Frequency of Leukemia types and Flowcytometric Immunophenotypic characterization of Acute Lymphoblastic Leukaemia presenting at the University Teaching Hospital, Lusaka

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A Dissertation Submitted to the University of Zambia, in partial fulfillment of the requirements for the award of the degree of the Master of Science Degree in Pathology (Haematology)

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DECLARATION

This work/dissertation in its present form has not been submitted or accepted previously for the award of a degree or diploma in this or any tertiary institution, and is not being submitted for a degree or diploma in any tertiary institution or for another degree or diploma at this institution. I declare that this Dissertation contains my own work except where specifically acknowledged. I further declare that I followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Master of Science in Pathology (Haematology), University of Zambia guidelines.

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CERTIFICATE OF APPROVAL

The dissertation of **Jonas Zimba** is approved as fulfilling part of the requirement for the award of the degree of the Masters of Science Degree in Pathology (Haematology) conferred by the **University of Zambia**

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ABSTRACT

The Frequency of Leukaemia types vary in different geographical locations and between age groups and gender. The diagnosis of Leukaemia at UTH is done by Morphological examination of blood or bone marrow smears only. Immunophenotyping is a further diagnostic tool that can be performed to diagnose Leukemias and in this study it was used to diagnose and sub type Acute Lymphoblastic Leukaemia (ALL).The aim of the study was to determine the frequency of the Leukaemia types and to characterize ALL at UTH by immunophenotypes and compare these patterns to those occurring in other parts of the world.A cross sectional descriptive study in which **72** consecutive cases of different types of leukemias occurring between June 2014 and June 2015 were analyzed from records and Laboratory results for frequency and epidemiology of Leukaemias Also 14 consecutive cases of ALL occurring between June 2015 and October 2015 were analyzed and characterized by flow cytometry of blood or bone marrow samples at UTH in Lusaka, Zambia.

The most frequent Leukaemia was chronic myeloid Leukaemia (CML) at 55.5%, followed by Acute lymphoblastic Leukaemia (ALL) at 15.3%, then 13.9 % for each, Acute Myeloid Leukaemia (AML) and chronic lymphocytic Leukaemia (CLL), and lastly 1.4% for other Leukaemias. The median age of all Leukaemia types was 36 and age range was from 1 to 82 years. Of the total 72, 12(16.7%) were children ($\leq$ 15 years) and 60 (83.3%) were adults (> 15 years). Of the 72 cases 32 (44.4%) were females and 40 (55.6%) were males (M: F ratio 1.3: 1). The 14 ALL had expressed CD markers and were immunophenotyped as follows; 6 (42.9%) T cell lineage ALL, 6 (42.9%) B cell lineage ALL and 2 (14.2%) were Mixed lineage ALL. B cell lineage was further sub classified as follows; 4 (66.7%) were Common ALL(C ALL) while 2 (33.3%) were Mature B cell ALL. The T cell lineage was also further sub typed as follows 1(16.7%) Precursor T ALL while the Mature T Cell ALL was 5(83.3). Of the 14 ALL cases 8 (57.1%) were children ($\leq$ 15 years) and 6 (42.9%) were adults (>15 years). Of the 14 ALL cases 3 (21.4%) were females and 11 (78.6%) were males with an overall M: F ratio of 2.5:1. The study showed that Leukaemias were more common in males than in females and occurred more between 20 to 44 years of age with a peak of 30 to 39 years. ALL was more commonly observed in children whereas both CML and CLL were mostly observed in adults. Further CML was the most common Leukaemia type at UTH. C ALL was the most common B cell lineage ALL while Mature T cell ALL was the most common T cell lineage ALL Flow Cytometric immunophenotyping is an added sensitive diagnostic tool to morphological diagnosis of Acute Lymphoblastic Leukaemia as it not only confirms but also subtypes the ALL.

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Key words: Leukaemia, Acute Lymphoblastic Leukaemia, Chronic Myeloid Leukaemia, B cell and T cell

DEDICATION

This thesis is dedicated to my late wife, **Mary Luhanga Zimba**, who before her death used to encourage me to pursue further education and be the best example to the Children in terms of determination to study hard.

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LIST OF ABREVIATIONS

ABL	Homolog of the Abelson gene
AL	Acute Leukaemia
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
B- ALL	B Lymphoid cell Acute Lymphoblastic Leukaemia
BCR	Breakpoint cluster region gene
BD	Becton Dickinson
CD	Cluster of Differentiation
Cig	Cytoplasmic immunoglobulin
C ALL	Common Acute Lymphoblastic Leukaemia
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
FAB	French American – British working group classification
FBC	Full Blood l count
FSC	Forward scatter
FITC	Fluorescein Isothiocyanate
HLA- DR	Human Leukocyte Antigen – DR
ID	Identity number
M/F Ratio	Male /Female Ratio
MPO	Cytoplasmic myeloperoxidase
MoAbs	Monoclonal Antibodies
NCCN	National Comprehensive Cancer Network
PBS	Phosphate buffer saline
PE	Phycoerythrin
Ph	Philadelphia Chromosomes
SSC	Side scatters
T- ALL	T Lymphoid cell Acute Lymphoblastic Leukaemia
TdT	Terminal deoxynucleotidyl Transferase
Thy- ALL	Thymic Acute Lymphoblastic Leukaemia
UNZA-BREC	University of Zambia Biomedical Research Ethics Committee

WHO	World Health Organization
UTH	University Teaching Hospital

CHAPTER 1: INTRODUCTION

1.1 Background

The Leukaemias are a group of disorders characterized by the accumulation of malignant white cells in the bone marrow and blood. There are four main types of Leukaemias, namely: Acute Lymphoblastic Leukaemia (ALL), Chronic Lymphocytic Leukaemia (CLL), Acute Myeloid Leukaemia (AML), and Chronic Myeloid Leukaemia (CML) (CancerResearchUK, 2011).

