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CORRELATION BETWEEN MORPHOLOGY, CYTOCHEMISTRY, AND FLOW CYTOMETRY IN THE DIAGNOSIS OF ACUTE LEUKEMIA AT TIKUR ANBESSA SPECIALIZED HOSPITAL, ADDIS ABABA, ETHIOPIA, 2018: A PROSPECTIVE CROSS-SECTIONAL STUDY

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A thesis submitted to the Department of Pathology, School of Medicine, College of Health Science of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Histotechnology

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List of abbreviations

AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
AL	Acute Leukemia
ALL	Acute lymphoblastic (lymphocytic) leukemia
AML	Acute myeloid (myeloblastic/myelogenous) leukemia
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
AUL	Acute undifferentiated leukemia
BCR-ABL	Breakpoint cluster region-Abelson
BD	Becton Dickinson
BM	Bone marrow
CALLA	Common Acute Lymphoblastic Leukemia Antigen
CAE	Chloroacetate esterase
CC	Cytochemistry
cCD	Cytoplasmic cluster of differentiation
CD	Cluster of differentiation
CI	Confidence interval
CL	Chronic Leukemia
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CR	Complete remission
CSF	Cerebrospinal fluid
DAB	3, 3'-diaminobenzidine
DNA	Deoxyribonucleic Acid
DPX	Distyrene Plasticizer Xylene
EDTA	Ethylene diamine tetra acetic Acid
EGIL	European Group for the immunological classification of leukemias
FAB	French-American-British group
FACS	Fluorescence-activated cell sorter/sorting
FC	Flow cytometry

FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
HLA-DR	Human leukocyte antigen-D related
Ig	Immunoglobulin
IH	Immunohistochemistry
mAb	Monoclonal antibodies
MPO	Myeloperoxidase
NPV	Negative predictive value
PAS	Periodic Acid Schiff reaction
PB	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy	Phycoerythrin-cyanine
PerCPCy	Peridinin-chlorophyll-protein Complex: CY5.5 Conjugate
PI	Principal Investigator
PPV	Positive predictive value
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SBB	Sudan Black B
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
t	Translocation
TdT	Terminal Deoxynucleotidyl Transferase
TASH	Tikur Anbessa Specialized Hospital
USA	United State of America
WBC	Whole Blood Cell
WHO	World Health Organization

Abstract

Background: Leukemia is a group of disorders characterized by the accumulation of abnormal white cells in the bone marrow. The main classification of leukemia is into acute and chronic leukemia. The diagnosis of leukemia has moved from evaluation of morphology and cytochemistry to assessment by modern methods such as immunophenotyping, cytogenetics, and molecular characterization.

Objective: To analyze the correlation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

Method: This prospective cross-sectional study was conducted from May 2017 to March 2018 in Tikur Anbessa Specialized Hospital and convenient sampling method was used. A total of 40 study participants were diagnosed with acute leukemia based on morphology. Cytochemistry was performed using Sudan Black B and Periodic Acid Schiff. Flow cytometry employing primary and secondary antibodies was performed using FACS Calibur (Becton Dickinson). Data were entered and analyzed using SPSS 20.0. Frequencies were calculated for socio-demographic data and for the morphologic and flow cytometry results. Cross tab was used to measure the association between morphology and cytochemistry and between morphology and flow cytometry and between cytochemistry and flow cytometry. Kappa value was calculated to show the measure of agreement between flow cytometry and morphology. The significant level was based on the p-value of less than 0.05.

Results: Based on BM morphology, 15(37.5%) of the 40 cases were classified as ALL and 21 (52.5%) as AML while 4(10%) cases remained unclassified. Based on Flow cytometry, 14(35%) were classified as ALL, 25(62.5%) as AML and 1(2.5%) case was classified as Bi-lineage. Out of 15 ALL cases which were detected by morphology, 2(13.3%) cases showed PAS reactivity and 8(53.3%) showed SBB positivity. Out of 21 AML confirmed cases based on morphology, 19(90.5%) were positive for SBB. Out of four unclassified cases, three cases (75%) were SBB positive.

Conclusion: FCA and cytochemistry helped in classifying the unclassifiable cases based on morphology. FCA helped in lineage assignment particularly in ALLs. It is important to use morphology, cytochemistry, and FCA together in classifying acute leukemia as it greatly influences the treatment and the prognosis.

1. INTRODUCTION

1.1. Background

Cancer, also known as a malignant neoplasm, is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. The latter process is referred to as metastasizing. Metastases are a major cause of death from cancer [1].

Cancers can be grouped according to the type of cell they start in. There are seven main categories; these are Carcinoma, Sarcoma, Leukemia, Lymphoma, Multiple Myeloma, Melanoma, and Brain and spinal cord tumors [2].

Leukemia is a type of cancer found in blood and bone marrow and is caused by the rapid production of abnormal white blood cells. These abnormal white blood cells are not able to fight infection and impair the ability of the bone marrow to produce red blood cells and platelets. These cancers do not form solid tumors. Instead, large numbers of abnormal white blood cells (leukemia cells and leukemic blast cells) build up in the blood and bone marrow, crowding out normal blood cells. The low level of normal blood cells can make it harder for the body to get oxygen to its tissues, control bleeding, or fight infections [2, 3].

Leukemia affects little more males than females, and whites than Blacks and the high socioeconomic class. The three main risk factors for leukemia and lymphoma are as follows; first, environmental factors, second, infectious agents, and last, genetic predisposition. Exposure to X-Ray, hydrocarbons, insecticides, gasoline, trichloroethylene, colors used for painting, thinners, solvents, benzene, engine fuel paints, and long-term exposure to plastic leads is related to high incidence of leukemia. The risk of leukemia is higher in mechanics and industrial workers [4].

1.1.1. Types of leukemia

Leukemias are classified into four main categories, based on the type of white blood cell affected and characteristics of the disease. Based on characteristics of the disease, Leukemia are classified as acute leukemia and chronic leukemia and based on types of WBCs affected, they are classified as myelogenous leukemia and lymphocytic leukemia [5].

The term “acute” means that leukemia can progress quickly, and if not treated, would probably be fatal within a few months. In acute leukemia (AL), the leukemia cells are similar to immature blood cells (called blasts), and like normal blasts divide quickly. Unlike normal blasts, however, leukemia cells divide uncontrollably due to dysregulation of proliferation mechanisms. AL arises from the dysregulated clonal expansion of immature lymphoid or myeloid progenitor cells that are blocked at a particular stage of differentiation [6, 7, and 8].

In chronic leukemia (CL), the leukemia cells are more mature cells, but they are not completely normal. They generally do not fight infection as well as normal white blood cells. Moreover, they survive longer, build up, and crowd out normal cells. CL tend to progress over a longer period of time, and most patients can live with it for many years. However, CL is generally harder to cure than AL [8].

1.1.2. Classification of Acute Leukemia

Acute leukemia is further subdivided into acute myeloid (myeloblastic/myelogenous) leukemia (AML) and acute lymphoblastic (lymphocytic) leukemia (ALL) based on morphology and cytochemistry. Such a classification of acute leukemias and further subtyping is done by many ways: (i) by morphology and cytochemistry supplemented by immunophenotyping, as proposed by French-American-British (FAB) group; (ii) Proposed World Health Organization (WHO) Classification of Acute Leukemia; (iii) by immunophenotyping alone, as proposed by the European Group for the immunological classification of leukemias (EGIL) [9,10].

The most important differences between WHO criteria and FAB criteria are (i) the lowering of the threshold for the percentage of blast cells to 20% in the blood or bone marrow versus 30% in FAB; and (ii) the recognition of cases of acute leukemia with an even lower blast count if specific acute leukemia-associated cytogenetic or molecular genetic abnormalities are present [11].

ALL is cancer that starts from the early version of white blood cells called lymphocytes in the bone marrow. Lymphoblasts are abnormal white blood cells that are dominating both in the bone marrow and the peripheral blood of patients with ALL. They are characterized by smaller size (10-15μm) than myeloblasts, thin rim of dark blue cytoplasm with no granules, a nucleus is

round or convoluted, and centrally located and has 1 or 2 nucleoli which are less prominent [7,12].

ALL is a malignant disorder, which originates in a single B –or T-lymphocytes progenitor because of somatic mutation in a single progenitor cell at one of the several discrete stages of development. It is the most common malignant disease affecting children, accounting for approximately 30% of all childhood cancers. ALL has a bimodal age distribution, peaking in children between 3 and 5 years of age and again in persons older than 65 years [13, 14].

Several types of chromosome changes may be found in ALL cells: translocation, deletions, and inversions. The most common translocation in ALL in adults is known as the Philadelphia chromosome, which is a swap of DNA between chromosomes 9 and 22, abbreviated as t(9;22) [7].

Furthermore, signs and symptoms of ALL can include fever, weakness, fatigue, breathlessness, opportunistic infections, weight loss, anorexia, easy bruising and bleeding, thrombosis, edema of the lower limbs and the abdomen, swollen liver or spleen, lymphadenopathy, or bone pain [15].

The FAB Cooperative Working Group defines three categories of lymphoblasts: L1 lymphoblasts are small cells characterized by a high nucleus-to-cytoplasm ratio. L2 lymphoblasts are larger, often in a more heterogeneous population, with a lower nucleus-to-cytoplasm ratio, prominent nucleoli (often with perinuclear chromatin condensation) and nuclear membranes that may be reniform or irregular. They may be indistinguishable from the M1 variant of myeloid leukemia, and the differentiation must be made primarily by myeloperoxidase (MPO) staining; the M0 variant of myeloid leukemia, which is MPO negative, may be indistinguishable from ALL without immunophenotyping [16].

L3 lymphoblast is a heterogeneous group of cells identical to Burkitt-like leukemia and characterized by deeply basophilic cytoplasm and prominent cytoplasmatic vacuolization. Approximately 85% of children with ALL have predominant L1 morphology, 14% have L2, and 1% have L3, while the L2 subtype is more common in adults [16].

AML has many other names, including acute myelocytic leukemia, acute myelogenous leukemia, acute granulocytic leukemia, and acute nonlymphocytic leukemia. Myeloblasts are abnormal

cells that predominantly makeup AML. These cells are larger than lymphoblasts and have lower nuclear to cytoplasmic ratio, prominent multiple nucleoli, Auer rods (stick-like structures in the cytoplasm) in 50% of AML, and granules in the cytoplasm [12, 17].

The original classification scheme proposed by the FAB Cooperative Group divides AML into 8 subtypes (M0 to M7). Although AML blasts evolve from common myeloid precursors, the eight subtypes differ in degree of maturation. M0 designates AML with minimal morphologic or cytochemical differentiation, M1–2 AML with minimal or moderate granulocytic differentiation, M3 acute promyelocytic leukemia (APL), M4 AML with mixed myelomonocytic differentiation, M5a and M5b monoblastic leukemia with minimal or moderate differentiation, M6a myeloid leukemia with dysplastic background erythropoiesis, M6b acute erythroblastic leukemia, and M7 acute megakaryoblastic leukemia [10].

In some instances, individual leukemic cells simultaneously express both lymphoid and myeloid surface antigens, showing characteristics of more than one hematopoietic lineage. This leukemia has been referred to as biphenotipic, mixed-lineage, or hybrid leukemia, and, depending on the criteria applied, the incidence of this subgroup of ALL ranges from 7%to 25% [16].

1.1.3. Diagnosis of Acute Leukemia

The diagnosis of leukemia has moved from evaluation of morphology and cytochemistry to assessment by modern methods such as immunophenotyping, cytogenetics and molecular characterization [18].

1.1.1.1.Cytochemical stains

Leukocyte cytochemistry encompasses the techniques used to identify diagnostically useful enzymes or other substances in the cytoplasm of hemopoietic cells. These techniques are particularly useful for the characterization of immature cells in the acute myeloid leukemias, and the identification of maturation abnormalities in the myeloproliferative disorders. The use of cytochemistry to characterize lymphoproliferative disorders has been largely superseded by immunological techniques [9].

Types of cytochemical stain for leukocyte diagnosis

Myeloperoxidase (MPO), a microbicidal protein in the primary granules, is the hallmark enzyme of the myeloid lineage. Expression of the MPO gene is specific for myeloid precursors and their leukemic counterparts, and transcription of the MPO gene is turned on only during the late myeloblast and promyelocyte stages of myeloid maturation. The diagnosis of acute myeloid leukemia (AML) is easy if more than 3% of blast cells are confirmed to be cytochemical MPO positive, although most blast cells are MPO negative in M0, M5, and M7 subtypes by the French–American–British (FAB) classification [19].

Sudan black B is a lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils, and some monocytes. It cannot be extracted from the stained granules by organic dye solvents and gives comparable information to that of MPO staining [9].

Periodic Acid-Schiff (PAS) Reaction: Periodic acid specifically oxidizes 1-2 glycol groups to product stable dialdehydes. These dialdehydes give a red reaction product when exposed to Schiff's reagent (leucobasicfuchsin). Positive reactions occur with carbohydrates, principally glycogen, but also monosaccharides, polysaccharides, glycoproteins, mucoproteins, phosphorylated sugars, inositol derivatives and cerebrosides. Glycogen can be distinguished from other positively reacting substances by its sensitivity to diastase digestion. ALL blasts show "block" positivity and the method can be used to differentiate ALL from AML [9].

1.1.1.2.Flow Cytometry

Flow cytometry measures optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA [20].

Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state when hit by the laser. Upon returning to their

resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously. Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein, although many other dyes are available [20].

Immunophenotyping is the identification and quantification of cellular antigens through fluorochrome-labeled monoclonal antibodies. It discovers T- and B-cells and provides prognostic information not available by other techniques. In addition, it provides a sensitive means to monitor the progress of patients after chemotherapy and aids in the detection of minimal residual disease [21].

Immunophenotype of B-lineage ALL

In B-lineage ALL the most important markers for diagnosis, differential diagnosis, and subclassification are CD19, CD20, CD22, CD24, and cytoplasmic CD79a (cCD79a). The earliest B-lineage markers are CD19, CD22 (membrane and cytoplasm) and cCD79a.A positive reaction for any two of these three markers, without further differentiation markers, identifies pro-B ALL (EGILB-I subtype). The presence of CD10 antigen (CALLA) defines the "common" ALL subgroup (EGILB-II subtype). Cases with the additional identification of cytoplasmic heavy mu chain constitute the pre-B group (EGIL B-III subtype), whereas the presence of surface immunoglobulin light chains defines mature B-ALL (EGIL B-IV subtype) [22].

Immunophenotype of T-lineage ALL.

T-cell markers are CD1a, CD2, CD3 (membrane and cytoplasmic), CD4, CD5, CD7, and CD8. CD2, CD5, and CD7 antigens are markers of the most immature T-cells, but none of them is absolutely lineage-specific so that the unequivocal diagnosis of T-ALL rests on the demonstration of surface and/or cytoplasmic CD3 (cCD3)[22].

Immunophenotype of AML

Cells in AML express two or more antigens chosen from a group that includes cytoplasmic myeloperoxidase (cMPO), CD13,CD33, CD64, CD65 and CD117[23].

Cytogenetics can be helpful in the identification of recurrent translocations, as well as gain and loss of gross chromosomal material. The major limitation of this technique is that in some cases leukemic cells fail to enter metaphase. However, fluorescence *in situ* hybridization (FISH) can enable the detection and direct visualization of virtually all investigated chromosomal abnormalities in ALL [22].

The results of the current methods of evaluation (i.e, morphology, staining, molecular analysis, flow cytometry) study not only differentiate ALL from AML but also categorize the subtypes of acute leukemia. This analysis divides B-cell ALL into 4 subtypes and T-cell ALL into 2 subtypes based on surface markers as determined by the degree of maturation. The subtypes of B–cell ALL are early pre-B ALL (also called pro-B ALL), common ALL, pre-B ALL, and mature B-cell ALL (Burkitt leukemia). And also, the subtypes of T-cell ALL are pre-T ALL and mature T-cell ALL [14].

1.2. Statement of the Problem

Cancer is the major cause of morbidity and mortality worldwide. According to the WHO, the global burden of cancer will continuously increase, during the next 20 years. Among the different types of cancers, leukemia has greatly increased in frequency. Leukemia is one of the most common cancers among children. However, recently the incidence has been increased in adults as well [24].

Leukemia ranked eighth for cancer incidence and ninth for cancer deaths at the global level in 2015. Leukemia incidence was ranked highest for low-SDI (sociodemographic index) and low-middle SDI countries at sixth place (leukemia was ninth and eighth for cancer deaths in low SDI and low-middle SDI countries, respectively). Leukemia was ranked lowest in high-SDI countries at 13th place (eighth for cancer deaths) [25].

In the United States, the incidence is higher in Caucasians than in Afro-Americans and Hispanics. The American Indians/Alaskan natives have the lowest incidence rates. Trends in overall incidence of leukemia have generally been stable or slowly increasing [26].

