ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF ALLIED HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

DIAGNOSTIC UTILITY OF IMMUNOPHENOTYPING BY FLOW CYTOMETRY FOR DIAGNOSIS AND CLASSIFICATION OF ACUTE LEUKEMIAS IN TIKUR ANBESSA SPECIALIZED HOSPITAL, ADDIS ABABA, ETHIOPIA

By: MetasebiaTegegn (BSc)

Advisors:

Aster Tsegaye (MSc PhD)

Rawleigh Howe (MD, PhD)

Fatuma Hassen (PhD candidate, MPH)

Amaha Gebremedhin (MD, Hematologist)

Collaborators:

Dr Abdulaziz Abubeker (MD, Hematologist)

Dr FissehatsionTadesse (MD, Hematologist)

Dr Daniel Hailu (MD, Pediatric Oncologist)

Dr Haileeyesus (MD, Pediatric Oncologist)

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Masters of Science in Clinical Laboratory Sciences (Laboratory Management and Quality Assurance Specialty Track)

Nov, 2016

Addis Ababa, Ethiopia

Acknowledgment

I would like to express my sincere gratitude to my school, AHRI, TASH and my advisers Dr Raweligh Howe, Dr Aster Tsegaye and Fatuma Hassen for their continuous assistance and encouragement throughout my study .The co investigators from hematology unit and pediatric department of School of Medicine, Addis Ababa University**,** the patients and all who directly or indirectly contributed to this study are gratefully acknowledged for their kind collaboration. Finally, I am highly indebted to my friends and ALERT Laboratory staff for all the support.

Table of Contents

List of Table

List of Figure

Abbreviations

ABL	Acute bilineal leukemia
AHRI	Armauer Hansen Research Institute
AL	Acute Leukemia
ALERT	All Africa TB and Leprosy Rehabilitation and Training Center
ALL	Acute Lymphoid (Lymphocytic) Leukemia
AML	Acute Myeloid leukemia
APC	Allophycocyanin
APL	Acute Promyelocytic Leukemia
BAL	Biphenotypic Acute Leukemia
B-ALL	B cell Acute Lymphoid leukemia
BD	Becton-Dickinson
BM	Bone Marrow Morphology
CD	Cluster of differentiation
cCD3	Cytoplasmic CD3
CLL	Chronic Myloid Leukemia
CML	Chronic Lymphoid Leukemia
EDTA	Ethylene diaminetetra acetic acid
EGIL	European Group for the Immunological Characterization of Leukemias
FAB	French-American-British
FACS	Fluorescent activated cell sorter/sorting
FCA	Flowcytometry Analysis
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HIV	Human immunodeficiency virus
HLA DR	Human Leukocyte antigen D reference antigen
MPO	Myeloperoxidase
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PE	Phycoerythrin
PM	Peripheral Morphology

PMT	Photomultiplayer Tube
PerCP	Peridinin chlorophyll cyanin
SBB	Sudan Black B
SPSS	Statistical Package for Social Science
SSC	Side scatter
T-ALL	Tcell Acute Lymphoid leukemia
TASH	Tikur Anbessa Specialized Hospital
Tdt	Terminal Deoxynucleotidyltransferase
WHO	World Health Organization

Abstract

Background: Immunophenotypic characterization of acute leukemia is an important clinical application of flow cytometery and has become a powerful tool contributing to proper diagnosis and classification.

Objective: To phenotype and classify acute leukemias by flow cytometry using commonly used markers for leukemia diagnosis.

Method: A total of 40 pediatric and adult patients diagnosed with acute leukemia were evaluated by flow cytometry with 17 surface and cytoplasmic markers known to be useful in discriminating different types of acute leukemia.

Results: 21 of 40 patients (52.5%) were classified as Acute Myeloblastic leukemia (AML) while 19 (47.5%) were identified as Acute lymphoblastic leukemia (ALL). Of all the ALL cases, 10 of the 19 (52.6%) were B-ALL and 47.4% (9/19) were T-ALL. Markers of immaturity HLA-DR and CD34 antigens were co expressed in 61% of AML cases, and 33% of T-ALL cases, whereas CD34 was expressed in 50% of the B-ALL cases. MPO and CD13 were the most commonly expressed markers of AML, whereas CD19 and cCD79a were present in all cases of B-ALL. Cytoplasmic CD3 and CD7 were the most sensitive markers for T-ALL. Discrimination of AML from ALL patients by flow cytometry was 80% concordant with traditional morphology. Notable discrepancies occurred in cases where leukemia cells expressed markers for more than one lineage.

Conclusion: Immunophenotyping by flow cytometry provides useful information to confirm diagnoses of standard morphology methodology, to provide classifications where morphology is indeterminate, and to provide further lineage and maturation information for ALL not obtainable by morphology is needed for ALL cases. It thus represents an important tool among many in leukemia classification and is realistic in the resource limited settings.

Key words: Leukemia, ALL, AML, Flow cytometry, Immunophenotyping

1. Introduction

1.1. Background information

Acute leukemia (AL) is a proliferation of immature bone marrow-derived cells (blasts) that may also involve peripheral blood or solid organs. 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 (1).

Acute leukemias are a heterogeneous group of malignancies with varying clinical, morphologic, immunologic, and molecular characteristics. AL is divided into 2 categories, depending upon their cell of origin. Leukemia evolving from the myeloid/granulocyte, erythroid and megakaryocyte cell line is called acute myelogenous leukemia (AML). Lymphocytic precursors give rise to acute lymphocytic leukemia (ALL). AML is the most common type of acute leukemia in adults, accounting for 80% of new cases. AML is uncommon in children. The incidence increases steadily with age, with a sharp increase after 45 years. ALL is the most common malignant disease affecting children, accounting for approximately 30% of all childhood cancers (2).

Different classification approaches have been proposed by different cooperative groups such as French-American-British (FAB), World Health Organization (WHO) and European Group for International Characterization of Leukemias (EGIL). FAB system was based on morphological and cytochemical criteria. The classification of AL proposed by EGIL was based on immunophenotyping, targeting a more precise delineation of the hematological linage and differentiation stage of specific types of leukemia. The WHO classification has incorporated cytomorphology, immunophenotyping, cytogenetic and molecular changes (3).

Timely and accurate diagnosis of hematologic malignancies is crucial to appropriate clinical management. Because it is extremely difficult to characterize leukemia as lymphoblastic or myeloid based on morphologic appearance of blasts, additional analyses of the blasts are necessary, including cytochemical staining, phenotypic analyses with flow cytometry, and molecular evaluation for chromosomal abnormalities (i.e, cytogenetic studies) (2).

Immunophenotyping allows an early confirmation of AL diagnosis and establishes lineage assignment. Adequate and comprehensive panels of monoclonal antibodies also allow detection

of aberrant immunophenotypic profiles of prognostic value or of use in detecting minimal residual disease. A number of unusual immunophenotypic features are also associated with prognosis (4).

Immunophenotypic analysis of the reactivity of leukemic cells with monoclonal antibodies has proved to be useful and nowadays essential in the diagnosis of acute leukemias. This was first shown to be relevant in the characterization and classification of acute lymphoblastic leukemias of various cell types as there is no specific and reliable cytochemical marker to recognize lymphoblasts. Subsequently, immunological markers have also been shown to be important in the diagnosis of acute myeloblastic leukemias, particularly when the nature of the blasts cannot be defined by morphology and cytochemistry. Cases of "undifferentiated" acute leukemias include those with poorly differentiated myeloblasts (AML-M0), or those derived from early erythroid and megakaryocyte precursors (5).

Flow cytometery is the methodology used to detect cell surface antigens using monoclonal antibodies conjugated with different fluorochromes. The value of characterization of leukemic cells is to establish the lineage (such as myeloid, T lymphoid or B lymphoid). It would also be used to establish the dual lineage in biphenotypic leukemia, unusual co expression of antigens or aberrant profile and demonstrate clonality (6).

Acute leukemia displays characteristic patterns of antigen expression, which facilitate their identification and proper classification. 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 cell. Immunophenotyping proved to be of great help in the distinction between lymphoid and myeloid acute leukemias, especially if equivocal morphology and cytochemistry are found. The technique enables sub classification of acute lymphoblastic leukemias, the diagnosis of minimally differentiated, erythroid and megakaryocytic acute myeloblastic leukemia subtypes and the recognition of both T and B cell chronic lymphoproliferative disorders. Immunophenotyping of hematological malignancies using multiparameter flow cytometry approaches has proven to be a highly sensitive and specific method for the identification of neoplastic cells, even when present at very low frequencies among a major population of normal hematopoietic cells (7).

Immunophenotyping using flow cytometry has become an essential component of acute leukemia (AL) diagnosis and had a role to play in ALL patients to confirm a definite and a probable diagnosis, to define therapeutically and prognostically groups such as B and T lineage ALL and to distinguish AML – M0 from ALL (8). Immunophenotyping of leukemia cells plays crucial role in identification of leukemia cell line, definition of maturation stage and finding possible aberrant antigens what in turn serves for individual treatment monitoring and detection of residual disease (9).

Flow cytometric immunophenotyping is an indispensable tool for quantitative and qualitative evaluation of antigen expression of hematopoietic cells. It is also a valuable tool for detecting aberrant antigen expression. Malignant blasts often have an abnormal phenotype that allows distinction from normal immature cells. Abnormalities include expression on myeloid blasts of markers usually not present on cells of that particular lineage (10). Flow cytometry offers a series of powerful tools for precise definition of cell populations in bone marrow or peripheral blood specimens. Furthermore, by identifying sequential steps of cell maturation, Flowcytometry is able to define different patterns of differentiation of individual lineages (11).

The importance of classifying acute leukemia into more homogeneous subgroups is related to differences in pathogenesis, natural history, and prognosis between the groups. Current modes of treatment for acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL) are sufficiently different that an imprecise diagnosis can adversely affect prognosis. Newer therapeutic approaches have also increased the importance of assigning certain cases to the correct subtype of AML or ALL. Immunophenotyping of acute leukemia involves the identification of surface, cytoplasmic, and nuclear antigens expressed by leukemic cells. This provides important information that can be used in conjunction with morphology, cytochemistry, cytogenetics, and clinicopathologic data to diagnose and classify cases of acute leukemia. Because cytogenetic investigations take more time to be performed, morphologic-cytochemical analysis and immunophenotyping are currently the primary tools used to diagnose and classify acute leukemia (12).

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 . In Immunophenotyping of leukemic cells, Flowcytometry plays essential role in identification of leukemia cell line,

maturation stage and detection of residual disease. The lineage of most cases of morphologically and cytochemically poorly differentiated AL and a few AML sub sets and biphenotypic acute leukemia (BAL) can be accurately characterized by immunophenotyping. Multiparametric flow cytometry is the preferred method for immunophenotyping of AL. The lineage of hematopoietic cells is defined both by antigens expressed and the absence of expression of antigens associated with a different lineage. Leukemia cells, however, may aberrantly express some antigens of another lineage or lack expression of an expected antigen. It is important, therefore, to use panels that include sufficient numbers of antibodies to assess a spectrum of both myeloid and lymphoid antigens. The imunophenotying analyses must undergo some steps: lineage assignment, maturational analysis, complete characterization of blast cells and normal cells (3).

1.2. Statement of the problem

In 2000, approximately 256,000 children and adults around the world developed a form of leukemia, and 209,000 died from it. In 2010, globally, approximately 281,500 people died of leukemia and in 2012 leukemia developed in 352,000 people globally and caused 265,000 deaths. ALL is most common type of cancer, with three quarters of leukemia cases in children. However, about 90% of all leukemias are diagnosed in adults, with AML and CLL being most common in adults. It occurs more commonly in the developed world (13).

In Ethiopia, Acute leukemia is one of the most serious public health problems. According to earlier study by Shamebo in the 1990's in Tikur Anbessa (Black Lion) Hospital, a teaching and referral hospital in Addis Ababa, the commonest type of leukaemia was chronic myeloid leukaemia (CML) 57.8%, acute leukaemias and chronic lymphatic leukaemia (CLL) accounted for 21.1% each. Of the acute leukaemias, 53.3% were ALL while 46.7% were AML. (14) (15) . Leukemias are common than previously thought, and only one referral hospital is available for the diagnosis and treatment of leukemia in Ethiopia. Currently morphology and sometimes Cytochemical technique is practiced for the examination and classification of acute leukemia.

The FAB classification of ALL and AML is based on morphology and cytochemical staining of blasts. However, the recent classification schemes proposed by the WHO require the additional evaluation of the leukemic blasts by molecular analysis and flow cytometry. The results of these four methods of evaluation (i.e., morphology, cytochemical staining, flow cytometry, molecular analysis), not only differentiate ALL from AML, but also categorize the subtypes of acute leukemia. Knowing the subtype of a patient's leukemia helps in predicting the clinical behavior of the disease and the prognosis and in making treatment recommendations. Recent advances in cytogenetic analysis has shown that various subtypes of AML and ALL behave differently and should not be treated similarly (2) .

