CLINICAL AND IMMUNOPHENOTYPIC PROFILES OF CHILDREN DIAGNOSED WITH ACUTE LEUKEMIA AT MOI TEACHING AND REFERRAL HOSPITAL, ELDORET, KENYA.

Olbara Kipruto Gilbert, MBChB (Moi)

SM/PGCHP/10/13

Thesis submitted in partial fulfillment of the requirements for the award of the degree of Masters of Medicine in Child Health and Pediatrics, School of Medicine, Moi University.

DECLARATION

This thesis is my original work and has not been presented for academic purposes in any other institution of higher learning. No part of this thesis may be reproduced without prior written permission of the author and/or Moi University.

Olbara, Kipruto Gilbert Signature…….**.....** Date……..

SM/PGCHP/10/13

This thesis has been presented with our approval as University supervisors.

Professor Constance Tenge (MBchB, MMED, MPH)

Associate Professor and Pediatrician,

Department of Child Health and Pediatrics, Moi University

Signature: …….. Date: ……

Dr. Kirtika Patel (BSc, MSc, PhD)

Senior Lecturer and Allergist,

Department of Immunology, Moi University.

Signature……. Date: ……

DEDICATION

I dedicate this research to my beloved wife Irene and our lovely children, Nailantei and Leshan for their unending patience, inspirational motivation and silent prayers as I journeyed through this path of knowledge.

CLINICAL AND IMMUNOPHENOTYPIC PROFILES OF CHILDREN DIAGNOSED WITH ACUTE LEUKEMIA AT MOI TEACHING AND REFERRAL HOSPITAL, ELDORET, KENYA

ABSTRACT

Background: Acute leukemia is a heterogenous group of diseases comprising of several subtypes that differ in their clinical manifestations and response to treatment. Appropriate diagnosis and risk stratification is important in improving treatment outcome. At Moi Teaching and Referral Hospital, there is paucity of data on immunophenotypic subtypes of acute leukemia.

Objectives: To determine the clinical and immunophenotypic profiles of children diagnosed with acute leukemia at Moi Teaching and Referral Hospital (MTRH).

Methods: This was a cross sectional study carried out between January 1st 2015 and January 1st 2016. Children with a presumptive diagnosis of acute leukemia based on hemogram and peripheral blood film had bone marrow aspirate (BMA) done. Acute leukemia was confirmed by immunophenotyping of the BMA samples using a four colour BD FACS calibur™ flow cytometry machine with a standardized panel of monoclonal antibodies for acute lymphoblastic and myeloblastic cells. Demographic, clinical and BMA morphological characteristics at diagnosis were documented. Data analysis was performed on the confirmed cases of acute leukemia using SPSS Version 21 and presented in tables and bar graphs. Tests of associations was done using chi square test while concordance between morphological and flow cytometry diagnosis was determined by Kappa coefficient.

Results: Fifty two children with a presumptive diagnosis of acute leukemia had BMA done, 34(65%) had acute leukemia on BMA morphology while 41(78.8%) were confirmed by flow cytometry. Twenty one (51.2%) were males, 29 (72.5%) were less than nine years while 24 (60%) had symptoms for more than one month. There were more ALL than AML participants presenting with symptoms for longer than one month prior to diagnosis (p=0.036). Fever was present in 31(75.6%) participants, anemia in 30 (73.2%), hepatomegaly in 21 (51.2%) while 7(18.2%) had central nervous system involvement at diagnosis. Generalized lymphadenopathy was more common in ALL than AML patients. In eighteen (45%) participants hemoglobin level was less than 7g/dl and in 13(32.5%) white blood cell count was above 50 x 10^9/uL. Platelet count was <50 x 10^9/uL in 84% of ALL patients and only 50% of AML patients. In 8/41(20%) cases, a conclusive diagnosis of acute leukemia could not be made on BMA morphology but were appropriately diagnosed on flow cytometry. On immunophenotyping, 26 (63 %) participants had ALL while 15(37 %) had AML.T cell ALL constituted 27% of all ALL cases.

Conclusion: Fever and anemia are the most common clinical feature at diagnosis of acute leukemia. Proportion of high risk leukemia immunophenotype at MTRH is relatively high. Up to one fifth of leukemia cases are not conclusively diagnosed when morphology alone is used.

Recommendation: Flow cytometry should be routinely used alongside morphology to improve diagnosis and risk stratification of acute leukemia.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am grateful to my supervisors Professor Constance Tenge and Dr. Kirtika Patel for providing guidance and mentorship.

I thank those who helped with data collection especially Sandra Langat and Celestine Tallam, all the children and parents/guardians who agreed to participate in the study.

I am grateful to Mr Nikol Kigen in the AMPATH reference laboratory for running all the flow cytometry samples.

I would like to acknowledge my biostatisticians Dr Ann Mwangi and Mr. Henry Mwangi, for their input in data analysis.

I would like to recognise the invaluable technical contribution of Prof Terry Vik, Dr Festus Njuguna, Dr Teresa Lotodo and Dr John Chumba.

I also thank the CHAP department, my friends and colleagues for their moral, intellectual and material support.

I would like to appreciate Moi University, School of Medicine, The Moi Teaching and Referral Hospital for sponsorship.

Lastly, I thank my family for all the support and patience during the study.

ABBREVIATIONS AND ACRONYMS.

AL	Acute leukemia
ALC	Absolute Lymphocyte count
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloblastic Leukemia
AMPATH	Academic Model for Providing Access to Healthcare
ANC	Absolute Neutrophil Count
APL	Acute Promyelocytic Leukemia
BFM	Berlin-Frankfurt-Munster protocol
BMA	Bone Marrow Aspirate
CBC	Complete Blood Count
CD	Cluster of differentiation
CI	Confidence Interval
CLL	Chronic Lymphocytic leukemia
CML	Chronic Myeloid Leukemia
CNS	Central Nervous System
COG	Children's oncology group
EDTA	Ethylenediaminetraacetic acid
EFS	Event Free Survival
FAB	French-American-British
FACs	Flow automated cytometer

FC	Flow cytometry
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
HLA DR	Human leucocyte antigen DR
IHC	immunohistochemistry
IREC	Institutional Research and Ethics Committee
MABs	monoclonal Antibodies
MicroL/µL	micro-litre
mm	millimetres
MPO	Myeloperoxidase
MTRH	Moi Teaching and Referral Hospital
MU	Moi University
PBF	Peripheral Blood Film
PI	Principal Investigator
RA	Research Assistant
SCC	Sick child clinic
SOM	School of Medicine
SPSS	Statistical Package for the Social Sciences
WBC	White Blood Cells
WHO	World Health Organisation
Yr	Year

OPERATIONAL DEFINATIONS

Paediatric AL	Acute Leukemia (AL) affecting children less than 15 years of age.
Presumptive leukemia	A case presenting with unexplained generalized lymphadenopathy on clinical exam and/or hemogram findings of bi- or pancytopenia, and/ or atypical cells or blasts in the Peripheral blood film.
ALL	Presence of more than 25% lymphoblasts in the bone marrow aspirate smear and/ or if more than 20% positive for lymphoblast markers on flow cytometry.
AML	Presence of more than 25% myeloblasts in the BMA smear and/or if more than 20% positive for myeloblast markers on flow cytometry.
T cell ALL	More than 20% reactivity/ positivity for T cell lineage antigens on flow cytometry; CD3, CD7 or CD5
B cell ALL	More than 20% reactivity/ positivity for B cell lineage antigens on flow cytometry; CD10, CD19 or CD22.

CHAPTER ONE

INTRODUCTION

1:1: Background Information.

Leukemia is a cancer of the bone marrow in which there is uncontrolled proliferation of abnormal, poorly functioning white blood cells called blasts[1]. These cells are derived from the bone marrow and can infiltrate the peripheral blood or solid organs leading to bone marrow failure and organ enlargement.

The cellular proliferation is usually monoclonal; frequently along the lymphoid lineage or alternatively along myeloid lineage. Biphenotypic leukemia, which has both lymphoid and myeloid lineage has gained some significance recently because of availability of objective diagnostic criteria[2] because of availability of objective diagnostic criteria[3]. Depending on the course of disease progression, leukemia may be classified as being acute or chronic. The acute types are often malignancies emanating from precursor cells while the insidious ones are often malignancies arising from relatively or fully matured cells[1 .]

For a long time, leukemia classification has been based on a combination of clinical presentation and morphological characteristics of blast cells. Four clinical entities have been identified; Acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML, also named acute non lymphocytic leukemia ANLL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL).

Current research on the pathobiologic mechanisms including morphology, immunology, growth regulation, cytogenetic and molecular abnormalities in leukemic cells have established that leukemia is actually a more heterogeneous disease than initially thought[4]

This complexity is further reflected in the widely varying clinical features, treatment pathways and outcome associated with these diseases. On the basis of these insights, various classifications systems for hematopoietic malignancies have been proposed. The 2016 revision of the World Health Organization (WHO) classification of hematological malignancies incorporates new evidence of diagnostic, prognostic and therapeutic importance [5].

Acute leukemia is the most common malignancy in children globally, representing 30 % of all cancers diagnosed in children under the age of 15 years[4.] Acute lymphoblastic leukemia (ALL) is five times more frequent than acute myeloblastic leukemia (AML) contributing to 78% of all childhood leukemia diagnoses[5, 6]. Chronic leukemia accounts for less than 5% of all childhood leukemia.

In Africa, the burden of childhood cancers including acute leukemia is largely unknown; mainly because of lack of statistics and under reporting[7]. Reasons for under diagnosis include; inadequate infrastructure and diagnostic facilities, insufficient technical skills, limited access to care as well as low quality of cancer data systems. Globally, the age standardized incidence for acute leukemia among children less than fifteen years is 46.4 per million person years while in Sub Saharan Africa, it is reportedly low at 12.5 per million person -years[8]. In terms of proportion, acute leukemia constitutes between 16.9% and 21.3% of all childhood cancers in most of the African studies[9-11].

Although the gold standard for leukemia diagnosis has been morphological identification of leukemic blasts from marrow aspirates in addition to cytochemical staining, more recent research has shown that there is only modest correlation between morphologic categories

and treatment responsiveness or prognosis[12]. For these reasons, WHO currently advocates for use of adjuvant methodologies in alongside morphology to determine the immunological, cytogenetic and molecular basis of each case of diagnosed leukemia[5, 13].

In acute leukemia, determining the good and poor prognostic groups at diagnosis is important. This can be achieved by a multifaceted approach to diagnosis and classification employing immunophenotypic and molecular studies to supplement the traditional methods.

Some studies have shown that the immunological subtypes of leukemia vary with geographical location and socio-economic status. For instance, proportion of T- Cell ALL in Pakistan, India, Thailand and Malaysia is similar [14] at 19 % indicating a similar socio-economic Asian Belt. In Egypt however, a rather high proportion (50% T cell ALL) has been documented[15]. It is estimated that in most developed countries, 80% of ALL is of the precursor B-cell subtype explaining the pronounced peak incidence in early childhood [16].The distribution of ALL and AML also appears to be variable. In one hospital based Nigerian study, the proportion of AML was found to be higher than that reported in other parts of the world[9].

Historically, survival among children with T ALL was inferior to those with B ALL[17], however with intensification of therapy outcome has improved[17, 19]. The five year event free survival rates for ALL now range between 76% and 86% in children receiving protocol based therapy in high income countries while those for AML range between 49% and 63 % in some of the more successful clinical trials. In Africa though, the outcome is still very poor[20].

1.2 Problem Statement

Acute leukemia contributes 30 percent of all childhood cancer worldwide[4], and is a leading cause of cancer related mortality in the developing countries. In Western Kenya, it is the second most prevalent childhood cancer after Non-Hodgkin's lymphoma[11].

The burden of acute leukemia in Africa is usually underestimated. In sub- Saharan Africa for instance, fewer than 20 cases per million person - years are reported[21] compared 40 cases per million in Western Europe and 30-35 cases per million person-years in Eastern Europe[7]. The under diagnosis and lack of statistics has been attributed to inefficient methods of detecting cases diagnosed by blood smear or cytology.

Acute leukemia is a heterogeneous group of diseases constituting several subtypes that differ markedly in their clinical, biologic characteristics and response to treatment. Proper diagnosis and stratification based on subtype is important in improving treatment outcome. At MTRH, risk stratification is not routinely done and ALL cases are treated with the same protocol irrespective of immunological subtype.

1.3. Justification

Acute leukemia contributes to 30% of childhood cancer globally and is a leading cause of cancer related morbidity in developing countries. In Kenya, has been reported as the second most prevalent cancer in children less than 15 years.

BMA cytomorphology which is the main diagnostic modality for suspected acute leukemia at MTRH can only diagnose upto 80% of the cases and has a limited role in correlating leukemia subtype with treatment response and prognosis. World health organization

(WHO) currently recommends the use of adjunct methods alongside morphology to determine the cytogenetic basis of each case of leukemia diagnosed.

There is paucity of data on epidemiological profiles and the immunological subtypes of acute leukemia at MTRH

This study was therefore designed to investigate the immunophenotypes of acute leukemia and determine any association between the leukemia subtypes and clinico - pathological characteristics.

1.4 Research Question

What are the clinical and immunophenotypic profiles of children diagnosed with acute leukemia at MTRH, Eldoret Kenya?

1.5 Objectives

1.5.1 Broad Objective

To determine the clinical and immunophenotypic profiles of children diagnosed with acute leukemia at MTRH.

1.5.2 Specific Objectives

1. To determine the clinical and laboratory characteristic at diagnosis of children with acute leukemia at MTRH.
2. To identify the morphologic and immunophenotypic characteristics of children diagnosed with acute leukemia at MTRH.
3. To determine the correlation between immunophenotypic subtypes with the clinical and laboratory features of children diagnosed with acute leukemia at MTRH, Eldoret.

CHAPTER TWO

LITERATURE REVIEW

2.1: Epidemiology

Childhood cancers are rare, with a reported incidence in the United State of one case per 7,000 children aged 15 years and younger. More than 40% of childhood cancers are hematologic[4]. Acute leukemia is the most frequent malignancy that occurs during childhood and comprises approximately 30% of all childhood in the United States. Out these, 80 % constitute ALL followed by AML then CML and other types[4,6].Chronic leukemia accounts for less than 5% of all childhood leukemia. Recent molecular studies have demonstrated that leukemia is more heterogeneous than suggested by these groupings[4].