AML is mainly an adult's disease with a median age at presentation of 64 years. It accounts for 30% of all leukemias in adults, and 18,000 new patients are diagnosed in Europe each year, representing 0.6% of all cancers. The annual incidence rate in Europe ranges from two per 100,000/year to four per 100,000/year. The disease is more common in males [27].

ALL is uncommon in adults, where it represents15% of leukemias, but is the most common form of leukemia in people <20, accounting for over 80% of all leukemia patients and for 30% of all cancers in children. The incidence rate of ALL among 1- to 4-year-old children is 10 times greater than in the young adults aged 20–24. About 10,000 new cases are diagnosed in adults in Europe each year, with incidence rates between two and four per 100,000/year, roughly similar to the rates in other developed continents. ALL is slightly more common in men than in women [26].

Leukemia ranks among the top most common cancers for both sexes in Iraq, Jordan, and Kuwait, while it is much less common in most other countries. In Iran, the incidence registration has shown an increase in recent years, which has reached to the 7th grade after cancers of skin, breast, stomach, colorectal, bladder, and prostate [25, 28].

In Croatia between 1988 and 2009, there were a total of 8035 leukemia patients of these 785 cases of ALL in men and 612 in women and 1084 cases of AML in men and 1057 in women. The most common leukemia type was CLL, comprising 42% of leukemias, followed by AML with 27%, ALL with 17%, and CML with 14% [29].

In Kenya, leukemia in children below the age of 15 years comprised 37% of leukemia in all ages. Childhood acute leukemia formed 52.3% of all acute leukemia. AML and ALL occurred, in almost equal proportions 42 % and 46 % respectively [27].

In Ethiopia, Leukemia ranked seventh for cancer incidence and ninth for cancerdeaths in 2015. Extrapolation from clinical records from Tikur Anbessa Radiotherapy Center estimates that there is 120,500 new cancer cases/year. Most patients present with advanced disease, and there is a high rate of abandonment of treatment. Furthermore, Leukemia/lymphoma is the 5th common type of cancer [25, 30].

The pattern of leukemias in adult Ethiopians admitted to TikurAnbessa (Black Lion) Hospital, from January 1982 to December 1987 was analyzed. There were 7969 medical admissions, of which 180 (2.3%) were for leukemia. The age range was 14 to 80 years, with a mean of 37.6 years. The male: female ratio was 2.3:1. The commonest type of leukemia was chronic myeloid leukemia (CML) 57.8%, acute leukemias and chronic lymphatic leukemia (CLL) accounted for 21.1% each. Of the acute leukemias, 53.3% were lymphoblastic (ALL) while 46.7% were acute myeloblastic (AML). The overall incidence of leukemia in Addis Ababa population was estimated at 1.95 +/- 0.79/100,000/year at 95% confidence interval [31].

The FAB classification failed to distinguish immunophenotypic groups of ALL, did not recognize the significance of myelodysplastic changes in AML or cytogenetic abnormalities in either leukemia type. Hence it resulted in some subcategories of little clinical significance; immunophenotyping provided very clear guidelines for classification. Identifying the lineage of the leukemic cells not only helps in assessing the course of the disease but also aids in rendering the most specific treatment to the patients [10,32].

1.3. Significance of the study

Leukemia is one of the most common malignancies and is ranked 10th for cancer incidence and 8th for cancer death in developing countries. The main aim of this study was to assess the agreement or the correlation of morphology, cytochemistry and flow cytometry for the diagnosis of acute leukemia and to investigate their usefulness. Currently, in Ethiopia, only the morphology and sometimes cytochemistry is used to diagnose leukemia. And mostly we may get an inconclusive result because of lack of flow cytometry and cytogenetics. The diagnostic gold standard and classification of leukemia involves various methods including morphology, cytochemistry, cytogenetics and molecular genetics, immunophenotyping, and molecular biology. In recent years, increasing emphasis has been place on the relative importance of flow cytometry in the diagnosis of acute leukemia. It also shows the clinical utility of flow cytometry in diagnosing leukemia. Thus, patients and physicians benefit from better leukemia diagnosis and classification. Hospital administration of TASH and other tertiary care hospitals can use the data for planning in the implementation of flow cytometry for leukemia diagnosis. Researchers and policy makers can also benefit by using the finding as baseline data [16, 25, and 33].

2. Literature Review

A study from India conducted by Belurkar *et al* analyzed 50 cases of acute leukemia and found concordance rate as high as 86% between morphologic/cytochemistry (CC) diagnosis and flow cytometry (FC) diagnosis. Of these, complete concordance was seen in 58% of the cases and partial concordance was seen in 22% of the cases. Non-concordance was seen in only 4% of their cases. In the remaining 16% of the cases, FC helped in sub-classifying acute leukemia where morphology and CC had failed to do so. CD19 and CD20 were found to be consistent B-cell markers and CD3 was a very specific marker for T-cell leukemia. CD13 and CD33 were important myeloid markers and were aided by another secondary panel of markers like CD14, CD117 and CD41 [32].

A study conducted by Selicean *et al* assessed the correlation of cytomorphology with flow cytometric immunophenotyping in AML. As a result, the degree of correlation between blast percentage determined by cytology and immunophenotyping was low (r=0.049). The degree of correlation between myeloperoxidase positivity in CC and immunophenotyping was also low, with better results for CC. Expression of immunophenotypic markers was consistent with the composition of the study group regarding FAB classes, except for HLA-DR (49.0%), TdT (3.77%), CD14 (5.66%), CD15 (5.66%). They also discussed the importance of interpreting with caution positivity for erythroid and megakaryocytic markers and differential diagnosis of cases simultaneously expressing CD7 and CD56 [34].

In a study of cytochemical analysis of leukemia performed by Ahirwar *et al*, only 71% cases were diagnosed on the basis of morphology only, but with the addition of CC along with morphology, the diagnosis was made in 96% cases. The other 4% were undiagnosed even after CC requiring immunophenotyping, for which they were referred to the higher centers [35].

In the USA, a study conducted by Mhawech *et al*, assessed the relative usefulness of cytochemical staining and FC methods as applied to the diagnosis of acute leukemia in the pediatric population. As a result, cell lineage classification derived from FC and cytochemical stains agreed in all cases. Definitive diagnoses were feasible using FC results alone in 120 of 122 patients (98.4%) as compared with only 99 of 122 patients (81.2%) when only cytochemical

staining results were considered. In two patients with inconclusive FC results, cytochemical staining alone provided information sufficient for diagnosis [36].

A study conducted by Liqaa M *et al* diagnosed a total of 79 bone marrow specimens as acute leukemia based on morphology, cytochemistry and by flow cytometry. On the basis of morphology 58.21% were diagnosed as AML, 37.9 % diagnosed as ALL and 3.79% as AUL. In AML, SBB was positive in 78.2 % of cases, subclassified from M1 to M4, while 21.7% had negative SBB subclassified as M5a, M5b, and M6. PAS were negative in all AML cases. In ALL, PAS was positive in 60% of cases and SBB was negative in all cases. AUL cases had negative results in both stains. Flow cytometric results are: AML; M0 (2.53%), M1(2.53%), M2 (24.05%), M3 (5.06%), M3v (5.06%), M4 (8.86%), M5a (7.59%), M5b (1.27%), M6 (2.53%) and M7 (1.27%). Among ALL; LB precursor (26.58%), LB common (6.33%), LT type (3.8%) and L3 in (2.53%). SBB is still an important and cheap method in diagnosis and classification of AML, while PAS can diagnose 60% of ALL. FC not only helps in confirming a morphologic diagnosis in AL but also helps in assigning specific lineage [37].

Biren P *et al* included 343 new cases of acute leukemias in which morphological diagnosis using special stains has been made and immunophenotyping usingprimary and secondary markers have been applied and a definitive diagnosis was made. In addition, they analyzed the sensitivity and specificity, and positive and negative predictive value of the special stains in establishing the diagnosis of acute leukemia. SBB was positive in 72.7% cases of AML and negative in 27.3% cases. PAS was positive in only 28% of cases of ALL and it was negative in 72%cases. Morphology plus cytochemical staining with PAS and SBB was able to correctly diagnose 93.38% cases of AML. PAS stain was positive in only 27.4% cases of ALL [38].

In Egypt, a study conducted by Moneim Deghady A.A *et al* showed that cytochemical stains (MPO, SBB, PAS and NSE), when coupled with morphology accurately diagnosed 93.3% of acute leukemia cases. A total of 13/15 (86.7%) ALL cases and 15/15 (100%) AML cases could be diagnosed correctly. MPO showed significant positive association with CD13 and CD33 and significant negative association with CD10, CD19 and CD2. SBB had significant positive association with CD13 and CD33 and negative association with CD10 and CD19. NSE showed

positive association with CD14 and negative association with CD13. PAS had only positive association with CD5 [39].

In a retrospective study conducted by Shrestha *et al* out of a total 52 cases of acute leukemia diagnosed by FC over a two year period, there were 31 cases (59.6 %) of ALL, 20 cases (38.4%) of AML and one case (1.9%) of bi-phenotypic AL. Leukemia was diagnosed among adults in 44.2% whereas among children with age less than or equal to 15 years in 55.7 %. Most 38 (73%) were male and 14 (27%) were female with a male: female ratio of 2.7:1. For AML, it was found that M0 (5.0%), M1 (20%), M2 (60%), M3 (15%), M4 (5.0%) were detected. CD13 and CD33 were the most useful markers in the diagnosis of AML. The most common subtype was AML-M2. Of the 31 cases with ALL, 20 cases (64.5 %) were identified as B-ALL and 11 cases (35.5%) as T-ALL. Cytoplasmic CD3 (cCD3) and CD7 were the most sensitive antigens present in all cases of T-ALL. All cases of B-ALL showed expression of pan B-cell markers CD19 and CD22, but 15 (75%) of 20 cases expressed CD10 [40].

Domingos Resende G.A *et al* assessed the role of cytochemistry in the diagnosis of acute leukemias. As a result, cytochemical staining (associated with morphology) diagnosed 89.5% of cases. MPO was positive in 30 of the 36 AML cases (83.33%). SBB was positive in 29 of 35 cases (82.9 %.). Both reactions (MPO and SBB) were negative for the 31cases (100%) with ALL. PAS was also positive in 31 cases (100%) of ALL and in one of AML (3.2%) which was also positive for MPO and SBB with monocytoid blasts to morphological analysis. Immunophenotyping confirmed that the staining of ANAE processed in 10 cases of suspected monocytic leukemia cases showed positivity in 7 of the 10 cases (70%) [41].

A study conducted by Samir *et al* assessed 93cases of AL using FC and CC and assigned to one of four categories: myeloid, lymphoid, bi-phenotypic, and non-diagnostic. In leukemias designated as ALL or AML by both methodologies, there was lineage agreement in all but 3 of 71 cases (95.8%). However, when non-diagnostic or bi-phenotypic diagnoses made by either methodology were included, complete agreement occurred in only 77.4% of cases. Of 37 cases designated myeloid origin by FC, 33 (89.2%) were read as myeloid by CC. The four discordant diagnoses were read as lymphoid (2) or as non-diagnostic (2). About 80% of lymphoid

leukemias were diagnosed as such by both FC and CC; one early B cell ALL was diagnosed as myeloid and 8 as non-diagnostic. Fifty percent (50%) of FC defined T-cell ALL were considered non-diagnostic by cytochemistry as compared to 17% of the total ALL group. Of the remaining four designated non-T cell ALL by FC and non-diagnostic by CC, three were read by FC to be standard pre-B ALL and one an early B-cell ALL. Only 2/9 leukemias considered biphenotypic by flow were identified as such by CC [42].

In a retrospective study, Abbasi N *et al* assessed a total of 340 cases of acute leukemia studiedusing multicolor flow cytometry. As a result, 164 cases (48.2%) were ALL, 176 (51.8%) were AML. Acute leukemia was diagnosed among adults in 51.8% whereas 48.2% were children. Of the ALL cases, 130 cases (79.3%) were B-cell type and 34 cases (20.7%) were T-cell type. All cases of B-ALL showed expression of pan B-cell markers (CD19, CD22 and cytoplasmicCD79a) and 117 (90%) of cases expressed CD10. On the other hand, cCD3 and CD5 were the most sensitive markers for diagnosis of T-ALL. Of the 176 cases of AML, 16 cases (9%) were identified as acute promyelocytic leukemia (APL), while the rest 160 cases showed expression of CD34 and HLA-DR in 41.4% and 68.7%, respectively. None of the cases of APL were positive for both CD34 and HLA-DR. CD13 and CD33 were expressed in all cases of acute myeloid leukemia studied [43].

In Kenya, a study conducted by Patel K *et al* assessed 33 patients who underwent both flow cytometry and bone marrow morphology tests for diagnosis of leukemia between July 2013 and June 2014. The ages of the patients ranged from 3 to 76 years. The ratio of male to female was almost one to one (1:1.1). Using the Bone marrow morphology, 17 patients had AML and 15 had ALL, one case was inconclusive. There were five categories for the flow cytometry. They consisted of 6 cases of B-ALL, 13 cases of T-ALL, 10 cases of AML, 1 case of Bi-phenotypic, and 1 inconclusive case. There was concordance between the morphological and flow cytometry on 25 out of the 33 cases. The authors concluded that Flow cytometry had a role to play to confirm a definite and a probable diagnosis of patients with acute leukemia [44].

Salem D.A *et al* retrospectively analyzed the immunophenotypic data of 164 de novo AL patients from 2009 and 2010. Among these patients, 68.9% were classified as AML while 31.1%

classified as ALL. The commonest FAB subtype in AML group was AML-M4/5 (34.5%) which may differ from most published data. Regarding ALL, there were 74.5% with B-ALL and 25.5% with T-ALL. It was found that the combined use of HLA-DR and CD34 was much more helpful in distinguishing APL from non-APL AML than either of these antigens alone. It was found that cCD79a and CD19 were the most sensitive markers for B-ALL while cCD3, CD7 and CD5 were the most sensitive antigens for T-ALL. This analysis of AL phenotypes indicated that the employed antibody panels are adequate for proper diagnosis and classification of AL. Flowcytometry was found to be especially useful in the identification of AML-M0 and differentiation of APL from non-APL AML. Immunophenotyping results and FAB classification of theseAL patients were comparable to internationally published studies apart from predominance of AML-M4/5 and more frequent APL [45].

A study conducted on Immunophenotypic patterns of childhood acute leukemias in Indonesiaby Supriyadi E *et al* showed that from the 498 patients with acute leukemia, 116 (23%) were AML and 381 (77%) were ALL. Among ALL samples, 315 (83%) samples were B-lineage ALL, 2 samples were mature B and 64 (17%) were T-lineage ALL. The study found mature-B cell phenotype in 2 cases (0.2% of all samples). Among the patients with leukemia, 417 patients had complete data of both morphology and immunophenotyping. For immunophenotyping, they used two different panels. CD10, CD19, CD22, IgM, CD2, CD7, CD33, TdT, and IgG1were used as old panel and CD10, CD19, CD22, cCD79a, CD2, cCD3, CD7, CD13, CD33, cMPO, CD34, CD45, cIgG1, and cTdT as a new panel. The results of morphology and immunophenotyping were cross-tabulated. Kappa scores increased from 0.43 (moderate) with a previous panel to 0.82 (very good) with a new panel. With the old panel, from morphological ALL cases (n=86), 5 (5.8%) were categorized as AML by immunophenotyping. From 13 AML cases by morphology, 7 (54 %) were categorized as ALL by immunophenotyping. With the new panel, from 239 morphology defined ALL cases, 9 (3.8%) samples were categorized as AML by immunophenotyping. From 79 AML patients as determined by morphology, 12 (15%) were categorized as ALL (9 B-lineage and 3 T-lineage ALL) by immunophenotyping [46].

A Study conducted by Jha S.C *et al* assessed flow cytometric evaluation, morphological, and cytochemical correlation of 150 cases of acute leukemia. As a result, AML, B-ALL and T-ALL comprised 38%, 49%, and 13% of all cases. Almost all blasts were expressing dimCD45 with no

significant differences between the subtypes. CD34 was expressed differentially in AML subtypes, usually negative in APML. Aberrant expression of CD7 and CD19 were foundin 5% and 3.4%of all cases of AML respectively. In 40% cases, morphology and cytochemical studies clinched the diagnosis. Nonetheless, 60% cases essentially needed flowcytometric evaluation for diagnosis and subtyping of acute leukemias [47].

In Iraq, a prospective study was done in a total of 122 ALL patients by Jawad R.T *et al* Of them, 122 patients 89(73%) were positive for PAS stain, 33(27%) patients were negative. All 122 patients were negative for SBB. The patients further sub-classified into L1, L2, and L3. The PAS positivity in ALL-L2 was 84%, and hence served as an additional valuable criteria in distinguishing different FAB types; L3 was rarely positive [48].