The acute Leukaemia (ALL and AML) are aggressive malignant disorders in which haemopoietic stem cell or a lymphoid or myeloid marrow precursor cell accumulate in the bone marrow and blood (Baruchel, 2012, Hoffbrand et al., 2006). Chronic lymphocytic Leukaemia is characterized by accumulation in the blood of mature lymphocytes of either B- or T-cell type. CML is characterized by an increase in neutrophils and their precursors in the peripheral blood (Hoffbrand et al., 2006).

The global incidence of Leukaemia is approximately 0.01%. The annual death rate is 8 per 100,000 persons (Qadir et al., 2006). Most cases of Leukaemia occur in adults in whom the common types are AML and CLL. The most common type of Leukaemia in children and adolescents younger than 20 years is ALL (Facts-spring, 2014). CML occurs at all ages but with peak age of between 25–45 years (Hoffbrand et al., 2006). In children Leukaemia is the most common malignancy accounting for 30% of all cancers diagnosed in children less than 15 years of age in developed countries (Qadir et al., 2006). The incidence rates of Leukaemia are high in developed nations of the Americas, Western Europe, Australia and Canada. Less developed nations of Africa and Asia have low incidence rates. International variations reflect differences in exposure to risk factors. In sub-Saharan Africa, recorded incidence rates of Leukaemia are considerably lower as a result of under diagnosis and under reporting (Parkin et al., 2003). This is because in most under developed nations including Zambia diagnosis of Leukaemia is made by morphological examination of blood and bone marrow smears. However this method maybe a source of misdiagnosis and misclassification between Leukaemia subtypes. In general most studies have shown that Leukaemia affects more men than women worldwide, and whites are more prone to Leukaemia than are blacks (Iqbal, 2012). In the USA approximately 33 % more males

are living with Leukaemia than females (Facts-spring, 2014). In South –South Nigeria Leukaemia was more common in males (52.1%) than in females (47.9%) (Nwannadi et al., 2009).

Acute lymphoblastic leukemia (ALL) presumably arises from malignant transformation of B- or T-cell progenitor cells. B-cell Leukaemia occurs more frequently than T-cell Leukaemia. The ALL arise from B-cell in 85% patients and from T-cell in 15% cases (Rathee. and Neelkamal., 2013). The two lineages can further be differentiated into subtypes according to their stage of maturation and differentiation (Baruchel, 2012)**.** ALL is the most common type of cancer in children and accounts for 80% of leukemia in children aged 0 – 14 years with an incidence of 40 cases per million and a peak of 2 to 5 years in developed Western Europe (Stiller, 2009). In adults, this disease is less common than AML, accounting for about 20% of all types of acute leukemia's (Krawczyk-Kuliś, 2012). In sub –Saharan Africa the annual incidence of ALL is fewer than 20 per million children (Stiller, 2009).Lower age-adjusted incidence rates of ALL have been reported in Africa, Asia, and Vietnam. High rates found in Hong Kong, United Kingdom (UK), United States of America (USA), and Japan. In the USA between 2003 and 2007 the incidence of ALL among the age group 0 -19 years was 46.0 per one million children (Mejía-Aranguré et al., 2011).Generally, ALL is more common in males than females in all study populations (Patil Okaly et al., 2012). ALL is diagnosed with a medical history, physical examination , Full blood count (FBC), blood and bone marrow smears (Rathee. and Neelkamal., 2013). A blood or bone marrow smears examination showing blast cell count of at least 20%. The World Health Organization (WHO) Classification system of 2008 incorporate flow cytometry-based immunophenotyping, cytogenetic, and molecular approaches in conjunction with morphology and cytochemistry for the diagnosis, prognosis, and lineage assignment of Acute Leukaemia (AL) cases (Patil Okaly et al., 2012)**.**

Flow cytometry Immunophenotyping is a standard diagnostic procedure based on the principle of cell differentiation antigen expression (Cell Markers) determined by a panel of monoclonal antibodies (MoAb) in the maturation process of T and B lymphocytes where these surface and cytoplasm antigens are acquired and lost (Alves et al., 2012, Saxena and Anand, 2008).

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1.2 Statement of the problem

At University Teaching Hospital (UTH), the diagnosis of Leukaemia is made by morphological examination of blood and bone marrow. No further characterization of the Leukaemia is made and thus the Leukaemia cannot be classified according to the WHO classification system of 2008 as the broad classification of Leukaemias is based on cell phenotype which together with Genetic and Cytogenic studies are not dome at UTH. Therefore, different leukaemia types and subtypes with different clinical and prognostic characteristics cannot be classified for the benefit of leukaemia patients such as preferred treatment protocol, genotype – specific treatment approaches and targeted therapies. Also the patterns of Leukaemia types in Zambia cannot be compared to other geographical areas.

In this study we employed morphology and flow cytometry to diagnose and classify acute lymphoblastic Leukaemia and thus provide a benefit to the patients and have a basis on which the patterns of acute Leukaemia in Zambia can be compared with other patterns from around the world.