Although cytochemical stains are essential to recognize the subtypes of AML, they are of limited use in differentiating the subtypes of ALL, thus limiting their application in a predominantly pediatric population, in which 85% to 90% of acute leukemia is of lymphoid lineage. Moreover, cytomorphology and cytochemical stains utilizes bone marrow sample, which is technically invasive and accurate examination of smears also require technical skills that are not uniformly

available in different hospitals and in rural areas. Because of the recent advances made by immunophenotyping in understanding the lineage commitment of leukemia cells, flow cytometry has become a standard tool for the assessment and management of patients with leukemia improve the cost effectiveness of diagnostic services. Thus, there is the need to introduce flow cytometry in diagnosis of leukemias in Ethiopia (16).

Several studies have been conducted to introduce the application of immunphenotyping in the diagnoses and classification of acute leukemias. Immunophenotyping by flow cytometry confirm the diagnosis of leukemia or establish the diagnosis in cases in which morphology and cytochemical stains are equivocal. However, in Ethiopia the essential role and diagnostic utility of flow cytometry for analysis of hematological abnormalities like Leukemia is not investigated. Therefore, this study was aimed to evaluate the diagnostic utility of Immunophenotyping by flow cytometry for diagnosis and classification of acute leukemia.

1.3. Significance of the study

Tikur Anbessa Specialized Hospital (TASH) currently is the only referral center for the management of hematological malignancies in Ethiopia, a country with an estimated population of more than 90 million. Currently morphology of peripheral and bone marrow and sometimes cytochemical technique is practiced for the examination and classification of acute leukemia in the hospital, while WHO incorporates cytomorphology, immunophenotyping, cytogenetic and molecular feature into the classification scheme.

On the other hand, Immunophenotyping is widely practiced in Ethiopia for CD4+ T cells counting and TASH is using its FACSCalibur flow cytometer —which could be used for leukemia phenotyping-- forCD4 counts. Timely and accurate diagnosis of hematologic malignancies is crucial to appropriate clinical management. The result of this study will be useful to establish the technique in TASH so that clinicians can quickly characterize the type of acute leukemia to indicate appropriate therapy and ultimately improve the management of such patients. In so doing they can also prevent avoidable deaths. In addition, the data can be used as baseline for future research and utilization of the technique for other malignancies.

The Hematology fellowship (both Adult and Pediatric) as well as the masters of Clinical Laboratory Science (Hematology/Immunohematology) programs could also benefit from the establishment of the technique in the hospital both for teaching and research in addition to improving the clinical service, thus achieving the three pillar objectives of a teaching hospital simultaneously. Policy makers and planners can also utilize the data for appropriate planning and expanding the service at least to the teaching hospitals which already have the technology and where management of hematological disorders service will be expanded.

2. Literature Review

A number of studies were conducted to assess the implementation of morphology, cytochemical and Flowcytometry methods applied to the diagnosis and classification of Acute Leukemia. Most have shown that cell lineage classification derived from flow cytometry and morphological diagnosis were in agreement in most cases of leukemia, and that flow cytometry studies can provide useful supplementary information for diagnosis and management of acute leukemia.

2.1. Flow cytometric Immunophenotypic profile of acute leukemia

In a retrospective study conducted by Dalia *et al*., in Mansoura University Egypt, (2012) and the diagnostic usefulness of commonly used immune-markers to be used for proper diagnosis and classification of AL was evaluated. This study analyzed immunophenotypic data of 164 new AL patients. Among these patients, flow cytometry classified 68.9% of patients as AML while 31.1% of patients were classified as ALL. The FAB subtype in AML group was AML-M4/5 (34.5%). With regard to ALL, there were 74.5% with B-ALL and 25.5% with T-ALL. It was found that combined use of HLADR and CD34 was much helpful in distinguishing acute promyelocytic leukemia (APL) from non-APL AML than either of these antigens alone. cCD79a and CD19 were the most sensitive markers for B-ALL while cCD3, CD7 and CD5 were the most sensitive antigens for T-ALL. The authors suggested that analysis of AL phenotypes using flow cytometry found to be useful in the identification and differentiation of APL from non-APL AML (17).

A retrospective study was conducted at Washington University Medical Center (St Louis, Mo) to evaluate the expression of commonly used immunomarkers and patterns in various acute leukemias to help define the best use and role of multiparameter flow cytometry in the diagnosis and proper classification of acute leukemias. The study analyzed the immunophenotypic data from 508 new adult and pediatric acute leukemia patients, as studied using multiparameter flow cytometry in addition to routine morphologic and enzyme cytochemical analysis. Cytogenetic and/or molecular data were correlated in all 41 cases of acute promyelocytic leukemia (APL) and in 203 other cases of acute leukemia where those data were available. The study also determined the positive and negative predictive values of a combined CD34 and HLA-DR expression pattern for the differentiation of APL from other myeloid leukemias. The result for this study revealed

that in acute myeloid leukemia (AML) other than APL, expression of CD34 was seen in 62% and expression of HLA-DR in 86% of all cases. Twenty-six (10%) of 259 cases of non-APL AML were negative for both CD34 and HLA-DR as opposed to 33 (80%) of 41 cases of APL. None of the cases of APL were positive for both CD34 and HLA-DR in contrast to 149 (58%) of 259 cases of non-APL AML. Fifty-three cases were found to be examples of minimally differentiated AML (AML M0) based on the lack of expression of cytoplasmic CD3 and cytoplasmic CD79a and expression of one or more myelomonocytic-associated antigens and/or myeloperoxidase. Expression of CD20 was seen in 40 (24%) of 168 cases of precursor B-cell acute lymphoblastic leukemia (pB-ALL) and 52 (29%) lacked CD34 expression. Five of 180 cases of pB-ALL and 2 cases of precursor T-cell ALL (pT-ALL) were negative for terminal deoxynucleotidyl transferase (TdT). Aside from cytoplasmic CD3, CD5 and CD7 were the most sensitive antigens present in all 21 cases of pT ALL. CD33 was more sensitive but less specific than CD13 for myeloid lineage. The study concluded that aside from identification of blasts, flow cytometry was found to be especially useful in the correct identification of AML M0, differentiation of APL from AML M1/M2, and correct identification of TdT-negative ALL and unusual variants, such as transitional B-cell ALL and undifferentiated and biphenotypic acute leukemias (18).

A study conducted on immunophenotypic patterns of childhood acute leukemias in Indonesia showed that among 498 patients with acute leukemia, 116 (23%) were AML, and and 381 (77%) were ALL. Of the ALL samples, 315 (83%) were B-lineage ALL, 2 samples were mature B ALL, and 64 (17%) were T-lineage ALL. 417 patients had morphology studies performed in parallel with two immunophenotyping panels. Kappa scores, indicating degree of concordance, increased from 0.43 (considered moderate) comparing morphology to the first (~~–o~~ld") panel to 0.82 (considered very good) comparing morphology to the second (~~–n~~ew") panel. With the old panel, of 86 cases of ALL defined by morphology, 5 (5.8%) were categorized as AML by immunophenotyping.Of the 13 AML cases diagnosed by morphology, 7 (54 %) were categorized as ALL by immunophenotyping. With the new panel, of 239 morphology diagnosed 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, with 9 B-lineage and 3 T-lineage cases, by immunophenotyping (19).

A study by John M. Peters and coworkers has been conducted to provide an overview of the application of flow cytometric immunophenotyping to the diagnosis and management of acute leukemias, including salient features of those entities described in the 2008 World Health Organization classification. The study concluded that Immunophenotypic evaluation is essential for accurate diagnosis and classification of acute leukemia. Multiparameter flow cytometry provides a rapid and effective means to collect this information, as well as providing prognostic information and a modality for minimal residual disease evaluation (20).

A retrospective study conducted in Mumbai India by Ghohosh S Shinde et al., on the hematologic and immunophenotypic profile of AML in AML diagnosed patients. The diagnosis of 260 cases of AML was based on peripheral blood and bone marrow examination for morphology cytochemistry and immunophenotypic studies. The study results showed that AML-M2 was the commonest FAB subtype, in both children and adults. The highest WBC counts were seen in AML-M1 and the lowest in AML-M3 (10-97 x 10^9/L, mean 53.8 x 10^9/L). The mean values and range for hemoglobin was 6.8 gm/l (1.8 gm/l to 9.2 gm/l), platelet count (63.3 x 10^9/L (32-83 x 10^9/L), peripheral blood blasts 41.4% (5 to 77%) and bone marrow blasts 57.6% (34-96%). Myeloperoxidase positivity was highest in the M1, M2 and M3 subtypes. CD13 and CD33 were the most useful markers in the diagnosis of AML. CD14 and CD36 were most often seen in monocytic (38%) and myelomonocytic (44%) leukemias. Lymphoid antigen expression was seen in 15% of cases. CD7 expression was the commonest lymphoid antigen detected. The study concludes that MPO, CD13 and CD33 were the most diagnostic myeloid markers (21).

2.2. Cytomorphological and flow cytometry methods applied for the classification and diagnosis of acute leukemia

Studies conducted by Mhawech et al compared results of cytochemical staining and flow cytometry analysis in122 patients with acute lymphocytic leukemia and 10 with acute myeloid leukemia. Patients were selected from among 320 patients with acute leukemia at Texas Children's Hospital in 1997 and 1998. Cell lineage classification derived from flow cytometry and cytochemical stains were in agreement in all cases. Definitive diagnoses were feasible using flow cytometry 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 flow cytometry results, cytochemical staining alone provided information sufficient

for diagnosis. The study concluded that in most cases flow cytometry studies can provide adequate information for diagnosis and management of acute leukemia in children. Nevertheless, cytochemical staining should be available for those cases in which flow cytometry results fail to allow a definitive diagnosis (16).

A study was conducted by Belurkar et al in Melaka Manipal Medical College India to compare the morphologic and cytochemical diagnoses with flow cytometric diagnoses of acute leukemia and to analyze the usefulness of FCA over morphology. The study analyzed 50 cases of acute leukemia and found concordance rate as high as 86% between morphologic/ cytochemical diagnosis and flow cytometric diagnosis. Of these, complete concordance was seen in 58% of the cases and partial concordance was seen in 22% of the cases. Only in the 4% of the cases non-concordance was seen. In the remaining 16% of the cases, flow cytometry helped in sub classifying the acute leukemia where morphology and cytochemical 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 33 were important myeloid markers and were aided by other secondary panel of markers like CD14, CD117 and CD41. The study concluded that flow cytometry not only helps in confirming morphologic diagnosis in acute leukemia but also helps in assigning specific lineage to the blasts, particularly in acute lymphoid leukemia, thereby influencing the treatment and the prognosis (22).

A study conducted in Siriraj Hospital Faculty of Medicine Thailand to compare the percentages of marrow blasts derived from two different approaches comprising routine morphology-based manual counting and flow cytometric analysis. Fifty-five marrow samples were collected from 38 acute leukemia patients (36 AML and 19 ALL) after hematologic recovery from chemotherapy. The blast percentages were enumerated manually and by flow cytomety using CD45 and side light scatter expression. A good correlation was found in all 55 samples ($r = 0.829$), as well as in the 36 AML samples ($r = 0.86$). The blast percentages derived from flow cytometer were higher than from morphologic counting in 46 samples (83.6%). Using a cut-off point of < 5% blasts to define complete remission (CR), 48 cases (87%) were classified as morphological CR (83% CR in AML and 95% CR in ALL). The study concluded that there was a good correlation between the percentages of blasts achieved by either method, particularly in AML samples, although some discordant results occurred when <5% blasts were used as a cut-

off point to determine CR. The authors recommended that both methods should be complementarily performed to ensure a truly complete response to chemotherapy (23).

A study conducted by Abbas Rezaei et al., to evaluate leukemia marker expression of peripheral blood (PB) vs. bone marrow (BM) blasts using flow cytometry analysis. The study evaluated 18 patients with Acute Myeloid Leukemia (AML) and 13 patients with Acute Lymphoid Leukemia (ALL). In all cases, the amount of blasts in PB was 30% or higher. The panel of monoclonal antibodies used in this study consisted of CD3, CD7, CD5 (for T lymphocyte lineage); CD19, CD22, CD20, CD10 (for B lymphocyte lineage); CD13, CD14, CD33 (for myeloid subsets); and terminal deoxynucleotidyl transferase (TDT), HLA-DR, CD45 (non-lineage restricted). Expression levels of PB and BM markers were compared by using statistical analysis. The results showed apparent discrepancies for some markers in the ALL group but in AML patients most of the selected markers showed considerable correlation between PB and BM samples. Most markers (CD3, CD5, CD13, CD14, CD19, CD45, HLA-DR, and TdT) showed strong correlation between PB and BM samples in AML group. The study conclude that a targeted gating strategy for blast populations as well as selection of a suitable panel of monoclonal antibodies may be essential for diagnosis of leukemia resulting in similar immunotyping pattern in PB and BM. Although the study results are preliminary, this study suggests that BM aspiration for leukemia may be minimized (24).

