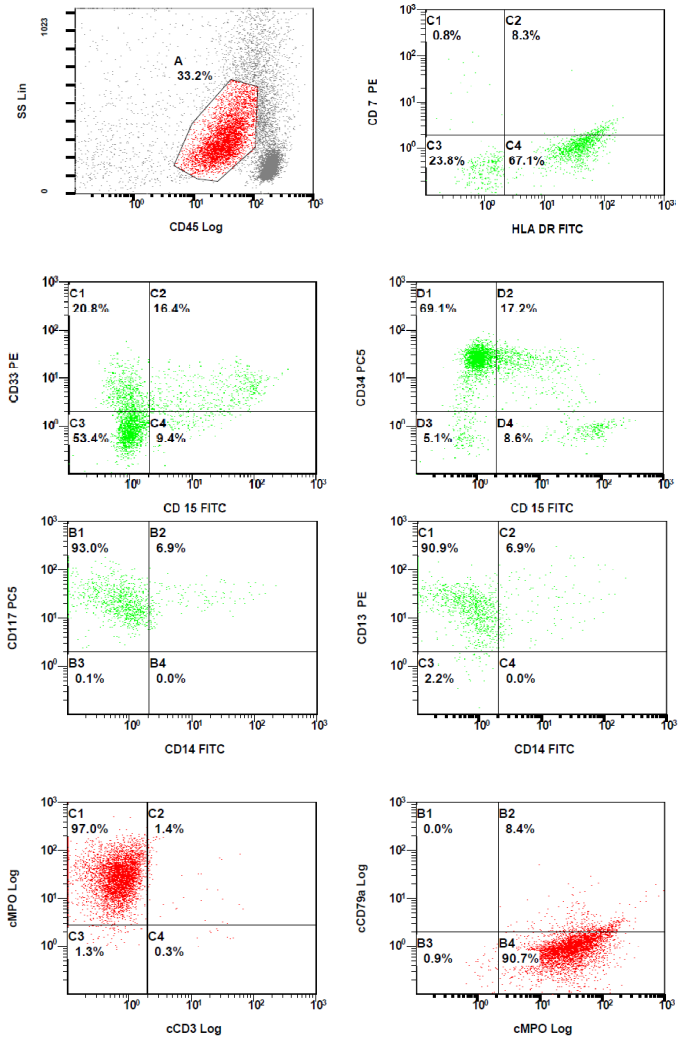
CD14 : 6.9%

B Cell Markers:

cCD79a : 8.4 %

T Cell Markers:

CD7 : 9.1%
cCD3 : 1.4%



Flow cytometric analysis in a case of ALL

Flow Cytometric Analysis Rep

Undifferentiated Leukemia Panel

Sample : Bone marrow

Gating Type : CD 45 VS Side Scatter (Atypical Cells-28 %)

Blast Markers:

CD34 : 18.7%

Myeloid Markers:

CD33 : 6.8%
CD15 : 0%
CD117 : 0%
CD13 : 3.6%
cMPO : 0.6%

Monocytic Markers:

CD14 : 0.2%

B Cell Markers:

cCD79a : 98.4 %
CD 10 : 54.4%
CD 19 : 67.8%

T Cell Markers:

cCD3 : 3.7%