There are relatively few epidemiologic studies on childhood acute leukemia from Africa because of lack of accurate population census figures, shortage of technical and infrastructural expertise[7]

The incidence of leukemia is highest in children aged 2 to 5 years of age. Sex differences have been reported in many studies; with a male predominance[22].Race differences in incidence have also been reported. Many studies have reported significantly greater incidence among African Americans than white population[4, 23].

2.2: Etiology and Pathogenesis.

Leukemia results from an expansion of malignant hematopoietic cells and the cellular proliferation is usually monoclonal. The single mutant hematopoietic progenitor cell is capable of indefinite self - renewal and gives rise to malignant, poorly differentiated hematopoietic precursors leading to hematopoietic insufficiency.

In most cases, acquired genetic abnormalities are associated with leukemia. However, inherited genetic abnormalities have been implicated in 5% of acute leukemia. Down's syndrome is the most common genetic syndrome associated with acute leukemia, with studies suggesting 10 - to - 20 fold increased risk of leukemia than in the general population[4].Patients with this condition have an increased risk of developing both ALL and AML. Other genetic disorders associated with acute leukemia include ataxia Telangiectasia, and other immunodeficiency syndromes, fanconi anemia, bloom syndrome, neurofibromatosis type I, Schwachman syndrome.

Confirmed clinical and epidemiological associations explain less than 10 per cent of childhood leukemia incidence leaving at least 90 per cent of cases with unknown etiological mechanism[24]. Current evidence suggests that leukemia results from chromosomal alterations and mutation that disrupt normal processes by which lymphoid or myeloid progenitor cells differentiate[23].The underlying trigger for molecular damage may be genetic or environmental. Published descriptions and investigations of geographic clusters of cases have raised concern that environmental factors may increase the incidence of acute leukemia.

Certain environmental factors have been associated with leukemogenesis. The role of chemical agents in the pathogenesis of leukemia is established best for the occurrences of

AML after benzene exposure in susceptible individual. The extent to which chemical exposures may contribute to the development of childhood ALL is less established. In a recent study by Children's Oncology Group (COG), it was found that ALL may be associated with parental exposures to hydrocarbons and specific medication[25].Parental occupational pesticide exposure have recently been implicated with an increased risk of AML and T-ALL in children[26]. Viral infections have been mentioned in pathogenesis of certain rare human leukemias but no viral agent has been demonstrated to have a definitive role in causing the more frequent subtypes of childhood ALL. In most children, the causal factors remain unknown.

2.3 Clinical Presentation

The initial presentation of leukemia is usually nonspecific and relatively brief. Anorexia, fatigue, irritability often are present, as is intermittent, low grade fever and may often be mistaken for more common ailments contributing to under diagnosis of acute leukemia in the sub Saharan Africa[7].

Bone pain, particularly affecting the long bones, and caused by leukemic infiltration of the periostium is a presenting symptom in up to 25 % of cases of acute leukemia[27].When bones of the lower extremity are involved, the child may exhibit a limp or refusal to walk[28]. Rarely, symptoms may be of several months' duration, may be localized predominantly to the bones or joints and may include joint swelling. Bone pain is deep, severe and awakes the patient at night. Tenderness may not be elicited. Bone pain may also be as a result of aseptic osteonecrosis because of malignant cell necrosis in the bone marrow involvement[29] .Bone radiographs demonstrate osteopenia, and in some instance lytic lesions. In some

children abnormalities are also detected on the radionuclide bone scan. Because some children can present without malignant leukemia cells on a routine blood count, mistaken diagnosis of juvenile idiopathic arthritis and osteomyelitis occasionally have been considered in children who have leukemia with bone pain[29]. Pathologic fractures in leukemic bone are uncommon.

As the disease progresses, signs and symptoms of bone marrow failure due to leukemic proliferation within the bone marrow become more apparent. The child presents with pallor, fatigue, bruising, or epistaxis, as well as fever, which may be caused by infection or disease process itself. These result from the decreased production of normal red blood cells, platelets and white blood cells. Approximately 50 per cent of children have WBC counts less than 10,000/microL and 20 percent have an initial leucocyte count more than 50,000/microL. One half of the children with leukemia will present with bleeding(including petichiae and purpura) and three quarters have platelet counts less than 100,000/microL at the time of diagnosis[28]. Even with a low platelet count severe hemorrhage is uncommon except in acute promyelocytic leukemia[30]. More than three quarter of the patients will present with anemia that is typically normochromic, normocytic and associated with normal to low reticulocyte count.

Other important sites of leukemia infiltration are the central nervous system (CNS) and the testes, usually sanctuary sites for the leukemic cells[31]. These have required particular attention in the development of treatments. Testicular leukemia (20%) usually manifests as painless, firm enlargement of one or both testes. Ovarian (30%) involvement may also occur.

CNS leukemia most frequently involves the meninges. Less than 5 per cent of children with leukemia involving the CNS can present with symptoms of raised intracranial pressure, which include headache, vomiting, lethargy, and/or nuchal rigidity[30, 31]. Others include, papilledema, retinal hemorrhages and cranial nerve palsies[32].Cranial nerve abnormalities are rare in leukemia.

On physical examination, findings of pallor, listlessness, purpuric and petechial lesions or mucous membrane hemorrhage may indicate bone marrow failure. The proliferative nature of the disease manifests as lymphadenopathy, spleenomegaly and less commonly hepatomegally. One half of the children with acute lymphoblastic leukemia will present with lymphadenopathy[28].

Respiratory distress usually is related to anemia but may also occur in children with obstructive airway problem due to a large anterior mediastinal mass (e.g. in the thymus or nodes). This problem is typically seen in adolescent boys with T – cell ALL.

Bone marrow studies in children who have leukemia are important in identifying specific subtype of leukemia. Bone marrow examination also serves to distinguish leukemia from other conditions involving severe bone marrow failure like aplastic anemia and myelofibrosis. In addition, bone marrow examination is important because not all children with leukemia will present with lymphadenopathy and hepato-spleenomegally, and may not have detectable cells in blood.

The major life threatening complication of a child with acute leukemia is overwhelming infection often sepsis or pneumonia. The risk of sepsis is directly correlated with severity

of neutropenia[2, 4].Other alterations of host immunity produced by leukemia also contribute to infection risk. Bleeding is mostly due to severe thrombocytopenia but may be augmented by severe coagulation factor deficiency. This is more pronounced in acute promyelocytic leukemia (APL) a subtype of AML. Approximately one- half of children with ALL present with bleeding (including petichiae and purpura) and three- quarters have a platelet count less than 100,000/microL at the time of diagnosis[7].

Clinical presentation of AML is identical to that of ALL except for a few subtle differences. Chloromas also called granulocytic sarcoma are a collection of leukemic cells outside the bone marrow forming an isolated tumor like mass. They are predominantly associated with AML, though their occurrence is rare. The median white cell count for AML is higher at 20 - 50 x 10^6 cells/uL.Up to one 70% of patients will present with white cell counts within this range. The median for ALL is 12 x 10^9 cells/uL. Hyper leukocytosis (> 100,000 white cells/uL) occurs in only 15% of the ALL children[33]. In AML, mediastinal masses are less common. However, about half of the children with T cell ALL; usually adolescent boys will have mediastinal masses at diagnosis.

2:4 Diagnosis

2.4.1. Morphology and Cytochemistry

The diagnosis of acute leukemia is based on morphologic and cytochemical investigations of bone marrow samples and/or peripheral blood smears. Historically, acute leukemia (AL) classifications used blast morphology and cytochemical stains to categorize the diseases broadly into ALL and AML.The use of morphology and cytochemistry alone correctly diagnosed up to 80% of acute leukemia cases[34].Within each lineage, distinct subtypes are

defined based on clinical and morphologic features in conjunction with immunophenotyping by immunohistochemistry (IHC) and/or flow cytometry (FC) and an emphasis toward classification by molecular genetics[5,35].

The Acute Leukemia diagnosis is suggested by peripheral blood findings indicative of bone marrow failure. Bone marrow examination should be performed for the following indications: Atypical cells in the peripheral blood; unexplained depression of more than one peripheral blood element (bicytopenia or pancytopenia); and unexplained lymphadenopathy or hepatospleenomegally associated with cytopenias.

Primary diagnosis rests on morphologic identification of leukemic blasts (either lymphoid or myeloid) in preparations of peripheral blood film and bone marrow stained with Wright - Giemsa or leishman stain. According to the WHO classification systems, presence of more than 25% leukemic blasts in a bone marrow aspirate and or PBF is required for definitive diagnosis of acute leukemia[5].

Myeloblasts have round to irregular nuclei, distinct nucleoli and very little cytoplasm (high nuclei: cytoplasm ratio). The cytoplasm frequently contains fine azurophilic granules and variable numbers of Auer rods (azurophilic granules within lysosomes). Lymphoblast's have round smooth nuclei, finely dispersed nucleoli with scanty cytoplasm. The cytoplasm rarely contains cytoplasmic granules.

2.4.2 Cytomorphological Classification of Acute Leukemia

The original classification scheme proposed by the French-American-British (FAB) Cooperative Group [35] divides AML into 8 subtypes (M0 to M7) and ALL into 3 subtypes (L1 to L3).More recently[36], an additional class AML M8 (acute basophilic leukemia) has

been described which was not in the initial FAB system. Acute basophilic leukemia is a rare and fatal entity of AML with primary differentiation to basophils. AML sub types are based on how leukemic blasts, the predominant cell in the disease process, replace normal hematopoiesis. Are blasts in a given case myeloblasts, monoblasts, megakaryoblasts, etc, and are they un-, minimally, or moderately differentiated.

- AML M0 AML with no evidence of differentiation
- AML M1 Myeloblastic leukemia with minimal maturation
- AML M2 Myeloblastic leukemia with maturation
- AML M3 Acute Promyelocytic leukemia (APL)
- AML M4 Acute Myelomonocytic leukemia (AMML)
- AML M5 Acute monoblastic leukemia (AMoL)
- AML M6 "Erythroleukemia"
- AML M6a AML with pure erythroid
- AML M6b Erythroleukemia
- AML M7 Acute Megakaryoblastic leukemia (AMkL).
- AML M8 Acute Basophilic Leukemia

For subcategorisation of AML into FAB types; M0 – M7, immunophenotyping is indispensible[37]. AML is also heterogenous with respect its morphology, immunology and germ line mutations[38] immunophenotyping therefore rapidly determines lineage and distinguishes between AML from acute lymhoblastic leukemia. Immunological stains positive for CD13, CD 15, CD1 and CD33 myeloid antigens.

Subtype M0 (negative for MPO activity rom cytochemistry and positive for myeloid markers like MPO and or CD13, CD33 and CD 117. FAB M7 on the other hand will be positive for platelet markers like CD 41 and CD61. FAB M3 is of significant clinical importance. It results in the fusion of truncated retinoic acid receptor alpha (RAR-alpha) on chromosome 17 releasing promyelocytic granules that containing a procoagulant that initiates disseminated intravascular coagulation[35, 38] The main clinical manifestation of this subtype is bleeding.

Acute lymphoblastic leukemia (ALL) is divided in French-American-British (FAB) classification L1, L2, and L3 based on cytologic features and degree of heterogeneity among leukemic cells. These features considered are cell size, chromatin, nuclear shape, nucleoli, and degree of basophilic in the cytoplasm and the presence of cytoplasmic vacuolation[35].

ALL-L1: Homogenous cells **(Small cell):** One population of cells within the case. Small cells predominant, nuclear shape is regular with occasional cleft. Nuclear contents are rarely visible. Cytoplasm is moderately basophilic. L1 accounts 70% of patients.

ALL-L2: Heterogeneous cells: Large cells with an irregular nuclear shape, cleft in the nucleus are common. One or more large nucleoli are visible. Cytoplasm varies in color and nuclear membrane irregularities. L2 accounts 27% of ALL patients. The FAB-L2 blast may be confused with the blasts of acute myeloid leukemia and is usually seen in older children and adults.

ALL-L3: Burkitt's lymphoma type: Cells are large and homogenous in size, nuclear shape is round or oval. One to three prominent nucleoli and sometimes to 5 nucleoli are visible. Cytoplasm is deeply basophilic with vacuoles often prominent. A high mitotic index is characteristic with presence of varying degrees of macrophage activity. Mature B lymphoid markers are expressed by most cases.

2.4.2: Immunological Classification

Currently, refinement in classification of acute leukemia is achieved by immunophenotyping. Differences in expression of surface membrane antigens or cytoplasmic (cluster of differentiation, CD) components are used to identify and classify lymphoproliferative disorders by cell of origin and stage of differentiation[39, 40.] This improves both accuracy and reproducibility of acute leukemia classification. It is useful for accurately identifying lineage of the malignant clone of leukemic blasts besides light microscopic diagnosis of childhood leukemia and resolve equivocal diagnosis of ALL versus AML[26], and to determine lineage of lymphoblasts along lines of B lineage-ALL and T-lineage ALL maturation. The latter is important for stratification into standard risk (SR) and High risk (HR) ALL, as well as determining mature B-ALL, which needs a different treatment schedule. In addition, a specific immunophenotype identified at diagnosis might be useful for evaluating minimal residual disease by flow cytometry during therapy. By this method more than 98% of acute leukemia cases can now be precisely allocated to their respective lineages[34,41].

Not only can acute myeloid leukemia (AML) be differentiated from the acute lymphoblastic leukemia (ALL) but B-cell or T-cell lineages can also be determined in the latter, which cannot be achieved by morphology and cytochemistry alone. For instance,

Jawaid et al. in their series reported that 11% cases of acute leukemia were unidentifiable in terms of their phenotype while 9% were identified incorrectly on morphological basis, all of which were correctly allocated to their lineages after flow cytometric analysis[41].

The most common B-lineage ALL is the B lineage phenotype positive for the following; B cell markers CD 19,CD 22,TdT,cytoplasmic CD79a,CD 34 and CD 10. B lineage ALL has been sub classified according to maturation stage into: early pre B (pro B), pre- B, transitional (or late) pre B and (mature) B-ALL[39]. In different regions, various incidences of B – lineage ALL have been reported.

T cell lineage can further be categorized into phenotypic subgroups, correlating to differentiation stages of thymic T cells. T cell markers are cytoplasmic CD3 and CD 7 plus CD 2 or CD5. This lineage can be further subdivided into early, mid or late thymocyte differentiation[42].