A retrospective study was carried out at Moi Teaching and Referral Hospital Kenya to compare morphological and flow cytometric diagnosis in patients previously diagnosed with leukemia. The findings were based on 33 patients who underwent both flow cytometry and bone marrow morphology tests for diagnosis of leukemia. 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: 6 B-ALL cases, 13 T-ALL cases, 10 AML cases, 1 Biphenotypic case and 1 inconclusive case. There was concordance between the morphological and flow cytometry data in 25 out of the 33 cases. The study conclude that flow cytometry was able to confirm definite and suggest probable diagnoses of patients with acute leukemia (25).

2.3 .The Ethiopian situation

In Ethiopia, Multi-parameter flow cytometric analysis has become routine in most referral hospitals, research and some specialized laboratories for the enumeration of CD4+ and CD8+ T cells in HIV/AIDS patients. Tikur Anbessa Hospital has four color flow cytometry which in addition to its current service for antiretroviral therapy monitoring can be used for leukemia diagnosis. However, to date there have been no studies which assessed the application of flow cytometry technique in the classification and diagnosis of acute leukemia. Currently morphology and sometimes cytochemical technique are applied in the hospital for the classification of subtypes of AML and ALL but the methods have difficulty in differentiating particular subtype of ALL. Therefore, the present study is designed to apply flow cytometric analysis of whole blood method in agreement with Wright stained peripheral blood and bone marrow smear for the diagnosis and classification of the acute leukemia at Tikur Anbessa Specialized hospital hematology unit, Addis Ababa, Ethiopia.

3. Objectives

3.1. General objective

- To initiate the diagnostic utility of immunophenotyping by flow cytometry for the diagnosis and classification of acute leukemias in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

3.2. Specific objectives

- To assess the phenotype of a set of peripheral blood samples from acute leukemia patients according to the expression of 17 markers, including MPO, CD2, CD3, CD4, CD7, CD8, CD10, CD13, CD14, CD19, CD33, CD34, CD45, CD56, CD79a, CD117, and HLA-DR.
- To select the best immune-markers to be used for proper diagnosis and classification of AL.
- To determine the overall agreement between morphology of Peripheral blood, bone marrow and Flow cytometric analysis of peripheral blood in the classification of acute leukemias.

4. Hypothesis

We hypothesize that classification of acute leukemia by flow cytometry will be concordant with that by morphology techniques. Accordingly, from statistical analysis, we expect between the two approaches, a high % agreement, high kappa value and very low p values indicating that observed concordance is highly unlikely be chance alone.

5. Materials and Methods

5.1. Study area and setting

The study was conducted with patients with acute leukemia diagnosed at Tikur Anbessa Specialized Hospital (TASH) hematology unit Addis Ababa, Ethiopia. TASH is one of the largest specialized tertiary referral and teaching hospitals in the country with over 700 beds under the administration of Addis Ababa University. The hospital serves 370,000-400,000 patients a year. It is the only tertiary referral hospital in the country in which hematological abnormalities like leukemia are diagnosed and treated. The hematology unit of the internal medicine department is devoted to this. The data from registration of hematology unit shows with in 2014 and 2015 over 1177 leukemia patients were diagnosed, of which 43% were ALL, 17% AML, 29% Chronic Myelogenous Leukemia (CML) and 11% Chronic Lymphocytic Leukemia (CLL). Typically at least 3-4 patients per week are diagnosed with suspected acute leukemia. Morphological examination of peripheral blood and bone marrow aspiration are the routine diagnostic methods currently used in the hospital; occasionally cytochemical staining techniques are also employed.

5.2. Study Design and period

A facility based cross sectional study was conducted to initiate the diagnostic utility of Immunophenotypic analysis of acute leukemia by Flowcytometry in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia from June 2016 to August 2016.

5.3. Source Population

Both adult and pediatric patients who were diagnosed with acute leukemia and who had an indication for morphological examination of peripheral blood and bone marrow aspiration at the hematology unit of Tikur Anbessa Specialized Hospital represented the source population.

5.4. Study Population

Patients diagnosed with acute leukemia who fulfill the inclusion criteria at the hematology unit of TASH were participants of this study.

5.5. Inclusion and Exclusion criteria

5.5.1. Inclusion criteria

- Patients who were diagnosed for acute leukemia and who had morphology analysis performed on peripheral blood and bone marrow aspirates.
- Patients who gave consent to participate in the study.
- Patient who were willing to gave whole blood.
- Patients of any age group.

5.5.2. Exclusion criteria

- Patients who had previous diagnosis of cancer or who have responded to chemotherapy.

5.6. Sample size

- Since detailed immunophenotypic screening is carried out using flow cytometry to select the best immune-markers to be used for proper diagnosis and classification of acute leukemia, 40 patients who were diagnosed of having AL by peripheral and bone marrow analyses were purposefully included in this study.

5.7. Sampling techniques

A convenient sampling method were used and patients who were newly diagnosed with acute leukemia enrolled in Tikur Anbessa specialized hospital hematology unit from June to Aug 2016 and who fulfilled the inclusion and exclusion criteria were consecutively included in the study.

5.8. Data collection technique

5.8.1. Study procedure

Consent and assent were obtained from acute leukemia diagnosed patients using a standardized and structured format. Socio-demographic data and morphologic results of the peripheral blood and bone marrow aspirate smears of the study participants were collected from the patient medical record by the data collector. Peripheral blood was collected by the assigned nurses and delivered immediately to the principal investigator for flow cytometry analysis at the ALERT and AHRI Laboratory.

5.9. Laboratory methods

5.9.1. Principle of Flowcytometry

Flow cytometry uses principles of light scatter, and light excitation and emission of fluorochrome molecules to generate specific multiparameter data from particles and cells in the size range of 0.5µm to 40µm diameter. The cells are hydrodynamically focused in a sheath of phosphate buffered saline before intercepting an optimally focused light source. Lasers are most often used as light source in flow cytometry. As the cells of interest intercept light, they scatter it, and fluorochromes are excited to a high energy state. This energy is released as photons of light with specific spectral properties unique to different fluorochromes.

Flow cytometry is used for analyzing large populations of single cells; cells are labeled with a fluorescent dye which is coupled to a monoclonal antibody (mAb) which binds to those cells coated with the antigen for which the antibody is specific. Cells travel in suspension one by one and a laser beam is directed at the stream. As each labeled cell passes through the beam, it's resulting fluorescence and angle diffusion is detected by a photocell. Each particle is characterized by different variables, Forward Angle Light Scatter (FSC) related to the cell size and SSC, the Side Scatter related to the cell granularity and complexity.

5.9.2. Flow cytometric procedure

For immunophenotyping PB samples were collected in EDTA tubes and immediately transported to the flow cytometry laboratory and processed and stained within 24 hrs. Flow cytometric analyses were done with BD FACSCalibur instrument (4-color) at ALERT/AHRI Laboratory.

For surface antigen staining, 100 micro liters of sample blood was stained with 10 micro liters of fluorescently labeled monoclonal antibody (mAb) and incubated in the dark at room temperature (RT) for 20 minute, lysed with BD FACS lysing solution, and incubated at room temperature for an additional 10 min. After centrifugation at 400 x g, the tube supernatants were decanted, the cells vigorously were resuspended with phosphate buffered saline (PBS) again centrifuged, supernatant decanted, and cells resuspended in appropriate amount of PBS (typically 400 ul), and acquisition done on a FASCalibur flow cytometer.

For detection of cytoplasmic antigens, peripheral blood samples were surface stained with 10 micro liters of anti-CD45 PerCP-Cy5.5 and incubated in the dark at RT for 20 minutes and lysed lysing solution, PBS was added to a final volume of 4cc and cells centrifuged as above. After the supernatant was decanted, 500 µl of permeabilization reagent (BD Perm-2 diluted with a 10 fold excess of distilled water to create a 1x stock solution) was added. After a 10 minute incubation at RT in the dark, cells were washed with PBS, 10 micro liters of conjugated antibodies specific for cytoplasmic antigens were added, and the cells vortexed and incubated for 20 min in the dark. The cells were then washed after centrifugation at 800x g for 12 minutes, decanted, resuspended in 400 ul of PBS and acquired on the FACSCalibure. The mAbs used were conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll cyanin 5.5 (PerCP-Cy5.5), or allophycocyanin (APC). Different combinations of mAb against the following antigens were used: cytoplasmic CD3 (cCD3), cytoplasmic CD79a (cCD79a), cytoplasmic MPO (cMPO), CD34, CD3, CD4, CD7, CD8, immunoglobulin kappa or lambda light chains, CD14, CD45, CD10, CD19, CD13, CD33, CD14, CD2, HLA-DR and CD117. Acquisition was performed on a FASCalibur flow cytometer. The cells were analyzed with the most appropriate blast gate using the combination of CD45 and side scatter with a prepared template. To assign molecular expression for categorical analysis, a marker was considered positive when at least 20% of the gated leukemia cells were positive. The following antibody panels were used for immumophenotyping:

1. Anti-CD8/CD4/CD3 (Dako catalog numberTC 660) with anti-CD45 PerCP-Cy5.5 (catalog number BD 564105)

2. Anti-CD19 /CD56/ CD3 (Dako catalog number TC 662) with anti-CD45 PerCP-Cy5.5 (as above)
3. Anti-CD19/ kappa/lambda (Dako catalog number DakoTC 669) with anti-CD45 PerCP-Cy5.5 (as above)
4. Anti-cMPO/cCD79a/cCD3 (Dako catalog number TC 667) with anti-CD45 PerCPCy5.5 (as above)
5. Anti-CD33/CD34/CD117 (Dako catalog number TC 686) with anti-CD45 PerCPCy5.5 (as above)

6. Anti-CD13-FITC (Dako catolog number F0831); anti-HLA-DR-APC (Dako); anti-CD10-PE (Dako), anti-CD45 PerCPCy5.5 (as above)
7. Anti-CD45-FITC and anti-CD14-PE (BD leukogate)
8. Isotype controls:IgG1-APC (BD catalog number 345818) , IgG1-FITC (BD 345818); IgG1-PE (BD 340013); with anti-CD45 PerCP-Cy5.5-for surface stain
9. Cytoplasmic Isotype controls: same as above. Surface stain-for cytoplasmic stain. Anti-CD2-FITC, (BD Catalog number #555326), anti-CD7-PE (BD catalog number #555361), CD45 PerCP-Cy5.5 (as above)

Gating of blasts

For the immunophenotypic analysis of acute leukemia, CD45 in combination with perpendicular light scatter was used for gating of leukemic blasts. The observations that leukemic blasts usually show less intense CD45 expression compared with normal blood leukocytes has simplified gating strategies for leukemic analysis (6). In cases when CD45 expression was not distinguishable from normal cells, the CD45-perpendicular light scatter gate was defined after back gating populations identified by the over expression of markers such as CD34 or CD117 which are considerably lower in frequency among normal blood leukocytes.

Acute lymphoblastic leukemia (ALL) was characterized as B or T-cell specific based on the expression of CD10, CD19, cyCD79a (B-cell markers) and CD3, CD4, CD8, CD2, cyCD3 and CD7 (T-cell markers). Acute myeloblastic leukemia cells were identified by their expression of cytoplasmic MPO, CD13 and CD33 and CD117. When a given leukemia cells expressed markers of more than one lineage (eg. myeloid and lymphoid), the final classification was based on recommendations from a reference textbook, itself based on likelihoods of aberrant expression in definitive cases. For example, a leukemia expressing both cCD3 and cMPO is much more likely to be T-ALL than AML based on known cases (26).

5.10. Data quality assurance

All the quality control measures were undertaken before starting the procedure. BD FACSCalibur Instrument photomultiplier tube and compensation settings were performed by using control blood samples stained with leukogate (anti-CD45-FITC and CD14-PE), and monostained with anti-CD3-FITC, anti-CD56PE, anti-CD45 PerCP-Cy5.5, and anti-CD3 APC.

Separate samples stained with surface or cytoplasmic isotype control antibodies were used to define gates categorizes a given marker as positive or negative. In addition, the dead cell stain 7-AAD was run as a separate sample for most all patients to assure that dead cells did not substantially contribute to the phenotypic profile.

5.11. Data interpretation

Marker expression was defined as percentage of leukemia gated cells above the threshold value defined by the negative isotype control tubes. Leukemia gates were defined by CD45 and perpendicular light scatter as defined above.

5.12. Data Analysis

SPSS version 20 was used for data entry and analysis. Concordance analysis was performed with the level of significance based on a p-value of less than 0.05. Kappa value was calculated for the morphology result versus flow cytometry. Agreement level was determined using kappa values. Agreement was considered "Fair" when kappa value lies between 0.21-0.40, moderate 0.41-0.60, Substantial 0.61-0.80, and Almost perfect when kappa is 0.81-1.00 (27).