In Korea a study conducted by Kang E.S *et al* determined the sensitivity and specificity of cytochemical staining through examining the blasts from 94 patients with newly diagnosed acute leukemia by morphology, cytochemical stains and immunophenotypic markers between September 1989 and July 1992. By surface phenotyping, 41 patients were classified into lymphoid leukemia, 51 into myeloid leukemia and two into mixed lineage. The PAS positivity was found in 36 of 41 ALL and in 29 of 53 AML and the mixed lineage leukemia. However, PAS-positive lymphoblastic leukemias were negative in other cytochemical stains: MPO, SBB, α- naphthol chloroacetate and α-naphthyl butyrate esterase. The PAS- positive myeloblastic leukemias were positive in at least one other stain except three cases. Thus, the sensitivity of combination of PAS positivity and MPO, SBB, and ANBE negativity in diagnosis of lymphoblastic leukemia was 52%, same as PAS alone. However, the specificity was 94%, significantly higher than PAS alone, 53%. These results suggest that this combination of cytochemical staining is useful for distinction of lymphoblastic from myeloblastic leukemia. Eleven (12%) were negative for all cytochemical stainings, while immunophenotyping grouped 6 cases into myeloid lineage and the remaining 5 into lymphoblastic [49].

In Ethiopia, Shamebo M *et al* studied eighty-two consecutive cases of acute leukemias in adult Ethiopians admitted to the Tikur Anbessa (Black Lion) Hospital, a teaching and referral hospital in Addis Ababa, Ethiopia, from January 1982 to December 1992. The age range was 13-78

(mean 29.6) years. The male to female ratio was 1.6:1. Acute myeloblastic (AML) and acute lymphoblastic (ALL) leukemias occurred in 53.7% and 46.3%, respectively [50].

Taken together, studies reviewed above demonstrated no single method is sufficient by itself and combination of methods improves diagnostic accuracy. In Ethiopia, there are no published reports on the correlation of morphology, cytochemistry, and flow cytometry. A flow cytometer is available in many health facilities like Regional Laboratories, referral hospitals and research centers in the country. TASH being the only center where patients with hematological malignancies are being treated, hence evaluating the usefulness of techniques other than a morphological examination on Wright's/ Giemsa stained smears and establishing them in this premier hospital is mandatory. This study tries to address the usefulness morphology and cytochemistry in collaboration with flow cytometry for diagnosis of acute leukemia and contribute to fill this gap.

3. Objectives

3.1. General Objective

- To evaluate the correlation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

3.2. Specific Objective

- To determine the proportion of concordance and discordance between morphology and cytochemical stain for diagnosis of acute leukemia.
- To determine the proportion of concordance and discordance between cytochemical stain and flow cytometry for diagnosis of acute leukemia.
- To determine the proportion of concordance and discordance between morphology and flow cytometry for diagnosis of acute leukemia.
- To determine agreement between morphology, cytochemical staining and flow cytometry for diagnosis of acute leukemia.

3.3. Hypothesis

- There will be a strong agreement between morphology, cytochemical stain, and flow cytometry for diagnosis of acute leukemia.

4. Method and Materials

4.1. Study Design

The Prospective cross-sectional study was conducted to evaluate the correlation between morphology, cytochemistry, and flow cytometry for the diagnosis of Acute Leukemia.

4.2. Study Area

The study was conducted in Tikur Anbessa Specialized Hospital (also known as Black Lion Hospital), which is one of the oldest, the largest and the sole cancer referral hospital in Ethiopia. Affiliated with the Addis Ababa University's College of Health Sciences, Black Lion Hospital is the training center for undergraduate and postgraduate medical students, dentists, nurses, pharmacists, Laboratory technicians and others. The hospital was constructed in 1973 according to Ethiopian calendar.

4.3. Study Period

The study was conducted from May 2017 to March 2018.

4.4. Population

4.4.1. Source population

The Source population was all patients who attended at Tikur Anbessa referral hospital during the study period.

4.4.2. Study Population

The study population was Acute Leukemia patients who visit Internal medicine Hematology unit during the study period.

4.4.2.1. Inclusion Criteria

- Patients who were diagnosed with acute leukemia and who had BM morphology result
- Patients who gave written consent to participate in the study
- Patient who was willing to give whole blood
- Both female and male patients
- Patients of all age group and who fulfilled the above inclusion criteria were recruited

4.4.2.2. Exclusion Criteria

- Patients who have the previous history of cancer, chemotherapy and radiation therapy.

4.5. Study Variables

4.5.1. Dependent Variables

The dependent variables are agreement between the different methods in the diagnosis and characterization of acute leukemias as AML, ALL and Bilineage

4.5.2. Independent Variables

- Age
- Sex
- Staining and method types
    - SBB
    - PAS
    - Morphology
    - Flow cytometry

4.6. Sample Size Calculation

Sample size at the required absolute precision level for sensitivity and specificity will be calculated using Buderer's formula

$$n = \frac{(Z_{1-\alpha/2})^2 \times S_N \times (1-S_N)}{L^2 \times Prevalence}$$

Where:

n = required sample size based on sensitivity

S_N = anticipated sensitivity

α= Level of significance (1 – α is the confidence level)

Z1-α/2 = standard normal deviate corresponding to the specified size of the critical region (α), and

L = absolute precision desired on either side of sensitivity or specificity

Then, we use the anticipated sensitivity (S_N) of 95%.

$Z_{1-\alpha/2}$= 1.96

L = 0.07

From previous study conducted in Addis Ababa, prevalence of AL was 53.3% for AML and 46.7% for ALL respectively (23).

Therefore, $n = \frac{(1.96)^2 \times 0.95 \times (1-0.95)}{(0.07)^2 \times 0.537}$

0.182476/0.0026313= 69 with 10% contingency the minimum sample size will be 76

The minimum sample size for this study will be 76 from AL patients.

4.7. Sampling Method

Convenient sampling method was used for this cross-sectional study.

4.8. Data Collection and Processing

Socio-demographic and clinical information of the study participants was obtained using standardized and structured format by the principal investigator. Then, after written consent and assent were obtained from those patients for whom peripheral blood analysis was indicated, trained physicians collected the blood sample. Peripheral blood was delivered immediately to the PI for Flow cytometry and special stain analysis at ALERT/AHRI Laboratory. Morphology result of the study participants was collected from the patient medical record by the data collector. Peripheral blood was routinely stained with PAS and SBB using a standard procedure.

4.9. Laboratory Methods

The laboratory analyses were including the following:

(1) Staining of the peripheral blood with cytochemical stains such as PAS and SBB, and microscopic examination and reporting was done by pathologist.

(2) Immunophenotyping of the peripheral blood sample with specified monoclonal antibodies.

Periodic Acid Schiff staining

For PAS stain, the air-dried PB was first fixed with Formalin-Ethanol then oxidized by periodic acid. The oxidative process results in the formation of aldehyde groupings through carbon-to-carbon bond cleavage. Free hydroxyl groups should be present for oxidation to take place. Oxidation was completed when it reaches the aldehyde stage. The aldehyde groups were detected by the Schiff reagent. A colorless, unstable dialdehyde compound is formed and when treated

with running tap water a final colored product is formed which is magenta colored and diagnostic.
Reagents used for PAS were periodic acid, Schiff reagent, sodium bisulfate, and Mayer's Hematoxylin.

Sudan Black B staining

Sudan Black B (SBB) is a lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils, and some monocytes. It cannot be extracted from the stained granules by organic dye solvents [51].

For SBB stain, following fixation, blood or bone marrow films are immersed in a buffered Sudan black B solution. After rinsing, slides are counterstained with Mayer's hematoxylin. Cells are examined microscopically for the presence of blue-black discrete granulation. Cells committed to the lymphoid pathway display negative staining reactions, whereas myeloid and monocytoid forms display characteristic positive reactions. The SBB usually parallels the MPO stain.
Reagents used were 90% formol alcohol, Sudan black B, 70% alcohol, and Mayer's hematoxylin.

Flow cytometric analysis

For immunophenotyping, PB samples were collected in EDTA tubes and immediately transported to the flow cytometry laboratory and processed within 24hrs. Flowcytometric analysis was done with 4-colour BD FACSCalibur instrument (Becton Dickinson Immunocytometry systems, San Jose, California) at ALERT/AHRI Laboratory.
The technique used for sample acquisition is lysed wash protocol using 100µl of peripheral blood which is collected in a 5ml EDTA tube and the respective antibody is added based on the assigned antibody panel.

Two types of staining techniques were used, surface staining and cytoplasmic staining. For surface antigen staining, 100µlof samples were stained with 10µl of the fluorescently labeled mAb and incubated in the dark at room temperature (RT) for 20 minutes. Following lysis with BD FACSlyzing solution the preparation was incubated at room temperature (RT for 10 min).

The samples were then washed twice by addition of PBS, centrifugation and supernatant decanting.The remaining cells were resuspended in an appropriate amount of PBS and acquisitions were done immediately on a flowcytometer.

For detection of cytoplasmic antigens, 100µlof PB were stained with 10µlof CD45 PerCp-Cy5.5,incubated in the dark at RT for 20 minutes then lysed with BD FACS lyzing solution and incubated at RT for 10 min, prior to two washes with PBS as above. After adding 500µl of permeabilization reagent (Perm-2), the cell suspension was incubated for 10min at RT in the dark, and washed twice with PBS. Then 10µl of the appropriate mAbs were added, vortexed, and incubated for 20 min. The stained cells were then washed once with PBS, resuspended in PBS and analyzed on theflowcytometer immediately. The BD FACSCalibur with Cell Quest Pro was used for all cytometry acquisition and analysis.

The mAbs were used in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll Protein Complex-Cyanine-5.5 (PerCP-Cy5.5) and Allophycocyanin (APC). Different combination of mAb against the following antigens were used: cCD3, cCD79a, cMPO, CD34, CD3, CD4, CD7,CD8, CD14,CD45, CD10, CD19, CD13, CD33, CD14, CD56, HLA-DR, TdT, and CD117. The immunophenotyping was performed on the flow cytometer. The cells were analyzed with the most appropriate blast gate using the combination of side scatter and CD45 expression. An antigen was considered positive when the expression is at least 20% of the gated cells. The following antibody panel was used for Immunophenotyping.

1. Dako-CD8 CD4 CD3 Dako(TC 660) with BD CD45 PerCP-Cy5.5 (BD 564105)
2. Dako-CD19 CD56 CD3 Dako(TC 662) with BD CD7 PerCP-Cy5.5 (BD 561602)
3. Dako-cMPO cCD79a cCD3 Dako (TC667) with BD CD45 PerCP-Cy5.5 (BD 564105)
4. Dako-CD33 CD34 CD117 Dako (TC 686) with BD CD45 PerCP-Cy5.5 (BD 564105)
5. Dako-CD13-FITC (Dako F0831); BD HLA-DR-APC; Dako CD10-PE, BD CD45 PerCP-Cy5.5 .
6. Dako TdT-FITC(F7139), BD-CD7-PE (332774) ; BD HLA-DR-APC (559866) ; BD CD45 Per-CPCy5.5
7. CD45-FITC and CD14 PE(BD leukogate)

8. Isotype controls, IgG1-APC (345818), BD-IgG1-FITC (345818); IgG1-PE (340013); with CD45 PerCP-Cy5.5 for surface stain
9. Isotype controls, IgG1-APC (345818) 100ug, BD IgG1-FITC (345818); IgG1-PE (340013); with CD45 PerCP-Cy5.5 for cytoplasmic stain.

Gating of blasts

For the immunophenotypic analysis of acute leukemia, CD45 expression in combination with SSC was used for gating of blasts. Typically leukemic blasts show reduced expression of CD45 and allow their identification independently of most normal blood cell subsets [52].

Blasts were identified based on side scatter and their dim expression of CD45, strong positivity for CD34 and HLA-DR, and the linage specific Markers.

More than 20% expressions of any antigen were considered as positive. The threshold for positivity was set based on isotope controls. ALL was sub-classified principally based on the expression of B cell markers CD10, CD19, and cCD79a and T cell markers CD3, CD4, CD8, cCD3 and CD7. AML was principally identified by the expression of MPO, CD13 and CD33 and CD117.

4.10. Quality Control

All the quality control measures were undertaken before starting the procedure for cytochemical stain and flow cytometry. Positive and negative control slides were used for the cytochemical stains. For the flow cytometry, BD FACSCalibur Instrument settings were performed by using BD-CD3-FITC, BD-CD8-PE, BD-CD45PerCP-Cy5.5, CD14-PE/CD45-FITC (BD-leukogate,) BD-CD3 APC. Control normal blood was used in every experiment to define machine settings (optical alignment, light scatter, photomultiplier voltage and spectral overlap compensation), antibody integrity and as a reference for normal monocytes, lymphocytes, and neutrophils, and in most experiments as a control for cytoplasmic staining for cMPO, cCD79a and cCD3. Surface and cytoplasmic protocols were used with negative isotype controls for all surface or cytoplasmic panels for all patient subsets. All protocols were strictly followed and acquisition and analysis were performed at AHRI under the supervision of a senior flow cytomter expert and discussion with advisors and collaborators.

4.11. Data Processing and Analysis

Data were entered and analyzed using SPSS software version 20.0 and different variables were analyzed. Frequencies were used for presenting socio-demographic data and for the morphologic and flow cytometry results. Cross tab was calculated to measure the association between morphology and cytochemistry and between morphology and flow cytometry. Kappa value was calculated to show the measure of agreement between flow cytometry and morphology. The significant level was based on the p-value of less than 0.05.

4.12. Data Interpretation

Data interpretation and reporting of the cytochemical staining of peripheral blood were done by pathologists. The cytochemical staining was confirmed as positive if more than 3% of blast cells are SBB or PAS positive. The data of flow cytometry were identified based on the CD antigen expressed on the cell surface. Results were obtained by gating CD45 Per-CP versus side-scatter and then the respective cell populations were analyzed. The flow cytometry results were interpreted by the principal investigator in collaboration with senior scientist.

4.13. Ethical Considerations

Ethical clearance was obtained from departmental research and ethics review committee of the department of pathology. This study was part of an ongoing previous study cleared by AHRI. Written informed consent and assent form as needed was obtained from each participant. Results and any information regarding patients were kept confidential during and after the completion of the research project by password protected electronic and locking hard copy files. Data generated were immediately used for patient care for free.

4.14. Dissemination of Results

The finding of this project will be publicly defended and submitted to Armauer Hansen Research Institute and Addis Ababa University, College of Health Sciences, School of Medicine, and Department of pathology. The results of the whole study will be disseminated to the concerned health institutions. This scientific work will be published in peer review journals and will be accessible for further study. Study findings will also be presented at scientific conferences.

4.15. Authorship and Intellectual Property Right

The authorship right is for the PI, advisors, and collaborators.

4.16. Operational Definition

Immunophenotyping: is a technique used to analyze heterogeneous populations of cells for the purpose of identifying the presence and proportions of the various protein expressed by cells.

Flow cytometry: is the measurement of cellular properties as they are moving in a fluid stream (flow), past a stationary set of detectors.

Antigen: Any substance capable, under appropriate conditions, of inducing a specific immune response and reacting with the products of that response that is with a specific antibody specifically.

CD: is marker used for the identification and investigation of cell surface molecules providing target for immunophenotyping of cells.