The current WHO classification divides ALL into two main groups only, i.e. B-lineage and T-lineage ALL, without further categorization. This study will limit itself to the two phenotypic categories only.

2.5 Flow Cytometry and Role in Immunophenotyping

Flow cytometry is a rapid analysis of multiple characteristics of single cells resulting in both qualitative and quantitative data [39] .Flow cytometry detects membrane, cytoplasmic or nuclear antigens at a rate of 100,000 cells per second [43] producing rapid measurements of physical characteristics of cells. Use of flow cytometry to diagnose and confirm leukemia is a standard procedure in developed countries but currently most developing countries rely on morphological and cytochemistry of cells in peripheral blood and bone marrow to diagnose leukemia.

There have been several published studies on implementation of flow cytometry in resource constrained countries in Central and South America[44]. In the diagnosis and characterization of acute leukemia, flow cytometry is more sensitive and less subjective a than BMA morphology giving reproducible results[43,45,46] .Current flow cytometric machines are much smaller, affordable and well suited for high volume operations. The high sensitivity of flow cytometry makes it possible to a very small number of neoplastic cells in the bone marrow, as a way of assessing minimal residual disease after leukemia treatment[47].

The major limitations of flow cytometry are the high costs of instruments, reagents and specialized skills needed to perform tests. For more than a decade, FACs machines have been available at MTRH and currently in use for monitoring response to antiretroviral therapy by quantifying CD4+ T Lymphocyte populations, and CD4: CD8 T Lymphocyte ratio in peripheral blood of HIV infected persons. Though the same BD FACs machine is a used for leukemia subtyping, the method of cell preparation (appendix), the types of monoclonal antibodies and software for interpretation of results differ significantly.

2.5.1 General Principles of Flow Cytometry

The basis of flow cytometric analysis rests on the difference in optical and fluorescence characteristics of single cells (or any other particle including nuclei, microorganisms, chromosome preparations, and latex beads).Physical properties such as size (represented by forward angle light scatter) and internal complexity (represented by right angle scatter) can resolve certain cell populations[37]Specific fluorescent dyes are used to label cells.

The fluorescent dye binds or intercalates with different cellular components such as DNA or RNA.

The nature of acute leukemia makes it amenable to flow cytometry analysis by targeting the many surface proteins and specific glycoprotein's on the cell surface of the abnormal leukocytes. This is made possible by the availability of monoclonal antibodies directed against these surface proteins. Wide arrays of monoclonal antibodies are currently available for flow cytometry. To diagnose acute leukemia, a panel of monoclonal antibodies has to be judiciously selected. This should be large enough to diagnose and subtype most of the acute leukemia but also limited so as to be cost effective. The monoclonal antibodies are selected to target specific category of antigens [46,47].

Lineage associated antigens: These target lineage cell antigen and help in identification of the subtype of leukemic cells; B- cell– CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79, CD138, immunoglobulin.

T cell; CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD45RA, CD45RO, TCR NK cell; CD16, CD56, CD57

Myeloid; CD13, CD14, CD15, CD33, CD117, anti MPO.

There are guidelines published by different working groups from different parts of the world for implementation of flow cytometry in leukemia[43,47,48] .None of them has arrived at a consensus on the way to select an ideal panel of monoclonal antibodies required to analyze cases for acute leukemia[43] due to the heterogeneity in the expression of antigens by acute leukemic blasts.

In their review of literature, Pamnani et al (2009) [43], recommends consideration of several factors when selecting a panel of monoclonal antibodies in Kenya and other resource constrained settings. These include, laboratory type, workload of leukemic samples,

technical expertise, number of antibodies used in the panel and indication for immunophenotyping (initial diagnosis, sub typing, follow up studies and detection of minimal residual disease[43]. The type of flow cytometer (three or more colors) will also help determine the panels. At MTRH, the Dutch Foundation for Immunophenotyping of Hematological Malignancies (SIHON) recommendation[48] of selecting monoclonal antibody panels has been locally adopted and is in current use.

2.6: Treatment and Prognosis

Before therapy is initiated however, it is necessary that several critical diagnostic distinctions are made. ALL must be distinguished from AML, myelodyspalstic syndrome (MDS) or AML arising in the setting of MDS because therapeutic strategies and prognosis vary considerably for these diseases[35].

AML can be distinguished from ALL by demonstration of definitive commitment to myeloid lineage through judicious use of morphological, immunohistochemical and immunological methods.

Morphological and genetic subtype of AML using FAB 5-9 based a particular myeloid lineage and the degree of leukemia cell differentiation. This study will only focus on distinguishing ALL from AML and determining the immunologic subtypes of ALL. The study will not further categorize AML cases due to limited availability of monoclonal antibodies during the study period.

CHAPTER THREE

METHODOLOGY

3.1 Study Site

This study was done in the pediatric wards of the Moi Teaching and Referral Hospital, Eldoret-Kenya. The Hospital is located in Eldoret town of the Uasin Gishu County in North Rift region, 310 Kilometres Northwest of Nairobi. This is also the teaching hospital for the Moi University School of Medicine, University of Eastern Africa, Baraton and the Kenya Medical Training College, Eldoret. It is the second largest national referral hospital in Kenya and serves patients from the Western part of Kenya and the north and south rift region. It has a catchment population of approximately 16.24 million people (42 % of Kenyan population) and approximately 20,000 admissions per year. Approximately 40,000 children are seen at the pediatric OPD per year.

The pediatric oncology ward is located within the new Shoe for Africa Children's Hospital. The oncology ward has 17 beds served with a multidisciplinary team of health care providers providing various oncology services. There are a total of 110 -125 new admissions annually to the pediatric oncology ward. On average two to three new cases of acute leukemia are diagnosed monthly.

The BD FACS Calibur flow cytometry machine is located within the Academic Model for Providing Access to Care (AMPATH) Reference laboratory and is run and operated by a technician alongside the hematopathology consultantant. Since the establishment of AMPATH, the FC machine has been used to determine the CD4: CD 8 ratios in the management of HIV/AIDs patients. From 2013, the machine has been calibrated through the partnership of Doctor to Doctor program and the Vu Medical University, Amsterdam,

the Netherlands, to be able to perform flow cytometry diagnosis of acute leukemia. The initial pilot and standardization of the FC technique at MTRH was done and published by Patel et al. [49] in 2015

3.2 Study Design

A cross sectional study

3.3 Study Population

The target population constituted all children admitted to the pediatric wards with a presumptive diagnosis of acute leukemia during the study period (1st January 2015 – 1st January 2016) while study population were those meeting the following criteria..

3.3.1 Inclusion criteria

1. Age less than fifteen years.
2. A presumptive diagnosis of acute leukemia based on clinical characteristics and a peripheral blood film.

3.3.2 Exclusion criteria:

1. Any child who had received chemotherapy previously. Any prior treatment would alter the clinical and pathological characteristics and diagnosis.

3.4 Sample Size

Based on previous MTRH pediatric oncology records and data from Eldoret cancer registry, 55 children were diagnosed with acute leukemia between January 1st 2012 and December 31st 2013. On the basis of the small expected sample size, this study enrolled all the participants meeting inclusion criteria between 1st January 2015 and 1st January 2016.

3.5. Outcome Measures

The primary outcome was the proportion of the morphological and immunological subtypes of acute leukemia diagnosed at MTRH. The secondary outcomes were the frequency of clinical and laboratory characteristics associated with the immunological subtypes of acute leukemia.

3.6. Data Collection

A pretested structured data collection form was used to collect data. The data collection form included demographic data including county of residence, clinical profiles, morphologic and immunophenotypic details including subtypes of acute leukemia.

3.7. Execution of the Study

Data was collected over a period of 12 months beginning 1st January 2015 to 1st January 2016. The principal investigator was assisted by a research assistant (medical officer) stationed at the oncology unit. Sensitization of the clinical officers, pediatric residents in the pediatric unit and research assistant was done during a two day pediatric oncology workshop. During the study period, the PI trained by apprenticeship all the new residents on bone marrow procedure techniques.

Any child seen by clinicians at Sick Child Clinic, with a history and physical examination findings suggestive of acute leukemia had their blood samples drawn for complete blood count (CBC) and peripheral blood film (PBF).

Approximately 2-3milliliters of blood was aseptically drawn from any visible peripheral vein (see appendix VII) into standard EDTA-anticoagulated vacutainer tube and transported to the laboratory within 45 minutes. Automated blood count was done using

Mindray 500 machine and peripheral blood smear prepared by hematology laboratory technicians who read and availed the results within 2 hours. Any suspicious hemogram or peripheral smear; showing leukocytosis with bicytopenia or pancytopenia on hemogram and atypical cells or blasts on the peripheral film, was sent to the pathologists for confirmation and reporting. Those with a presumptive diagnosis of acute leukemia based on initial peripheral blood film were admitted to the pediatric wards for a diagnostic bone marrow.

The point of entry of participants in this study was the pediatric wards. The investigators were informed by a phone call of any child clinically suspected to have acute leukemia with a suggestive peripheral blood film (presence of blasts or atypical cells) admitted to the ward for a diagnostic bone marrow. In the wards, the PI or research assistant sought informed consent (assent if child older than 7 years) from all the parents of children meeting the inclusion criteria and enrolled them into the study prior to the diagnostic BMA. A detailed history and physical examination was done and findings recorded into the data collection form (see appendix III). Initial hemogram and peripheral blood film findings were then abstracted from the patients file. The scheduled diagnostic BMA (see appendix IV) was done by the PI using standard procedure (appendix V) in the presence of a laboratory technician. Morphological diagnosis was made by the pathologist after looking at the Giemsa stained BMA samples under both low and high power to determine morphology (Appendix X). The PI also facilitated any logistical and technical issues including providing disposable BMA kits. These were provided for by Doctor 2 Doctor program in conjunction with Vu Medical University, Amsterdam the Netherlands for routine patient care in pediatric oncology unit as part of the collaboration with MTRH in

clinical care. The first 0.5 – 1 ml of marrow aspirate was drawn and mounted on the slides, a BMA smear prepared by the technician for morphological analysis. A new 20 milliliter syringe was then used to obtain a second marrow aspirate of 2-3 milliliter from the same site, and collected in a standard EDTA-anticoagulated vacutainer tube and transported to the AMPATH Reference laboratory within 45 minutes for FC analysis. Immunophenotyping was done using a BD FACS Calibur™ (Beckman, United States of America) four color flow machine. The result was made available within 3 days of taking sample . Where a dry tap was encountered or the bone marrow unsuccessful, 2 -3ml of blood aseptically collected in an EDTA vacutainer tube from any visible peripheral veins for immunophenotyping.

Documentation of the diagnostic laboratory findings which included CBC with differentials, PBF, BMA and flow cytometry was done by the principal investigator and research assistant in the data collection form (appendix III). Parameters abstracted from the patient charts on the CBC included the absolute white blood cells count (WBC), absolute lymphocyte count (ALC) and absolute granulocyte (ANC) count, the hemoglobin (Hb) level and the platelet count.

Outcomes was measured as the immunophenotypic subtype of acute leukemia, whether myeloid lineage (AML) or lymphoid (ALL) and their proportions. The cases of ALL were further stratified into those of precursor B – cell ALL and those precursors T cell- ALL based on immunophenotyping using FC and their proportions determined. The PBF and BMA were read by two general pathologists independently. A pathological diagnosis was made by the first pathologist who sent it to a second senior pathologist within MTRH for confirmation and approval. A pediatric haemato pathologist from Vu University

Amsterdam was used to break a deadlock for difficult cases where the two pathologists were not in agreement (tie breaker) by sending photomicrographs of the blood smears.

Flow cytometry diagnosis was based on pattern of cells staining for a particular lineage. For diagnosis, lineage specific monoclonal antibodies were considered positive if the blast cells stain for more than 20% lineage specific cell markers as mentioned in the appendix.

The MTRH diagnostic laboratory has both internal and external inspection mechanisms to ensure adherence to the necessary quality control measures. External quality controls are done in conjunction with the American Proficiency Institute and the Kenyan-based Human Quality Assessment Services every quarterly. The turn-around time for the investigation after receiving a specimen is approximately one day but may be longer depending on availability and workload of the pathologists.

3.8 Data Management and Analysis

Data was collected in a pretested questionnaire, cleaned and subsequently entered into a computer data base using double entry to ensure accuracy. All patient details were kept confidential and non-coded data was only available to the PI and research assistant. Copies of all diagnostic results were returned to the patient's file and guardians informed appropriately.

Patient data was tabulated and processed using SPSS (21) for windows software. Qualitative data were expressed as frequency and percentages, quantitative data as mean with or without standard deviation and median.

The chi square test was used for comparative analysis between frequencies of clinical or laboratory features and immunophenotypic subtypes. The fisher's exact test was used if

more than or 25% of the cells had expected counts less than 5%. The differences were considered significant if p was ≤ 0.05.The level of agreement between bone marrow morphology and flow cytometry diagnosis was tested using Cohen kappa coefficient

3.9 Ethical Considerations

Approval to carry out the study was sought from Institutional Research and Ethics committee (IREC) of MTRH and Moi University. Written informed consent was obtained from the parent or guardian of all subjects included in this study. Assent from children older than 7 years was also sought. All procedures to be done were explained including possible benefits and potential risks. Any adverse event during the study was duly reported. Confidentiality was maintained throughout the study. All participants received the same level of care as all other patients. No inducements or incentives were used to convince patients, parents or guardians to participate in the study.

Results of the study will be made available to all persons concerned with the care of children with acute leukemia.

3.10 Study Limitations

Further stratification of the AML cases into sub types M0 – M8 could not be done because of limited reagents and budgetary constraints. No chest imaging was done in this study that would have otherwise helped differentiate the leukemia subtypes.

CHAPTER FOUR

RESULTS

Figure 1 shows a summary of flow of participants during the study. Fifty two children with a presumptive diagnosis and peripheral blood film suggestive of acute leukemia were recruited and had BMA done. Out of the 52, eleven participants did not have leukemia; 7 were found to have hypoplastic bone marrow, 3 dyserythropiesis and 1 with visceral leishmaniasis. These patients were excluded from further analysis. Thirty three were confirmed to have acute leukemia by both BMA morphology and flow cytometry diagnosis while 8 had flow cytometry diagnosis only. The additional eight cases included 5 participants with ALL and 3 with AML and were appropriately assigned lineage on immunophenotyping. One of the ALL cases had mature B cell ALL subtype consistent with ‘burkitt leukemia’ on morphology and ‘lymphocytosis’ on flow cytometry(the preselected monoclonal antibody panel was specific to precursor B cells). Data of the 41

patients confirmed to have acute leukemia were analyzed in this study.