5.13. Ethical considerations

This project proposal were approved and ethically cleared by Department Ethics and Research Committee (DERC) of Addis Ababa University (AAU), College of Health Science (CHS), School of Allied Health Sciences (SAHS), Department of Medical Laboratory Science (DMLS) and AHRI/ ALERT Ethical Review Committee (AAERC) and AUU department of Internal medicine and pediatric. Each patient were notified about the purpose of the study, their right to refuse to participate in the study, and confidentiality of the information gathered. Written informed consent was obtained from study participants (see annex III & IV). Blood specimens were collected study procedure. The morphology result of PM and BM were extracted from the Medical record of the patient. The FC results were given to the study participants for free. Patient information was kept confidential by keeping records locked and electronic files password protected.

5.14. Result dissemination

Flow cytometric phenotyping results has been communicated to the referring clinician from TASH, and in addition, has and will be reviewed critically in the context of individual patient management as well as prior to the commencement of other related leukemia phenotyping studies planned. The findings will also be published in peer reviewed journals.

5.15. Work Flow

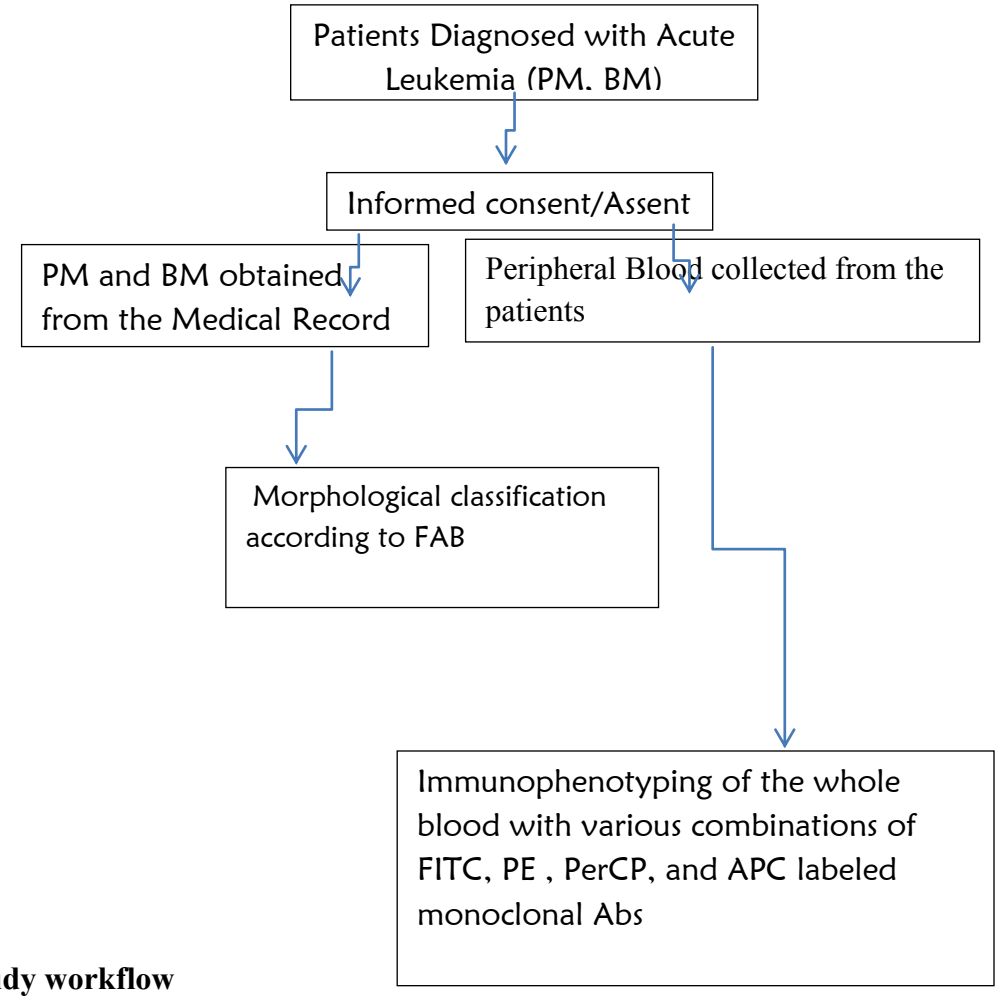

Figure 1. Study workflow

5.16. Operational definitions

Immunophenotyping: is a technique used to analyze heterogeneous populations of cells for the purpose of identifying the presence and proportions of the various markers (eg proteins) expressed by cells.

Flow cytometry: is the methodology used to detect cell surface markers or antigens using monoclonal antibodies conjugated with different fluorochromes.

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

Intensity of antigen expression: antigen binding capacity of a given fluorochrome antibody conjugate.

CD: is an abbreviation for **C**luster **D**ifferentiation which classifies identical molecules recognized by different clones of antibodies. Practically speaking it typical defines cell surface proteins or markers which are differentially expressed on different populations of lymphoid and non-lymphoid cells, and hence is very useful for immonophenotyping leukemia cells.

Aberrant Antigen Expression: deviating from normal expression; the expression of common myeloid markers on lymphoid cells and vice versa.

Kappa coefficient: is a statistic which measures inter-rater agreement for qualitative (categorical) items.

Strength of the Agreement: Based on strength of Kappa coefficient: < 0.00 -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; and 0.81-1.00 Almost Perfect.

6. Result

6.1. Gating of Leukemic cells and definition of marker positive and negative cells

Figure 2 depicts plots defining multiple parameters after staining of leukemia containing peripheral blood. Leukemia cells, outlined in the black polygon (upper right panel) typically exhibited reduced CD45 expression compared to normal leukocytes. Depicting CD45 expression (x-axis) as a function of multiple other markers (eg CD13, CD10, and HLA-DR in this example) thus can readily reveal the expression of such markers on leukemia cells (blue circles), as opposed to normal peripheral blood cells with higher CD45 density (Fig 2, middle panels). Gating (electronically selecting) leukemia cells on the basis of CD45 and SSC, allows for the visualization of the marker on interest on the leukemia cells exclusive of non-leukemia cells (lower set of panels).

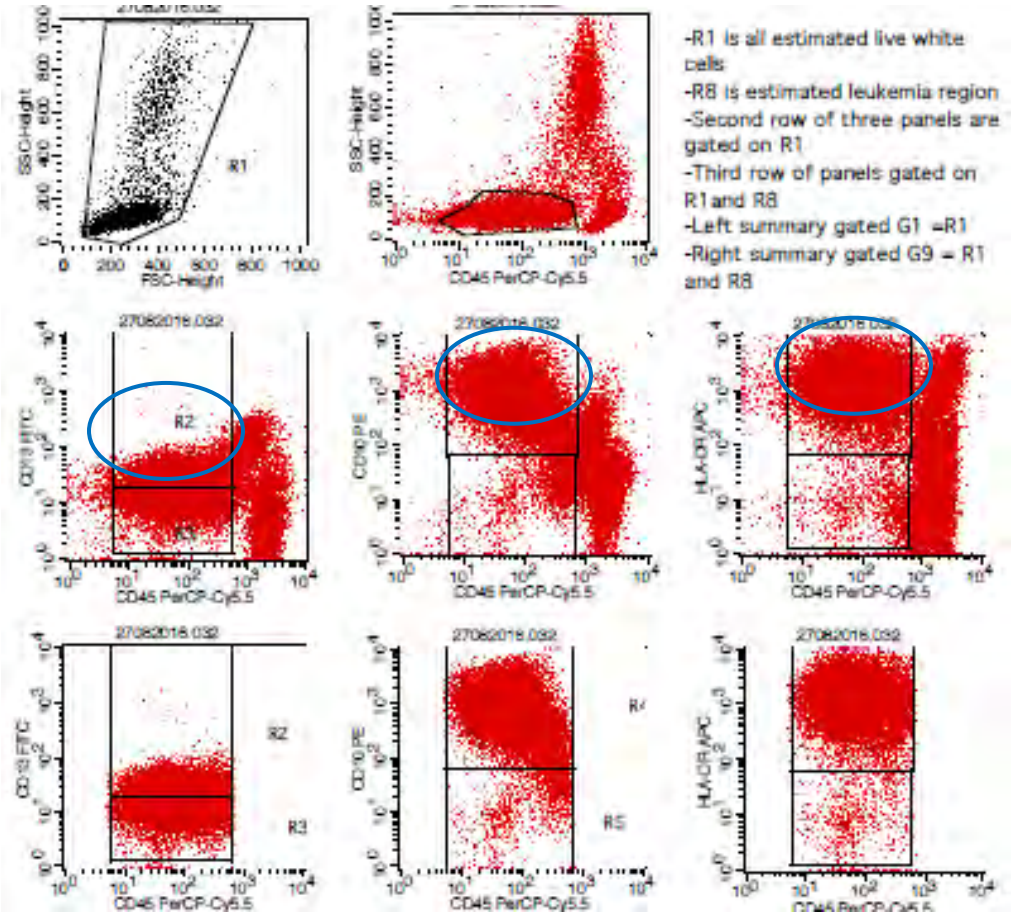

Figure 2 Definition of leukemia cells by expression of CD45 and side scatter and phenotyping of such gated cells.

The definition of cut off values or regions to define marker positive and negative cells is done with an independent control tube containing fluorochrome conjugated antibodies without reactivity to human cells. Such stained cells typically exhibit a small shift in non-specific staining relative to unstained cells, and thus serve as a more suitable means to define cutoff values. Fig 3 depicts such control antibody staining by anti-CD45 stained leukemia cells. By convention, a region is defined, eg R3 in the lower left plot, encompassing nearly all the control stained cells, and an adjacent region, eg R2, which includes all values along the y-axis higher than the first region (R3). These region settings are applied to all stained samples within the experiment, and thus define the marker negative population (eg R3) and marker positive population (eg R2) for each marker in the experiment. Typically, separate positive and negative regions are defined for each fluorochrome to be used in combination with CD45. In this example, PerCP-Cy5.5 is the fluorochrome conjugated to the anti-CD45 which defines the leukemia, whereas FITC, PE and APC are fluorochromes conjugated to control antibodies. Since all other marker antibodies are conjugated to either of these fluorochromes, the positive and negative regions defined in this control will serve as adequate controls for all other marker-antibody conjugates used in the experiment.

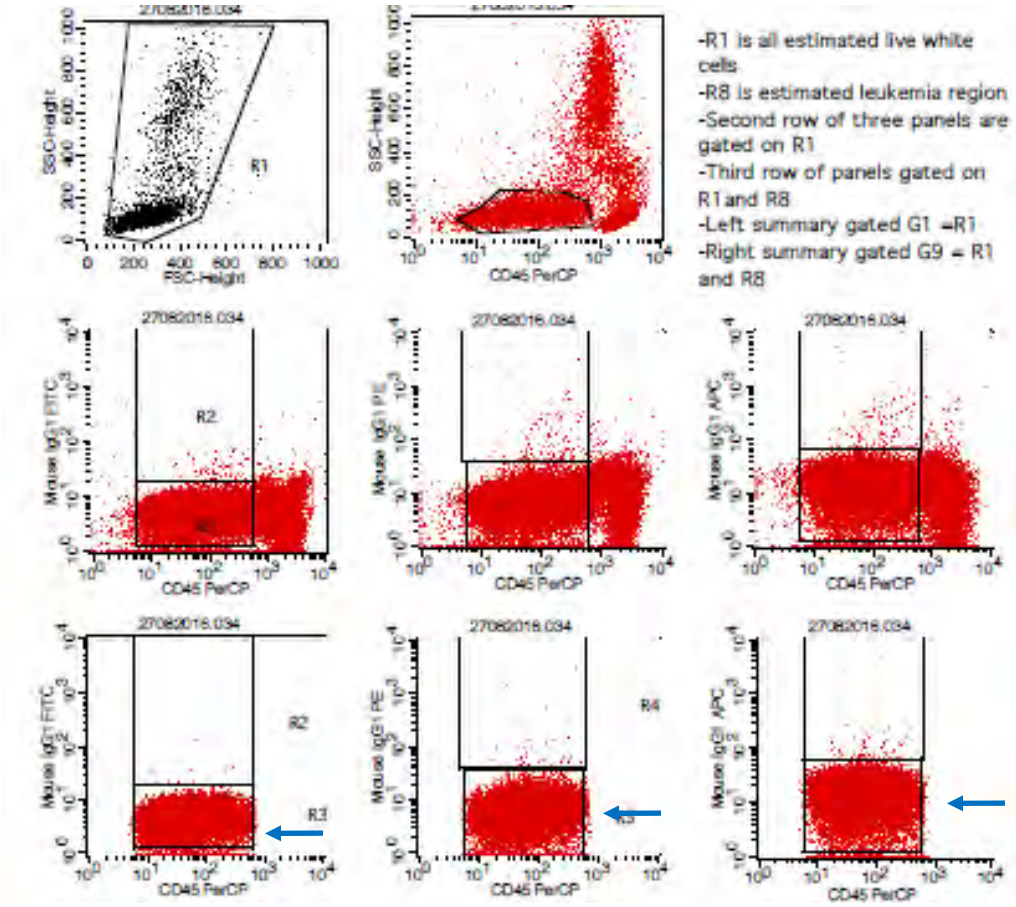

Figure 3 Creation of regions to define marker positive and negative cells using IgG control antibodies.