1.3 Study Justification

In Zambia, flow cytometry has been in place for over a decade now with many public and private health facilities owning a flow cytometer. In UTH alone, there are 6 flow cytometers (3 FACS caliburs and 3 FACS counts) supported by the Ministry of Health. However, these instruments are only used for CD4 enumeration. There has been no capacity to use them as an investigative tool in the diagnosis of Leukaemia. Flow Cytometric Immunophenotyping is the WHO recommended diagnostic and lineage assignment tool for Leukaemia. Correct diagnosis and Lineage assignment of ALL is helpful as different lineages may have different clinical features and prognosis. Therefore lineages which are aggressive clinically can effectively be managed. Such descriptive studies can help to provide a foundation in elucidating the etiology of ALL by other further investigative strategies. Questions such as why a particular region has more of a particular type of leukaemia than other regions may be asked and followed up.

1.4 Research Question

What are the frequencies of different types of leukemia's and what are the immunological Characteristic of Acute Lymphoblastic Leukaemia presenting at the University Teaching Hospital (UTH).

1.5 Objectives

General

To determine the frequencies of Leukaemias and to characterize Acute Lymphoblastic Leukaemia presenting at UTH, Lusaka, Zambia by immunophenotyping

Specific

1. To determine the frequency of diagnosis and epidemiological features of Leukemia types presenting at the University Teaching Hospital.
2. To classify ALL subtypes by immunophenotyping using flow cytometry at the University Teaching Hospital.
3. To determine the epidemiological features of ALL subtypes presenting at the University Teaching Hospital.

CHAPTER 2: LITERATURE REVIEW

2.1 Biology of Acute Lymphoblastic Leukaemia

ALL is a biologically heterogeneous disease represented by distinct clinical and biological subtypes. Developments in analyzing chromosome structure such as karyotype, and polymerase chain reaction methodologies together with gene expression profiling, genome –wide analyses and the development of monoclonal antibodies allows the biological characterization of ALL (Carroll et al., 2011, Lo Nigro, 2013).Recurrent chromosomal and molecular abnormalities characterize ALL subtypes in both adults and children though frequencies of certain subtypes differ in these two groups. Many reports indicate that the most common chromosomal abnormality in children with B cell ALL is hyperdiploidy (> 50 Chromosomes) accounting for 25% of childhood ALL and only 7 % in adults (NCCN, 2012). The chromosomal translocation t(12, 21), which results in TEL-AML1 subtype is also common in children accounting for 22% of cases but only account for 2% in adults (NCCN, 2012). Both the TEL and the AML1 genes are important regulators of haemopoietic-cell development, essential for definitive haemopoiesis. The TEL/AML1 fusion probably inhibits the transcription activity of the normal AML1 gene involved in proliferation and differentiation of hematopoietic cells (Pieters and Carroll, 2008) .TheTEL-AML1 fusion gene serves as a first-hit mutation by endowing the preleukaemic cell with altered self-renewal and survival properties (Pui et al., 2008).The Philadelphia Chromosome (Ph) is more common in adults accounting for 25% of cases while in children it accounts for 3% of ALL (NCCN, 2012). Chromosomal translocation t (9; 22) results in a Breakpoint Cluster Region – Abelson Tyrosine kinase fusion gene (BCR-ABL fusion gene). The fusion of the BCR signaling protein to the ABL non-receptor tyrosine kinase, results in constitutive tyrosine kinase activity associated with increased proliferation and decreased apoptosis (Pieters and Carroll, 2008). Other chromosomal and molecular abnormalities in the B cell ALL include hypodiploidy (< 46 chromosomes) which is only observed in 2% of adults and 1% in children; translocations in the Mixed lineage Leukaemia (MLL) gene, for example the t (4, 11) account for 10% in adults and 8% in Children; translocation t (1, 19) which result in E2A – PBX1, account for 3% in adults and 5% in children (NCCN, 2012)

More than 50% of cases of T-cell acute lymphoblastic Leukaemia have activating mutations that involve NOTCH1, a gene encoding a transmembrane receptor that regulates normal T-cell development (Pui et al., 2008).The chromosomal abnormalities in the T cell ALL include the

translocation t (1, 14), the T cell acute Leukaemia 1;(TAL- 1), which account for 12% in adults and 7% in children; translocation t (10, 14), the HOX11, account for 8% adults and 1% children and the translocation t (5, 14), the HOX11L2, account for 1% in adults and 3% in children (NCCN, 2012).Several of these recurrent, nonrandom chromosomal abnormalities are associated strongly with prognosis (Reddy and Perkins, 2004)**.** The hyperdiploidy and TEL – AML which are common in children are associated with favourable outcome, while the Ph – positive ALL mostly found in adults is associated with poor prognosis (Pieters and Carroll, 2008). The MLL gene, hypodiploidy and chromosomal abnormalities found in T cell ALL are also associated with poor prognosis (NCCN, 2012).