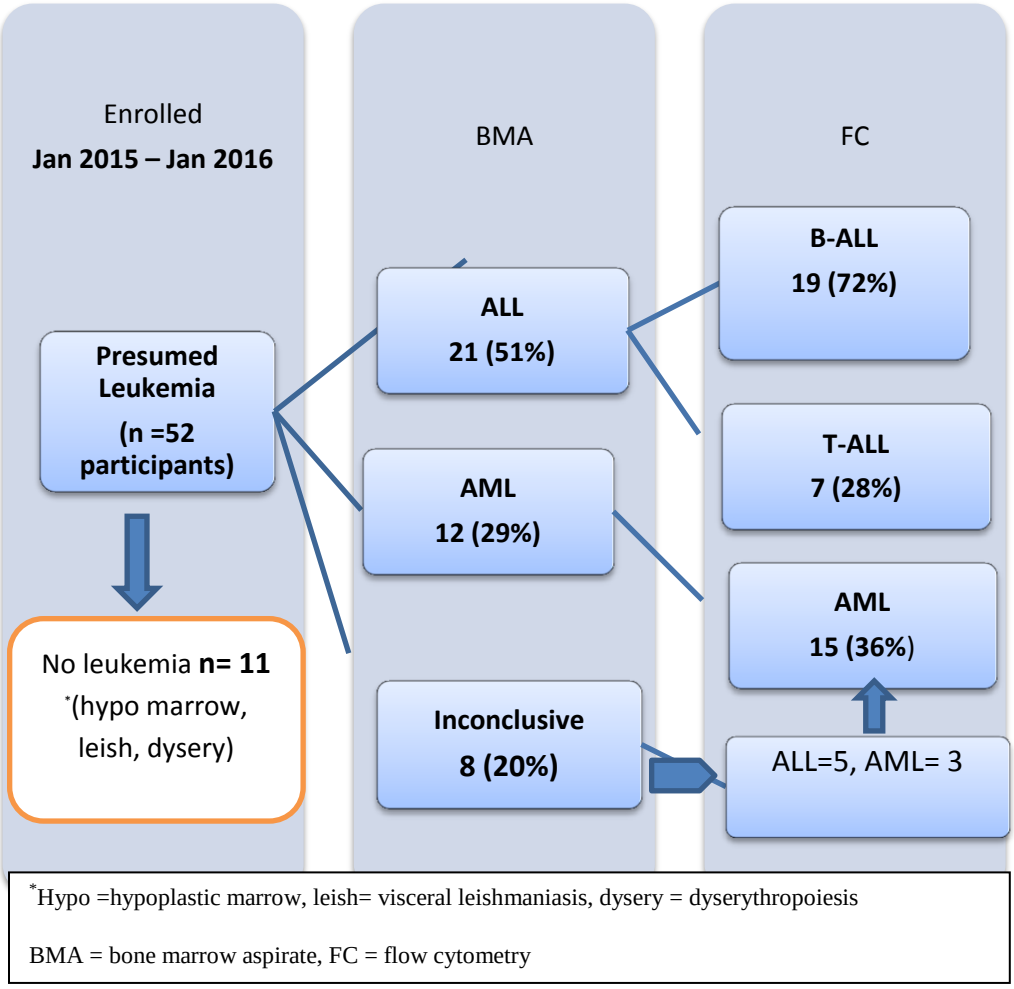
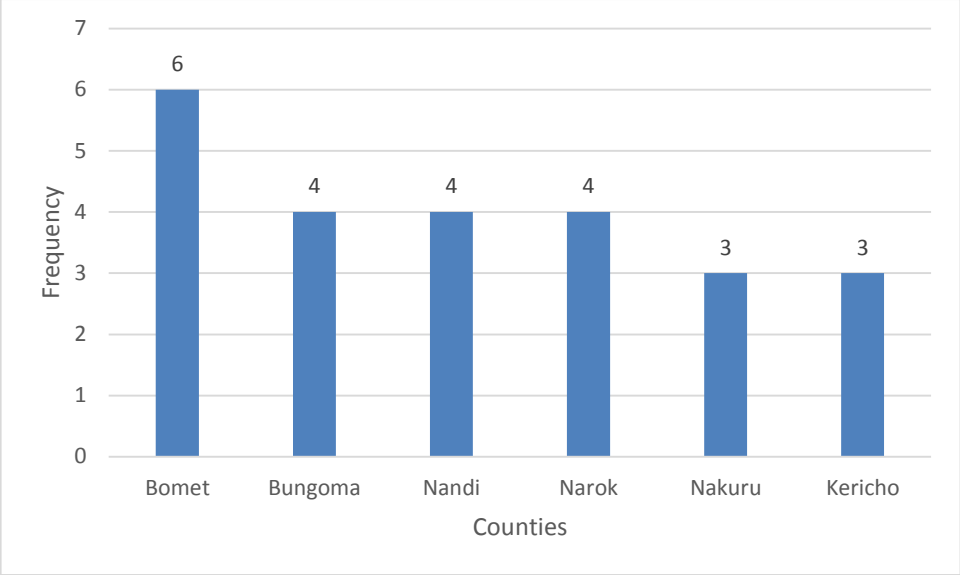


Figure 1: Consort diagram showing summary of flow of study participants.

Figure 2 shows regional distribution of the study participants. Of the 41 cases, majority of the study participants were from Bomet county.



*Residing in that county for a period of six months or more.

Figure 2: Showing top 6 counties* with highest number of cases

Others participants were 2 each from, Baringo, Turkana, Transnzoia, Kakamega,Nyamira and one each from Elgeyo Marakwet, Kisii, Wajir, Kajiado. Migori and Vihiga counties.

Table 1 shows a summary of demographic characteristics of the study participants at diagnosis. There were 21 males (51.2%) and 20 (48.7%) females with acute leukemia giving a male to female ratio of 1.05:1. The age ranged from 1 -14 years, with the mean age at diagnosis of acute leukemia being of 7.3 ± 3.8 years.

Table 1: Demographic characteristics of the study participants

.

Demographic characteristics	ALL (n=26) n(%)	AML (n=15) n(%)	P value
Age at diagnosis, mean (SD) years	6.7 ±3.6	8.3 ± 4.2	0.2007**
Range(yrs)	**2-13**	**1-14**	
<2	0	1 (6.7)	
2-9	20 (76.9)	8 (53.3)	
>9	6 (23.1)	6 (40)	
Gender			
Male	15 (57.7)	5 (33.3)	0.133*
Female	11 (42.3)	10 (66.7)	
Sex ratio (m:f)	1.4:1	1:2	

**Chi square test, **Independent sample t-test*

The median age at diagnosis of for B ALL was 6.4 ± 3.89 years while that of T ALL was 7.71 ± 2.498 years .

Table 2: Bivariate analysis of clinical characteristics associated with acute leukemia subtypes

Variable	Category	ALL n=26 n(%)	AML n=15 n(%)	p-value*
Duration of illness	≤1 month	7 (26.9)	9 (60)	0.036*
	>1month	19 (73.1)	6 (40)	
†Functional status	median (IQR)	80 (40)	70 (40)	0.565**
†Categorized functional status	>90	8 (30.8)	3 (20)	
	71 -90	8 (30.8)	4 (26.7)	
	51 -70	4 (15.4)	4 (26.7)	
	<50	6 (23.0)	4 (26.7)	

**Chi square test, ** Mann-Whitney U test*

† Lansky play functional status; 100% = Fully active, normal 90% = Minor restriction in physically strenuous activity 80% = Active, but tires more quickly 70%= Both greater restriction of, and less time spent in play activities 60%= Up and around, but minimal active play , keep busy 50% = Gets dressed but lies around much of the day ; no active play; able to participate in all quite play and activities 40% = Mostly in bed participates in quit activities 30% = In bed; needs assistance even for quiet play 20%= Often sleeping „ play entirely limited to very passive activities with quieter activity 10%= No play; does not go out of bed.

Out of 41, only 8(19.5%) participants had symptoms for more than 3 months. Most participants had functional status between 71-90% with minimal restriction in physical activity at the time of diagnosis.

4.2. Clinical characteristics at diagnosis of acute leukemia.

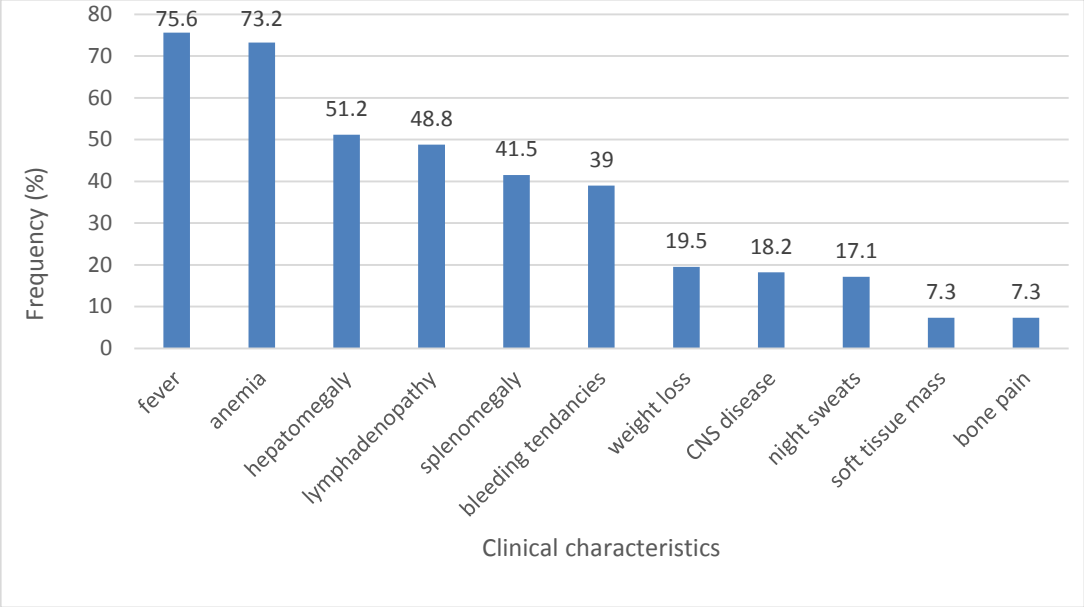


Figure 3: Showing frequency of clinical characteristics at diagnosis of acute leukemia.

Figure 4 shows the clinical characteristics of the acute leukemia subtype at diagnosis of acute leukemia. Fever (87% vs 72%) and weight loss (33% vs 12%) were more frequent among AML than ALL participants respectively. Bone pain was present in 3/25 (12%) study participants with ALL but no AML patient had bone pain at diagnosis. Generalized lymphadenopathy was present in n=16(64%) of ALL patients and only n=4(26%) of AML patients.

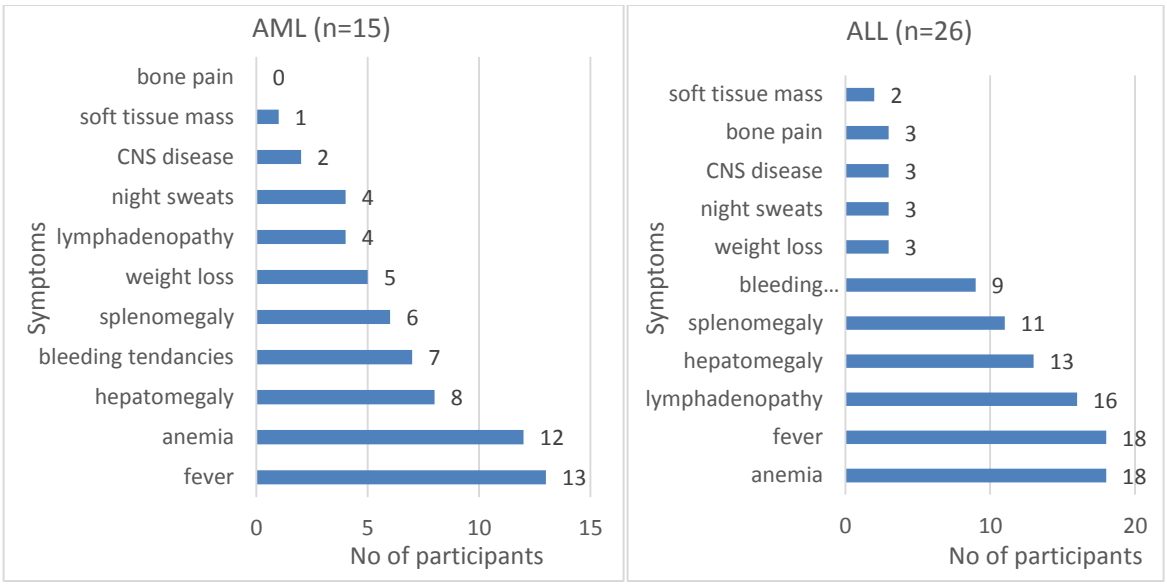


Figure 4: Frequency of clinical characteristics associated with the immunophenotypic subtypes of acute leukemia.

4.3 Laboratory characteristics at diagnosis.

Table 3 shows the frequency of laboratory characteristics associated with immunophenotypic subtypes of acute leukemia. Hemoglobin levels ranged from 3.9 g/dL to 15.5g/dL with median of 7.4g/dL (IQR, 4). In 18/39 (46%) patients the hemoglobin level was <9g/dl, only 7(17.9%) of the patients had hemoglobin levels above 10g/dl. Two cases did not have data on hemoglobin levels.

The total white blood cell count ranged from 0.3 $x10^9$/uL to 306 $x10^9$/uL. while platelet counts ranged from 3 x 10^9/uL to 411 $x10^9$/uL..Laboratory findings of the study participants are summarized in table 3.

Table 3: Laboratory characteristics of study participants at diagnosis of acute leukemia.