6.2. Leukemia case summary and demographics

A total of 40 acute leukemia cases were phenotyped by flow cyomtery; 21 were classified as acute myelogenous leukemia (AML) and 19 as acute lymphocytic leukemia (ALL). Of the ALL cases, there were 10 (52.6 %) identified as B lineage leukemia cells (B-ALL), and 9 (47.5%) defined as T lineage cells (T-ALL). With respect to gender frequency, females represented 47.5% and males 52.5% of the cases. 25% of the patients were less than 18 years of age while 75% were 18 and above.

Table 1 Demographic of acute leukemia patients at Tikur Anbessa Specialized Hospital, Addis Ababa, June 2016 to August 2016 (n=40)

Variable*	Number	Per cent (%)
Male	21	52.5
Female	19	47.5
Age <18 years old	10	25
Age 18 and above	30	75.0
ALL classification*	19	47.5
AML classification	21	52.5

* ALL is acute lymphocytic leukmemia; AML is acute myelocytic leukemia; classification as defined flow cytometry

6.3 .Phenotypic analysis of AML, B-ALL, and T-ALL

Myeloid markers evaluated included CD33, CD13, CD117 and MPO. A given marker expressed by >20 % of the gated leukemia cells were defined as positive, and the percentage of positive cases among all AML for each marker determined. MPO, the most commonly expressed marker was expressed in 20 of 21 (95%) AML cases, CD13 was expressed in 19 of 21 (90%) of the cases and CD33 and CD117 in 18 (85.7%) and 16 (76%) cases, respectively. The total marker positive cells were also defined for each leukemia case and each marker, and the mean and standard error for each marker determined among the entire set of AML cases. The mean %

MPO, % CD13, % CD33 and % CD117 positive cells was 86.6 ± 20.8, 60 ± 30.0, 65.7± 32 and 53 ±32.7, respectively (Table2).

Among acute leukemia cases classified as B-ALL, cCD79a and CD19 were expressed by at least 20% of leakemic cells for all cases, whereas CD10 was positive in 80 % of the cases. The % mean + standard deviation for cCD79a, CD19, and CD10 for the B-ALL group was 82.2 ± 16.2 and 77.4± 25.1, 60.2 ± 35.9, respectively. (Table 2).

All cases of T-ALL were positive for cytoplasmic CD3 with a mean of 86.9 ±11.7 % positive cells; CD7 was positive in 7 of 8 cases, and among all cases of T-ALL, the mean % CD7 positive cells was 71.9 ± 35.8. More variable in expression among T-ALL cells was surface CD3, positive in only 66% of the cases, and CD4 and CD8, positive in 55% and 44% of the T-ALL cases, respectively. Similarly variable, the mean % surface CD3, CD4, and CD8 positive cells among the 10 cases of T-ALL was 40.4 ±27.7, 24.3 ±25.4, and 25.1± 23.6, respectively. CD2, a marker normally expressed by nearly all mature T cells and other non-T cells, was positive in 5 of 7 cases (71%), and the mean among all cases defined with this marker was 59.1 ± 36.4 % positive cells (Table 2).

6.4. Expression of Markers of Immaturity

We evaluated markers of immaturity including CD34, known to be present on early progenitor cells within AML, B-ALL, and T-ALL lineages; CD117, expressed principally on early AML lineage cells; and HLA-DR, detectable on early AML and T-ALL, but constitutively expressed on all B-ALL lineage cells. Among AML cases, CD34 was expressed by 13 of 21 cases (62%), whereas HLA-DR was positive in 14 of 21 cases (66.7%) (Table 3) . All 13 of the patients positive for CD34 were also positive for HLA-DR, whereas 7 of 21 (33%) were negative for both markers, and 1 positive for HLA-DR but CD34 negative. Moreover, CD117 was simultaneously expressed in 11 of the 13 HLA-DR+CD34+ cases, and an additional 5 AML cases which were HLA-DR-CD34-. In the B-ALL group of leukemias 5 of 10 (50%) exhibited CD34 expression, and, as expected, all 5 co-expressed HLA-DR (Table 3). In the T-ALL group, 1 case was CD34+HLA-DR+, 3 cases were CD34-HLA-DR- (and 5 additional cases expressed either CD34 or HLA-DR, not shown in Table 3). One T-ALL leukemia expressed CD117 without HLA-DR or CD34 (Table 3).

Table 2 Expression of phenotypic markers by different categories of leukemia.

CD Marker	AML			B-ALL			T- ALL		
	% Pos Cases ⁑ (n = 21)	Mean	SD	% Pos Cases ⁑ (n=10)	Mean#	SD	% Pos Cases ⁑ (n=9)	Mean	SD
CD3	0%	2.0	2.8	0%	2.0	2.3	6 (66.6%)	40.4	27.7
CD4	3(14%)	12.1	14.8	0%	3.6	3.5	5 (55.6%)	24.3	25.4
CD8	0%	1.4	2.7	0%	1.5	1.1	4(44.4%)	25.1	25.6
CD19	1(4.7%)	5.7	9.2	10(100%)	77.4	25.1	1(11%)	14.6	26.8
cCD79a	4(19%)	12.7	19.8	10(100%)	82.2	16.2	0%	4.6	3.8
cCD3	0%	1.3	1.9	0%	4.5	5.6	9(100%)	86.9	11.7
CD10	3(14.2%)	9.8	21.6	8(80%)	60.2	35.9	4(44.4)	40.4	44.7
CD33	18(85.7%)	65.7	32	1(10%)	7.4	8.8	2(22%)	22.6	40.7
CD13	19(90%)	60	30.0	4(40%)	9.4	11.8	2(22%)	19.7	34.1
cMPO	20(95%)	86.6	20.8	6(60%)	45.6	39.9	2(22%)	12.7	18.1
CD117	16 (76%)	53	32.7	0%	0.41	0.5	1(11%)	3.5	7.7
CD2	-	-	-	0%*	2.6*	1.3*	5(71%)‡	59.1‡	36.4‡
CD7	-	-	-	1(10%)	11.2	16	7(87.5)†	71.9†	35.8†
CD34	13(62%)	52	42.0	5(50%)	35	41.8	3(33%)	15.6	22.9
HLA-DR	14(66.6%)	54	43.2	10(100%)	86	14.8	3(33%)	13.1	18.5

The percentage positive cells determined for each marker and each case, and the mean and standard deviation of marker positive cells determined for the entire group of leukemia cases

⁑ A leukemia case was scored positive for a given marker if more than 20 % of the leukemia cells were positive for that marker

* n = 6

‡ n = 7

† n = 8

Table 3 Co-expression of HLA-DR, CD34 and/or CD117 in acute leukemias

CD Markers	AML (n = 21)	B-ALL (n = 10)	T-ALL (n = 9)
CD34+/HLADR+	13 (62%)	5 (50%)	1 (11%)
CD34-/HLADR-	8 (38%)	0 (0%)	3 (33%)
CD34+/HLA-DR+/CD117+	11 (52%)	0 (0%)	0 (0%)
CD34-HLA-DR-/CD117+	5 (24%)	0 (0%)	1 (11%)

6.5. Aberrant Antigen Expression

In our study, aberrant expression of myeloid antigens was seen in some ALL cases (Table 2). Within the B-ALL group, CD33, CD13 and cMPO was observed in 10%, 40% and 60% of the cases, respectively. In none of the cases was more than one myeloid marker detected. 2 of 9 cases of T-ALL displayed cMPO, 1 case were positive for CD19, and 1 positive for CD117. 44 % of T-ALL cases showed CD10 expression and these were all negative for CD19. Out of 9 cases of T-ALL, two expressed CD33 and CD13.

Aberrant expression of lymphoid antigens was also seen among AML leukemias (Table 2). One case was positive for the B lineage marker CD19, and 4 of 21 (19%) were positive for the B lineage marker cCD79a.

6.6. Agreement of acute leukemia classification by morphology vs flow cytometry

The traditional FAB classification of ALL and AML is based on morphology and cytochemical staining of blasts, the recent classification schemes proposed by WHO require the additional evaluation of the leukemic blasts by flow cytometry and molecular analysis of chromosomal rearrangements and mutations, while the EGIL classification has proposed that acute lymphocytic leukemia subtypes be classified on the basis of immunophenotype alone. In order to provide perspective on the flow cytometry based classifications for the leukemias in our study, we compared them with classification provided by pathologists at Tikur Anbessa Specialized Hospital after review of peripheral blood and bone marrow aspirate smears. Of the 40 leukemia

cases we phenotyped by flow cytometry, 23 were classified as AML, 15 as ALL, and two as indeterminate by morphology. This contrasted with the flow classification of 21 AML and 19 ALL.

As depicted in Table 4, among the 21 cases of AML classified by flow cytometry, 19 (90.4%) were concordantly diagnosed as such by morphology, while 2 were classified as ALL. Conversely of the 23 cases of AML by morphology, 19 were classified similarly by flow cytometry. Of the 15 cases of ALL defined by morphology, 13 were defined as such by flow cytometry, while 2 were classified as AML by flow phenotyping. Finally, the two indeterminant cases by morphology were typed as ALL by flow cytometry analysis. The degree of statistical concordance is summarized in Table 5, illustrating an overall 80% agreement between morphology and flow cytometry classification (kappa = 0.6154, p = 0.0000).

Table 4 Agreement of acute leukemia classification by morphology vs flow cytometry

		Flow Cytometry		
	Classification*	**AML**	**ALL**	**Total**
Morphology	**AML**	**19**	**4**	**23**
	ALL	**2**	**13**	**15**
	Inconclusive	**0**	**2**	**2**
	Total	**21**	**19**	**40**

*The degree of association between morphology and flow cytometry was evaluated by chi-square testing (Pearsons Chi-square = 14.6 Person, p = 0.001)

Table 5 Statistical Agreement between morphology and flow cytometry in leukemia classification.

Agreement	Expected Agreement	Kappa	Std . Err.	Z	Prob>Z
80.00%	48.00%	0.6154	0.1448	4.25	0.0000

6.7. Flow cytometry phenotype among leukemias cases with discordant classification.

To explore possible reason(s) for discrepancies between morphology and flow evaluation of leukemias, we considered the possibility that discordant cases may have preferentially occurred in leukemias which expressed markers of more than one lineage. Table 6 summarizes cell surface phenotype of some of the discordantly classified leukemias stratified by markers which were interpreted to be lineage specific (and hence defined the flow based classification) and those interpreted to be aberrantly expressed. Two cases of T-ALL were defined by flow cytometry which had been classified as AML by morphology. Both cases were strongly positive for cytoplasmic CD3 and variably expressed other T cell markers. However, both leukemias also expressed some myeloid antigens including MPO, CD33 and/or CD13. According to the literature, myeloid antigen expression is commonly observed among cases of T-ALL, but conversely cCD3 expression uncommon among AML cases (26). Consequently, the lineage was assigned to T lymphoid rather than myeloid in these cases. Similarly, another case occurred in which the acute leukemia was diagnosed as AML by morphology, but B-ALL on the basis of flow, and in this case myeloid markers CD13 and CD33 were also present, the final flow classification again based on the likely lineage origin after consultation of the literature (26).

Table 6 Cell surface phenotype among some acute leukemias cases with discordant classification.

PM Result	BM Result	FCA Result	Lineage specific Phenotype	Aberrant Expression
AL	Inconclusive	B-ALL	CD19,cCD79a,CD10,HLA-DR	cMPO
ALL	Inconclusive	AML	CD33,CD13,CD117,cMPO,CD34,HLA-DR	No
AML	AML	T-ALL	CD8,cCD3, CD2,CD7	cMPO
AML	AML	T-ALL	CD3,cCD3,CD7,CD2,CD34	CD10,CD33,CD13
AML	AML	B-ALL	CD19,cCD79,CD10, CD34, HLA-DR	CD13,CD33

7. Discussion

The capacity to quantitative numerous unique molecules on individual cells by flow cytometry is ideal for the study of leukemic cells. Thus, such immunophenotyping has become an important and sensitive tool contributing. with clinical, morphological, cytochemical and cytogenetic analyses, to the classification, prognosis, and disease monitoring of acute leukemia (28). The classification into B- and T-lineage ALL is important for risk stratification and therapy of the patients (29).

Classification of Acute Leukemia

Based on flow cytometry phenotyping, 40 blood samples of acute leukemia were classified into 21 AML (52.5%) and 19 ALL (47.5%) cases. This distribution is similar to a previous study in Ethiopia by Shamebo et al (15) , which demonstrated 46.3% AML and 53.7% A LL among acute leukemia cases during a 10 year period from 1982-1992. However, both studies have been based on patients diagnosed in a tertiary care referral center, and hence can only be considered approximate estimates for the incidence of these leukemias nation wide. ALL cases were further sub classified into B-cell (10 cases) and T-cell (9 cases) ALL.

Our results are also comparable to a study by Salem *et al* in Egypt which demonstrated, except that they observed a higher fraction of AML (69%) than ALL, and a higher proportion of B-ALL (75%) relative to T-ALL among ALL cases (17).