2.2 Immunophenotypic features of ALL

Currently in most of the developing countries, characterization and classification of leukemia's is made by morphological and cytochemical analysis of cells in peripheral blood and bone marrow (Pamnani, 2009).Even with experience, morphologic examination can separate only about 70% to 80% of acute leukemia's as ALL or AML, (Reddy and Perkins, 2004). Immunophenotyping of leukaemic lymphoblast's by flow Cytometry is essential to establish the correct diagnosis and define cell lineage (Pui et al., 2008).Furthermore certain immunophenotypes show close association with overt or cryptic cytogenetic abnormalities and thus constitute stronger predictors of prognosis and drug resistance. For instance, t (1; 19) carries bad prognosis in pre- B – All, but not in early pre B ALL (Pamnani, 2009).Precursor B-cell ALL typically expresses Human leukocyte antigen (HLA-DR+) , Terminal deoxynucleotidyl Transferase (TdT+) , Cluster of Differentiation 19 (CD19+) , and/or CD79a+, and/or CD22+, and/or CD34+ (Abdulsalam, 2011, Rathee. and Neelkamal., 2013). This type of ALL is subdivided into 4 groups: (a) Early Pre – B ALL based on CD34, HLA-DR and CD19 positivity and CD10 negativity, account for 9 – 11% in adults and 60% in children , (b) Common – ALL based on CD10 positivity in addition to the presence of other markers CD19, HLA-DR and varying percentage of CD34 , account for more than 50% in adults and 10% in children , (c) Pre – B ALL, if cytoplasmic immunoglobulin (cytIg) is demonstrated, Accounting for 12% in adults and 20- 25 % in children , and (d) B – ALL , if surface immunoglobulin (sIg) is detectable , CD34 is negative, account for 5% in adults and 2% in children (Abdulsalam, 2011, Hoffbrand et al., 2006, Matutes, 1995, Orfao et al., 2004). Precursor T-cell ALL Cells are TdT+ in addition to cyt CD3+ and CD34+ {Abdul-Hamid,

2013).This type of ALL is also subdivided into 4 groups. (a) Pro T-ALL based on CD7 positivity and CD2, CD4 and CD8 negativity, account for 7% in adults (b) Pre T-ALL based on CD2 and CD7 positivity ,and CD4 and CD8 negativity , (c) Cortical T-ALL or Thymic ALL (Thy ALL) is CD1a , CD7, CD2, CD5, CD4, CD8 Positive , most common T ALL accounting for 17% in adults and , (d) Mature T-ALL are surface CD3, CD2, CD7, CD4 or CD8 positive and negative for TdT, CD34,CD1a (Abdulsalam, 2011, Hoffbrand et al., 2006, Reddy and Perkins, 2004).

2.3 Immunophenotypic studies

A number of studies have been carried out to determine the outcome of various combinations of panels of monoclonal antibodies on ALL In Brazil, Natal, Rio Grande do Norte, a study on immunophenotyping in patients with ALL concluded that immunophenotyping is an important method in the diagnosis, monitoring and prognostic assessment in determining the pathological mechanisms of evolution of ALL. A panel of monoclonal antibodies against CD1a, CD2, CD3, CD4, CD7, CD8, CD10, CD13, CD33, CD14, CD19, CD22, CD79a, CD117, CD34, IgM, TdT, HLADr, and human kappa and lambda Light chains were used. The expression of the markers was similar to what has been documented in literature (Alves et al., 2012). A similar study by Salem et al, in Egypt to evaluate the diagnostic usefulness of commonly used immune-markers for immunophenotyping of AL and to define the best immune-markers to be used for proper diagnosis and classification of AL and to recognize the frequency of different AL subtypes and the antigen expression profile in Egyptian patients found that cytoplasmic CD79a (cytCD79a) and CD19 were the most sensitive markers for B-ALL while cytCD3, CD7 and CD5 were the most sensitive antigens for T-ALL. Their analysis of AL phenotypes proved that employed antibody panels were adequate for proper diagnosis and classification of AL. Their Immunophenotyping results of classification of ALL patients were comparable to internationally published studies (Salem and Abd El-Aziz, 2012). In the study by Alves et al study the Immunophenotyping results of classification of ALL were also comparable to internationally published studies. All B-lineage ALL were diagnosed when leukaemic cells were expressing pan-B cells markers CD19 and cytCD79a. Further sub classification of B cell ALL showed that (a) early pre-B ALL expressed only CD19, HLA-Dr, CD34, and TdT (b) Common ALL expressed CD10, as well as expression of CD19, cytCD22, and HLA-Dr in most cases. (c) Pre-B expressed cytoplasmic immunoglobulin and (d) mature B cell expressed surface Immunoglobulin. T-cell ALL diagnosis was based on

expression of surface CD7 and cytCD3 antigens (Alves et al., 2012).The correlation between sex and immunological subtypes in all study populations shows that males are more affected by the disease, regardless of immunological classification (Alves et al., 2012). However a study by Bachir et al in Morocco found that though the frequency of T- ALL is significantly higher in males (M/F ratio: 2.93: 1) the frequency of T-ALL CD10+ subset is more in females when compared with the T-ALL CD10– subset (Bachir et al., 2009).The correlation between age and immunological subtype's shows that the B-lineage ALL occurs more frequently in patients aged 15 and below while the T-cell ALL subtype is frequent in adults (Alves et al, 2012). A study by Bachir in Morocco reported a peak in incidence between 3 and 5 years among the B-cell ALLs subtype in children (Bachir et al., 2009). In a study in America on relationship to morphologic and molecular classification and proposal for a minimal screening program highly predictive for lineage discrimination in 62 adult patients with ALL showed, 48 (77%) were B cell ALL and 14 (23%) were-T cell ALL . Further immunologic sub classification of B cell–lineage ALLs showed; 6 (12.7%) Early-pre-B-cell All, 23(48.9%) common ALLs, 12 (25.5%) pre-B-cell ALLs and 6 (12.7%) mature B-cell ALLs. The T cell ALLs were further classified as 2(14.3%) pro-T-cell ALL, 7 (50%) pre-T-cell ALL, 2 (14.3%) cortical T-cell ALL, 2 (14.3%) mature T-cell ALL. (Thalhammer-Scherrer et al., 2002). In the above study B cell and T cell ALL prevalence were as reported in international publications, however a study in Saudi Arabia, Bahrain, to determine the Immunophenotypic pattern of ALL using a panel of monoclonal antibodies found that of the 32 analyzed cases, 30 cases (93.8 %) were B cell ALL and 2 (6.2 % were T cell ALL suggesting a lower prevalence of T cell ALL in Arabs compared to reported frequency of 14% and 24% respectively in European and American studies (Al-SheikhIman, 1999). In another study in Brazil on immunophenotyping in patients with ALL using a panel of monoclonal antibodies found that 71 (56.4%) of the 126 cases were B-lineage ALL and 55 (43.6%) were T-cell suggesting a high prevalence of T –cell in Brazil (Alves et al., 2012). In the American study above the common ALL subtype was the most prevalent (at 48.9% of B cell ALL) conforming to international reports (Thalhammer-Scherrer et al., 2002). This was also the case with the Saudi Arabia study in which the prevalence was at 68.7%. However in the Saudi Arabian study the 6.2% reported for early pre B was much lower than the reported frequency of 12.7% in the American study, but the common ALL phenotype was as prevalent in Arabs as it is in the rest of the world (Al-SheikhIman, 1999).