Variable	category	ALL n=26 n(%)	AML n=14[ŧ] n(%)	P value
Hemoglobin g/dl	Median(IQR)	7.7 (4.7)	7.2 (2.6)	0.766†
	Range g/dl	**4 – 11.9**	**3.9 -15.4**	
Hemoglobin g/dl (classes)	< 7g/dl	11 (42.3)	7 (50)	0.745*
	7-10g/dl	11 (42.3)	4 (28.6)	
	>10g/dl	4 (15.4)	3 (21.4)	
Leucocyte counts $x10^9$/L	median(IQR)	12.8 (71.8)	24.5 (83.5)	0.257†
Leucocyte counts $x10^9$/L (classes)	$\leq 50 \times 10^9$/L	19 (73.1)	8 (57)	0.480*
	$> 50 \times 10^9$/L	7 (26.1)	6 (43)	
	Range	**3 – 262**	**3-119**	
Platelets $x10^9$/L	$\leq 50 \times 10^9$/L	21(80.8)	7(50)	0.071*
	$> 50 \times 10^9$/L	5(19.2)	7(50)	

†Mann-Whitney U test, *Fisher's Exact test, [ŧ] one AML patient missing laboratory data

Half of the participants with AML had platelet counts less than 50 $x10^9$/uL while more than 80% of ALL cases had platelet counts below 50 $x10^9$/uL (p=0.071).

4.4. BMA morphological and Flow cytometry findings at diagnosis.

The morphological findings of this study were based on 34 results for patients who had a conclusive BMA morphology and flow cytometry report at diagnosis. Figure 5 shows a summary of the morphological diagnoses. There were 21(51%) cases of ALL and 12 (35%) cases of AML and 1(3%) case of 'Burkitt like' leukemia (a mature B cell leukemia).

In 7/41(17%) cases the pathologists were not able to make a morphological diagnosis because the BMA sample was hemodilute with few bone marrow particles.

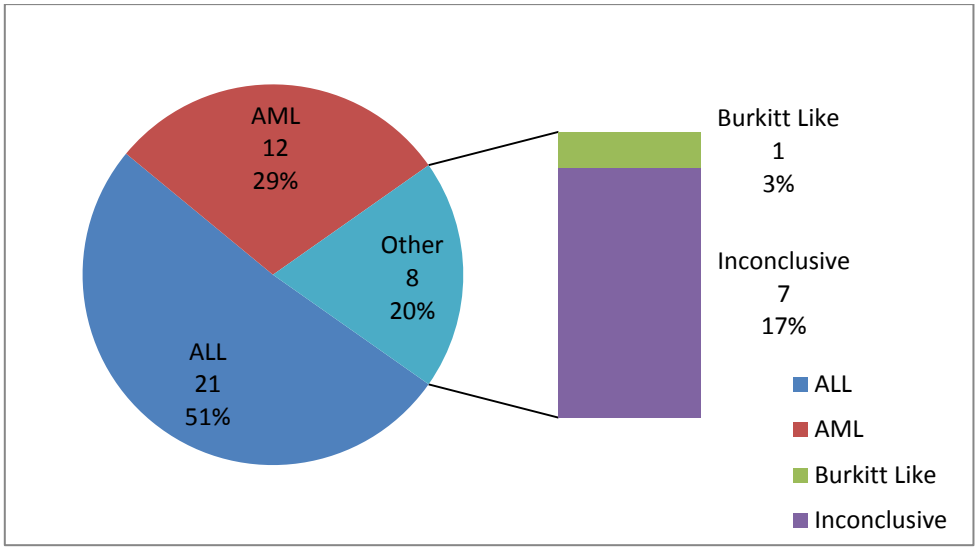


Figure 5: Frequency of morphological subtypes of acute leukemia among newly diagnosed patients.

On flow cytometry immunophenotyping (n=41), the proportions of acute leukemia subtypes observed were: B-ALL 19 (46%), T-ALL 7 (17%) and AML 15(37%). ALL constituted 63% of all acute leukemia cases diagnosed while AML was 37%.

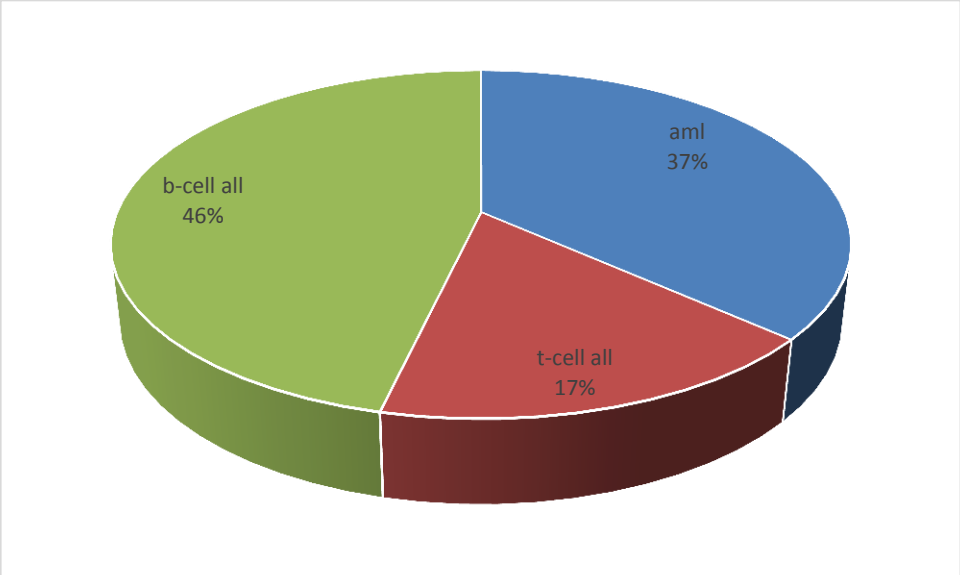


Figure 6: Frequency of immunophenotypic subtypes of acute leukemia among study participants.

The 8 (19.5%) cases that were not conclusively assigned a morphological diagnosis were correctly diagnosed on flow cytometry. Out of the eight unassigned cases, 3 were AML, 5 cases of ALL. Figure 6 shows a summary of the immunophenotypic subtypes of acute leukemia among study participants.

Amongst the 33/41 (80.4%) who had both confirmed morphological and immunological diagnosis of acute leukemia, there was agreement between morphological and flow cytometry diagnosis (kappa = 0.94).

CHAPTER FIVE

DISCUSSIONS

This is among the first studies in the Western Kenya region investigating the clinical and immunophenotypic profiles of children diagnosed with acute leukemia. The key findings are that 63% of acute leukemia patients in MTRH have ALL subtype with the proportions of T ALL and AML being relatively high. Three quarter of the acute leukemia participants had fever and anemia at diagnosis, while two fifths had severe thrombocytopenia at diagnosis. No clinical characteristic could equivocally differentiate the various subtypes of acute leukemia. BMA morphology was inconclusive in diagnosing one –fifth of the acute leukemia cases but these were confirmed and appropriately assigned lineage on flow cytometry immunophenotyping.

5.1 Demographic characteristics

No difference in gender was observed in our study .This contrasts most studies in literature that have reported a predominance of male gender irrespective age [5,50] A possible explanation for the difference is the small sample size in our study. At diagnosis, one third of the patients were in the high risk age group. This corroborates reports of an earlier systematic review by Macharia W N in 1996, who observed that the proportion of children with acute leukemia below two years or above nine years constituted 26% in developing countries and among black population[10]. Age at diagnosis remains a critical determinant of treatment outcome for pediatric ALL patients, even after adjusting for other biological factors [51] It is possible that a significant proportion of leukemia patients referred at MTRH already have high risk biologic factors for poor outcome at diagnosis.

5.2 Acute leukemia immunophenotypic subtypes.

On immunophenotyping, B ALL was the most common subtype accounting for 44% while 37% of acute leukemia diagnosed at MTRH. Similar patterns have been documented in literature [4, 52,53] However, the proportion of T ALL and AML reported in our study was relatively high. Unlike studies from developed countries, where T ALL subtype, accounts for less than 20% of ALL cases[39,54] most studies in Eastern Asia and Africa have reported high proportions of T ALL subtype. In south India for instance, Abitha et al in 2011 reported a frequency of 52.6% with a selective deficit of common ALL subtype [55]. Zahid et al in Morocco found that 21% of ALL were T ALL subtype[56] More recently Dakka et al reported a much higher proportion of 37% in the same population[57] This study however, included participants 16 years of age. In Indonesia, a relatively high proportion of AML subtype was observed[53]The difference in frequency of T ALL subtype in Africa compared to western data has been attributed to etiological causes[58,59] .These environmental exposures are related to socioeconomic status and pattern of infections in early infancy[16, 55, 60].For instance, a recent meta-analysis by Bailey et al has linked maternal pesticide exposure during pregnancy and paternal occupational exposure to increased incidence of AML and a slight rise in ALL particularly T ALL subtype[26] It is possible that environmental and infectious exposures during antenatal and early childhood could be one of the factors contributing to the observed pattern of leukemia subtype distribution.

Similarly, the high proportions of AML observed could also be a result of environmental factors. However, a more probable explanation in our case relates to under diagnosis of ALL cases, a situation common to Africa[21].ALL is frequently misdiagnosed for more common tropical illness like malaria and leishmaniasis that have similar presentation –

anemia, fever and malaise[7,61]. Additionally, presenting clinical features in AML (fever, anemia and bleeding) are more dramatic than those of ALL prompting an earlier referral by first contact physician to higher level health facilities. This is evidenced by the significantly shorter time delay to diagnosis among AML patients observed in our study when compared to ALL participants. This concept of referral and pre admission bias is common in countries with constrained resources[52]. The significance of determining immunophenotypic subtype prior to initiating treatment is well documented. About 30 % of T cell ALL patients are likely to relapse on current treatment protocols[62] while treatment outcomes of AML, particularly in Africa are dismal in the absence of good supportive care[20].Therefore, risk stratification by immunophenotyping and intensifying the treatment in T ALL subtype is likely to result in better outcomes[19].

5.3 Clinical and laboratory characteristics at diagnosis of acute leukemia.

With respect to clinical characteristics, fever was the most common clinical characteristic for all subtypes of acute leukemia followed by anemia and hepatomegaly respectively. This is similar to the findings of the study by Zahid ,M. et al(1994) in Pakistan, where fever was the presenting feature in 79% of ALL patients and 67% of AML patients[56.] Among ALL patients however, anemia was more frequent than fever (72% vs 68%) at the time of diagnosis consistent earlier findings Ahoya, A (unpublished master's thesis) in MTRH and Rana AZ et al [63, 64.] Ahoya, A. reported that 93% of newly diagnosed ALL patients at MTRH had anemia while only 83% had fever.

Only 12% of the acute leukemia patients had bone pain at diagnosis with AML patients were unlikely to have bone pain at diagnosis. This corroborates an earlier unpublished

thesis study by Ahoya A who observed that only 10% of ALL patients had bone pain at diagnosis[63]. The prevalence of bone pain in our study is lower than that documented in literature where bone pain is a presenting feature in 21-38% of the cases of ALL[65] Lima et al (2016) in Brazil observed that bone pain was present in 21.8% of pediatric AML patients while Zahid et al in Morocco found a much higher rate of 67%[27,56] .A possible reason of the low occurrence of bone pain in our study is the small sample size of 41 compared to 62 in the Morocco study. Nevertheless, it is possible that some of the patients with bone pains at MTRH are diagnosed and treated for other more common conditions like osteomyelitis or sickle cell disease particularly if blasts are absent from the initial peripheral blood films[29].

When bivariate analysis was performed to correlate clinical features with immunophenotypic subtypes, the only significant difference between ALL and AML was duration of illness (64% vs 36.3%, p=0.048). No significant difference was observed between the ALL subtypes underlying the importance of using adjunct diagnostic methods in classifying acute leukemia subtypes[39].

With respect to ALL subtypes, the median leucocyte count in our study was lower than that found in other similar studies. Sidhom et al in 2008 for instance[66], observed a median T cell count of 110 $x10^9$/uL while Bachir et al in Morocco observed a median T cell leucocyte count of 116 x $x10^9$/uL[67] .Both studies recruited patients up to the age of 18 years. The wider age range in the two studies could explain the difference in the median TLC between these studies and our study. In the morocco study, Bachir et al observed that distribution of ALL subtype was significantly influenced by the clinical features. Compared with B ALL, male sex (p=0.008), older age (p=0.048), higher leucocyte count

(p=<0.0010 and mediastinal mass (p=0.0011) are typical features of T cell ALL. In our study however, we did not do any imaging to look for mediastinal enlargement. Mediastinal enlargement has been consistently associated with in T ALL subtype.

The median platelet count for acute leukemia in our study was similar to what was found by Fleming AF who reported that three quarter of acute leukemia patients in Africa have platelet counts less than 100,000 x 10^9/uL at diagnosis [7]. In our study, 80% of ALL cases had platelet counts below 50 000 x 10^9/uL compared to only half of AML cases. Though severe thrombocytopenia was more common in ALL than in AML, bleeding tendencies was more frequent among the AML patients. It is therefore possible that bleeding in acute leukemia, particularly AML could be a consequence of a qualitative platelet defect rather than inadequacy in platelet numbers alone. Margolin et al (2006) observes that even with low platelet counts, severe hemorrhage is uncommon except in acute promyelocytic leukemia (APML) [28].

5.4 BMA Morphological diagnosis

One third of the BMA morphological diagnoses were acute myeloid leukemia consistent flow cytometry findings. Notably, 8/34 cases could not be conclusively characterized on BMA morphology. These were however, appropriately assigned lineage on immunophenotyping. Out of the eight, five were ALL; including one case of mature B ALL which was classified as 'Burkitt like' leukemia on morphology and 'lymphocytosis' (since the preselected monoclonal antibody panel was programed to identify precursor B cells rather than mature B cells) on flow cytometry. This is similar to findings by jawaid et al[41] and Belurkar, et al who observed that even when used alongside cytochemical

staining, morphology could confirm diagnosis in only 80% of acute leukemia cases [34.] In the study by Belurkar et al, cytomorphology did not correctly assigned lineage in 11/33 (33.3%). ALL cases and 1/12(8.3%) AML cases but these were correctly classified on immunophenotyping underlining the role of flow cytometry immunophenotyping in diagnosis and classification of acute leukemia.