Immunophenotypic profile of of AML

From the antigens expressed in AML linage, MPO was nearly universally expressed, with CD13, CD33 and CD117 also expressed in the major proportion of patients. These phenotypic findings are quite similar to those reported by Salem *et al* (17). Our results are also in agreement with that by Paredes-Aguilera *et al* (30) who concluded that in comparison with CD13, CD14 and CD33, MPO was the most sensitive marker for AML.

Immunophenotypic Profile of B-ALL

All cases of B-ALL expressed cCD79a and CD19. CD10 was variable expressed (80% positive). Of note, according to the EGIL classification system, CD10 is not expressed by the most immature B-ALL cells (B1 or pro B cells), but is expressed by B-ALL at intermediate stages

(B2/common B cells or B3/pre-B cells). CD10 expression is reduced in the most mature EGIL stage (B4). The latter commonly express surface Immunoglobulin (Kappa and Lambda) which was uniformly negative in our cases, suggesting that, at least by the EGIL classification, our B-ALL cases all represented immature B cells (B1, B2 and B3) (20). Our findings are quite consistent with those of Zahid Kaleem (18) *et al* as well as Salem *et al* (17).

Paredes-Aguilera *et al* (30) compared the sensitivity of B lineage markers cCD79a, cyCD22, CD19, CD20 and CD22 in their study of 74 cases of B-ALL and showed that cCD79a has 100% sensitivity and 80% specificity followed by cCD22, which showed 97% sensitivity and 88% specificity. They concluded that these are highly sensitive markers for B-cell ALL cases. The study concludes that the cytoplasmic CD79a, CD3, and MPO were highly sensitive markers for B-ALL , T-ALL and AML respectively.

Aberrant expression of myeloid antigens, CD33 (10%), CD13 (40%) and cMPO (60%) were seen in some cases of B-ALL. The study by Salem *et al* also observed aberrant expression of CD13 and CD33 myeloid markers among B-ALL cases, but markers MPO and CD117 were rarely expressed (17).

Immunophenotypic profile of T-ALL

All cases of T-ALL were positive for cytoplasmic CD3. In addition, the T cell marker CD7 was observed in all but one case. Surface CD3 was observed in only 66% of the cases, an expected finding of immature T lineage cells which have expressed CD3 but incomplete production T-Cell receptor proteins which normally associate in a complex with CD3 and are required for CD3 to be expressed on the surface. CD4 (55%) and CD8 (44%) were variably expressed in T-ALL. Similarly, studies by Salem et al **(17)** and Zahid Kaleem et al (18) showed universal expression of cytoplasmic CD3, high CD7, but variably percentages of surface CD3, CD4 and CD8.

In our study, aberrant expression on T-ALL was seen for myeloid markers CD33 (22%), CD117 (11%), CD13 (22%) and MPO (22%), whereas B cell markers CD10 (44%) and CD19 (11%) were inappropriately expressed. The study by Salem *et al* (17) and others observed aberrant expression of CD10 ranging from 19-43% and CD19 varying from 0 to 2%.

Expression of Maturation Antigens

Expression of HLA-DR and CD34 was seen in about two thirds of patients with AML, with nearly all cases either co-expressing these two immature markers or expressing neither molecule. The study by Salem *et al* exhibited, among non-Acute Promyelocytic Leukemia (FAB M3 AML) cases, similar CD34 but higher HLA-DR. Most of the cases of AML in their study were classified as either M1 or M2 AML leukemia, which would be expected to have high CD34 and HLA-DR. Although we did not classify AML cases by morphology according to the FAB system, the predominance of CD34 and HLA-DR in our cohort would be consistent with a high fraction of M0, M1 or M2 cases of AML. Further work in Ethiopia will be needed to determine more precisely FAB classification in comparison with flow cytometry.

About half of the B-ALL cases were positive for CD34. HLA-DR is expressed at all stages of B cell maturation, and as expected, all of our B-ALL cases were HLA-DR positive. These findings on a limited number of B-ALL cases concur with those of Zahid Kaleem et al (18) who observed about 70% of B-ALL expressing CD34 and virtual all positive for HLA-DR. Only about a third of T-ALL cases were positive for both HLA-DR and CD34, suggesting that these represent very immature forms of T-ALL.

Morphology and Flow cytometry

In general we observed about 80% agreement between flow cytometry analysis and cell morphology as determined by evaluation of peripheral blood and bone marrow aspirate smears. Others have reported similar or higher concordance. Patel *et al* (25) showed a nearly identical concordance of 76% between flow cytometry and bone marrow morphology in acute leukemia cases in Kenya, with a study by Suprihadil et al (31) exhibiting somewhat higher concordance. In contrast to these studies, Belurkar et al (22) observed a concordance rate as high as 86% between morphologic/cytochemical diagnosis and flow cytometric diagnosis among 50 cases of leukemia, and Khalil et al., (32) reported 97% concordance with immunophenotyping and morphology together with cytochemistry. Importantly, the latter two studies combined morphology with cytochemistry in acute leukemia diagnosis, and no cytochemistry was done in either our study or the Kenyan study. Cytochemistry is particularly useful at specifically identifying myeloid.

Summary and Synthesis

In summary, we performed a pilot study to explore the feasibility of flow cytometry in the Ethiopian setting for the diagnosis of acute leukemia, and to compare results with currently used morphology techniques. We observed that AML, B-ALL and T-ALL could be confidently discriminated. In addition use of maturational markers by flow cytometry could readily classify leukemias into those which were relatively immature from those which were relatively mature.

A notable finding in this study was the inappropriate or aberrant expression of myeloid lineage markers on lymphoid leukemias and conversely the expression of lymphoid markers on myeloid leukemia. Aberrent expression of markers has been repeatedly documented in the literature. The underlying mechanism remains to be explored, but a classic example of phenotypic abnormalities has been observed with a demonstrated with the M2 AML leukemia associated with the t(8,21) translocation, which is almost always associated with the expression of lymphoid markers CD19 and sometimes CD79a as well as CD56 among AML leukemia cells. On the molecular level, the translocation results in the production of the fusion transcript RUNX1-RUNX1T1 which induces proteins which specifically activate B lineage molecules (17). These findings underscore the importance of including several myeloid and lymphoid markers rather than a single lineage marker, and that in general the more positive markers are present for a given lineage the more likely the leukemia can be assigned to that lineage (17).

While there was good concordance between classifications by flow cytometry compared with traditional morphology, we did, however, observe several discrepancies in assigning acute leukemia to either myeloid or lymphoid lineages. In several of the discordant cases, the leukemias expressed markers of more than one lineage. For example, flow cytometry phenotyped lymphoid leukemias expressed myeloid lineage molecules; hence it is conceivable that expression of these and other myeloid lineage markers contributed to a myeloid morphological appearance upon microscopy and subsequent AML rather than ALL classification. The discrepancies we observed here are in fact mirrored in many previous studies and reinforce the well accepted view that diagnosis and prognosis of leukemia for optimal clinical management ideally combines traditional morphological, cytogenetics and cytochemical approaches with new mythologies including flow cytometry and fluorescent in situ hybridization (FISH), with none of these approaches alone being adequate for a gold standard diagnosis and prognosis. While

capacity for cytochemistry already exists in the country, we are, as part of a larger initiative, introducing capacity for cytogenetics and FISH in the country, so that collectively all these methodologies will be available.

8. Strength and Limitation of the study

8.1 Strength

The study strengths included extensive lab protocol optimization prior to study initiation, use controls to estimate non-specific staining; performance in an ISO accredited laboratory, and local availability advice from flow cytometry experts.

8.2.Limitations

Despite the relatively large number of markers used in this study, many more markers have been used in many leukemia phenotyping studies (26) . As stated above, the study would be improved by parallel analysis by cytochemistry, cytogenetic or FISH, but these methodologies were either unavailable or not yet developed in the country. Markers for AML leukemias M6 (erythroleukemia) or M7 (megakaryoblastic leukemia) were not included hence such leukemias if present may have been missed. Final more extensive patient clinical information was not captured.

9. Conclusion

It was found that MPO and CD13 were the most commonly expressed markers of AML. CD19 and cCD79a were found to be the most sensitive markers for B-ALL while cCD3 and CD7 were the most commonly expressed markers for T-ALL. In general there was good agreement between flow cytometry and morphology in the discrimination of myeloid from lymphoid leukemias. The ability of flow cytometry to confirm morphological diagnoses, to provide an alternative when morphology results are indeterminant and to more precisely define lymphoid leukemias suggests it can represent a valuable supplementary methodology for clinical management of leukemia in this country.

10. Recommendation

The study demonstrates the feasibility of application of flow cytometry towards the diagnosis of leukemia in Ethiopia. Further research including use of expanded panels of markers for more precise diagnosis, analysis of bone marrow aspirates, as well as analysis of leukemized versions of lymphoma should be undertaken. In addition, development of complementary diagnostic modalities such as cytogenetics and FISH should be undertaken in order to strength the capacity of leukemia diagnosis and prognosis in the country.

11. Reference

1. Pui C-H, Evans WE, editors. A 50-year journey to cure childhood acute lymphoblastic leukemia. Seminars in hematology; 2013: Elsevier.
2. Mittal P, Meehan KR. The acute leukemias. Hospital Physician. 2001;5:37-44.
3. ANGELESCUn S, BERBEC NM, COLITA A, BARBU D, LUPU AR. Value of Multifaced Approach Diagnosis and Classification of Acute Leukemias. Mædica. 2012;7(3):254.
4. Béné MC, Bernier M, Castoldi G, Faure GC, Knapp W, Ludwig W, et al. Impact of immunophenotyping on management of acute leukemias. Haematologica. 1999;84(11):1024-34.
5. Rowan R, BAIN B, England J, Hyde K, Matutes E, Reilly J, et al. Immunophenotyping in the diagnosis of acute leukemias. BRITISH MED JOURNAL PUBL GROUP BRITISH MED ASSOC HOUSE, TAVISTOCK SQUARE, LONDON, ENGLAND WC1H 9JR; 1994.
6. Saxena R, Anand H. Flow cytometry in acute leukemia. Indian Journal of hematology and blood transfusion. 2008;24(4):146-50.
7. Orfao A, Chillón MC, Bortoluci AM, López-Berges MC, García-Sanz R, Gonzalez M, et al. The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of PML-RARalpha gene rearrangements. Haematologica. 1999;84(5):405-12.
8. Sengar M, Rai AK, Saxena A, Singh A, Raina V, Seth T, et al. Acute leukemia: Diagnosis improved by flow cytometry in addition to morphology. Asia-Pacific Journal of Clinical Oncology. 2009;5(1):55-65.
9. Wozniak J, Kopec-Szlezak J. Standard immunophenotyping of leukemia cells in acute myeloid leukemia (AML). Central European Journal of Immunology. 2008;33(1):24.
10. Ossenkoppele GJ, van de Loosdrecht AA, Schuurhuis GJ. Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms. British journal of haematology. 2011;153(4):421-36.
11. Del Vecchio L, Brando B, Lanza F, Ortolani C, Pizzolo G, Semenzato G, et al. Recommended reporting format for flow cytometry diagnosis of acute leukemia. haematologica. 2004;89(5):594-8.
12. Wang JC, Beauregard P, Soamboonsrup P, Neame PB. Monoclonal antibodies in the management of acute leukemia. American journal of hematology. 1995;50(3):188-99.

13. Mathers CD, Boschi-Pinto C, Lopez AD, Murray CJ. Cancer incidence, mortality and survival by site for 14 regions of the world. Geneva: World Health Organization. 2001;8.
14. Shamebo M. Acute leukaemias in adult Ethiopians in a teaching hospital. Ethiopian medical journal. 1994;32(1):17-25.
15. Shamebo M. Leukaemia in adult Ethiopians. Ethiopian medical journal. 1990;28(1):31-7.
16. Mhawech P, Buffone GJ, Khan SP, Gresik MV. Cytochemical staining and flow cytometry methods applied to the diagnosis of acute leukemia in the pediatric population: an assessment of relative usefulness. Journal of pediatric hematology/oncology. 2001;23(2):89-92.
17. Salem DA, El-Aziz SMA. Flowcytometric immunophenotypic profile of acute leukemia: mansoura experience. Indian Journal of Hematology and Blood Transfusion. 2012;28(2):89-96.
18. Kaleem Z, Crawford E, Pathan MH, Jasper L, Covinsky MA, Johnson LR, et al. Flow cytometric analysis of acute leukemias: diagnostic utility and critical analysis of data. Archives of pathology & laboratory medicine. 2003;127(1):42-8.
19. Purwanto YM, Gunawan S, Selvi Nafianti D, Purnomosari US, Westra G, Sutaryo JC. Immunophenotypic patterns of childhood acute leukemias in Indonesia. Asian Pacific Journal of Cancer Prevention. 2011;12:3381-7.
20. Peters JM, Ansari MQ. Multiparameter flow cytometry in the diagnosis and management of acute leukemia. Archives of pathology & laboratory medicine. 2011;135(1):44-54.
21. Ghosh S, Shinde S, Kumaran G, Sapre R, Dhond S, Badrinath Y, et al. Haematologic and immunophenotypic profile of acute myeloid leukemia: an experience of Tata Memorial Hospital. Indian journal of cancer. 2003;40(2):71.
22. Belurkar S, Mantravadi H, Manohar C, Kurien A. Correlation of morphologic and cytochemical diagnosis with flowcytometric analysis in acute leukemia. Journal of cancer research and therapeutics. 2013;9(1):71.
23. Wongprajun S, Auewarakul CU. A method comparison study of flow cytometry and cytomorphology to determine the percentages of blasts in patients with acute leukemia after induction and consolidation chemotherapy. J Med Assoc Thai. 2010;93(1):S157-64.
24. Rezaei A, Adib M, Mokarian F, Tebianian M, Nassiri R. Leukemia markers expression of peripheral blood vs. bone marrow blasts using flow cytometry. Medical Science Monitor. 2003;9(8):CR359-CR62.