A study done in India to collect, analyze, and correlate morphologic, cytochemical, Immunophenotypic, and cytogenetic data from patients diagnosed with ALL at an Indian specialty cancer Centre found that cell markers CD34 and HLADR were not only expressed in the B-ALL (58% and 93% of the patients, respectively, but also in the T-ALL (40% and 30% of the patients, respectively) (Patil Okaly et al., 2012). However another similar study in Egypt found that of the 13 (25.5%) T- ALL none of T-ALL cases were positive for HLADR or CD34 (Salem and Abd El-Aziz, 2012).Associations have been established between certain immunophenotypes and certain recurrent chromosomal abnormalities. Marchesi et al in a review article on pathogenetic, clinical, and prognostic features of adult t (4; 11)(q21;q23) (which results in a fusion gene MLL-AF4)reported that this chromosomal abnormality is associated with the multipotent or very early CD10 negative B-progenitor cells with a frequent co expression of CD15 and CD65 myeloid antigens. CD19, CD22, cyCD79a, HLA-DR, TdT, and CD34 are frequently and strongly expressed (Marchesi et al., 2011). Thalhammer – Scherrer et al had earlier reported an association between Early-pre-B cell–specific phenotypes and co expression of myeloid antigens, particularly CD15/CD65s. The common ALL phenotype was associated with BCR-ABL translocation (Thalhammer-Scherrer et al., 2002).

CHAPTER 3: METHODOLOGY

3.1 Study Design

The study was a cross sectional study of all age group patients who have been attended to or where being attended to at the haemato-oncology and Pediatric –Oncology clinics and wards at UTH, Lusaka Zambia. The target Populations were; Leukaemia Patients of all age groups who were attended to at UTH in the study for frequency of leukaemia and. Suspected and smear microscopy diagnosed ALL patients being attended to at UTH for Immunophenotypic characterization of ALL. The Study Population were persons diagnosed by blood or bone marrow smear morphology as leukaemia patients at the haemato-oncology and Pediatric –Oncology clinics at UTH from June 2014 to October 2015 for study on frequency of different types of Leukaemia and all patients diagnosed by blood or bone marrow immuno flow cytometry as ALL patients from the haemato-oncology and Pediatric –Oncology clinics at UTH from July 2015 to October 2015 for study on Acute lymphoblastic Leukaemia Immunophenotypic characterization. The study was conducted at the UTH virology and Haematology Laboratories in the Department of Pathology and Microbiology.

3.2 Sample Size calculation and sampling method

Sample size for Leukaemia Frequencies

Based on the 2008 Global Prevalence of all Leukemia's (1991 – 1995) at 2.5 (Bray et al, 2013).

We needed to enrol 38 or more participants in order to identify the true prevalence with precision of +/-5% and 95% confidence interval.

$$N = \frac{Z^2 \times P(1-P)}{(d)^2} = 1.962 \frac{\times 0.025(1 - 0.025)}{(0.05)^2} = 38$$

N=sample size, z=statistic for 95% = 1.96, p=expected prevalence = 2.5% and d=0.05

Sample size for acute lymphoblastic Leukaemia immunophenotyping

Based on the age-standardized (world) incidence per million

Acute lymphoid Leukaemia (1991 – 2001) Malawi, at 0.5% (Parkin et al., 2003).

We needed to enrol 8 or more participants in order to identify the true prevalence with precision of +/-5% and 95% confidence interval.

$$N = \frac{Z^2 \times P(1-P)}{(D)^2} = \frac{1.962 \times 0.005(1 - 0.005)}{(0.05)^2} = 8$$

N=sample size, z=statistic for 95% =1.96, p=expected prevalence= 0.05% d=0.05

Sampling Method

Convenience sampling in which consecutive individuals presenting themselves to UTH haemato-oncology or pediatric oncology clinics and found to meet the inclusion criteria were included in the study.