5. Work Flow

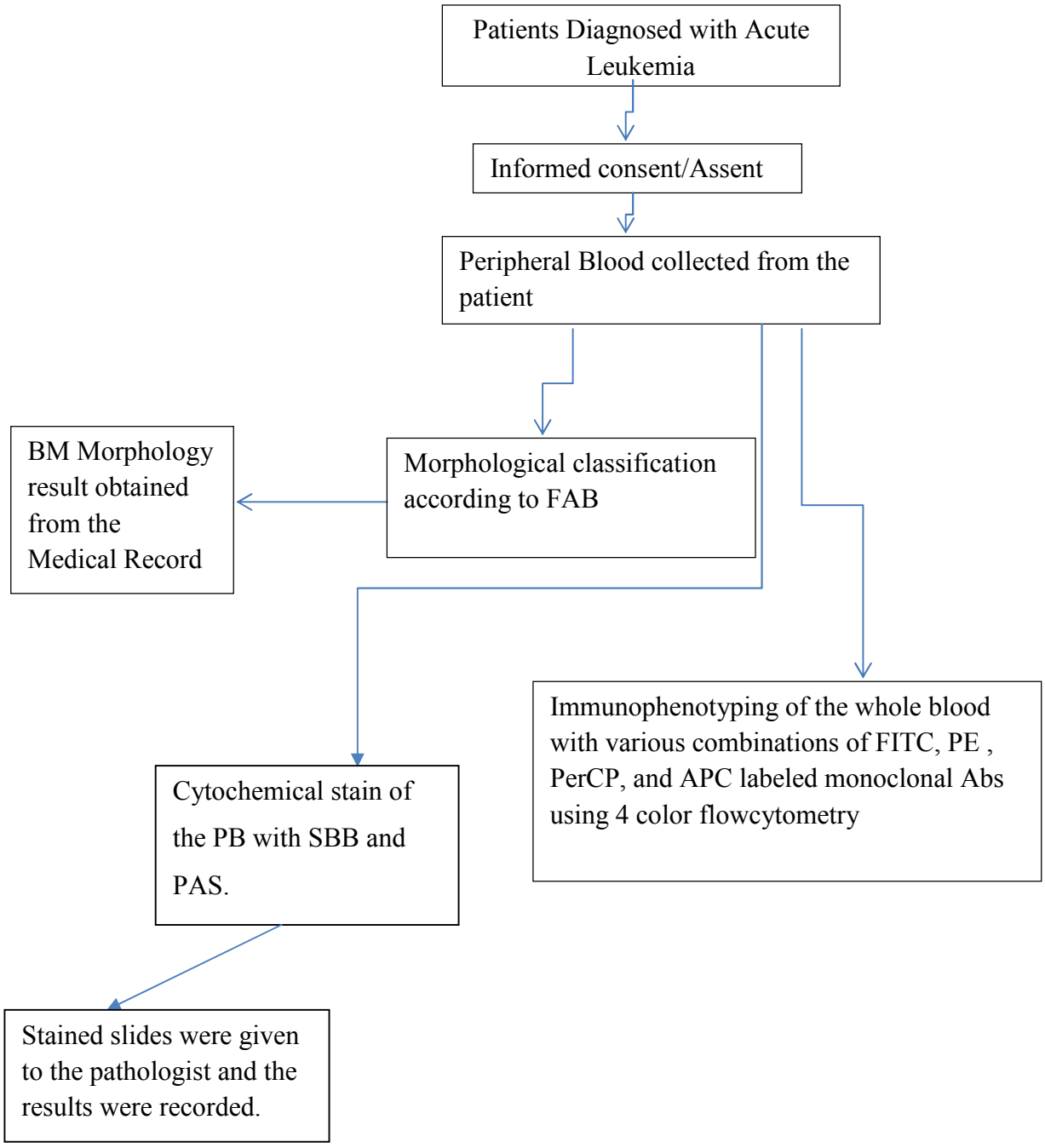

Figure 1: Study workflow

6. Results

6.1. Socio-demographic characteristics

A total of 40 participants diagnosed with acute leukemia based on bone marrow morphology were recruited from Tikur Anbessa Specialized Hospital (TASH). Of them, 26 participants (65%) were male and 14 (35%) were female and majority (72.5%) were from rural areas. Ten participants (25%) were in the age group 0 to 15 years, 19 participants (47.5%) were in the age group 16 to 30 years, 6 participants (15%) were of age group 31 to 45 years and 5 participants (12.5%) were above 45 years as shown in Table 1.

Table 1: Socio-demographic characteristics of the study participants, TASH, Addis Ababa, Ethiopia, 2018 (n=40).

Variables		**Number**	**Percent**
Sex	Male	26	65
	Female	14	35
Age* (Years)	0-15	10	25
	16-30	19	47.5
	31-45	6	15
	>45	5	12.5
Residency	Rural	29	72.5
	Urban	11	27.5

6.2. Morphology and Flow Cytometry Finding

Morphologic and flow cytometric classification of leukemia is summarized in Table 2. Based on Flow cytometry, 14(35%) of the 40 cases, were classified as ALL, 25(62.5%) were classified as AML and 1(2.5%) case was classified as Bi-lineage. Based on BM morphology, of these 40 cases, 15(37.5%) were classified as ALL and 21 (52.5%) were classified as AML. Whereas, 4(10%) cases remained unclassified.

Table 2: Classification of Acute leukemia (ALL and AML) using flow cytometry and morphology, TASH, Addis Ababa, Ethiopia, 2018 (n=40).

	Flow cytometry		Morphology	
	Number	Percent	Number	Percent
ALL	14	35	15	37.5
AML	25	62.5	21	52.5
AL	0	0	4	10
Bilineage	1	2.5	0	0
Total	40	100	40	100

AL= Acute Leukemia, ALL=acute lymphoblastic leukemia, AML=Acute myeloid leukemia

6.3. Cytochemical staining Versus Morphology

Morphology detected 15 ALL cases; of these lymphoblasts only 2 cases (13.3%) showed PAS reactivity. In addition, 8(53.3%) of ALL cases showed SBB reactivity. And of 21 AML cases based on morphology, 19(90.5%) showed positivity for SBB. And out of four unclassified cases, 3(75%) showed positivity for SBB (Table 3). Fig 2 &3 shows PAS positive lymphoblasts and SBB positive myeloblasts respectively of our case.

Table 3: Results of the cytochemical staining versus morphology result of the study participants at TASH, Addis Ababa, Ethiopia, 2018 (n=40).

	PAS		SBB		Total
	Negative	**Positive**	**Negative**	**Positive**	
ALL	13(86.7%)	2(13.3%)	7(46.7%)	8(53.3%)	15(100%)
AML	21(100%)	0	2(9.5%)	19(90.5%)	21(100%)
AL	4(100%)	0	1(25%)	3(75%)	4(100%)
Total	38(95%)	2(5%)	10(25%)	30(75%)	40(100%)

AL= Acute Leukemia, ALL=acute lymphoblastic leukemia, AML=Acute myeloid leukemia, SBB=Sudan BlackB, PAS=Periodic acid Schiff

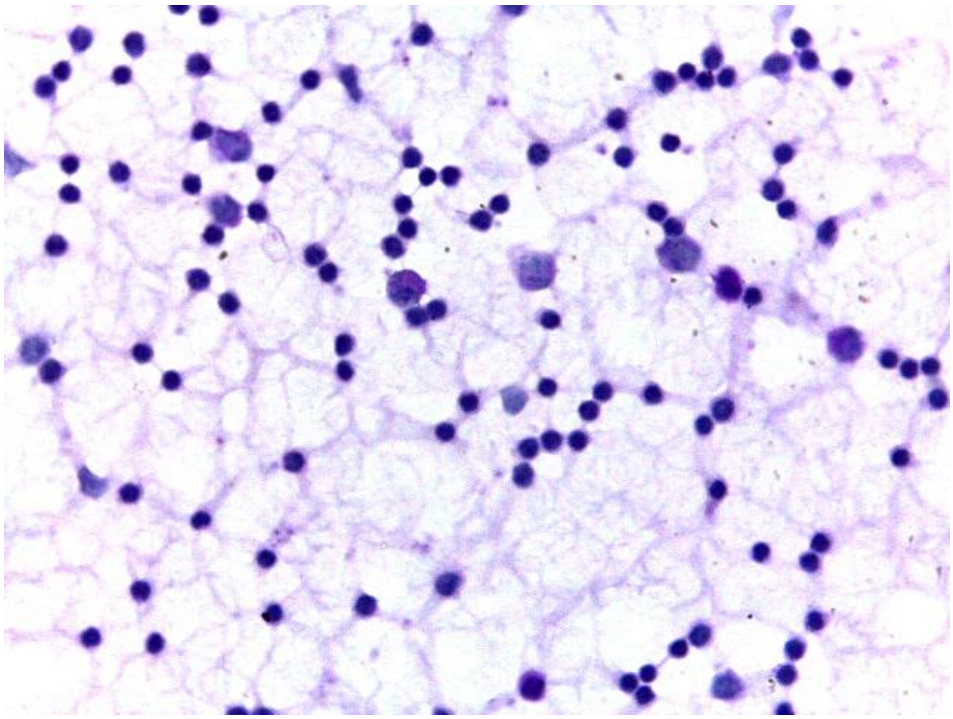

Fig 2: PAS positive lymphoblasts

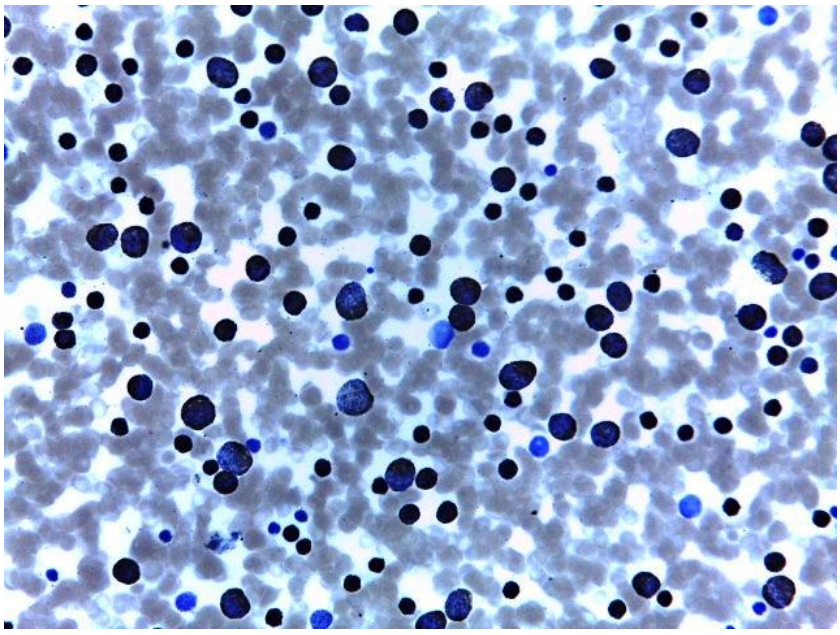

Fig 3: SBB positive myeloblasts

6.4. Cytochemical staining Versus Flow cytometry

As shown in Table 4, flow cytometry detected 14 ALL cases, and of these lymphoblasts only 2 cases (14.3%) showed PAS reactivity. In addition, 6(42.9%) of ALL cases showed SBB reactivity. And out of 25 AML cases based on flow cytometery, 23(92%) were positive for SBB. One Bi-lineage leukemia (T-ALL and AML), was SBB positive.

Out of 7 B-ALL cases, only one case (14.3%) showed PAS reactivity. In addition, 3(42.9%) of B-ALL cases showed SBB reactivity. And similarly, out of 7 T-ALL cases, only one case (14.3%) showed PAS reactivity and 3(42.9%) showed SBB positivity (Table 5).

Table 4: Results of the cytochemical staining versus Flow cytometry result, of the study participants, TASH, Addis Ababa, Ethiopia, 2018 (n=40).

	PAS		SBB		Total
	Negative	**Positive**	**Negative**	**Positive**	
ALL	12(85.7%)	2(14.3%)	8(57.1%)	6(42.9%)	14(100%)
AML	25(100%)	0(0%)	2(8%)	23(92%)	25(100%)
Bi lineage	1(100%)	0(0%)	0(0%)	1(100%)	1(100%)
Total	38(95%)	2(5%)	10(25%)	30(75%)	40(100%)

ALL=acute lymphoblastic leukemia, AML=Acute myeloid leukemia, SBB=Sudan Black B, PAS=Periodic acid Schiff

Table 5: Results of the cytochemical staining versus Flow cytometry result for ALL cases, of the study participants, TASH, Addis Ababa, Ethiopia, 2018 (n=14).

	PAS		SBB		Total
	Negative	**Positive**	**Negative**	**Positive**	
T-ALL	6(85.7%)	1(14.3%)	4(57.1%)	3(42.9%)	7(50%)
B-ALL	6(85.7%)	1(14.3%)	4(57.1%)	3(42.9%)	7(50%)
Total	12(85.7%)	2(14.3%)	8(57.1%)	6(42.9%)	14(100%)

ALL=acute lymphoblastic leukemia, AML=Acute myeloid leukemia, SBB=Sudan Black B, PAS=Periodic acid Schiff

6.5. Flow cytometric Immunophenotyping

Flow cytometric immunophenotyping identified AML in 25(62.5%) cases, B lineage ALL in 7 (17.5%) cases, T lineage ALL in 7 (17.5%) cases, and Bi lineage in 1 (2.5%) case. In AML, all cases expressed cMPO while CD117, CD33, CD13, and TdT were present in 84%, 80%, 72%, and 20% respectively. Aberrant expression of T cell antigens (CD4 and CD7) was observed in 12(48%) and 5(20%) cases, respectively. And also the expression of B cell antigens (CD19 and cCD79a) was observed in 2(8%) of AML cases, while the progenitor cell markers CD34, and HLA-DR were expressed in 17 (68%) each.

In T lineage ALL, cCD3, and CD7 were expressed in all cases, followed by CD8, TdT, CD4, CD3, and HLA-DR in 3(42.9%), 3(42.9%), 2 (28.6%), 2 (28.6%), and 2(28.6%) cases, respectively. The expression of myeloid-associated antigens (CD33, CD117, CD13, and cMPO) was observed in 1(14.3%), 1(14.3%), 2(28.6%) and 3(42.9%) cases, respectively. Progenitor cell markers CD34was expressed in 3(42.9%) cases.

In B lineage ALL, CD19, CD10, cCD79a and TdT were expressed in all cases. Myeloid-specific markers CD33 and cMPO were present in 1(14.3%) and 2(28.6%) subjects, respectively. The T and monocyte marker CD14 was observed in 2(28.6%) of cases. Finally, the progenitor cell marker CD34 was expressed in 4(57.1%) of cases. The HLA-DR marker is a progenitor marker for the myeloid lineage, but is expressed by all immature and mature B cells and B cell ALL.

In Bilineage leukemia, one population was positive for T-cell markers (CD3 and CD4) and the other was positive for AML markers(CD33, CD13, and MPO). Immunophenotypic profile of the 40 AL study participants is summarized in Table 6.

Table 6: Immunophenotypic profile of the 40 AL study participants, TASH, Addis Ababa, Ethiopia, 2018.

	AML (N=25)	B- ALL(N=7)	T-ALL (N=7)	Bilineage (N=1)
CD3	0 (0%)	0 (0%)	2 (28.6%)	1 (100%)
CD4	12 (48%)	2 (28.6%)	2 (28.6%)	1 (100%)
CD8	0 (0%)	0 (0%)	3 (42.9%)	0 (0%)
CD19	2 (8%)	7 (100%)	0 (0%)	0 (0%)
CD56	2 (8%)	0 (0%)	0 (0%)	0 (0%)
CD33	20 (80%)	1 (14.3%)	1 (14.3%)	1 (100%)
CD34	17 (68%)	4 (57.1%)	3 (42.9%)	0 (0%)
CD117	21 (84%)	0 (0%)	1 (14.3%)	0 (0%)
CD13	18 (72%)	0 (0%)	2 (28.6%)	1 (100%)
CD10	0 (0%)	7 (100%)	0 (0%)	0 (0%)
HLA-DR	17 (68%)	7 (100%)	2 (28.6%)	0 (0%)
TdT	5 (20%)	7 (100%)	3 (42.9%)	0 (0%)
CD7	5 (20%)	0 (0%)	7 (100%)	0 (0%)
cMPO	25 (100%)	2 (28.6%)	3 (42.9%)	1 (100%)
cCD79a	2 (8%)	7 (100%)	0 (0%)	0 (0%)
cCD3	0 (0%)	0 (0%)	7 (100%)	0 (0%)
CD14	0 (0%)	0 (0%)	0 (0%)	0 (0%)

ALL= Acute lymphoblastic leukemia, AML= Acute myeloid leukemia, T-ALL= T lineage acute lymphoblastic leukemia, B-ALL= B lineage acute lymphoblastic leukemia, CD= Cluster of differentiation, cCD3= Cytoplasmic cluster of differentiation, TdT= Terminal Deoxynucleotidyl Transferase, HLA-DR= Human leukocyte antigen-D related, MPO= Myeloperoxidase

6.6. Gating leukemia cells and use of Isotype control

In Fig 4, Flow cytometric finding are shown for one panel of our case. Leukemia cells are identified in the black polygon; typically leukemic blasts show reduced expression of CD45. This panel includes CD33 FITC, CD34 PE, CD45 PerCP-Cy5.5 and CD117 APC. The expressions of these markers are represented in blue circles, according to either of two gates. In the middle set of plots (dashed blue circles), all cells within the leukocyte gate (R1) are being analyzed, whereas, with the lower set of plots (solid blue circles), only cells corresponding to the leukemia-specific gate (black polygon, R8) are visualized.