25. Patel K, Lotodo T, Njuguna F, Emonyi W, Mining S, Buziba N, et al. Use of Flow Cytometry Immunophenotyping for Diagnosis of Acute Leukemia at Moi Teaching and Referral Hospital, Eldoret, Kenya. American Scientific Research Journal for Engineering, Technology, and Sciences (ASRJETS). 2015;13(1):72-80.

26. Ortolani C. Flow cytometry of hematological malignancies: John Wiley & Sons; 2011.

27. Viera AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. Fam Med. 2005;37(5):360-3.

28. Garand R, Robillard N. Immunophenotypic characterization of acute leukemias and chronic lymphoproliferative disorders: practical recommendations and classifications. Hematology and cell therapy. 1996;38(6):471-86.

29. Van Dongen J, Macintyre E, Gabert J, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease Report of the BIOMED-I Concerted Action: Investigation of minimal residual disease in acute leukemia. Leukemia. 1999;13:1901-28.

30. Paredes-Aguilera R, Romero-Guzman L, Lopez-Santiago N, Burbano-Ceron L, Monte CD, Nieto-Martinez S. Flow cytometric analysis of cell-surface and intracellular antigens in the diagnosis of acute leukemia. American journal of hematology. 2001;68(2):69-74.

31. Supriyadi E, Widjajanto PH, Purwanto I, Cloos J, Veerman AJ, Sutaryo S. Incidence of childhood leukemia in Yogyakarta, Indonesia, 1998–2009. Pediatric blood & cancer. 2011;57(4):588-93.

32. Khalil S, Jackson J, Pyle R, Robichaud M. Immunophenotyping of acute leukemia at King Faisal Specialist Hospital and Research Centre. Annals of Saudi medicine. 1995;15(2):137-9.

Annexes

Annex I.Information Sheet in English

Addis Ababa University, Collage of health science, School of Allied Health Science, Department of medical laboratory science
E-mail: SMLT@ethionet.et
Tel. +251 112-75-51-70

Participant Information sheet

1. **Study title:**

Diagnostic utility of Immunophenotyping by flow cytometry for diagnosis and classification of acute leukemia

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 time to read the following information carefully. Ask 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 Diagnostic utility of Immunophenotyping by flowcytometry for diagnosis and classification of acute leukemia. I hope that this will help to understand more about the disease classification and its specific therapy.

5. Why you have 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 . In this study a total of 76 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 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, the doctor in charge to treat your case (acute leukemia) will ask you some questions which you are expected to answer. These are: history of treatment related to your current case, sign and symptoms with clinical findings and time of the onset of the disease. In addition you will be asked your age, current address. You will be asked these questions as a normal treatment procedure even if you will not take part in this study.

As routine diagnosis procedure you will be asked to give bone marrow aspirate for morphologic examination which will be aspirated with a 10- or 20-ml plastic syringe from the iliac crest and this study gone take the result morphologic examination of your bone marrow aspirate. And you will give peripheral blood, which will be collected by inserting a small needle into a vein in your arm. If you take part in the research you will be asked to give your consent for the leftover sample to be used for the study.

8. What is the study procedure

If you take part in the research the demographic data assembled by your physician, morphologic and cytochemical 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. However, you will be given 100 ETB for transportation during participation in the project. 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 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 avoided, 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 is 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 give?

As already described, during the laboratory analysis we will use your given code not your 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 as your 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 Doctor, so as your 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 AHRI/ALERT Research Ethics committee and by the Addis Ababa university Research Ethics committee.

17. **How to give my consent**

    If you have 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

Metasebia Tegegen , MSc fellow

Mobile: 0912009896

AHRI/ALERT Ethical Committee:

Tel No: 0113 481285

AAU, Department of Medical Laboratory Science Research and Ethical Committee

Tel: 011 275 5170

Annex II.Information sheet in Amharic version

የመረጃ ቅጽ

1. የጥናቱ ማጠሪያ

የፍሉሳይቶሜትሪ ዘዴን አጣዳፊ የደም ካንሰር በሽታን ለማከም ና አይነቱን ለመለየት ማጠቃለል

2. በጥናቱ እንድሳተፉ ስለመጋበዝ

በዚህ ጥናት ላይ እንዲሳተፉ እንጋብዘዎታለን ነገር ግን በጥናቱ ከመሳተፍዎ በፊት የጥናቱን አላማና አስፈላጊነት በቅድሚያ መረዳት ያስፈልገዎታል፡፡ እባክዎ ጊዜ ወስደው የሚከተለውን መረጃ ያንብቡ፡፡ ማንኛውም ጥያቄ ወይም ግልፅ ያልሆነ ነገር ካለ ማጠየቅ ይችላሉ፡፡

3. የበሽታው ምንነት

አጣዳፊ የደም ካንሰር በሽታ መቅኔ በደንብ ያልደረሱ(ያላደጉ) የደም ሕዋሳትን ያለ አግባብ ሲያመርት ይከሰታል ፡፡ እነዚ ህዋሳት ወደ ደም ዝውወር በመግባት ህመም ያስከትላሉ ፡፡ በሽታውን በፍጥነት መለየትና አይነቱን መለየት ትክክለኛ ህክምናና መድኃኒት ለማግኘት ይረዳል፤ በተጨማሪም በሽታው የሚያመጣውን ህመም ና ጉዳት ይቀንሰዋል፡፡

4. የጥናቱ ዓላማ

እኔ አሁን የማጠናው አጣዳፊ የደም ካንሰርን በፍሉሳይቶሜትሪ ዘዴ ለማከም ና አይነቱን ለመለየት ነው ፡፡ የዚህም ጥናት ዉጤት ስለ በሽታው የተሻለ እውቀት እንዲገኝ እንዲሁም የሽታውን አይነት በጊዜ ለመለየት ና ለመከላከል በሚጠቅም ጥረት ጉልህ ድርሻ ይኖረዋል፡፡

5. እርሶ ለምን በዚህ ጥናት እንዲሳተፉ ተመረጡ

እርሶ በዚህ ጥናት ላይ እንዲሳተፉ የተመረጠበት ምክንያት አጣዳፊ የደም ካንሰር በሽታ ህመምተኛ በመሆንዎና በጥናቱ ሊካተቱ የሚችሉት አጣዳፊ የደም ካንሰር በሽታ ህመምተኞች ብቻ በመሆናቸው ነው፡፡ በዚህ ጥናት 76 የሚሆን አጣዳፊ የደም ካንሰር ህመምተኞች ይሳተፋሉ

6. በዚህ ጥናት ላይ ለመሳተፍ የግድ ያስፈልጋል?

በጥናቱ ላይ ለመሳተፍ የግድ አያስፈልግም፤ በፍላጎት ላይ ብቻ የተመሠረተ ነው፡፡ በጥናቱ ላይ ለመሳተፍ ከወሰኑ ይህ መረጃና መስማማትዎን የሚልጽ ቅጽ ይሰጠዎታል፡፡ መረጃውን ካነበቡና የሚከይቁት ጥያቄ ካለም በማጠየቅ በሚባ ከተረዱ በኃላ መስማማትዎን ይገልጻሉ፡፡ ከጥናቱ በፈለጉት ጊዜና ሰዓት ያለምንም ቅድመ ሁኔቴ ማቋረጥ ይችላሉ፡፡ እራስዎን ከጥናቱ በማገለልዎ ምክንያት ለህመምዎ የህክምና እርዳታ ከማግኘት አያግድም፡ እንደማንኛውም ታካሚ አስፈላጊውን የህክምና እርዳታ ያገኛሉ፡፡

7. በጥናቱ ላይ ከተሳተፍኩ ከእኔ ምን ይፈለጋል?

በጥናቱ ላይ ለመሳተፍ ከተስማሙ እርስዎን ለማከም የተመደበው ሀኪም ስለ በሽታዎ አንዳንድ እርስዎ እንዲመልሱ የሚከበቁ ጥያቄዎች ይጠይቅዎታል፡፡ ይህ ጥያቄ በመደበኛ የሕክምና ጊዜ የሚከይቅ በመሆኑ የዚህ ጥናት ብቻ

ጥያቄ አይደለም፡ ከወሰኑ የመቅኔ ምርመራ ወጪዎትን ጥናቱ ወስዶ ይጠቅማል በተጨማሪም አንድ የሾርባ ማንኪያ ደም ከክንድዎ ላይ እንዲሰጡ ይጠየቃሉ፡፡ እዚህ ጥናት ላይ ባይሳተፉም ቢሳተፉም በህጉ መሰረት አስፈላጊወን ህክምና ያገኛሉ::

8. ከእርስዎ ምን ይጠበቃል?

በጥናቱ ለመሳተፍ ከተስማሙ ዶክተርዎ እርስዎን በመጠየቅ የወሰደው ሜዲታወሽ ለጥናቱ ይወላል እንዲሁም ከላይ የተገለፀወን የመቅኔ ምርመራ ወጩን እና ደም እንዲሰጡ ይጠበቃል፡፡እነዚ ወጩች እና ናመፃዎች ለጥናቱ ምርምር ይወላሉ፡፡

9. በጥናቱ ላይ በሰተፉ ጥቅማ ጥቅም አገኛለሁኝን?

በዚህ ጥናት ላይ በመሳተፍዎ የተለየ ጥቅም በግል አያገኙም፡ ነገር ግን የትራንስፖርት ወጭዎ ይሸፈንልዎታል(100 ብር) በተጨማሪም በጥናቱ ወቅት የተገኘወን የላቦራቶሪ ወጩ ለእርስዎና ለዶክተርዎ ይገለጻል፡፡ እንዲሁም የእርስዎ በጥናቱ መሳተፍ ስለበሽታው አይነት ያለወን ጥሩ ግንዛቤ እንዲኖረንና በሽታወን ለማከም ጠቃሚ ሚጃ በመሳጠት ይጠቅመናል፡፡

10. በጥናቱ ላይ በመሳተፌ የሚደርስብኝ ጉዳት አለ?

ጥናቱ ላይ በመሳተፍዎ የሚደርስብዎ ትልቅ ጉዳት የለም፤ ነገር ግን የደም ናመፃ በሚወሰድበት ወቅት ለፈጠር የሚችለወን አነስተኛ ህመማና የደም መፍሰስ ለሚከወን ልምድ ባላቸወና እና ስልጠፍ በተሰጣቸው ባለሙያዎች ይከናወናል፡፡

11. ጥናቱ ሲያልቅ ከእኔ ምን ይጠበቃል?

ምንም አይጠበቅም፡

12. በዚህ ጥናት መሳተፍ በሚኒጥር ይያዛልን?

አዎን ለዚህ ጥናት የሚነበሰበው ናመፃ እና የናመፃ ወጩ በሚኒጥር ይያዛል፡፡ ስለ እርስዎ የሚልጽ ማንኛወም ነገር በናመፃም ሆነ በወጩ ላይ አይጻፍም፡ ወጩ ሲገለጽ ስም አልባ ይሆናል፡፡ ለእያንዳንዱ ናመፃ ልዩ መሊያ ቁጥር ወይም ምልክት ይሰጠዋል፡፡ የትኛው ቁጥር የማን እንደሆነ ዋና ተመራማሪው ብቻ ያወቃል፡፡ ስለ ናመፃዎ ወጩ ከዋና ተመራማሪው በተጨማሪ እርሶን የሚታተለው ህክምም ሊያወቀው ይችላል፡፡

13. እኔ የምነግሰው ናመፃ ምን ይሆናል?

የርስዎ ወጩና ናመፃ የተለያ ቁጥር ይሰጠዋል፡፡ በናመፃው ላይ የርስዎ ስም አይጻፍም፡ አብዛኛው ናመፃ ወዲውኑ ስራ ላይ ይወላል፡፡በጥናት የሚገኘው ሚጃ በህትመት ሚዲከ ለጠፍ ባለሙያና ለሳይንትስቶች ይደርሳል፡፡ ወጩ በጅምላ በሚለጽበት ጊዜ የማንንም ወጩ አይወከልም፡

14. ያልተጠበቀ የላቦራቶሪ ወጩ ካለ?