Inclusion Criteria and Exclusion Criteria

Documented Individuals who were diagnosed with Leukaemia by blood or bone marrow smear morphological examination from June 2014 to June 2015 for study on frequency of different types of Leukaemia and Individuals diagnosed with Acute lymphoblastic Leukaemia by blood or bone marrow immuno flow cytometry from July 2015 to October 2015 and gave consent to participate in the study without being forced Were included in the study. Individuals who did not provide informed consent and were not immunophenotypically diagnosed as ALL were excluded to participate in study for immunophenotyping#

3.3 Data management

For Frequency of different Leukaemia type's demographic data of participants of all age groups was collected from laboratory results register and UTH statistics center after getting permission from the Head of Laboratory services. For immunophenotype characterization of ALL participants of all age groups were recruited from UTH haemato-oncology or paediatric oncology clinic and wards during routine clinicians examination of patients and during blood or bone marrow sample collection on all suspected acute leukaemia patients Thereafter information on the participant's demographic data was collected and compiled using a questionnaire. The demographic data included the participants' age and sex. . The patients' files were also reviewed for any further relevant data to the research and also to confirm the accuracy of information provided by the participant and recorded in the questionnaire.

Blood and/ or Bone marrow specimens were collected in EDTA by clinicians or nurses as routine for patient evaluation i.e. specimens already collected and brought to the laboratory for Full blood

count (FBC) and smear microscopy from patients already diagnosed or suspected to have acute lymphoblastic Leukaemia were used . The remainder specimens were used for Immunophenotyping by flow cytometry as a part of basic patient work up for definitive diagnosis and sub typing in addition to FBC and Smear microscopy performed as routine tests.

3.4. Specimen Preparation and Storage

Upon collection, specimen tubes were bar coded with laboratory serial numbers. Whole blood was used for FBC while smears were prepared from whole blood and bone marrow and was performed in Haematology laboratory. After these two tests have been performed in Haematology laboratory the specimens were then taken to Virology laboratory and given participants study identity numbers corresponding to the one on the questionnaire. This remainder specimen is the one that was used to perform immunophenotyping procedure within 24 hours from time of collection i.e. the specimens were stored for only 24 hours at 18 – 25 degree Celsius. In the Virology laboratory, each specimen study identity number was recorded on to a compilation summary sheet.

3.5 Quality Control of Equipment

To ensure reliable results, quality control was performed on all the analytical instruments and analyzers used for any purpose during specimen analysis according to the UTH quality control guidelines. Quality control included equipment calibrations and analytical control runs on every analyzer before each test analysis.

3.6 Specimen Analysis

Full blood count

The test was run as a routine procedure on samples from already diagnosed or suspected ALL cases for review or for diagnosis respectively on an automated Haematology analyzer called Sysmex X T 2000 machine. Whole blood was used within 24 hours from time of collection. Results were sent to the requesting clinician and a copy of the results were then assigned a study ID corresponding to the one on the questionnaire,

Peripheral blood smear

Well mixed whole blood from EDTA Vacutainer tubes collected from already diagnosed or suspected cases was used to make a thin smear as a routine procedure for review or diagnosis respectively. The basis of suspicion of a patient as having Leukaemia was clinical presentation and the smears were then stained with May – grunwald and Giemsa working solutions and examined by light microscope. Results were sent to the requesting clinician and a copy of the results were then assigned a study ID corresponding to the one on the questionnaire.

Bone marrow smear

Well mixed Bone marrow aspirate from EDTA Vacutainer tubes collected from suspected cases of Acute Leukaemia was used to make a thin smear as a routine procedure for diagnosis of Leukaemia. They were then being stained with May – Grunwald and Giemsa working solutions, kept in a clean rack and examined under a light microscope. Results were sent to the requesting clinician and a copy of the results were then assigned a study ID corresponding to the one on the questionnaire

Flow Cytometric immunophenotyping

The primary purpose of the peripheral blood or bone marrow smear morphological examination was to separate the acute leukemia into acute myelogenous leukemia (AML) and ALL. Also for the diagnosis of ALL, a blast percentage of at least 20% in bone marrow or peripheral blood is required. Immunophenotyping was used to distinguish definitely ALL from AML in cases were microscopy could not distinguish the two and classify ALL into B – or T – cell lineage. B- Cell ALL was further sub- classified. Immunophenotypic studies was performed on blood collected in an EDTA Vacutainer tube and /or on bone marrow aspirates collected in EDTA Vacutainer tube. Both these samples were originally used for the routine FBC enumeration and bone marrow or blood smear microscopy from previously diagnosed or suspected cases of ALL in order to have a definitive diagnosis and sub classify ALL. Appropriate monoclonal antibodies (MoAbs) labeled in appropriate fluorochrome were used to stain the leukaemic cells. The stained leukaemic cells were then run on a Fluorescence-Activated Cell Sorter (FACS) flow cytometry machine (BD Biosciences, Heidelberg, and German).Monoclonal antibodies required for immunophenotyping of ALL were acquired from Becton Dickinson (BD) Immuno- cytometry system (South Africa

office) (with varying batch numbers and expiry dates, CD3/MPO/CD79 had batch number 4302962 , expiry 29th February 2016; CD5 had 4322745 ,expiry 31st October 2016 ; CD117 had 4344915 , expiry 30th November 2016 ; CD7 had 4316845 , expiry 30th November 2015; Kappa/Lambda/CD19 had 5226172 , expiry 31st October 2016; CD5/CD10/CD19 had 5247896 , expiry 29th February 2017;CD34 had 432680, expiry 30th June 2016; CD20 had 4212770 , expiry 30th April 2016; Anti TdT had 5613550, expiry30th June 2016; CD2 had 5002999, expiry 30th November 2016 and CD33 had 4344875, expiry 31st June 2016). The Three colour cytometry analysis was carried out using Fluorescein Isothiocyanate (FITC) – labeled antibodies against CD3, CD5, CD2, and kappa light chain, CD34, CD33 and CD4. Phycoerythrin (PE) – labeled antibodies against CD10, Lambda heavy chain, CD7, MPO, CD117 and TdT and Peridinin Chlorophyll Protein (PerCP) – labeled antibodies against CD79a, CD45, CD19 and CD20. This panel of monoclonal antibodies distinguished Lineage –specific markers which are expressed in Haemopoietic progenitor cells – CD34, and TdT: Myeloid markers – CD33, CD117 and Cytoplasmic myeloperoxidase (MPO) : B – Lymphoid markers – CD19 , CD10 , CD20 , and CD79a : T- Lymphoid markers - CD2 , CD7 , CD3 **,** CD4 , and CD5