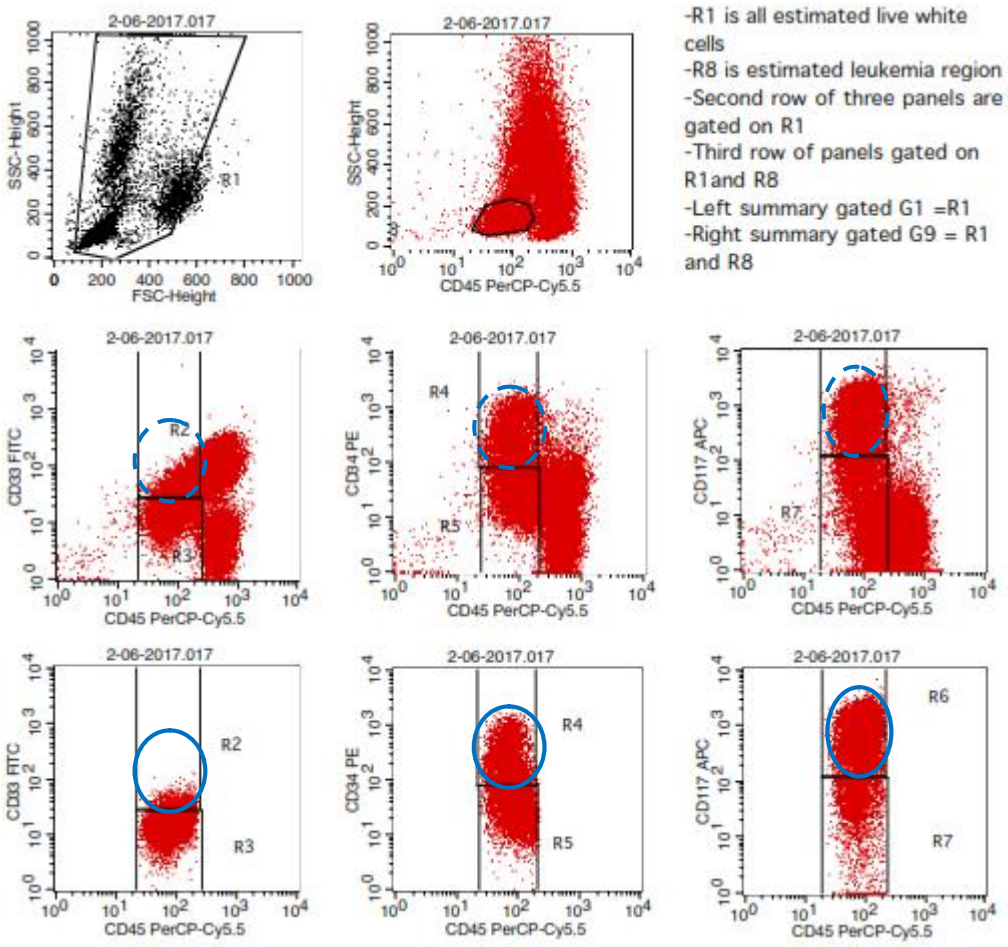

Fig 4: Example of AL immunophenotype.

The definition of marker-positive and marker-negative cells depends on regions defined by the isotype control panel, shown in Fig 5. Isotype controls are an antibody without specificity for human cells and are utilized to define the level of background (non-specific staining) staining. Non-specific staining can occur for multiple reasons, including the binding of antibodies by their constant Fc region rather than their unique antigen binding region, to Fc receptors on cell surfaces.

Fig 5 represents isotype control antibody staining by anti-CD45 stained leukemia cells. The leukemia cells are again identified by the cells within the CD45 intermediate black polygon (R8). The regions R2, R4, and R6 are then defined as marker-positive regions (but negative for the isotype controls), whereas the regions R3, R5, and R7 are defined as negatively staining cells,

expected to approach 100% among the isotype control panel. Once such isotype control regions are defined, they are then identically applied to all other marker panels. Note the identity in the y-axis positions of regions R2 through R7 between Fig 4 and Fig 5.

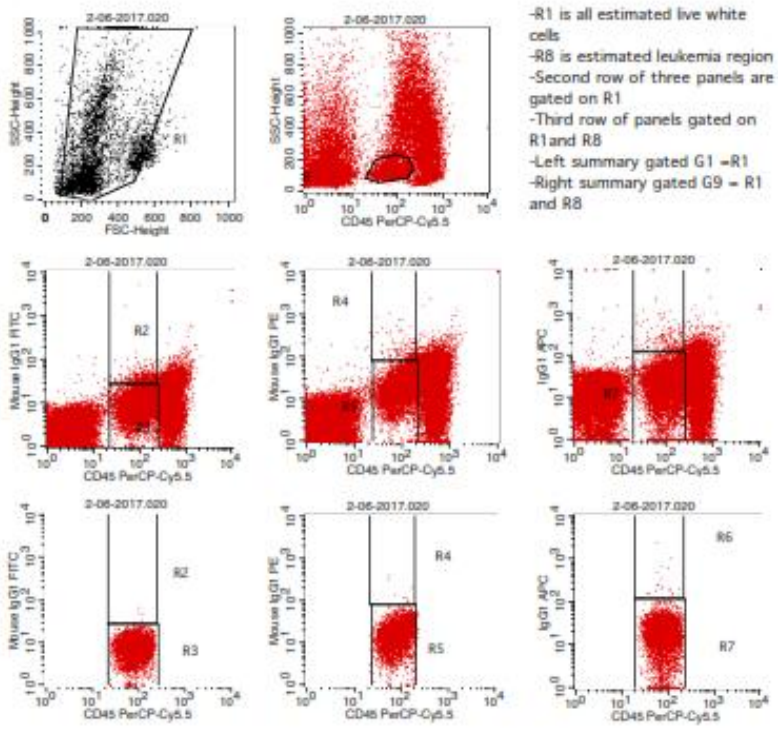

Fig 5: Example of Isotype control (IgG antibody) staining

6.7. Correlation of Morphologic diagnosis versus Flow diagnosis

Table 7 summarizes correlation between morphological diagnoses from BM versus flow cytometry findings. Flow cytometry and morphology showed an agreement in 12/15(80%) of ALL cases, i.e. 3/15(20%) of the morphology results were discordant with FCA result. Of these three discordant cases, one was reported as Bilineage leukemia (T-ALL and AML) and the others as AML by flow cytometry.

In AML, flow cytometry and morphology were in agreement in 19/21(90.5%) of the cases, i.e. 2/21(9.5%) of the morphology results were discordant with FCA result. The two discordant cases were reported as ALL by flow cytometry.

Four cases were unclassified based on morphology; they were reported as acute leukemia. With flow cytometry, they were reported as AML (Table 7) and with cytochemistry, three cases were positive for SBB as displayed in Table 2 earlier.

Table 7: Correlation of morphologic diagnosis versus flow diagnosis in the 40 cases of the study participants, TASH, Addis Ababa, Ethiopia, 2018 (n=40).

		Morphology			Total
Flow cytometry		ALL	AML	AL	
	ALL	12	2	0	14
	AML	2	19	4	25
	Bilineage	1	0	0	1
Total		15	21	4	40

AL= Acute Leukemia, ALL=acute lymphoblastic leukemia, AML=Acute myeloid leukemia

6.8. Results with discrepancy between Flow cytometry and Morphology

Table 8 summarizes results with a discrepancy between Flow cytometry and morphology, the lineage-specific markers as well as aberrant expressions. In total, there were nine discrepancies between flow cytometry and morphology result. Two cases of T-ALL were defined by flow cytometry which had been classified as AML by morphology. Both cases were strongly positive for cCD3 and also expressed other T cell markers. However, it also aberrantly expressed some myeloid antigens including MPO, CD117, and CD13. Six cases of AML were diagnosed by flow cytometry which had been classified as ALL (2) and AL (4). Most cases were positive for CD13, CD33, and MPO. On the other hand, aberrant expressions of lymphoid antigen were seen including CD4, CD19, and CD7. Moreover, one Bi-lineage case was found based on flow cytometry which was ALL based on morphology. It was positive for sCD3, CD4, CD13, CD33, and MPO. The discrepancies may be due to aberrant expression of markers.

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Table 8: Results with a discrepancy between Flow cytometry and morphology, TASH, Addis Ababa, Ethiopia, 2018.

Morphology Result	**FCA Result**	**Cytochemistry Result**	**Lineage specific markers**	**Aberrant expression**
ALL	AML	SBB+/PAS -	CD13, CD33, MPO, CD34, HLA-DR	CD4
ALL	AML	SBB+/PAS -	MPO, CD34, CD117, HLA-DR	None
AL	AML	SBB -/PAS -	CD13, CD33, MPO, TdT	None
AL	AML	SBB+/PAS -	CD13, CD33, MPO, CD34, CD117, HLA-DR	CD19, CD7
AL	AML	SBB+/PAS -	CD13, CD33, MPO, CD34, CD117	CD4
AL	AML	SBB+/PAS -	CD13, CD33, MPO, CD34, CD117, HLA-DR, TdT	CD4
AML	T-ALL	SBB+/PAS -	CD7, cCD3, CD34, HLA-DR, TdT	CD13, MPO, CD117
AML	T-ALL	SBB-/PAS -	CD7, cCD3, CD8, CD13, CD34	MPO
ALL	Bi lineage	SBB+/PAS -	CD3, CD4, CD13, CD33, MPO	None

6.9. Agreement between Morphologyand Flow cytometry

Interpretation of Kappa Agreement

< 0 Less than chance agreement

0.01–0.20 Slight agreement

0.21– 0.40 Fair agreement

0.41–0.60 Moderate agreement

0.61–0.80 Substantial agreement

0.81–0.99 Almost perfect agreement [53].

The kappa value of morphology and flow cytometry was 0.584 (Table 9) which shows that the agreement level between these two methods was moderate based on the above interpretation criteria.

Furthermore, since $p = .000$ (which actually means $p < .0005$), our kappa (κ) coefficient is statistically significantly different from zero.

Table 9: Agreement level between morphology and flow cytometry results in the 40 cases of the study participants, TASH, Addis Ababa, Ethiopia, 2018.

Agreement		Std . Err.	Z	Prob>Z
	Kappa			
77.5%	0.584	0.110	4.487	0.0000

7. Discussion

In this cross-sectional study, FCA and cytochemistry were done in 40 cases of acute leukemia at TASH and compared with their morphologic diagnosis. Based on the BM morphology, 15(37.5%) were classified as ALL and 21 (52.5%) as AML. 4(10%) cases remained unclassified. With Flow cytometry, 14(35%) were classified as ALL, 25(62.5%) as AML and 1(2.5%) case as Bi-lineage. Out of the 14 cases of ALL, there were 7 (50%) B lineage ALL cases and 7 (50%) T lineage ALL cases.

In our study predominant cases were of AML (52.5% based on morphology and 62.5% based on FCA) which correlates well with 53.7% findings of Shamebo *et al* in the same hospital [50], 51.5% Patel *et al* [44], 68.9% Salem *et al* [45], 51.8% Abbasi *et al* [43], and 54.3% Kang *et al* [49]. Conversely, Belurkar *et al* [32] showed a predominance of ALL 68%.

Cytochemical staining Versus Morphology or FCA

In the present study, morphology detected 15 ALL cases, 13.3% showed PAS reactivity and 53.3% showed SBB positivity. And also flow cytometry detected 14 ALL cases, of these 14.3% showed PAS reactivity and 42.9% showed SBB reactivity.

Biren *et al* [38] demonstrated a frequency of PAS positive ALL (28%) which was slightly higher than our study. In the present study, SBB positive ALL was high in comparison to 0.96% reported by Biren *et al* [38] and 13.3% by Deghady *et al* [39].

In the current study, out of 21 AML confirmed cases based on morphology, 90.5% showed positivity for SBB. Additionally, out of 4 unclassified cases, 3(75%) cases were only positive for SBB. Furthermore, out of 25 AML confirmed cases based on a flow cytometry, 23(92%) showed positivity for SBB. We observed one Bi-lineage (T-ALL and AML), which was SBB positive.

In the study of Belurkar *et al* [32] 91.6% of AML cases were SBB positive which is consistent with our current study. However, several studies showed SBB positivity lower than our results, including, 72.8% by Biren *et al* [38], 78.2% by Liqaa *et al* [37], 75% by Kang *et al* [49], and 82.9% by Resende *et al* [41]. Conversely, Deghady *et al* [39] showed 100% SBB positive AML cases which is slightly higher than our current study.

In our study, myeloblasts of all AML cases were negative for PAS stain which is similar to the findings of Liqaa *et al* [37] and Ahirwar *et al* [35]. Conversely, frequencies of PAS-positive AML in Deghady *et al* [39] and Kang *et al* [49] were 33.3% and 55%, respectively.

Flow Cytometric Immunophenotyping of AML

In our study, all cases of AML expressed cMPO while CD117, CD33, CD13, and TdT were present in 84%, 80%, 72%, and 20% respectively. Aberrantly, the expression of T cell antigens (CD4 and CD7) was observed in 12(48%) and 5(20%) cases, respectively. Furthermore, the expression of B cell antigens (CD19 and cCD79a) was observed in 2(8%) cases each. In AML, the progenitor cell markers CD34, and HLA-DR were expressed in 17 (68%) each.

Similarly, Selicean *et al* showed that, among their 53 AML cases, the most frequently expressed markers were: CD13 (83.02%), CD33 (81.13%), CD117 (75.47%), and cMPO (54.72%). CD7 was present in 15.1% of all cases. Progenitor cell markers CD34, TdT, and HLA-DR were expressed in 79.25%, 3.77% and 49% of all cases, respectively [34].

Additionally, Shrestha *et al* discovered the most frequently expressed markers in AML were: CD13 (100%), CD33 (90%), cMPO (85%), CD117 (80%), and CD14 (5%). Progenitor cell markers CD34 and HLA-DR were expressed in 75% and 70% respectively. Aberrant expression of lymphoid antigen was seen in 6/20 cases (30 %) of AML including CD7 (5%), cCD79a (5%), and CD22 (20%) [40].

Flow Cytometric Immunophenotyping of T-ALL

In the present study, cCD3, and CD7 were expressed in all cases of T-ALL, followed by CD8, TdT, CD4, CD3, and HLA-DR in 3(42.9%), 3(42.9%), 2 (28.6%), 2 (28.6%), and 2(28.6%) cases, respectively. The expression of myeloid-associated antigens CD33, CD117, CD13, and cMPO was observed in 14.3%, 14.3%, 28.6% and 42.9% of the cases, respectively. Progenitor cell marker CD34was expressed in 3(42.9%) cases.

In a similar study Shrestha *et al* showed out of 11 cases of T-ALL, the positivity rates of stem cell marker CD34 and HLA-DR were 45.4% and 0%, respectively. cCD3, surface CD3, and CD7 were positive in all cases. Aberrant expression of the myeloid antigen CD13 was seen in 1/11 cases (9.1 %) [40].

In the study of Salem *et al*, all T-ALL cases were negative for HLA-DR, and for CD34 which contrasts with our study. Similarly all cases of T-ALL were positive for cCD3 while CD7, CD5, CD3, TdT, CD4, and CD8 were expressed in 92.3%, 84.6%, 38.5%, 76.9%, 30.8% and 53.8% cases, respectively. Only 1 of 13 cases of T-ALL showed CD33 expression and none expressed CD13 [45].

Flow Cytometric Immunophenotyping of B-ALL

Result in this study showed CD19, CD10, cCD79a and TdT were expressed in all B-ALL cases. Myeloid-specific markers like CD33 and cMPO were present in 14.3% and 28.6% of cases, respectively. The T-cell and monocyte lineage-specific marker CD4 was observed in 28.6% of cases. Finally, the progenitor cell marker CD34 was expressed in 4(57.1%) of cases. The HLA-DR marker is a progenitor marker for the myeloid lineage, but is expressed by all immature and mature B cells and B cell ALL.

A similar study was done by Salem *et al* where all cases of B-ALL expressed cCD79a and CD19 while CD20, CD10, and TdT were expressed in 26.3, 89.5 and 97.4% of cases, respectively. B-ALL showed HLA-DR and CD34 expression in 97.4% and 76.3% of cases respectively. A few cases of B-ALL expressed CD33 (10.5%) and CD13 (7.9%) without the expression of cMPO or CD117. None of the B-ALL cases expressed T-cell associated antigens [45].
In the study of Abbasi *et al*, all cases of B-ALL showed expression of pan B-cell markers (CD19, CD22, and cCD79a) and 90% of cases expressed CD10. Aberrant expression of myeloid markers CD13 and CD33 was seen in 7% of B-ALL cases respectively [43].

However, a study by Shrestha *et al* found 20 cases of B- cell lineage ALL, which included 75 % of CD10 positive ALL. All cases of B-ALL showed expression of B-cell markers cCD22 (100%), CD19 (85%), and cCD79a (75%), respectively, which is lower than our study. The positivity of stem cell markers CD34 and HLA-DR was seen in 90% and 85%, respectively. Aberrant expression of myeloid antigen CD13 and CD33 was seen in 15% and 5%, respectively [40].

Flow Cytometric Immunophenotyping of Bilineage leukemia

In the present study, one Bilineage leukemia was found. One population was positive for T-cell markers (CD3 and CD4) and the other was positive for AML markers (CD33, CD13, and MPO). A similar study was done by Shrestha *et al* [40] where one case of biphenotypic acute leukemia showed expression of both cMPO and cCD3 in addition to CD34, HLA-DR, CD13, CD117, CD2, CD3, and CD7.

Correlation of Morphologic diagnosis versus Flow diagnosis

In the current study, complete concordance between morphologic and flow cytometric diagnosis was seen in 31 (77.5%) cases, of which 12/15 (80%) were ALL and 19/21 (90.5%) were AML.

A similar study by Patel *et al* revealed concordant results in 25 (75.6%) cases, whereas in 8 cases the results were discordant; comparisons were done between bone marrow aspirate morphology and immunophenotyping only [44].