For those participants with both BMA morphological and flow cytometry diagnosis, the level of agreement was good with Kappa score at 0.94. This compares well with study by Supriyadi et al in Indonesia[53] .In this large multicenter prospective study, there was concordance between morphology and flow cytometry (Kappa=0.82). The high kappa score in our study could be attributed to the small sample number of participants.

5.4. Study Limitations

A limitation of this study is the small sample frame within the various subtypes of acute leukemia. Being a rare heterogeneous disease, it was not possible to get sufficient participants in each category for comparative analysis and generalization of study results into the population. However, this study provides significant descriptive insights on the various immunological and clinical aspects of acute leukemia at MTRH. This is a basis for future research on acute leukemia. Due to lack of reagents AML cases were not categorized into their respective immunophenotypic subtypes.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study found that anemia, fever and thrombocytopenia and the commonest clinical characteristics at diagnosis of acute leukemia in children at MTRH.

There was no clinical characteristic that could equivocally differentiate the various subtypes of acute leukemia. When BMA morphology is used as the only diagnostic modality; up to one fifth of the acute leukemia cases at MTRH are not conclusively diagnosed.

The proportion of high risk acute leukemia subtype (T cell ALL, and AML) at MTRH is relatively high.

Routine use of flowcytometry will improve the diagnosis and stratification of acute leukemia into prognostic groups.

6.2 Recommendations

Proper risk stratification of acute childhood leukemia subtypes may help optimize treatment while reducing chemotherapy toxicity resulting in superior outcomes. We therefore, recommend the routine use of flow cytometry alongside BMA morphology to improve the diagnosis and classification of acute childhood leukemia at MTRH.

REFERENCES

1. Huh YO, Ibrahim S. Immunophenotypes in adult acute lymphocytic leukemia. Role of flow cytometry in diagnosis and monitoring of disease. *Hematol Oncol Clin North Am*. 2000;14(6):1251-1265.
2. Amal S. Al-Seraihy, Tarek M. Owaidah, Mouhab Ayas, Hassan El-Solh, Mohammed Al-Mahr, Ali Al-Ahmari, and Asim F. Belgaumi.Clinical characteristics and outcome of children with biphenotypic acute leukemia. Haematologica. 2009 Dec; 94(12): 1682–1690.
3. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995 Oct;9(10):1783-6
4. Hutter JJ, Schulze-Rath R, Hammer G, et al. Childhood leukemia. *Pediatr Rev*. 2010;31(6):234-241. doi:10.1542/pir.31-6-234.
5. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.
6. Pui CH.Acute lymphoblastic leukemia.Pediatr Clin North Am. 1997 Aug;44(4):831-46. Review
7. Fleming AF. Leukaemias in Africa. *Leukemia*. 1993;7(2):138-141.
8. Eva Steliarova-Foucher, Murielle Colombet, Lynn A G Ries, Florencia Moreno, Anastasia Dolya, Freddie Bray, Peter Hesseling, Hee Young Shin, Charles A Stiller, and the IICC-3 contributors*.International incidence of childhood cancer, 2001–10:a population-based registry study.Lancet Oncology,2013.

9. Ochicha O, Gwarzo AK, Gwarzo D. Pediatric malignancies in Kano, Northern Nigeria. *World J Pediatr*. 2012;8(3):235-239.
10. Macharia WM. Childhood cancers in a referral hospital in Kenya: A review. *East Afr Med J*. 1996;73(10):647-650.
11. Mostert S, Njuguna F, Kemps L, et al. Epidemiology of diagnosed childhood cancer in Western Kenya. *Arch Dis Child*. 2012;97(6):508-512. doi:10.1136/archdischild-2011-300829.
12. McKenna RW. Multifaceted approach to the diagnosis and classification of acute leukemias. In: *Clinical Chemistry*. Vol 46. ; 2000:1252-1259.
13. Hospital CC. Value of Multifaced Approach Diagnosis and Classification of Acute Leukemias. 2012;7(3).
14. Arora RS, Eden TOB, Kapoor G. Epidemiology of childhood cancer in India. *Indian J Cancer*. 2009;46(4):264-273.
15. Kamel AM, Assem MM, Jaffe ES, Magrath I, Aboul Enein MI, Hindawy DS. Immunological phenotypic pattern of acute lymphoblastic leukaemia in Egypt. *Leuk Res*. 1989;13(7):519-525.
16. Greaves MF, Colman SM, Beard ME, et al. Geographical distribution of acute lymphoblastic leukaemia subtypes: second report of the collaborative group study. *Leuk Off J Leuk Soc Am Leuk Res Fund, UK*. 1993;7(1):27-34.
17. Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, Reaman GH, Carroll WL.Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. J Clin Oncol. 2012 May 10;30(14):1663-9.

18. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. *Lancet*. 2013;381(9881):1943-1955.

19. Pui CH, Yang JJ, Hunger SP, Pieters R, Schrappe M, Biondi A, Vora A, Baruchel A, Silverman LB, Schmiegelow K, Escherich G, Horibe K, Benoit YC, Izraeli S, Yeoh AE, Liang DC, Downing JR, Evans WE, Relling MV, Mullighan CG. Childhood Acute Lymphoblastic Leukemia: Progress Through Collaboration. J Clin Oncol. 2015 Sep 20;33(27):2938-48.

20. Kersten E, Scanlan P, Dubois SG, Matthay KK. Current treatment and outcome for childhood acute leukemia in Tanzania. *Pediatr Blood Cancer*. 2013;60(12):2047-2053.

21. Togo B, Traoré F, Togo AP, et al. [Epidemiology and prognosis of childhood cancers at Gabriel-Touré Teaching Hospital (Bamako, Mali)]. *Med Sante Trop*. 2014;24(1):68-72.

22. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin*. 2008;58(2):71-96.

23. Pui C-H. Childhood leukemias, third edition. *Child Leuk Third Ed*. 2010.

24. Greaves MF. Aetiology of acute leukaemia. *Lancet*. 1997;349(9048):344-349.

25. Shu XO, Perentesis JP, Wen W, et al. Parental exposure to medications and hydrocarbons and ras mutations in children with acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Cancer Epidemiol Biomarkers Prev*. 2004;13(7):1230-1235.

26. Bailey HD, Fritschi L, Infante-Rivard C, et al. Parental occupational pesticide exposure and the risk of childhood leukemia in the offspring: Findings from the childhood leukemia international consortium. *Int J Cancer*. 2014;135(9):2157-2172.

27. Lima MC De, Bousfield D, Paula A, et al. Acute Myeloid Leukemia : analysis of epidemiological profile and survival rate ଌ. *J Pediatr (Rio J)*. 2016;(xx).

28. Margolin, JF, Steuber, CP, Poplack D. Acute lymphoblastic leukemia. In: *Principles and Practice of Pediatric Oncology*. ; 2006:538.

29. D. K, M. B, V. E, et al. Aseptic osteonecrosis in children with acute lymphoblastic leukemia. *Pediatr Hematol Oncol*. 1998;15(4):307-315. Rubnitz JE. Childhood acute myeloid leukemia. *Curr Treat Options Oncol*. 2008;9(1):95-105. doi:10.1007/s11864-008-0059-z.

30. Pui C-H. Central nervous system disease in acute lymphoblastic leukemia: prophylaxis and treatment. *Hematology Am Soc Hematol Educ Program*. 2006:142-146.

31. Chen CY, Zimmerman RA, Faro S, Bilaniuk LT, Chou TY, Molloy PT. Childhood leukemia: Central nervous system abnormalities during and after treatment. *Am J Neuroradiol*. 1996;17(2):295-310.

32. Pui CH, Behm FG, Singh B, et al. Heterogeneity of presenting features and their relation to treatment outcome in 120 children with T-cell acute lymphoblastic leukemia. *Blood*. 1990;75(1):174-179.

33. Belurkar S, Mantravadi H, Manohar C, Kurien A. Correlation of morphologic and cytochemical diagnosis with flowcytometric analysis in acute leukemia. *J Cancer Res Ther*. 2014;9(1):71-79.

34. Vardiman J. The classification of MDS: From FAB to WHO and beyond. *Leuk Res*. 2012;36(12):1453-1458.

35. Servitzoglou M, Grenzelia M, Baka M, et al. A novel karyotype in acute myeloid leukemia with basophilia. *Pediatr Hematol Oncol*. 2014;31(2):149-156.

36. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, Dworzak MN, Adachi S, de Bont E, Harbott J, Hasle H, Johnston D, Kinoshita A, Lehrnbecher T, Leverger G, Mejstrikova E, Meshinchi S, Pession A, Raimondi SC, Sung L, Stary J, Zwaan CM, Kaspers GJ, Reinhardt D; AML Committee of the International BFM Study Group. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood. 2012 Oct 18; 120(16):3187-205.

37. de Rooij JD, Zwaan CM, van den Heuvel-Eibrink M. Pediatric AML: From Biology to Clinical Management. J Clin Med. 2015 Jan 9;4(1):127-49.

38. Pui CH, Robison LL, Look AT Acute lymphoblastic leukaemia.Lancet. 2008 Mar 22;371(9617):1030-43. doi: 10.1016/S0140-6736(08)60457-2. Review

39. Szczepański T, van der Velden VHJ, van Dongen JJM. Classification systems for acute and chronic leukaemias. *Best Pract Res Clin Haematol*. 2003;16(4):561-582.

40. Chana J. Shani T, Hashmi K. Role of immunophenotyping in diagnosis of acute leukemia. J Coll. Physician Surg. Pak 2000;158-60.

41. Szczepański T, Van Der Velden VHJ, Van Dongen JJM. Flow-cytometric immunophenotyping of normal and malignant lymphocytes. *Clin Chem Lab Med*. 2006;44(7):775-796.

42. Pamnani R. Immunophenotyping of acute leukaemias by flow cytometry: a review. *East Afr Med J*. 2009;86(12 Suppl).

43. Ruiz-Argüelles A, Rivadeneyra-Espinoza L, Duque RE, Orfao A. Report on the Second Latin American Consensus Conference for flow cytometric immunophenotyping of hematological malignancies. In: *Cytometry Part B - Clinical Cytometry*. Vol 70. ; 2006:39-44.

44. Brown BJ, James BO, Ajayi SO, Ogun OA, Oladokun RE. Factors influencing time to diagnosis of childhood cancer in Ibadan, Nigeria. *Afr Health Sci*. 2009;9(4):247-253.
45. Brown M, Wittwer C. Flow cytometry: Principles and clinical applications in hematology. *Clin Chem*. 2000;46(8 II):1221-1229. doi:1530-8561.
46. Greig B, Oldaker T, Warzynski M, Wood B. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: Recommendations for training and education to perform clinical flow cytometry. In: *Cytometry Part B - Clinical Cytometry*. Vol 72. ; 2007.
47. van Wering ER, van Lochem EG, Leenders M, et al. Three-color flowcytometric analysis of mature and immature hematological malignancies. A guideline of the Dutch Foundation for Immunophenotyping of Hematological Malignancies (SIHON). *J Biol Regul Homeost Agents*. 2004;18(3-4):313-326.
48. Patel K, Lotodo T, Njuguna F, et al. Use of Flow Cytometry Immunophenotyping for Diagnosis of Acute Leukemia at Moi Teaching and Referral. (July 2013):72-80.
49. Mushtaq N, Fadoo Z, Naqvi A. Childhood acute lymphoblastic leukaemia : experience from a single tertiary care facility of Pakistan. :1399-1404.
50. Hossain MJ, Xie L, McCahan SM. Characterization of pediatric acute lymphoblastic leukemia survival patterns by age at diagnosis. *J Cancer Epidemiol*. 2014;2014:865979. doi:10.1155/2014/865979.
51. Gujral S, Badrinath Y, Kumar A, et al. Immunophenotypic Profile of Acute Leukemia : Critical Analysis and Insights Gained at a Tertiary Care Center in India. 2009;205(April 2008):199-205.
52. Supriyadi E, Widjajanto PH, Veerman AJP, et al. Immunophenotypic Patterns of

Childhood Acute Leukemias in Indonesia. 2011;12:3381-3387.

53. Kamps WA, van der Pal-de Bruin KM, Veerman AJP, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. *Leuk Off J Leuk Soc Am Leuk Res Fund, UK*. 2010;24(2):309-319.
54. AR Abitha, N Anuratha, KR Rajalekshmy, TG Sagar.Time trend in frequency of occurrence of major immunophenotypes in paediatric acute lymphoblastic leukemia cases as experienced by Cancer Institute, Chennai, south India during the period 1989-2009. *Indian Journal of Cancer, Vol. 48, No. 3, July-September, 2011, pp. 310-315*
55. Zahid M, Khalid A, Ahmed Z. Acute Leukemias of Childhood : A Retrospective Analysis of 62 Cases. 1994:15-18.
56. Dakka N, Bellaoui H, Khattab M, et al. Immunologic profile and outcome of childhood acute lymphoblastic leukemia (ALL) in Morocco. *J Pediatr Hematol Off J Am Soc Pediatr Hematol*. 2007;29(8):574-580.
57. Greaves MF. Subtypes of Acute Lymphoblastic Leukemia, Implications for the pathogenesis and epidemiology of leukemia. In: Magrath IT, O' Connor GT, Ramot B, editors. Pathogenesis of Leukemias and lymphomas. Environmental influences. New York: Raven Press; 1984. p. 129.
58. Fleming AF. Possible aetiological factors in leukaemias in Africa. *Leukemia Research*. 1988; 12(1): 33-43.
59. Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. Nat Rev Cancer. 2006;6:193–203.
60. Raul C. Ribeiro, Federico Antillon, Francisco Pedrosa, Ching-Hon Pui. Global Pediatric Oncology: Lessons From Partnerships Between High-Income Countries and

Low- to Mid-Income Countries. J Clin Oncol. 2016 Jan; 34(1): 53–61.

61. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006;354(2):166-178.
62. Ahoya A.(2014). Response to induction chemotherapy in paediatric acute lymphoblastic leukemia at Moi Teaching and Referral hospital, Eldoret.(Unpublished master's thesis).Moi University,School of Medicine.Eldoret,Kenya.
63. Rana ZA, Rabbani MW, Sheikh MA, Khan AA.Outcome of childhood acute lymphoblastic leukaemia after induction therapy--3 years' experience at a single paediatric oncology centre. J Ayub Med Coll Abbottabad. 2009 Oct-Dec;21(4):150-3.
64. Sinigaglia R, Gigante C, Bisinella G, Varotto S, Zanesco L, Turra S. Musculoskeletal manifestations in pediatric acute leukemia. *J Pediatr Orthop*. 2008;28(1):20-28.
65. Sidhom I, Shaaban K, Soliman S, et al. Clinical significance of immunophenotypic markers in pediatric T-cell acute lymphoblastic leukemia. *J Egypt Natl Canc Inst*. 2008;20(2):111-120.
66. Bachir F, Bennani S, Lahjouji A, et al. Characterization of acute lymphoblastic leukemia subtypes in moroccan children. *Int J Pediatr*. 2009;2009(October 2003):674801.