Reagent Preparation

All reagents, MoAbs, BD Intrasure – Fixing, Permealizing and Lysing reagent, Solution A and solution B, and BD Calibrite beads were brought to room temperature before use. The cytofix concentrate 10X was diluted 10 fold in distilled water. The lysing solution concentrate (Becton Dickinson's FACS Lysing Solution) was also diluted 1:10; v: v with distilled water.

Flow cytometry- Standardization

Flow cytometry was performed on a three- colour FACSCallibur flow cytometer (Becton Dickinson Immuno cytometer Systems) which was calibrated with a set of standardized beads (Becton Dickinson Calibrate i.e. unlabeled beads; and FITC, PE and PerCP antibody labeled Calibrite beads.

Flow cytometry -Manual gating

Manual gating was performed with IgG three colour conjugated fluorochromes to include more than 90% of blast cell or Population. This fraction (gate) was defined by analysis of forward and side scatter as shown in Figure 1.

Flow cytometry -*Compensation*

Compensation was done to adjust for spectral overlap which occur when samples are stained with two or more fluorochromes. This was done using the sample and the IgG three colour conjugated fluorochromes by adjusting the compensation network using the appropriate slider.

Flow cytometry - *Quality control*

For each test (surface and cytoplasmic analysis), an isotype-matched MoAb was used as negative Control.

Flow cytometry- Assay Procedure

Eight test tubes each to contain the monoclonal antibodies with their appropriate conjugated fluorochrome (FITC, PE and PerCP) and a patient Blood or Bone marrow sample were set up labeled as follows:

1. IgG
2. CD3/MPO/CD79a
3. Kappa/Labda/CD19
4. CD5/CD10/CD19
5. CD34/TdT
6. CD2/CD7/CD20
7. CD3/CD4/CD45
8. CD33/CD117

For surface antigens, (CD2,CD4,CD4,CD7,CD10,CD19,CD20,CD33,CD34,CD45 and CD117) 100 µL of whole blood and/or Bone Marrow cells previously well mixed were incubated with 10 µL of MoAb for 15 minutes at room temperature in the dark. Furthermore, 1ml of lysing solution was added to the cell suspension, and then incubated for 10 min at room temperature in the dark.

Then, the cell suspension was centrifuged for 5min at 1500 revolutions per minute (rpm), the supernatant fluid was discarded and the cell pellet was resuspended in 0.4ml of cold PBS (pH 7.2) and centrifuged again. The last step was repeated. Finally, the cell pellet was resuspended in 0.5 ml of cytofix, and cell suspension was kept in the dark at 4◦C until flow cytometry analysis.

In the intracytoplasmic cell markers staining assay (TdT, CD3, CD79a, and MPO), cells samples (100 microliter) were first incubated for 15 min at room temperature with 0.1ml of Becton Dickinson's (BD) solution A (Fixative).Then, cell suspensions were centrifuged for 5 min at 1500 rpm . The supernatant was discarded and the cell pellet was resuspended in cold PBS, and washed twice by centrifugation for 5 minutes at 1500 rpm .Then 100 microliters of BD solution B (lysing agent) and an appropriate amount of fluorochrome conjugate MoAb (10microlitre) was added and incubated in the dark at room temperature for 15 minutes. Thereafter the suspension was washed twice in PBS and then centrifuge for 5 minutes at 1500 rpm. The cell pellet was resuspended in 0.5 ml of cytofix solution and kept in the dark at 4◦C until flow cytometry analysis.

Leukaemic Samples were considered positive for a particular antigen if 20% or more of leukaemic cells reacted with a particular antibody as shown in Figure 1.