A study done by Supriyadi *et al* showed, complete concordance between morphologic and flow cytometric diagnosis in 297 (93.4%) patients which is higher than our study. In their study, 230/239 (96%) were ALL and 67/79 (84.8%) were AML [46].

In a study by Belurkar *et al* complete concordance between morphologic and flow cytometric diagnosis was seen in 29 (58%) cases, of which 17/34 were ALL and 12/15 were AML which is lower than our study [32].

In the current study, the kappa value of morphology and flow cytometry was 0.584 which shows that the agreement level between these two methods was moderate.

A study done by Supriyadi *et al* showed concordance with morphology was very good (κ=0.82) using the three-color method with a panel of 15 monoclonal antibodies (n=387) which is higher than our study [46].

Taken together, our findings and the various studies discussed above showed different levels of concordancy between morphologic and flow cytometric analysis emphasizing for the need to have a combination of methods such as morphology, cytochemistry, and FCA together.

8. Strength and limitation

8.1 Strength

- Control samples from healthy individuals and BD FACS Calibur Instrument setting were performed in an ISO accredited laboratory for flow cytometry.
- Positive and negative controls were used for the cytochemistry stains.
- Cytochemistry results were done by a senior pathologist.
- Flow cytometry was performed with a help of a local specialist.

8.2 Limitation

- The diagnosis of leukemia has moved from evaluation of morphology and cytochemistry to assessment by modern methods such as immunophenotyping, cytogenetics, and molecular characterization. Cytogenetics and molecular characterization were not included in this study.
- Additional cytochemical stains like MPO, Acid phosphatase, oil red O, α- naphthol chloroacetate, and α-naphthyl butyrate esterase were not included in our study but it was used in other studies [32, 49].
- Markers for AML leukemias M6 (erythroleukemia) or M7 (megakaryoblastic leukemia) were not included hence such leukemias if present may have been missed.

9. Conclusion and recommendation

9.1 Conclusion

Cytochemical stains are important in developing countries for the diagnosis of acute leukemia especially in AML due to low cost and simplicity. Our study with SBB positivity in AML correlates well with that of the results obtained by flow cytometry (92%) and morphology (90.5%). However, our study with PAS revealed a low sensitivity of this stain for the diagnosis of ALL cases, a finding corroborating many other studies.

On comparison of morphologic and flow diagnoses, it was found that there was complete concordance in 77.5% cases. Out of 4(10%) unclassified cases based on morphology, the three cases were SBB positive and it was diagnosed as AML by flow cytometry.

In AML, all cases expressed cMPO while CD117, CD33, and CD13 were present in 84%, 80%, and 72% respectively. CD19, CD10, and cCD79a were found to be the most useful markers in the identification of B-ALL, whereas cCD3 and CD7 were consistently expressed by T-ALL.

Flow cytometry is very important in lineage assignment particularly in ALLs, and also helps in confirming the morphologic diagnosis in acute leukemia. It is important to use morphology, cytochemistry, and FCA together in classifying acute leukemia as it greatly influences the treatment and the prognosis.

9.2 Recommendation

- We strongly recommend for further studies to use many more markers and also additional cytochemical stains.
- It will be very helpful if cytogenetics and molecular characterization can be undertaken.

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Annex 1. Participant Information sheet

1. **Study title:**

Correlation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia, 2018: A Prospective cross-sectional study.

2. **Invitation paragraph:**

You have been invited to take part in this research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take a time to read the following information carefully. Ask a question if there is anything that is not clear or if you would like more information.

3. **Introduction of the disease**

Acute leukemia (AL) is a proliferation of immature bone marrow-derived cells (blasts) that may also involve peripheral blood or solid organs and it is characterized by a rapid increase in the number of immature blood cells. Crowding due to such cells makes the bone marrow unable to produce healthy blood cells and causes serious illness. The immunophenotypic categories of acute leukemia are particularly important because they identify distinctive treatment and prognostic groups

4. **The purpose of the study**

I am investigating the Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia. I hope that this will help to understand more about the disease classification and its specific therapy.

5. **Why have you been chosen?**

You are invited to participate in this study as suspected acute leukemia patient. We want to identify and classify the type of acute leukemia by flow cytometry and cytochemistry if acute leukemia is confirmed. In this study, a total of 40 acute leukemia suspected patients will participate.

For Parents/guardian---your child is invited to participate in this study as suspected acute leukemia patient. We want to identify and classify the type of acute leukemia by flow cytometry and cytochemistry if acute leukemia is confirmed. In this study, a total of 40 acute leukemia suspected patients will participate.

6. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign the consent form. You are free to withdraw at any time, without giving a reason. A decision not to take part or to withdraw at any time, will not affect the standard of care you receive.

7. What will happen to me if I take part?

Your role in the study:

If you agree to participate, we will take your morphologic result, your age and current address from your record data. And you will give peripheral blood, which will be collected by inserting a small needle into a vein in your arm.

8. What is the study procedure

If you take part in the research the demographic and clinical investigation data will be assembled by your physician, the morphologic result of your bone marrow aspirate and 5 ml blood will be used for the study.

9. What are the possible benefits of taking part and incentives?

You will not be provided any incentive to take part in this research. If you participate in this research, you may not get any direct benefit but anything found in the study based on your laboratory results will be communicated to you and your physician. In addition, your

participation is likely to help us in the utilization of flowcytometry for the better diagnosis and classification of acute leukemia.

10. What are the possible disadvantages and risks of taking this part?

There is no major risk in participating in this research, but the minor pain and bleeding that may occur during blood collection will be minimized, as the procedure is carried out by trained and experienced health professionals on the standard good clinical practice.

11. Will my taking part in the study be kept confidential?

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept separately in a password protected data management file and it will not be revealed to anyone except the principal investigator and your treating physician. Your personal information will not be disclosed even during the reporting of the findings. Reports will be written and disclosed anonymously.

12. What will happen to any samples I or my child give?

As already described, during the laboratory analysis we will use your or your child given code, not your or your child's name for your sample. The samples are immediately processed and analyzed. If there is any abnormal result, it will immediately be communicated to you and your Doctor, so your child's Doctor will take the appropriate action. The data collected will be written and published in peer-reviewed scientific journals.

13. If there is any abnormal result?

If there is any abnormal result, it will immediately be communicated to you and your child's Doctor, so as the Doctor will take the appropriate action.

14. What will happen to the results of the study?

Data from this study will be analyzed and published in scientific journals but your identity will not be revealed. Data will also be presented at seminars at national meetings. No information containing your name will be disclosed.

15. Who is organizing and funding the project

The cost of this research project is covered by AHRI.

16. Who has reviewed the study?

This study was given a favorable ethical opinion by the Pathology department Ethics committee and by the Addis Ababa university Research Ethics committee.

17. How to give my consent

If you have the interest to take part in this research, the PI or the delegated person will be available at the hematology clinic and will provide you the consent form which you can sign if you agree to participate.

You will be given a copy of the information sheet and a signed consent form to keep

Thank you in advance for considering taking part in this study

Study coordinator and Principal investigator

Kiyya Dessalegn, MSc fellow

Mobile: 0913821401

Annex 2. Information Sheet for Control Group

1. **Study title:**

   Correlation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia, 2018: A Prospective cross-sectional study.

2. **Invitation paragraph:**

You have been invited to take part in this research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take a time to read the following information carefully. Ask a question if there is anything that is not clear or if you would like more information.

3. **Introduction of the disease**

Acute leukemia (AL) is a proliferation of immature bone marrow-derived cells (blasts) that may also involve peripheral blood or solid organs and it is characterized by a rapid increase in the number of immature blood cells. Crowding due to such cells makes the bone marrow unable to produce healthy blood cells and causes serious illness. The immunophenotypic categories of acute leukemia are particularly important because they identify distinctive treatment and prognostic groups.

4. **The purpose of the study**

   I am investigating the Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia. I hope that this will help to understand more about the disease classification and its specific therapy.

5. **Why have you been chosen?**

You are invited to participate in this study you are expected as a healthy individual so as you are selected to participate as a control group for the study.

For Parents/guardian---your child is invited to participate in this study as your child is expected as a healthy individual so that she /he is selected to participate in the study as a control group.

6. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you or your child will be given this information sheet to keep and be asked to sign consent form. You or your child is/are free to withdraw at any time, without giving a reason.

7. What will happen to me if I take part?

Your role in the study:

If you agree to participate, You/your child will be asked to give peripheral whole blood, which will be collected by inserting a small needle into a vein in your /your Childs arm.

8. What are the possible benefits of taking part and incentives?

You/your child will not be provided any incentive to take part in this research. If you participate in this research, you/your child may not get any direct benefit but anything found in the study based on your laboratory results will be communicated to you. In addition, your participation is likely to help us in co-relation between morphology, cytochemistry, and flow cytometry, for the better diagnosis and classification of acute leukemia.

9. What are the possible disadvantages and risks of taking this part?

There is no major risk in participating in this research, but the minor pain and bleeding that may occur during blood collection will be minimized, as the procedure is carried out by trained and experienced health professionals on the standard good clinical practice.

10. Will my taking part in the study is kept confidential?

The information that we collect from this research project will be kept confidential. Information about you/your child that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept separately in a password protected data management file and it will not be revealed to anyone except the principal investigator and physician. Your personal information will not be disclosed even during the reporting of the findings. Reports will be written and disclosed anonymously.

11. What will happen to any samples I or my child give?

As already described, during the laboratory analysis we will use your or your child given code not your or your child's name for your sample. The samples are immediately processed and analyzed. If there is any abnormal result, it will immediately be communicated to you. The data collected will be written and published in peer reviewed scientific journals.

12. If there is any abnormal result?

If there is any abnormal result, it will immediately be communicated to you in order to communicate physcian.

13. What will happen to the results of the study?

Data from this study will be analyzed and published in scientific journals but your identity will not be revealed. Data will also be presented at seminars at national meetings. No information containing your name will be disclosed.

14. Who is organizing and funding the project

The cost of this research project is covered by AHRI.

15. Who has reviewed the study?

This study was given a favorable ethical opinion by the ethical committee of pathology department and by the Addis Ababa university Research Ethics committee.

16. How to give my consent

If you have the interest to take part in this research, the data collector will provide you the consent form which you can sign if you agree to participate.

You will be given a copy of the information sheet and a signed consent form to keep

Thank you in advance for considering taking part in this study

Study coordinator and Principal investigator

Kiyya Dessalegn, MSc fellow **Mobile: 0913821401**

Annex 3. Information sheet Amharic version - የመረጃ ቅጽ ለተሳታፊ

1. <u>የጥናቱመጠሪያ</u>

ለአጣዳፊ የደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪን ዘዴን ማመሳሰል ወይም ማዋቀር::

2. <u>በጥናቱእንድሳተፋስለመጋበዝ</u>

በዚህ ጥናት ላይ እንዲሳተፉ እንጋብዘዎታለን ነገርግን በጥናቱ ከመሳተፍዎ በፊት የጥናቱን አላማና አስፈላጊነት በቅድሚያ መረዳት ያስፈልገዎታል፡፡ እባክዎ ጊዜ ወስደው የሚከተለውን መረጃ ያንብቡ፡፡ ማንኛዉም ጥያቄ ወይም ግልፅ ያልሆነ ነገር አለመጠየቅ ይችላሉ፡፡

3. <u>የበሽታውምንነት</u>

አጣዳፊየደምካንሰርበሽታመቅኔበደንብያልደረሱ(ያላደጉ)
የደምሕዋሣትንያለአግባብሲያመርትይከሰታል::እነዚህዋሳትወደደምዝውውርበመግባትህመምያስከትላሉ::በሽታውንበፍጥ ነትመለየትናአይነቱንመለየትትክክለኛህክምናናመድኃኒትለማግኘትይረዳል፤በተጨማሪምበሽታውየሚያመጣውንህመምናጉ ዳትይቀንሰዋል::

4. <u>የጥናቱዓላማ</u>

እኔ አሁን የማጠናዉ ለአጣዳፊ የደም ካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪን ዘዴን ማመሳሰል ወይም ማዋቀር:: የዚህም ጥናት ዉጤት ስለበሽታው የተሻለ እውቀት እንዲገኝ እንዲሁም የሽታውን አይነት በጊዜ ለመለየትና ለመከላከል በሚደለገዉ ጥረት ጉልህ ድርሻ ይኖረዋል::

5. <u>እርሶለምንበዚህጥናትእንዲሳተፋተመረጡ?</u>

እርሶበዚህጥናትላይእንዲሳተፉየተመረጡበትምክንያትየአጣዳፊየደምካንሰርበሽታተጠርጣሪተጠቂበመሆንዎናበጥናቱሊካ ተቱየሚችሉትበአጣዳፊየደምካንሰርበሽታህመምየተጠረጠሩተጠቂዎችብቻበመሆናቸውነው::በዚህጥናት40የሚሆንበአ ጣዳፊየደምካንሰርህመምየተጠረጠሩተጠቂዎችይሳተፋሉ::

ለአሳዳጊ----

ልጆበዚህጥናትላይእንዲሳተፍየተመረጠበትምክንያትየአጣዳፊየደምካንሰርበሽታተጠርጣሪተጠቂበመሆኑ/ንዋበጥናቱሊካ ተቱየሚችሉትበአጣዳፊየደምካንሰርበሽታህመምየተጠረጠሩተጠቂዎችብቻበመሆናቸውነው::በዚህጥናት40የሚሆንበአ ጣዳፊየደምካንሰርህመምየተጠረጠሩተጠቂዎችይሳተፋሉ::

6. <u>በዚህጥናትላይለመሳተፍየግድያስፈልጋል?</u>

በጥናቱላይለመሳተፍየግድአያስፈልግም፤በፍላጎትላይብቻየተመሠረተነው::በጥናቱላይለመሳተፍከወሰኑይህመረጃናመስ ማማትዎንየሚገልጽቅጽይሰጠዎታል::መረጃውንካነበቡናየሚጠይቁትጥያቄካለምበመጠየቅበሚገባከተረዱበኃላመስማማ

ትዎንይገልጻሉ፡፡ከጥናቱበፈለጉትጊዜናሰዓትያለምንምቅድመሁኔቴማቋረጥይችላሉ፡፡እራስዎንከጥናቱበማግለልዎምክንያ ትለህመምዎየህክምናእርዳታከማግኘትአያግድም፡፡እንደማንኛውምታካሚአስፈላጊውንየህክምናእርዳታያገኛሉ፡፡

7. <u>በጥናቱላይከተሳተፍኩከእኔምንይፈለጋል?</u>

በጥናቱላይለመሳተፍከተስማሙወይምልጆእንዲሳተፍከተስማሙእድሜ፣አድራሻ፣እናየመቅኔምርመራውጤቶትንጥናቱወ ስዶይጠቀማልበተጨማሪምአንድየሾርባማንኪያደምከከንድዎወይምከልጆከንድላይእንዲሰጡይጠየቃሉ፡፡እዚህጥናትላይ ባይሳተፉምቢሳተፉምበህጉመሰረትአስፈላጊውንህክምናያገኛሉ::

8. <u>ከእርስዎምንይጠበቃል?</u>

በጥናቱለመሳተፍከተስማሙዶክተርዎእርስዎንወይምልጆንበመጠየቅየወሰደውማስታወሻለጥናቱይውላልእንዲሁምከላይየ ተገለፀውንየመቅኔምርመራውጤቱንእናደምእንዲሰጡወይምልጆእንዲሰጥይጠበቃል፡፡እነዚውጤቶችእናናሙናዎችለጥና ቱምርምርይውላሉ፡፡

9. <u>በጥናቱላይቢሳተፉጥቅማጥቅምአገኛለሁኝን?</u>

በዚህጥናትላይበመሳተፍዎየተለየጥቅምበግልአያገኙም፡፡በጥናቱወቅትየተገኘውንየላቦራቶሪውጤትለእርስዎናለዶክተርዎ ይገለጻል፡፡እንዲሁምየእርስዎ/የልጆበጥናቱመሳተፍስለበሽታውአይነትያለውንጥሩግንዛቤእንዲኖረንናበሽታውንለማከም ጠቃሚመረጃበመስጠትይጠቅመናል፡፡

10. <u>በጥናቱላይበመሳተፌየሚደርስብኝጉዳትአለ?</u>

ጥናቱላይበመሳተፍዎ/ልጆበመሳተፉየሚደርስብዎ/የሚደርስበትትልቅጉዳትየለም፤ነገርግንየደምናሙናበሚወሰድበትወቅ ትሊፈጠርየሚችለውንአነስተኛህመምናየደምመፍሰስለማስወገድልምድባላቸውናእናስልጠናበተሰጣቸውባለሙያዎችይከ ናወናል፡፡