በጥናቱ ላይ የተገኘ ማንኛውም ያልተጠበቀ ውጤት ካለ ለዶክተርዎ እንዲሁም ለእርስዎም ይገለፃል፤ ዶክተርዎ ሚባውን ህክምና ያረግልዎታል፡፡

15. የጥናቱ ውጤት?

የጥናቱ ውጤት በሳይንሳዊ መፅሔት ላይ ይታተማል እንዲሁም የጥናቱ ውጤት በተለያዩ ስብሰባዎች ላይ ይቀርባል፡፡ ነገር ግን የእርስዎ ማንነት በምን መልኩ አይገለፅም፡፡

16. የጥናቱን ወጪ የሚሸፈነው?

የጥናቱ ወጪ የሚሸፈነው በ አርሞር ሀንሰን የምርምር ማእከል ነው

17. ፈቃደኝነቴን ለመግለፅ?

ጥናቱ ላይ የመሳተፍ ፍላጎት ካለዎት ከሚሄዱበት ክፍል ከጥናቱ ዋና አስተባባሪ ወይም ከተወከለው ሰው የስምምነት ቅፅ በመሙላድ ፊርማዎትን በማኖር ስምምንዎትን ሚጋገጥ ይችላሉ፡፡

18. ይህ ጥናት ተቀባይነትን አግኝቷል?

ይህ ጥናት በአህሪ/አለርት የስነ-ምግባር ኮሜና በአዲስ አበባ ዩንቨርስቲ የስነ-ምግባር ኮሜ ተገምግሞ ተቀባይነትን አገኝቶ ፀድቋል፡፡

ተጨማሪ መረጃ ከፈለጉ የሚከተሉትን ባለሞያዎች ማጋገር ይችላሉ

1. የጥናቱ አስተባባሪና ዋና ተመራማሪ መታሰቢያ ፤ ስልክ ቁ. 0912009896
2. አህሪ/አለርት ስልክ ቁ. 0113481285

Annex III.Consent form in English

Study title: Diagnostic utility of Immunophenotyping by flowcytometry for diagnosis and classification 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 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 unable to sign. 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: Metasebia Tegegn-0912009896

Assent Form for Children 12-17 years

Study title: Diagnostic utility of Immunophenotyping by flowcytometry for diagnosis and classification 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 and cytochemical result 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 unable to sign. 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: Metasebia Tegegn-0912009896

Annex IV.Consent form for parental/guardian

Study title: Diagnostic utility of Immunophenotyping by flow cytometry for diagnosis and classification of acute leukemia

I _____being parent/guardian of _____ have been requested to let my child participate in this study, which plans to evaluate the Diagnostic utility of Immunophenotyping by flow cytometry for diagnosis and classification 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 V.Consent form Amharic Version

ተሳታፊ የሚፈርሙት የስምምነት ቅጽ

የጥናቱን አላማና ሂደት በዝርዝር ከተረዱ በኃላ የሚከተለውን ቅጽ በጥንቃቄ ይፈርሙ፡፡

- የጥናቱ ተሳታፊ እንደሆን በሙሉ ፈቃድ ወስጃለሁ
- ከዚህ ጋር የተያያዘውን የመለጫ ቅጽ በትክክል አንብቤ ተረድቻለሁ፡፡በእኔ ላይም ስለሚደረግ ማንኛውም ጥናት ተገንዝቤአለሁ፡ በተጨማሪም አስፈለቃጊውን ገለጻና ማብራሪያ ከላይ በተጠቀሱት ሰው ተደርጎልኛል፡፡ ደም ከከንድዎ ላይ እንዲሰጡ ይጠየቃሉ
- አጥኚዎቹ የመቅኔ ምርመራ ወጤንትን ወስደው ይጠቀማሉ በተጨማሪም አንድ የሾርባ ማኪያ የደም ናሙና እንደሚወስዱ በማወቅ ተረድቻለሁ፡፡

- ጥያቄ የማየቅ መቦት የመያየት እድል ከላይ ከተጠቀሱት አጥኚዎች ወይም ከነሱ ተወካይ ጋር ተሰጥቶኝ በጥናቱ ላይ በቂ ምክር ና ወይይት አድርጌያለሁ፡፡
- በተመራመሪዎቹ የጥናቱን ወጪ ይፋ እንዲያደርጉ እፈቅዳለሁ፡፡ነገር ግን ስም ማጠቀስ የለበትም፡
- ተመራመሪዎቹ በጠፍዬ ላይ ያለን ችግር እንዲነግሩኝ ፈቅጀላቸኃለሁ፡፡
- በማንኛውም ጊዜ ከጥናቱ እራሴን ማገለል እንደምችል አወቄያለሁ፡፡
- ከእኔ የሚሰበሰበው ማንኛውም መረጃ በጥንቃቄ ናሚስጥራዊነቱ በተጠበቀ ቦታ እንደማቅመጥ አወቄያለሁ፡፡

ስም_____ ፊርማ_____

ቀን_____

ይህበጥናቱየሚተፈወሰወመጪረምስለሚችልከላይየተዘረዘሩትመረጃዎችለተሳታፊወየተሰጠፍተሳታፊወምላመስተፍመስሞሞዙንገለል ተኛታዛቢ በመሆንአረጋግጣለሁ፡፡

_____ _____

የገለልተኛታዛቢስምፌርማዙን

ስለጥናቱ ዝርዝር መረጃ ሰስለማስጠቱ አረጋግጣለሁ .....

የመተማመኛ ቅጹን ከስምምነት ቅጽ ጋርአያይዤ ሰጥቻለሁ

ፊርማ..... ስም.....

Annex VI. Parental/guardian consent in Amharic version

የስምምነት ማጠየቂያ ቅጽ

የጥናቱ ርዕስ ፡የፍለጣይቶሜትሪ ዘዴን አጣዳፊ የደም ካንሰር በሽታን ለማከምና ለመለየት ማጠቀመ

እኔ----------የልጄ _____ ወላጅ/አሳዳጊ ስሆን አጣዳፊ የደም ካንሰርን በፍለጣይቶሜትሪ ዘዴ ለማከም ና ለመለየት በሚደረግ ጥናት ላይ ልጄ የጥናቱ ተሳታፊ እንድትሆን/እንዲሆን በመሉ ፈቃድ ወስጃለሁ

- ከዚህ ጋር የተያያዘወን የመለጫ ቅጽ በትክክል አንብቤ/ተነቦልኝ ተረድቻለሁ፡፡ልጄ ላይ ስለሚደረግ ማንኛውም ጥናት ተገንዝቤአለሁ፡ በተጨማሪም አስፈለቃጊወን ገለጻና ማበራሪያ ከላይ በተጠቀሱት ሰው ተደርጎልኛል፡፡
- አጥኚዎቹ የልጄን የመቅኔ ምርመራ ወጪ ወስደው እንደሚጠቀሙ በተጨማሪም አንድ የሾርባ ማንኪያ የደም ናመፃ ከልጄ ከንድ ላይ እንደሚወስዱ በሚባ ተረድቻሉ፡፡ነገር ግን የደም ናመፃ በሚወሰደበት ወቅት

ለፈጠር የሚችለውን አነስተኛ ህመሞና የደም መፍሰስ ለሚከሰድ ልምድ ባላቸውና እና ስልጠና በተሰጣቸው ባለሞያዎች እንደሚከናወን ተረድቻለው

- ጥያቄ የማየቅ መብት ና የመያየት እድል ከላይ ከተጠቀሱት አጥኚዎች ወይም ከነሱ ተወካይ ጋር ተሰጥቶኝ በጥናቱ ላይ በቂ ምክር ና ወይይት አድርጌያለሁ፡፡
- በተሞሙሜዎቹ የጥናቱን ወጪ ይፋ እንዲያደርጉ እፈቅዳለሁ፡፡ነገር ግን የልጄ ስም ማጠቀስ የለበትም፡
- ተሞሙሜዎቹ በልጄ ጠፍ ላይ ያለን ችግር እንዲነግሩኝ ፈቅጀለቸኃለሁ፡፡
- በማንኛወም ጊዜ ከጥናቱ ልጄን ማገለል እንደምቸል አወቄያለሁ፡፡
- ከልጄ የሚነበሰበው ማንኛወም መረጃ በጥንቃቄ ና ሚስጥራዊነቱ በተጠበቀ ቦታ እንደሚቀመጥ አወቄያለሁ፡፡ ስለዚህም ልጄን በጥናቱ ወስጥ ለማሳተፍ በፍፀም ፈቃደኝነት የስምማት ቃሌን ማስጠቴን በፊርሜ አረጋግጠለሁ፡፡

_____ _____

የተሳታፊው ሥም ፊርማ

ቀን /ወር/ዓ.ም

_____ _____

_____/_____/____

ምስክር (ማንበብና መፃፍ ለሚቸሉ) የምስክር ፊርማ

ቀን/ወር/ዓ.ም _____

_____/_____/____

የተሞሙሜው ስም

ፊርማ _____

ቀን /ወር/ዓ.ም

Annex VII.Clinical data collection sheets

Alert No…… Study number…….

Entry criteria: Patient history; Patient examination; Consent form

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

Clinical data required:

Full clinical investigation report on first day

Date the patient enrolled in the study dd / mm /yy E.C.

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

Data collection Form

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

Age Adult / Pediatrics ----------

Sex M/F ----------

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

Cytochemical result ----------

Flowcytometry result ----------

Annex VIII Protocol

Procedure for Flow cytometric Analysis

FlowCytometry Analysis: The BD FACS Calibur (Becton, Dickinson Fluorescence Activated Cell Sorter will be used for immunophenotyping analysis. The BD FACSCalibur is 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 fluoresceine isothiocyanate (FITC) conjugated monoclonal antibody, 10µl of Phycoerythrin (PE) conjugated monoclonal antibody, 10µl 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,with the respective pannel

- ✓ Dako-CD8 CD4 CD3 dako(TC 660) with BD CD45 PerCPCy5.5 (BD 564105, 100t)
- ✓ Dako-CD19 CD56 CD3 dako(TC 662) with BD CD7 PerCPCy5.5 (BD 561602, 50t)
- ✓ Dako-CD19 kappa lambda dako (TC669) with BD CD45 PerCPCy5.5 (BD 564105, 100t)
- ✓ Dako -cMPO cCD79a cCD3 dako (TC667) with BD CD45 PerCPCy5.5 (BD 564105, 100t)
- ✓ Dako-CD33 CD34 CD117 dako (TC 686) with BD CD45 PerCPCy5.5 (BD 564105, 100t)
- ✓ Dako-CD13-fitc (dako F0831); BD HLA-DR-APC; Dako CD10-PE , BD CD45 PerCPCy5.5 .
- ✓ CD45PerCP and CD14 PE((BD leukogate)
- ✓ Isotype controls ,IgG1-APC (345818) 100 ug , BD- IgG1-fitc (345818); IgG1-PE (340013); IgG1-PerCP (345817) with CD45 PerCPCy5-for surface stain
- ✓ Isotype controls, IgG1-APC (345818) 100 ug , BD- IgG1-fitc (345818); IgG1-PE (340013); IgG1-PerCP (345817) with CD45 PerCPCy5-for surface stain-for cytoplasmic stain.
- ✓ BD CD2 ,BD CD7, CD45
- ✓ 7-AAD (Dead cell stain)

Suface stain procedure

- ✓ After adding the respective antibody based on the assigned panel
- ✓ Add 100µL of whole blood in each tube.
- ✓ The mixture was Vortexed tenderly and incubated about 20 minutes in the dark area at room temperature (20- 25˚C).
- ✓ Tow ml of 1X FACS lysing buffer was added to incubated mixture. Then it was vortexed tenderly and incubated for 10 minutes in the dark area at room temperature again; after that Centrifuge at 1500 rp for 5 minutes was done.

- ✓ The supernatant was removed. Subsequently 2-3 ml of PBS (washing buffer was) added and centrifuged at 1500 rpm for 5 minutes and the supernatant was removed. 1ml of PBS added and mixed completely, analysis can be done immediately or can be stored at 2-8˚C until analysing them.
- ✓ Analysis was done by FACS flow cytometer. Samples vortexed thoroughly prior to acquisition

Cytoplasmic stain procedure

- PB sample were stained with 10 micro CD45 percp and incubated in the dark at room temperature (RT) for 20 minute
- Sampel Lyzed with BD Facs lyzing solution and incubated at room temperature (RT for 10 min).Wahing with PBS and centrifugation1700RPM for 5 min were done as per the protocoal.
- The mixture is washed with PBS and 50 microliter of Permealization reagent is mixed with the cells and incubatedfor 10 min at RT protected from light, and centrigugation was done 2000RPM for 12 mint and washed with PBS
- 10 microliter of the mAb were added, vortexed, and incubated for 20 min in case of cytoplasmic antigens Then, the mixture was centrifuged (2000RPM for 12 mint0and washed and resuspended in PBS and analyzed on the flowcytometer immediately.