APPENDICES

Appendix I: Consent form

I, Dr. Gilbert Kipruto Olbara am a resident in the Department of Child Health and Pediatrics, Moi University. I am carrying out a study on the Clinical and Immunological patterns among children diagnosed with acute Leukemia at Moi Teaching and referral Hospital, Eldoret.

The study involves follow up of a child suspected to have cancer of the blood during the period of getting the diagnosis to time of starting the initial treatment and documenting all findings during this period. This will involve documenting all tests done and physical diagnosis. No additional investigations will be ordered for the purpose of this study.

There will be no direct benefits of participating in this study. Study subjects will be accorded same quality management as non –study subjects.

All information obtained in this study will be treated with utmost confidentiality and shall not be divulged to any unauthorized person.

Participation in this study is voluntary, there is freedom to refuse to take part or withdraw at any time.

Sign or make a mark if you agree to take part in the study

Parent /Guardian; Investigator: Date:

…… …….. … …….

Appendix II: Assent Form

I, Dr. Gilbert Kipruto Olbara, am a resident in the Department of Child Health and Pediatrics, Moi University. I am carrying out a study on the Clinical and Immunological profiles among children diagnosed with acute Leukemia at Moi Teaching and referral Hospital, Eldoret.

The study involves follow up of a child suspected to have cancer of the blood during the period of getting the diagnosis to time of starting the initial treatment and documenting all findings during this period. This will involve documenting all tests done and physical diagnosis. No additional investigations will be ordered for the purpose of this study.

There will be no direct benefits of participating in this study. Study subjects will be accorded same quality management as non –study subjects.

All information obtained in this study will be treated with utmost confidentiality and shall not be divulged to any unauthorized person.

Participation in this study is voluntary, there is freedom to refuse to take part or withdraw at any time.

Sign or make a mark if you agree to take part in the study

Parent /Guardian; Investigator: Date:

…… ……. …….

Appendix III: Data Collection Form

DEMOGRAPHICS

Study Number:…… Medical Record:……

Date of Birth:…… Sex: 0 Male 0 Female

County……..

CLINICAL PRESENTATION

Duration of symptoms before presenting to Moi Teaching and Referral Hospital

<2 weeks 0 2-4 weeks 0 1-3 months 0 >3months 0

HIV serology:

0 Negative 0 Positive 0 Unknown

Lansky play scale:

0 100% **0** 90% **0** 80% **0** 70% **0** 60%

0 50% **0** 40% **0** 30% **0** 20% **0** 10%

0 Bleeding tendencies 0 Night Sweats 0 Weight loss

0 Fever 0 Hepatomegaly 0 Splenomegaly

0 Anaemia 0 Bone pain 0 Soft tissue mass

0 Lymphadenopathy

Site of lymphadenopathy……

0 CNS findings……..CNS disease: 0 absent 0 present

Lansky Scale

100% = Fully active, normal

active

90% = Minor restriction in physically strenuous activity

80% = Active, but tires more quickly

70%= Both greater restriction of, and less time spent in play activities

60%= Up and around,but minimal active play , keep busy

passive activities with quieter activity

50% = Gets dressed but lies around much of the day ; no

play; able to participate in all quite play and activities

40% = Mostly in bed participates in quit activities

30% = In bed; needs assistance even for quiet play

20%= Often sleeping ,, play entirely limited to very

10%= No play; does not go out of bed·

INITIAL LABORATORY FINDINGS

Total WBC count……x 10^9/uL

0< 5.0 x 10^9/uL **0** 5 – 12 x10^9/uL **0**13 – 50 x10^9/uL **0** > 50 x 10^9/uL

Absolute Lymphocyte count…….x 10^9/uL

Absolute Neutrophil count……x 10^9/uL

Platelet Counts …….x 10^9/ul Haemoglobin

level……..g/dl

PBF blasts……%

0 Lymphoblasts 0 Myoblasts 0 Other

Comment……..

BONE MARROW ASPIRATE DIAGNOSIS

a) **Morphology**.

Date of BMA procedure……0 Successful 0 Dry tap

BMA Blasts 0 Hemodilute 0 Absent 0 Present

0 Blasts……% 0 Lymphoblast 0 Myeloblast

BMA diagnosis by pathologists Date of diagnosis……

0 ALL 0 AML 0 Other ……..

Comments……

b) **Immunophenotype by flow cytometry**

BMA diagnosis by Flow Cytometry. Date of diagnosis ……

0 ALL 0 AML 0 B-Cell ALL 0 T - Cell ALL 0 Other

Comments……..

Challenges or complications faced if any …….

……

……

Appendix VI: Procedure for Taking a Blood Sample for blood cell counts

1. Sterilise the skin with spirit(>70% alcohol)
2. Allow the spirit to air-dry
3. Use a new sterile needle or cannula for venous puncture and collect 2-3 milliliters of blood in a standard EDTA anti-coagulated sample collection bottle.
4. Safely discard the needle and complete a laboratory request form

Appendix IV: Peripheral Blood Film Preparation

Slide preparations —peripheral blood smears will be prepared using either the wedge technique or the particle crush technique, manually.

A clean slide, free from dust, dirt, grease, and fingerprints, is used.

The slides will be alcohol-cleaned.

Smears will be made from one small drop of blood which has not been allowed to clot, and which has been completely mixed, from an anti-coagulated blood sample.

Procedure:

The Wedge technique —the drop of blood is placed at the end of one slide and spread evenly down the length using a second glass slide. The second slide should be angled at 30 degrees to the base slide; the drop should be pushed using a rapid, even motion.

Particle crush technique — a small drop of blood is placed at the end of a glass slide. A second glass slide is held parallel and directly over the drop of the aspirate. The second slide is then pressed against the drop of aspirate and pulled across the full length of the first slide.

Staining procedure:

- The slides will be stained with leishman stain.
- The slide is flooded with the stain (7-10 drops) for 5 minutes.
- Buffer of distilled water is added (10-15 drops) for 20-30 minutes.
- Slide is washed and cleaned around the periphery (back) with the buffer.
- Then air dried.

Appendix V: Bone Marrow Aspirate Procedure

Materials:

- Sterile gloves
- Povidone-iodine antiseptic solution for cleansing the chosen site, along with the necessary drapes to maintain sterility at the site
- A 1 or 2 percent lidocaine solution for local anaesthesia, along with a sterile syringe, a 23-gauge and a 21-gauge needle
- Sufficient quantities of sterile gauze and bandages to clean the biopsy site and to apply local pressure to insure haemostasis when the procedure has been completed
- Jamshidi bone aspirate and biopsy needle with stylet and a device (obturator) for removing the biopsy core from the needle without damage to the specimen or disposable plastic BMA needles.
- Four to five 20 cc and 10 cc sterile syringes for aspirating the bone marrow.

Choice of aspiration site:

- The posterior superior iliac crest and spine is the favored site of examination in the child and in most infants as it offers the least discomfort to the patient.
- The anterior iliac crest may be used for bone marrow aspiration and biopsy when access to the posterior iliac crest is limited (for example, the patient is unable to be moved for proper access to the chosen aspiration site, morbid obesity, skin diseases, or previous radiation). An initial attempt to sample the posterior iliac bone may be worthwhile, even in neonates.
- Sternal aspiration should not be undertaken in young children due to the risk of perforating the sternal plate. For the same reasons, marrow biopsy at sternal sites is contraindicated.

PREPARATION OF SAMPLES — Slides of the aspirated marrow will be prepared at the bedside. Depending on the clinical scenario, up to 9 bone marrow direct smears may be prepared.

When performed at the bedside, slides should be prepared rapidly to avoid clotting. Adequacy of the specimen is determined by the presence of "spicules," which appear as fatty droplets, granules, or small chunks of bone, which allow assessment of marrow

cellularity. Spicules tend to concentrate at the feathered edge when a smear of the bone marrow is made.

Materials:

- Glass slides and cover slips. Both should be clean and free of dirt, grease, oil, or fingerprints

Procedure — Place the glass slides in a convenient location before the procedure to allow quick and easy access. Place approximately 0.5 mL of marrow aspirate on one glass slide; if only one or two aspirate samples are to be prepared, the remainder may be added to an EDTA-anticoagulated sample collection tube, mixing well. This should be done immediately to prevent clotting of the specimen. Preparations are then made using one or more of the following two techniques:

i. Wedge technique — A few "particles" (spicules) are placed at the end of one slide and spread evenly down the length using a second glass slide. The second slide should be angled at 30 degrees to the base slide; the drop should be pushed using a rapid, even motion, ending in a particle (spicule)-rich feathered edge.

ii. Particle crushes technique — a small drop of aspirated marrow is placed at the end of a glass slide. A second glass slide is held parallel and directly over the drop of the aspirate. The second slide is then pressed against the drop of aspirate and pulled across the full length of the first slide to crush open and spread the marrow particles.

The slides will then be taken to the MTRH laboratory for staining with leishman stain (see in appendix IV under staining procedure).

POST-PROCEDURE INSTRUCTIONS — following the procedure, the patient should lie in a supine position, so as to apply body weight to the biopsy site, for at least 10 to 15 minutes. The site should then be inspected to ensure that there is no further bleeding. The patient should be advised that the procedure site may be slightly tender for several days. The following additional routine instructions should be given:

- For pain control, a non opiod analgesia for 24 to 48-hour period for example: Paracetamol given orally at 15mg/kg/dose every 6-8 hours, or Ibuprofen orally 10mg/kg/dose every 8 hours.
- The patient be directed to contact the physician or clinic if swelling, marked tenderness, increased pain, and/or further bleeding is observed.
- The patient should avoid overexertion (for example, heavy activity or exercise) for at least 24 hours, to avoid potential pain or bleeding at the site of the procedure. The area of the aspiration/biopsy should be kept dry during this time to minimize the chance of infection or bleeding.

If the patient continues to bleed from the aspirate/biopsy site after an initial observation period of 10 to 15 minutes, it may be prudent to reapply pressure to the site and have the patient lie supine for at least one hour. If bleeding continues after this additional time of observation, it may be necessary to transfuse platelets if the patient is severely thrombocytopenic or if platelet function is suspected to be compromised.

COMPLICATIONS

Risk factors for an adverse event included diagnosis of a myeloproliferative disorder, treatment with aspirin or warfarin, obesity, or disseminated intravascular coagulation. In general, however, when complications do occur, they tend to be minor, mainly consisting of bleeding at the biopsy/aspiration site or infection. This information should be obtained during the process of consenting.

Bleeding — Haemorrhage from bone marrow aspiration can occur at any site, is more likely in the individual with thrombocytopenia and/or abnormal platelet function, and is associated most commonly with the myeloproliferative disorders. In most cases, bleeding is controlled by manual application of pressure to the site. Pressure dressings should be applied to the site following the procedure in patients with thrombocytopenia. If bleeding continues, platelet transfusions may have to be given if the patient is severely thrombocytopenic, or if platelet function is compromised.

Infection — Infections are usually minor, requiring only topical medications. There is a potential risk of contracting infections from a patient, and some recommend double-gloving. However, universal precautions should be applied in all cases, and the operator should always take care to avoid needle penetration of the skin.

Tumour seeding — There have been rare case reports of tumour seeding from the bone marrow into the needle track (for example, into muscle, subcutaneous tissue, skin) following bone marrow biopsy, in patients with small cell lung carcinoma, multiple myeloma, and lymphoma.

Needle breakage — rarely, a bone marrow needle may break. If this occurs, an attempt to extract the distal segment with a haemostat should be made. If this maneuver is unsuccessful, a surgeon should be consulted.

Other — Seldom, patients may experience persistent discomfort at the site of biopsy. Exceedingly rare complications have included transient neuropathy with gluteal compartment syndrome secondary to post-biopsy bleeding, fracture due to underlying osteoporosis, and osteomyelitis. There may be abnormal radiologic studies of the pelvis post-biopsy, including lytic lesions surrounded by a sclerotic border, exostoses, or increased bone-seeking isotope uptake.

Adapted from UpToDate Version 21.3: Topic last updated, 2013.

Appendix VII: Ampath Reference Laboratory ProcedureFor Cell Preparation And Analysis Of Cell Surface And Intracellular Antigens For Leukemia Or Lymphoma

Procedure for Staining of Surface Antigens

1. Determine the appropriate number of 5ml falcon tubes required per sample.
2. Label these tubes with the patients ID no. and tube no. (e.g. 1,2,3…)
3. Add the appropriate amount of fluorescently labeled antibodies in the corresponding tubes according to the scheme in APPENDIX 1. Add wash buffer+ protein to balance the volume in each tube to 20µl (see APPENDIX 1). Add 30µl of cell sample, mix gently. Droplets of the sample that are not at the bottom of the tube, can be removed by absorbing these in the cotton wool of an ear stick.
4. Incubate for 15 - 45 min. at room temperature in the dark.
5. Add 1ml of FACS lysing solution to remove the erythrocytes.
6. Incubate for 10 minutes at room temperature in the dark.
7. Add 2 ml of wash buffer+protein and centrifuge at 1500rpm for 5 min.
8. When the cell pellet is rather "red" discard the supernatant and repeat the lysing step (step 6); otherwise discard the supernatant and resuspend the cell pellet in approximately 300µl wash buffer. When necessary an additional wash step can be performed.
9. The stained cells are now ready for analysis on the flow cytometer. Proceed to *Acquisition of data*.