11. <u>ጥናቱሲያልቅከእኔምንይጠበቃል?</u>

ምንምአይጠበቅም፡፡

12. <u>በዚህጥናትመሳተፍበሚስጥርይያዛልን?</u>

አዎንለዚህጥናትየሚሰበሰበውናሙናእናየናሙናውጤትበሚስጥርይያዛል፡፡ስለእርስዎ/ስለልጆየሚገልጽማንኛውምነገርበና ሙናምሆነበውጤቱላይአይጻፍም፡፡ውጤትሲገለጽስምአልባይሆናል፡፡ለእያንዳንዱናሙናልዩመለያቁጥርወይምምልክትይ ሰጠዋል፡፡የትኛውቁጥርየማንእንደሆነዋናተመራማሪውብቻያውቃል፡፡ስለናሙናዎውጤትከዋናተመራማሪውበተጨማሪ እርሶንየሚከታተለውህኪምምሊያውቀውይችላል፡፡

13. <u>እኔ/ልጄየምለግሰው/የሚለግሰውናሙናምንይሆናል?</u>

የርስዎ/የልጆውጤትናናሙናየተለያቁጥርይሰጠዋል፡፡በናሙናውላይየርስዎወይምየልጆስምአይጻፍም፡፡አብዛኛው ናሙናወዲውኑስራላይይውላል፡፡በጥናትየሚገኘውመረጃበህትመትመልክለጤናባለሙያናለሳይንትስቶችይደርሳል ፡፡ውጤትበጅምላበሚገለጽበትጊዜየማንንምውጤትአይወክልም፡፡

14. <u>ያልተጠበቀየላቦራቶሪዉጤትካለ?</u>

በጥናቱላይየተገኘማንኛውምያልተጠበቀውጤትካለለዶክተርዎወይምልጆንለሚከታተለውዶክተርእንዲሁምለእርስዎይገለ ፃል፤ዶክተሩምሚገባውንህክምናያደርጋል፡፡

15. <u>የጥናቱውጤት?</u>

የጥናቱውጤትበሳይንሳዊመፅሔትላይይታተማልእንዲሁምየጥናቱውጤትበተለያዩስብሰባዎችላይይቀርባል፡፡ነገርግንየ እርስዎማንነትበምንመልኩአይገለፅም፡፡

16. <u>የጥናቱንወጪየሚያወጣው?</u>

የጥናቱወጪየሚደገፈውበአርሞርሀንሰንየምርምርማእከልነው

17. <u>ፈቃደኝነቴንለመግለፅ?</u>

ጥናቱላይየመሳተፍፍላጎትወይምልጆእንዲሳተፍፍላጎትካለዎትከሚታዩበትከፍልከጥናቱዋናአስተባባሪወይምከተወከለ ውሰውየስምምነትቅፅበመውሰድፊርማዎትንበማሰፈርስምምንዎትንማረጋገጥይችላሉ፡፡

18. <u>ይህጥናትተቀባይነትንአግኝቷል?</u>

ይህጥናት በፓቶሎጂ የስነ ምግባር ኮሚቴና በአዲስ አበባ ዩንቨርስቲ የስነ-ምግባር ኮሚቴ ተገምግሞ ተቀባይነትን አገኝቶ ፀድቋል፡፡

<u>ተጨማሪመረጃከፈለጉየሚከተሉትንባለሚያዎችማነጋገርይችላሉ</u>

የጥናቱአስተባባሪናዋናተመራማሪ

ኪያ ደሳለኝ

ስልክቁ. 0913821401

Annex 4. Information sheet in Amharic version for control group

(የመረጃቅጽለኮንትሮልግሩፕ)

1. **<u>የጥናቱመጠሪያ</u>**

ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪን ዘዴን ማመሳሰል ወይም ማዋቀር::::

2. **<u>በጥናቱእንድሳተፋስለመጋበዝ</u>**

በዚህጥናትላይእንዲሳተፋእንጋብዘዎታለንነገርግንበጥናቱከመሳተፍዎበፊትየጥናቱንአላማናአስፈላጊነትበቅድሚያመረዳትያ ስፈልገዎታል::እባክዎጊዜወስደውየሚከተለውንመረጃያንብቡ::ማንኛዉምጥያቄወይምግልፅያልሆነነገርካለመጠየቅይችላ ሉ::

3. **<u>የበሽታውምንነት</u>**

አጣዳፊየደምካንሰርበሽታመቅኔበደንብያልደረሱ(ያላደጉ)
የደምሕዋሣትንያለአግባብሲያመርትይከሰታል::እነዚህዋሳትወደደምዝውውርበመግባትህመምያስከትላሉ::በሽታውንበፍጥ ነትመለየትናአይነቱንመለየትትክክለኛህክምናናመድኃኒትለማግኘትይረዳል፤በተጨማሪምበሽታውየሚያመጣውንህመምናጉ ዳትይቀንሰዋል::

4. **<u>የጥናቱዓላማ</u>**

እኔ አሁን የማጠናዉ ለአጣዳፊ የደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪን ዘዴን ማመሳሰል ወይም ማዋቀር:::: የዚህም ጥናት ዉጤት ስለበሽታው የተሻለ እውቀት እንዲገኝ እንዲሁም የሽታውን አይነትበጊዜለመለየትናለመከላከልበሚደለገዉጥረትጉልህድርሻይኖረዋል::

5. **<u>እርሶለምንበዚህጥናትእንዲሳተፉተመረጡ?</u>**

እርሶበዚህጥናትላይእንዲሳተፉየተመረጡበትምክንያትጤናማኖትተብሎተገምቶነው::

ለአሳዳጊ---- ልጆበዚህጥናትላይእንዲሳተፍየተመረጠበትምክንያትልጆጤናማነው /ናትተብሎተገምቶነው::

6. **<u>በዚህጥናትላይለመሳተፍየግድያስፈልጋል?</u>**

በጥናቱላይለመሳተፍየግድአያስፈልግም፤በፍላጎትላይብቻየተመሠረተነው::በጥናቱላይለመሳተፍከወሰኑይህመረጃናመስ ማማትዎንየሚገልጽቅጽይሰጠዎታል::መረጃውንካነበቡናየሚጠይቁትጥያቄካለምበመጠየቅበሚገባከተረዱበኃላመስማማ ትዎንይገልጻሉ::ከጥናቱበፈለጉትጊዜናሰዓትያለምንምቅድመሁኔቴማቋረጥይችላሉ::

7. **<u>በጥናቱላይከተሳተፍኩከእኔምንይፈለጋል?</u>**

በጥናቱላይለመሳተፍከተስማሙወይምልጆእንዲሳተፍከተስማሙአንድየሾርባማንኪያደምከክንድዎወይምከልጆክንድላይ እንዲሰጡይጠየቃሉ::

8. ከእርስዎምንይጠበቃል?

በጥናቱእንዲሳተፉከተስማሙደምእንዲሰጡወይምልጆእንዲሰጥይጠበቃል፡፡እነዚውጤቶችእናናሙናዎችለጥናቱምርምር ይውላሉ፡፡

9. በጥናቱላይቢሳተፉጥቅማጥቅምአገኛለሁኝን?

በዚህጥናትላይበመሳተፍዎየተለየጥቅምበግልአያገኙምግንበጥናቱወቅትየተገኘውንየላቦራቶሪውጤትለእርስዎናእንዲሁምየ እርስዎ/የልጆበጥናቱመሳተፍስለበሽታውአይነትያለውንጥሩግንዛቤእንዲኖረንናበሽታውንለማከምጠቃሚመረጃበመስጠት ይጠቅመናል፡፡

10. በጥናቱላይበመሳተፌየሚደርስብኝጉዳትአለ?

ጥናቱላይበመሳተፍዎ/ልጆበመሳተፉየሚደርስብዎ/የሚደርስበትትልቅጉዳትየለም፤ነገርግንየደምናሙናበሚወሰድበትወቅ ትሊፈጠርየሚችለውንአነስተኛህመምናየደምመፍሰስለማስወገድልምድባላቸውናእናስልጠናበተሰጣቸውባለሙያዎችይከ ናወናል፡፡

11. ጥናቱሲያልቅከእኔምንይጠበቃል?

ምንምአይጠበቅም፡፡

12. በዚህጥናትመሳተፍበሚሰጥርይያዛልን?

አዎንለዚህጥናትየሚሰበሰበውናሙናእናየናሙናውጤትበሚስጥርይያዛል፡፡ስለእርስዎ/ስለልጆየሚገልጽማንኛውምነገርበና ሙናምሆነበውጤቱላይአይጻፍም፡፡ውጤትሲገለጽስምአልባይሆናል፡፡ለእያንዳንዱናሙናልዩመለያቁጥርወይምምልክትይ ሰጠዋል፡፡የትኛውቁጥርየማንእንደሆነዋናተመራማሪውብቻያውቃል፡፡

13. እኔ/ልጄየምለግሰው/የሚለግሰውናሙናምንይሆናል?

የርስዎ/የልጆውጤትናናሙናየተለያቁጥርይሰጠዋል፡፡በናሙናውላይየርስዎወይምየልጆስምአይጻፍም፡፡አብዛኛውናሙናወ ዲያውኑስራላይይውላል፡፡በጥናትየሚገኘውመረጃበህትመትመልክለጤናባለሙያናለሳይንትስቶችይደርሳል፡፡ውጤትበጅ ምላበሚገለጽበትጊዜየማንንምውጤትአይወክልም፡፡

14. ያልተጠበቀየላቦራቶሪዉጤትካለ?

በጥናቱላይየተገኘማንኛውምያልተጠበቀውጤትለእርስዎይገለፃል፤ዶክተርእንዲያምክሩምይደርጋል፡፡

15. የጥናቱውጤት?

የጥናቱውጤትበሳይንሳዊመፅሔትላይይታተማልእንዲሁምየጥናቱውጤትበተለያዩስብሰባዎችላይይቀርባል፡፡ነገርግንየ እርስዎማንነትበምንመልኩአይገለፅም፡፡

16. የጥናቱንወጪየሚደግፈው?

የጥናቱወጪየሚደገፈውበአርሞርሀንሰንየምርምርማእከልነው

17. <u>ፈቃደኝነቴንለመግለፅ?</u>

ጥናቱላይየመሳተፍፍላጎትወይምልጆእንዲሳተፍፍላጎትካለዎትከጥናቱዋናአስተባባሪወይምከተወከለውሰውየስምምነት ቅፅበመውሰድፊርማዎትንበማሰፈርስምምንዎትንማረጋገጥይችላሉ::

18. <u>ይህጥናትተቀባይነትንአግኝቷል?</u>

ይህ ጥናት በፓቶሎጂ የስነ-ምግባር ኮሚቴና በአዲስ አበባ ዩንቨርስቲ የስነ-ምግባር ኮሚቴ ተገምግሞ ተቀባይነትን አገኝቶ ፀድቋል::

<u>ተጨማሪመረጃከፈለጉየሚከተሉትንባለሚያዎችማነጋገርይችላሉ</u>

የጥናቱአስተባባሪናዋናተመራማሪ

ኪያ ደሳለኝ

ስልክቁ. 0913821401

Annex 5. Consent Form for Adult /age above 18

Study title: Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

Please read this form and sign it once the above named or their designated representative has explained fully the aims and the procedures of the study to you.

- I voluntarily agree to take part in this study.
- I confirm that I have been given a full explanation by the above named and that I have read and understood the information sheet given to me which is attached.
- I understand that the investigators will take my morphologic result of bone marrow aspirate and 5 ml of a blood sample.
- I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result.
- I authorize the investigator to disclose the results of my participation in the study, but not my name.
- I authorize the investigators to disclose to me any abnormal test result
- I understand that I am free to withdraw from the study at any time
- I understand that information recorded during the study will be kept in a secure database.

Name:_____ Signature:_____ Date: _____

The participant is illiterate. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

Name of Impartial Witness*(if required)*	**Signature**	**Date**

I confirm that I have fully explained the purpose of the study and what is involved to:

…….

I have given the above named copy of this form together with the information sheet.

Signature: ……. Name: ……..

Contact Address: Kiyya Dessalegn-0913821401

Annex 6. Assent Form for Children 12-17 year

Study title: Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

Please read this form and sign it once the above named or their designated representative has explained fully the aims and the procedures of the study to you.

- I voluntarily agree to take part in this study provided that my parents/guardian gives their consent.
- I confirm that I have been given a full explanation by the above named and that I have read and understood the information sheet given to me which is attached.
- I understand that the investigators will take my morphologic result of bone marrow aspirate and 5 ml of blood sample.
- I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result.
- I authorize the investigator to disclose the results of my participation in the study, but not my name.
- I authorize the investigators to disclose to me any abnormal test result
- I understand that I am free to withdraw from the study at any time
- I understand that information recorded during the study will be kept in a secure database.

Name: _____ **Signature:** _____ **Date:** _____

The participant is illiterate. As a witness, I confirm that all the information about the study was given and the participant consented/assented to taking part.

Name of Impartial Witness*(if required)*	**Signature**	**Date**

I confirm that I have fully explained the purpose of the study and what is involved to:

…….

I have given the above named copy of this form together with the information sheet.

Signature: ……. Name: ……..

Contact Address: -Kiyya Dessalegn 0913821401

Annex 7. Consent Form for parental/guardian, age below 12

Study title: Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

I _____being parent/guardian of _____ have been requested to let my child participate in this study, which plans to evaluate the Co-relation between morphology, cytochemistry and flow cytometry in the diagnosis of acute leukemia. I have read the information sheet /it has been read for me that the study involves collecting whole blood from the vein of my child and utilizing the bone marrow result of my child. During collection of the specimen I have been told that there is no harm except little pain during blood collection. I understand that I will be interviewed about my child's demographic and medical information related to his/her acute leukemia infection and that all information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and drop out of the study if I want and that none of my actions will have any bearing at all on my child's overall health care and hospital access.

I was also told that my child's results would be reported timely to the requesting physicians for the appropriate treatment and management of the acute leukemia. Thus I have given my consent freely to let my child participate in the study, and I_____ hereby approve my consent with my signature.

.

_____	_____	____/____/___
Name of adult parent	Signature	Day/month/year
_____	_____	____/____/___
Witness (Illiterate)	Signature	Day/month/year
_____	_____	____/____/___
Name of the researcher	Signature	Day/month/year

Annex 8. Consent Form for control group

Study title:Co-relation between morphology, cytochemistry, and flow cytometry in the diagnosis of acute leukemia.

Selection of the control group: The study participant is selected as aberrantly healthy individual.

Please read this form and sign it once the above named or their designated representative has explained fully the aims and the procedures of the study to you.

- I voluntarily agree to take part in this study as a control group
- I confirm that I have been given a full explanation by the above named
- I understand that the investigators will take 5 ml of blood sample.
- I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result.
- I authorize the investigator to disclose the results of my participation in the study, but not my name.
- I authorize the investigators to disclose to me any abnormal test result
- I understand that information recorded during the study will be kept in a secure database.

Name: _____ **Signature:**_____ **Date:** _____

The participant is illiterate. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

Name of Impartial Witness*(if required)*	**Signature**	**Date**

Signature: ……. Name: ……..

Contact Address: Kiyya Dessalegn - 0913821401

Annex 9. Consent Form for control group (Amharic version)

(የኮንትሮልግሩፕተሳታፊየሚፈርሙትየስምምነትቅጽ)

የጥናቱርዕስ፡ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴንማመሳሰል ወይም ማዋቀር::

የጥናቱንአላማ-እኔአሁንየማጠናዉለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማመሳሰል ወይም ማዋቀር ነው፡፡የዚህምጥናትዉጤትስለበሽታውየተሻለእውቀትእንዲገኝእንዲሁምየሽታውንአይነትበጊዜለመለየትናለመከላከልበሚደረገ ዉንጥረትጉልህድርሻይኖረዋል፡፡እርሶምበዚህጥናትየኮንትሮልግሩፕሆነውተመርጠዋል፡፡

የኮንትሮልግሩፕሆነውበጥናቱየሚመረጡትጤናማናቸውተብለውየሚገመቱናቸው

- በጥናቱውስጥየኮንትሮልግሩፕተሳታፊእንድሆንበሙሉፈቃድወስጃለሁ
- በተጨማሪምአስፈላጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል፡፡ደምከከንድዎላይእንዲሰጡይጠየቃሉ
- አጥኚዎቹአንድየሾርባማንኪያየደምናሙናእንደሚወስዱበሚገባተረድቻሉ፡፡
- ጥያቄየመጠየቅመብትየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂምክ ርናውይይትአድርጌያለሁ፡፡
- በተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ፡፡ነገርግንስምመጠቀስየለበትም፡
- ተመራማሪዎቹበጤናዬላይያለንችግርእንዲነግሩኝፈቅጄላቸኃለሁ፡፡
- በማንኛውምጊዜከጥናቱእራሴንማግለልእንደምችልአውቄያለሁ፡፡
- ከእኔየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ፡፡

ስም_____ ፊርማ_____ ቀን_____

ይህበጥናቱየሚሳተፈውሰውመፈረምስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊውምለመሳተፍመስ ማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ፡፡

_____ _____

የገለልተኛታዛቢስምፊርማቀን

ስለጥናቱዝርዝርመረጃሰስለመስጠቴአረጋግጣለሁ .....