Procedure for Staining of Intracellular Antigens

1. Identify the tube(s) for surface and intracellular staining.
2. Follow step 1-5 above for surface staining procedure.
3. Add 2-3 ml of wash buffer+protein and centrifuge at 1500rpm for 5 min to remove the un-bound antibodies. The cell pellet will be red because of the presence of erythrocytes; these will be lysed in the next step.
4. Discard the supernatant and add 1ml of FACS lysing solution to remove the erythrocytes. The diethyleneglycol in the FACS lysing solution is a rather stringent way to remove erythrocytes. A side effect of this solution is that is permeabilizes the cells in the tubes, thereby enabling intracellular staining in a second incubation step .
5. Incubate for 10 minutes at room temperature in the dark
6. Add 2 ml of wash buffer+protein and centrifuge at 1500rpm for 5 min
7. Discard the supernatant and add the appropriate amount of fluorescently labeled antibody and buffer according to tube 7B in APPENDIX 1.
8. Incubate for 15- 45 minutes at room temperature in the dark.
9. Add 2-3 ml of wash buffer+protein and centrifuge at 1500rpm for 5 min.
10. Discard the supernatant and resuspend the cell pellet in approximately 300µl wash buffer.
10. The stained cells are now ready for analysis on the flow cytometer; Proceed to *Acquisition of data*

Appendix VIII: IREC Approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2014/179
Approval Number: 0001275

19th September, 2014

Dr. Gilbert Olbara,
Moi University,
School of Medicine,
P.O. Box 4606-00200,
ELDORET-KENYA.

Dear Dr. Olbara,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"Clinical and Immunophenotypic Profile of Children Diagnosed with Acute Leukemia at Moi Teaching and Referral Hospital, Eldoret."

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1275*** on 19th September, 2014. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 18th September, 2015. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc Director - MTRH Dean - SOP Dean - SOM
Principal - CHS Dean - SON Dean - SOD

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

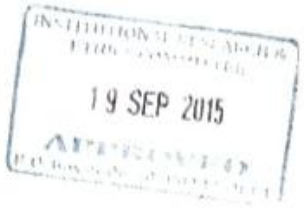
MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471/2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2014/179
Approval Number: 0001275

19th September, 2015

Dr. Gilbert Olbara,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.



Dear Dr. Olbara,

RE: CONTINUING APPROVAL

The Institutional Research and Ethics Committee has reviewed your request for continuing approval to your study titled:-

"Clinical and Immunophenotypic Profile of Children Diagnosed with Acute Leukemia at Moi Teaching and Referral Hospital, Eldoret".

Your proposal has been granted a Continuing Approval with effect from 17th August, 2015. You are therefore permitted to continue with your study.

Note that this approval is for 1 year; it will thus expire on 16th August 2016. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

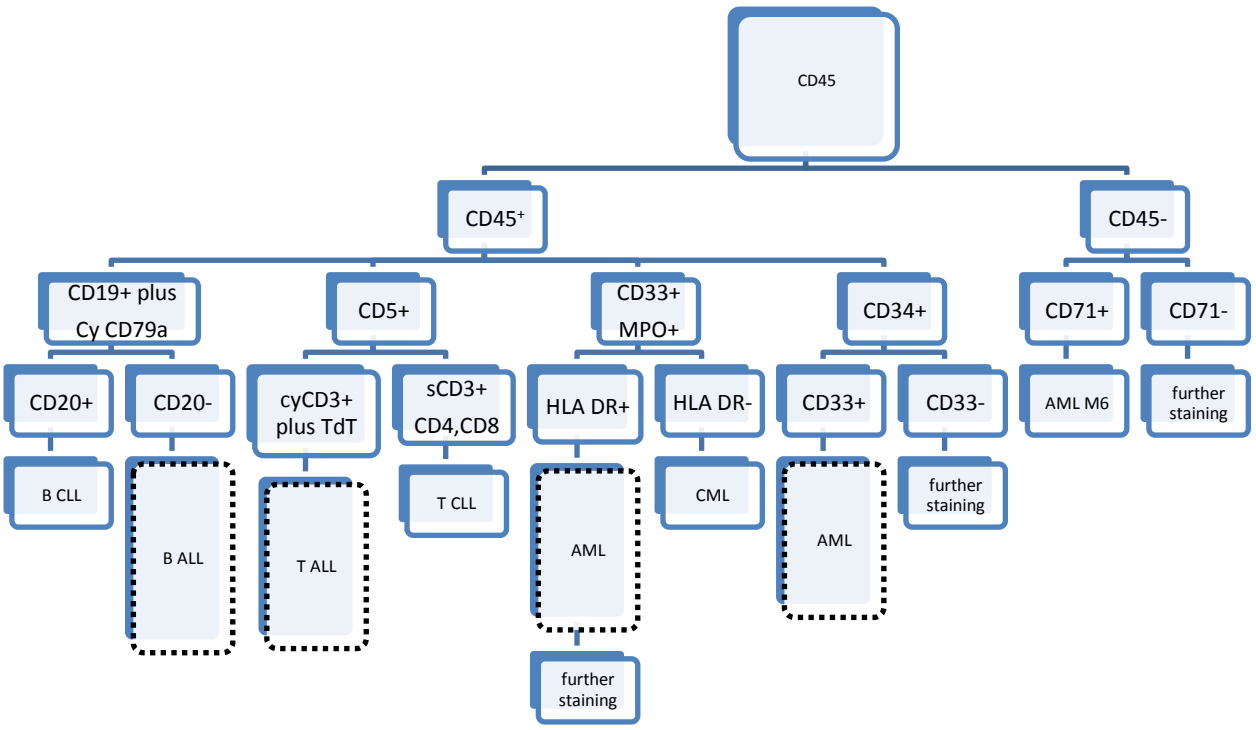
Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

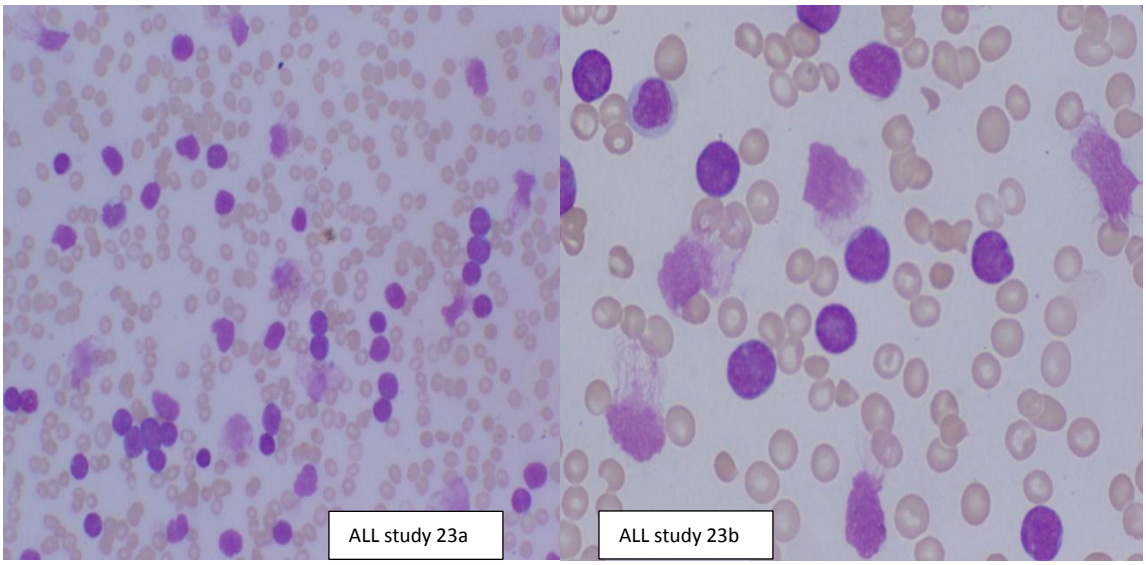
cc:	Director	-	MTRH	Dean	-	SOD
	Principal	-	CHS	Dean	-	SPH
	Dean	-	SOM	Dean	-	SON

Appendix IX: Monoclonal Antibody Panel.

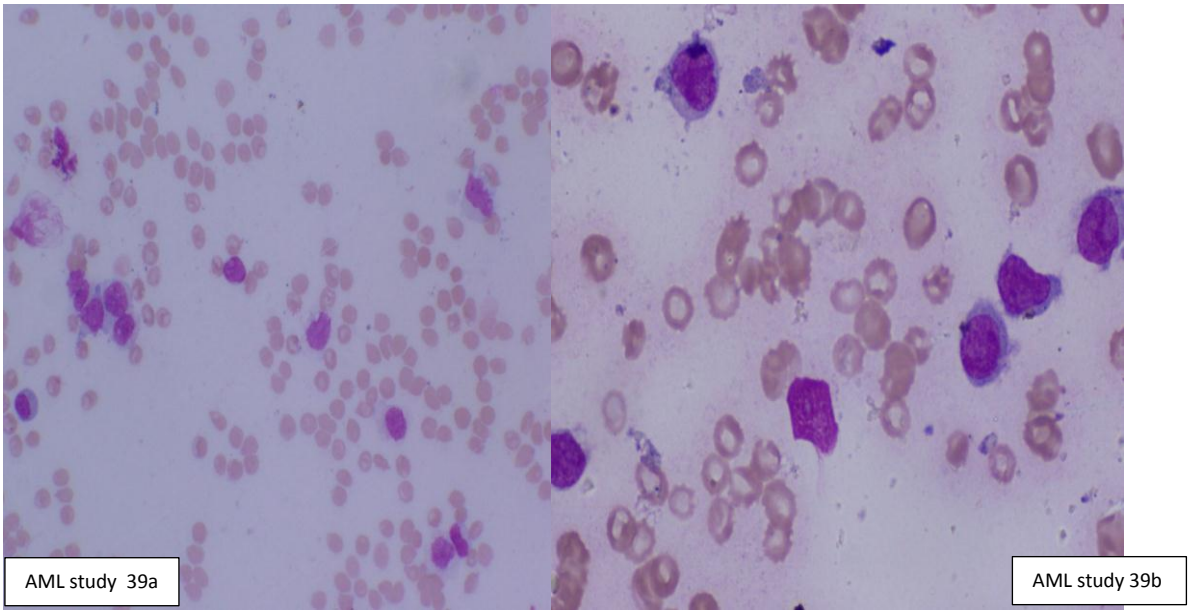
Algorithm chart proposed for immunophenotyping using minimum monoclonal antibody panel at MTRH.



Appendix X. Photomicrographs showing BMA morphology in selected participants with acute leukemia.

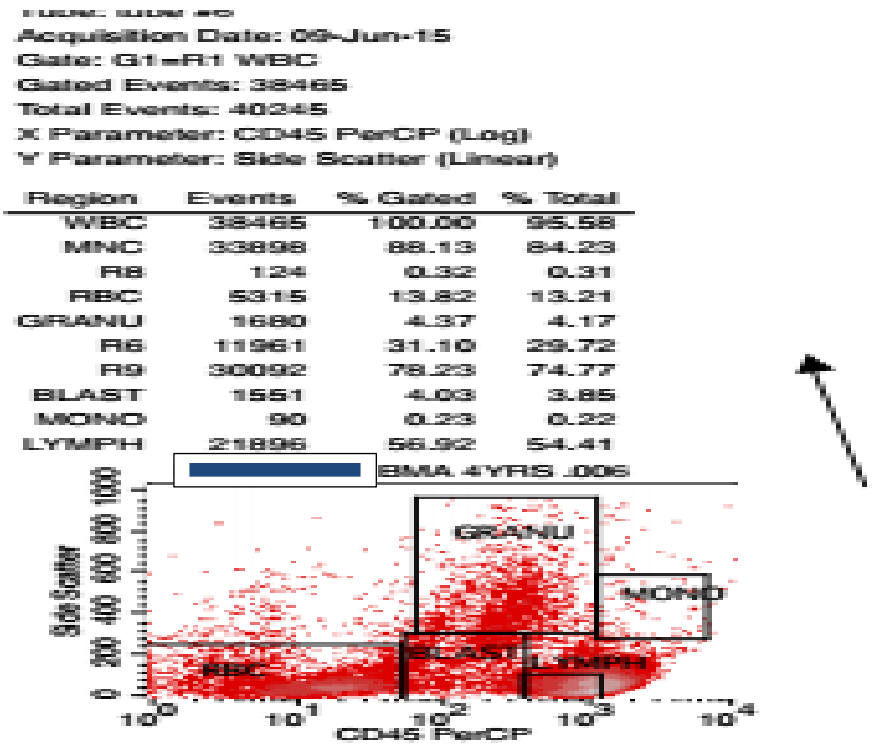


Photomicrograph of the BMA of study participant ALL 23a (low power) and 23b (high power) showing lymphoblasts morphology.



Photomicrograph of the BMA of study participant 39 a(low power) and 39b (high power) showing myeloblast(AML) morphology.

APPENDIX XI. Photomicrographs showing side scatter plots of a participant with blast cells on the BMA when flow cytometry immunophenotyping was done.



Photomicrograph showing side scatter analysis when gating using CD 45 for a 4 year old participant with blast of the bone marrow aspirate. The patient had ALL on both morphology and flow cytometry immunophenotyping.

Appendix XII: Human Resource and Contribution

Staff	Role	Station
Investigator/ Researcher	Wrote the proposal, collected data, analyzed and wrote the study the report.	Moi University, School of Medicine, Department of Child Health and Paediatrics.
Supervisors	Guided in proposal writing, supervised data collection, analysis and report writing. Edited the final manuscript.	Moi University, School of Medicine; [1] Department of Child Health and Paediatrics. [2] Department of Immunology.
Research Assistant	Assisted in data collection	Moi teaching and Referral Hospital, department of pediatric oncology.
Biostastician	Assisted in sample size calculation and data cleaning and analysis.	Moi University, School of Public Health, Department of Epidemiology and preventive health.

Appendix XIII: Time Line

Activity	**Time**	**Duration**
Concept development	November2013 - February	4 months
Proposal writing	March 2014 – June 2014	4 months
IREC Review and Approval	July 2014 – September 2014	3 months
Data collection	December 2014 – Jan 2016	14 months
Data analysis	Jan 2016 – March 2016	2 months
Thesis writing	March 2016 – June 2016	4 months

Appendix XIV: Study Budget

Item	Cost Ksh
Complete blood count @ Ksh 700	35,000
PBF	25000
BMA @ 1000	50,000
Flow Analysis@ 3000	150,000
Laptop	50,000
Printer	14,000
Printing paper 12 reams	5,000
Flask disk 1	2,000
Stationery	15,000
Research assistant	12,000
Biostastician	20,000
Contingency 10%	18,400
Total	396,400

*Cost for 50 tests