የመተማመኛቅጹንከስምምነትቅጽጋርአያይዤሰጥቻለሁ

Annex 10. Consent Form Amharic Version for Adult

ተሳታፊየሚፈርሙትየስምምነትቅጽ

የጥናቱርዕስ፡ ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማመሳሰል ወይም ማዋቀር::

የጥናቱንአላማናሂደትበዝርዝርከተረዱበኃላየሚከተለውንቅጽበጥንቃቄይፈርማሉ፡፡

- የጥናቱተሳታፊእንድሆንበሙሉፈቃድወስጃለሁ
- ከዚህጋርየተያያዘውንየመግለጫቅጽበትክክልአንብቤተረድቻለሁ፡፡በእኔላይምስለሚደረግማንኛውምጥናትተገንዝቤአ ለሁ፡፡በተጨማሪምአስፈላጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል፡፡ደምከክንድዎላይእንዲሰጡይ ጠየቃሉ
- አጥኚዎቹየመቅኔምርመራውጤቴንትንወስደውይጠቀማሉበተጨማሪምአንድየሾርባማንኪያየደምናሙናእንደሚወስ ዱበሚገባተረድቻሉ፡፡
- ጥያቄየመጠየቅመብትየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂምክ ርናውይይትአድርጌያለሁ፡፡
- በተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ፡፡ነገርግንስምመጠቀስየለበትም፡
- ተመራማሪዎቹበጤናዬላይያለንችግርእንዲነግሩኝፈቅጇላቸኃለሁ፡፡
- በማንኛውምጊዜከጥናቱእራሴንማግለልእንደምችልአውቄያለሁ፡፡
- ከእኔየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ፡፡

ስም_____ ፊርማ_____ ቀን_____

ይህበጥናቱየሚሳተፈውሰውመፈረምስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊውምለመሳተፍመስ ማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ፡፡

_____ _____

የገለልተኛታዛቢስምፊርማቀን

ስለጥናቱዝርዝርመረጃሰስለመስጠቴአረጋግጣለሁ .....

የመተማመኛቅጹንከስምምነትቅጽጋርአያይዤሰጥቻለሁ

ፊርማ..... ስም.....

Annex 11. Parental/guardian consent in (Amharic version)

የስምምነትመጠየቂያቅጽለወላጆችወይምአሳዳጊዎች

የጥናቱርዕስ፡ ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማመሳሰል ወይም ማዋቀር::

እኔ----------የልጄ _____ወላጅ/ አሳዳጊስሆንለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየትፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማወዳደርጥናትላይልጄየጥናቱተሳታፊእንድትሆን/እንዲሆንበሙሉፈቃድወስጃለሁ

- ከዚህጋርየተያያዘውንየመግለጫቅጽበትክክልአንብቤ/ተነቦልኝተረድቻለሁ፡፡ልጄላይስለሚደረግማንኛውምጥናትተገን ዝቤአለሁ፡፡በተጨማሪምአስፈለቃጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል፡፡
- አጥኚዎቹየልጄንየመቅኔምርመራውጤትወስደውእንደሚጠቀሙበተጨማሪምአንድየሾርባማንኪያየደምናሙናከልጄ ከንድላይእንደሚወስዱበሚገባተረድቻሉ፡፡ነገርግንየደምናሙናበሚወሰድበትወቅትሊፈጠርየሚችለውንአነስተኛህመ ምናየደምመፍሰስለማስወገድልምድባላቸውናእናስልጠናበተሰጣቸውባለሙያዎችእንደሚከናወንተረድቻለው
- ጥያቄየመጠየቅመብትናየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂም ክርናውይይትአድርጌያለሁ፡፡
- በተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ፡፡ነገርግንየልጄስምመጠቀስየለበትም፡
- ተመራማሪዎቹበልጄጤናላይያለንችግርእንዲነግሩኝፈቅጄላቸኃለሁ፡፡
- በማንኛውምጊዜከጥናቱልጄንማግለልእንደምችልአውቄያለሁ፡፡
- ከልጄየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ፡፡
- ስለዚህምልጄንበጥናቱውስጥለማሳተፍበፍፁምፈቃደኝነትየስምምነትቃሌንመስጠቴንበፊርማዬአረጋግጠለሁ፡፡

የተሳታፊውሥም_____ፊርማ ----------ቀን /ወር/ዓ .ም----------
ምስክር(ማንበብናመፃፍለማይችሉ)-_____ የምስክርፊርማ -------ቀን/ወር/ዓ.ም ----------

የተመራማሪውስም_____ ፊርማ ---------- ቀን /ወር/ዓ.ም- ----------

Annex 12. Assent Form for Children 12-17 years (Amharic Version)

የስምምነትመጠየቂያቅጽለህጻናትእድሜ--12-17

የጥናቱርዕስ፡ ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማመሳሰል ወይም ማዋቀር::

የጥናቱንአላማናሂደትበዝርዝርከተረዱበኃላየሚከተለውንቅጽበጥንቃቄይፈርማሉ፡፡

- የጥናቱተሳታፊእንድሆንቤተሰቦቼወይምአሳዳጊዎቼሙሉፈቃድሰጥተዋል
- ለጥናቱየሚሆንየደምናሙናለመስጠትበሙሉፈቃድወስጃለሁ
- ከዚህጋርየተያያዘውንየመግለጫቅጽበትክክልአንብቤ /ተነቦልኝተረድቻለሁ፡፡በእኔላይምስለሚደረግማንኛውምጥናትተገንዝቤአለሁ፡፡በተጨማሪምአስፈላጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል፡፡ደምከክንድዎላይእንዲሰጡይጠየቃሉ
- አጥኚዎቹአንድየሾርባማንኪያየደምናሙናእንደሚወስዱበሚገባተረድቻሉ፡፡
- ጥያቄየመጠየቅመብትየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂምክርናውይይትአድርጌያለሁ፡፡
- ተመራማሪዎቹበጤናዬላይያለንችግርእንዲነግሩኝፈቅጄላቸኃለሁ፡፡
- ከእኔየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ፡፡

ስም_____ ፊርማ_____ ቀን_____

ይህበጥናቱየሚሳተፈውሰውመፈረምስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊውምለመሳተፍመስማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ፡፡

_____ _____

የገለልተኛታዛቢስምፊርማቀን

ስለጥናቱዝርዝርመረጃሰስለመስጠቴአረጋግጣለሁ .....

የመተማመኛቅጹንከስምምነትቅጽጋርአያይዤሰጥቻለሁ

ፊርማ..... ስም.....

Annex 13. Parental/guardian consent in (Amharic version) for Children 12- 17 years

የስምምነትመጠየቂያቅጽለወላጆችወይምአሳዳጊዎችለህጻናትእድሜ--12-17

የጥናቱርዕስ፡ ለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየት የሚረዱትን ሞርፎሎጂ፣ ፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማመሳሰል ወይም ማዋቀር::

እኔ----------የልጄ _____ወላጅ/ አሳዳጊስሆንለአጣዳፊየደምካንሰርን ለመመርመርና አይነቱን ለመለየትፍሎሳይቶሜትሪንና ሳይቶኬሚስትሪንዘዴን ማወዳደርጥናትላይልጄየጥናቱተሳታፊእንድትሆን/እንዲሆንበሙሉፈቃድወስጃለሁ

- ከዚህጋርየተያያዘውንየመግለጫቅጽበትክክልአንብቤ/ተነቦልኝተረድቻለሁ::ልጄላይስለሚደረግማንኛውምጥናትተገን ዝቤአለሁ::በተጨማሪምአስፈሊቃጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል::
- አጥኚዎቹየልጄንየመቅኔምርመራውጤትወስደውእንደሚጠቀሙበተጨማሪምአንድየሾርባማንኪያየደምናሙናከልጄ ክንድላይእንደሚወስዱበሚገባተረድቻሉ::ነገርግንየደምናሙናበሚወሰድበትወቅትሊፈጠርየሚችለውንአነስተኛህመ ምናየደምመፍሰስለማስወገድልምድባላቸውናእናስልጠናበተሰጣቸውባለሙያዎችእንደሚከናወንተረድቻለው
- ጥያቄየመጠየቅመብትናየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂም ክርናውይይትአድርጌያለሁ::
- በተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ::ነገርግንየልጄስምመጠቀስየለበትም፡
- ተመራማሪዎቹበልጄጤናላይያለንችግርእንዲነግሩኝፈቅጄላቸኃለሁ::
- በማንኛውምጊዜከጥናቱልጄንማግለልእንደምችልአውቄያለሁ::
- ከልጄየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ::
- ስለዚህምልጄንበጥናቱውስጥለማሳተፍበፍፁምፈቃደኝነትየስምምነትቃሌንመስጠቴንበፊርማዬአረጋግጠለሁ::

የተሳታፊውሥም_____ፊርማ ----------ቀን /ወር/ዓ .ም----------
ምስክር(ማንበብናመፃፍለማይችሉ)-_____ የምስክርፊርማ -------ቀን/ወር/ዓ.ም ----------

የተመራማሪውስም_____ ፊርማ ---------- ቀን /ወር/ዓ.ም- ----------

Annex 14. Clinical data collection sheets

Card No……Study number…….

Entry criteria: Patient history; Patient examination; Consent form

Morphology result of bone marrow aspirate and 5 ml blood sample for Flowcytometry and Cytochemical stain

Clinical data required:

Full clinical investigation report on the first day

PI in charge - Name and signature ----------.

Data collection Form

Patient ID **----------**

Age ----------

Sex ----------

Residential area ----------

Morphology result ----------

Hemoglobin ----------g/dl

WBCs count ----------/ml

RBC ----------/µl

Platelet count ----------/ml

Cytochemical result

- PAS ----------
- SBB ----------

Flow cytometry result ----------

Annex 15. Principle and procedure of the methods

1. Principle and procedure of different types of cytochemical stains.

1.1.Principle and procedure of Periodic Acid Schiff reaction (PAS)

Principle

Periodic acid specifically oxidizes 1-2 glycol groups to product stable dialdehydes. These dialdehydes give a red reaction product when exposed to Schiff's reagent (leucobasic fuchsin). Positive reactions occur with carbohydrates, principally glycogen, but also monosaccharides, polysaccharides, glycoproteins, mucoproteins, phosphorylated sugars, inositol derivatives and cerebrosides. Glycogen can be distinguished from other positively reacting substances by its sensitivity to diastase digestion. In hemopoietic cells, the main source of positive reactions is glycogen.

Procedure

1. Fix air-dried blood films for 1 minute at room temperature in Formalin-Ethanol Fixative Solution.
2. Rinse slides 1 minute in slowly running tap water.
3. Immerse slides in Periodic Acid Solution for 5 minutes at room temperature.
4. Rinse slides in several changes of distilled water.
5. Immerse slides in Schiff's reagent for 15 minutes at room temperature. NOTE: Immediately after use, cap Schiff's reagent and return to the refrigerator (2–8°C).
6. Wash slides in running tap water for 5 minutes.
7. Counterstain slides in Hematoxylin Solution, Gill No. 3, for 90 seconds.
8. Rinse slides in running tap water for 15–30 seconds, air dry and examine microscopically under the oil immersion (900x) lens. Slides may be mounted in toluene or xylene based mounting media.

Results

PAS positive substances stain -> pink to red

Nuclei -> blue.

A Diastase (α-Amylase) Extraction slide -> no visible PAS staining of glycogen

1.2.Staining Procedure for Sudan Black B (SBB)

Principle: Sudan Black B (SBB) is a lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils and some monocytes. It cannot be extracted from the stained granules by organic dye solvents [47].

Specimen: Fresh whole or anticoagulated blood or bone marrow smears may be used. The slides must be fixed as soon as possible.

Procedure

1. Cool the fixative solution (formol-alcohol) in the refrigerator.
2. Place blood smears into the cool fixative for 1 minute. Gently agitate during this minute. Rinse or dip in deionized water.
3. Immerse in sudan black B stain for 5min. gently agitate.
4. Rinse or dip in 70% ethanol until no more dye washes out.
5. Rinse thoroughly in distilled water.
6. Immerse in Mayer Hematoxylin solution for 5 min. Rinse thoroughly in tap water.
7. Air-dry and examine slides microscopically using oil (100x) immersion objective.

Result

Neutrophils and their precursors show blue-black intracellular granulation. Monocytes stain less intensely and lymphocytes do not stain with Sudan black B.

2. Principle and procedure of Flow cytometry

Principle

Prepared single cell or particle suspensions are necessary for flow cytometric analysis. Various immunofluorecent dyes or antibodies can be attached to the antigen or protein of interest. The suspension of cells or particles is aspirated into a flow cell where surrounded by a narrow fluid stream, they pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence if the cell contains a naturally fluorescent substance or one or more fluorochrome-labeled antibodies are attached to surface or internal cell structures.

Light scatter is dependent on the internal structure of the cell and its size and shape. Fluorescent substances absorb light of an appropriate wavelength and reemit light of a different wavelength. Fluorescein isothiocyanate (FITC), Texas red, and phycoerythrin (PE) are the most common fluorescent dyes used in the biomedical sciences. Light and/or fluorescence scatter signals are detected by a series of photodiodes and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector. The resulting electrical pulses are digitized, and the data is stored, analyzed, and displayed through a computer system. The end result is quantitative information about every cell analyzed. Since large numbers of cells are analyzed in a short period of time (>1,000/sec), statistically valid information about cell populations is quickly obtained [prin of flow].

Procedure

FlowCytometry Analysis: The BD FACS Calibur (Becton, Dickinson Fluorescence Activated Cell Sorter will be used for immunophenotyping analysis. The BD FACSCalibur is a dual-laser, four-color, bench-top and fully integrated multiparameter system that is designed particularly to support a variety of applications.

Lysing and Staining:

- ✓ Ten microliters of fluorescein isothiocyanate (FITC) conjugated monoclonal antibody, 10µl of Phycoerythrin (PE) conjugated monoclonal antibody, 5µl of Allophycocyanin (APC) conjugated monoclonal antibody, and 5µl of Peridinin-chlorophyll-protein complex (perCP) conjugated monoclonal antibody will be added to 12 *75-mm tubes,

Dako-CD8 CD4 CD3 dako (TC 660) with CD45 PerCPCy5.5 (BD 564105, 100t)

Dako-CD19 CD56 CD3 dako(TC 662) with CD7 PerCPCy5.5 (BD 561602, 50t)

Dako-cMPO cCD79a cCD3 dako (TC667) with CD45 PerCPCy5.5 (BD 564105, 100t)
Dako-CD33 CD34 CD117 dako (TC 686) with CD45 PerCPCy5.5 (BD 564105, 100t)
Dako-CD13-fitc (dako F0831); HLA-DR-APC (BD 559866); CD10-PE (Dako R0848); CD45 PerCPCy5.5 (BD 564105, 100t)
Dako-CD7-fitc (Dako); BD HLA-DR-APC; Dako CD TdT-PE , BD CD45 PerCPCy5.5

Dako CD45PerCP and CD14 PE((BD leukogate)

- ✓ Afterward, 100µL of whole blood was in each tube.
- ✓ The mixture was Vortexed tenderly and incubated about 20 minutes in the dark area at room temperature (20- 25˚C).
- ✓ Tow 1000µl of 1X FACS lysing buffer was added to incubated mixture. Then it was vortexed tenderly and incubated for 20 minutes in the dark area at room temperature again; after that Centrifuge at 500g for 5 minutes was done.
- ✓ The supernatant was removed. Subsequently 2-3 ml of washing buffer was added and centrifuged at 500g for 5 minutes and the supernatant was removed. 1 ml of 1% cell fix (paraformaldehyde solution) was added and mixed completely, analysis can be done immediately or fixed cells can be stored at 2-8˚C until analyzing them.
- ✓ The analysis was done by FACS brand flow cytometer. Samples vortexed thoroughly prior to acquisition

Bead (sating for compensation)

1. Surface stain beads (follow extracellular staining procedure)
2. Use cells for live/dead compensation. Do not use beads
3. For $FoxP_3$either use beads whose host is rat otherwise, use cells
4. Get anti-mouse Comp Beads or anti-rat Comp beads
5. Vortex for few seconds
6. Take 1 drop from negative and positive beads (1 drop approximately)
7. Dilute 1 in 3 with FACS buffer. Add 200µl buffer to the beads
8. Mix well and add 50 to each compensation tubes containing appropriate antibodies (CD3-V450, CD4-780, CD8-PerCy5.5, cd25 PECy7, CD 127 APC, and CD161 PE). Add a maximum of$1/10^{th}$ of the Ab used for cells. Beads easily bind Ab in small quantity, unlike cells.

   Notice: Use anti- rat Comp Beads. Or cells for Foxp3 FITC
9. Incubate as you would for your cells
10. Wash, spin, decant as you would for your cells

11. Fix as you would for your Sample
12. Wash, spin, decant as you would for your cells
13. Finish by adding 300µl FACS buffer
14. Read