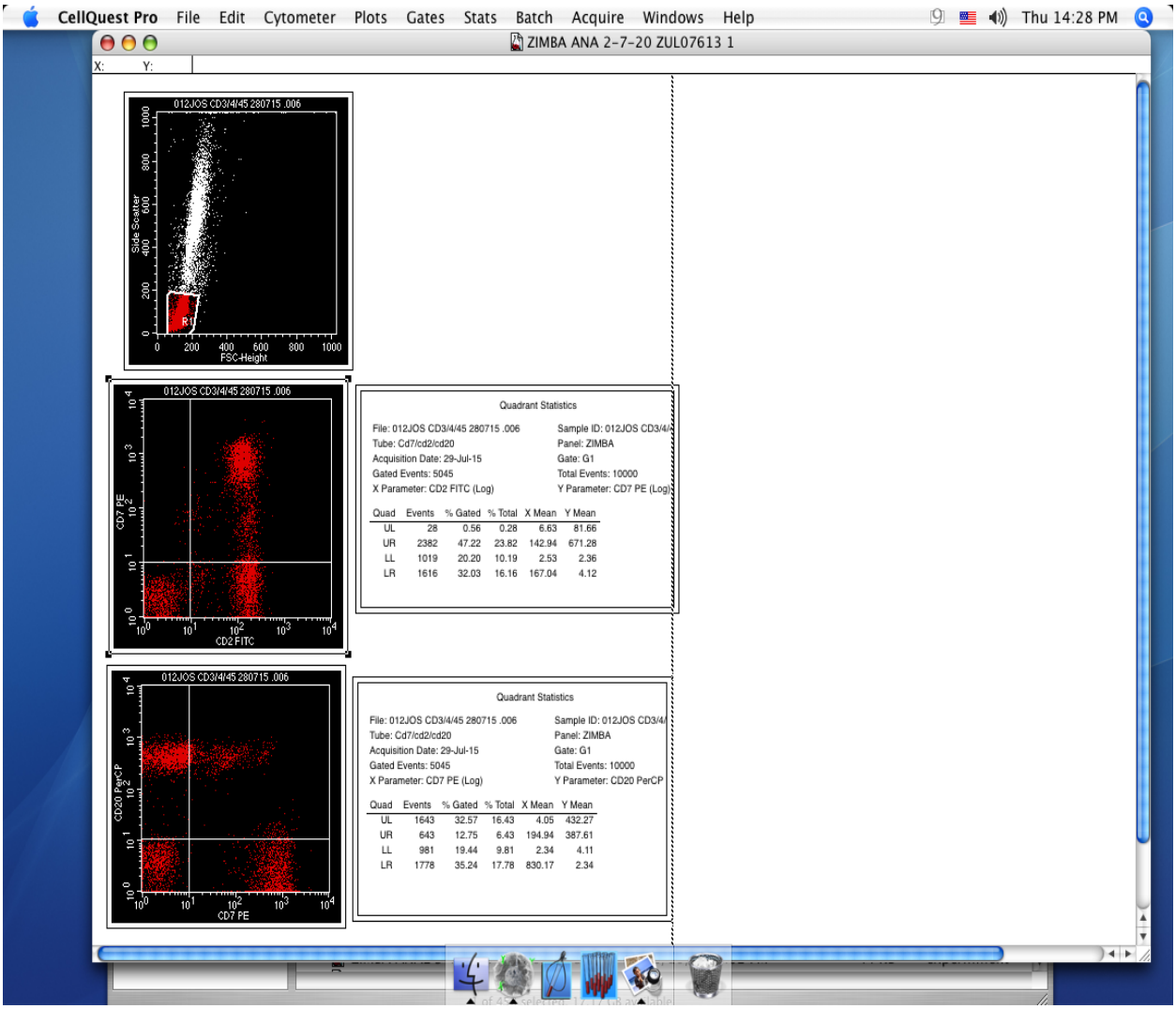

Figure 1: **T**hree- colour FACSCallibur flow cytometry dot plots with population of interest in top plot manual gated to includes ALL blast cell population defined by Forward and Side scatter; Middle plot showing FITC CD2 verses PE CD7, demonstrates positivity for both CD2 and CD7; Bottom plot showing PE CD7 verses PERCP CD20 , demonstrates positivity for both CD7 and CD20

3.7 **Determining Immunophenotypes of ALL**

All was divided into B-lineage and T – lineage leukemia's according to cell surface and cytoplasmic antigen expression. Assignment to a particular lineage or sub group was done when 2 or more markers were positive i.e. if only one marker was expressed by a blood or bone marrow

it was considered negative lineage Leukaemia was categorized as: (a) Early Pre- B ALL – based on CD34, TdT and CD19 positivity and CD10 negativity (b) Common – ALL based on CD10 positivity in addition to the presence of other markers e.g. CD19, CD20, CD79a and Varying percentages of CD34 and TdT, **(c)** Pre B ALL. based on CD10 +/- and heavy Mu (Cytoplasmic immunoglobulin (cμ) and surface immunoglobulin IgM (sIgM)) which were not investigated for (d) B Cell ALL – which expressed either the Kappa (κ) or Lambda (λ) immunoglobulin light chains in addition to the presence of other markers e.g. CD 19, CD20 , and CD79a but CD10 , CD34 and TdT negative.T-cell ALL diagnosis was based on T-cell reactivity to various MoAbs anti-T antigens: CD3, CD7, CD5, and CD2. Those with TdT + were classified as Pre cursor T **cell** and those with TdT – were classified as Mature T cell leukaemia. Acute myelogenous Leukaemia was diagnosed by presence of MPO, CD33 and CD117.

3.8 Data Processing and Statistical Analysis

Data were expressed as mean for normally distributed continuous variables or median (interquartile range) for non-normally distributed variables. Data were analyzed in IBM SPSS Statistics version 21 and Microsoft Excel 2010 for Windows. Results were summarized on to tables and graphs as given below. All statistical tests were considered significant if the p value was less than 0.05 or 95% confidence interval.

3.9 Ethical Considerations and Permissions

As researchers met the participants or participant's parents or their legal guardian they informed and explained to them about the study**.** The researchers also provided the participants with the study information sheet. If patients autonomously agreed to participate in the study, they were required to sign the consent form and if they are in the age group 7 - 17 years and understand what is happening they were signing an Assent form. All patients' records were protected and kept confidential. The questionnaire had captured the participants' file number, which was assigned a serial number hence specimen containers were identified by serial numbers. The file number was obtained for the purpose of retrieving the FBC and Smear microscopy results. The information obtained was used only for research purpose and access to this information was restricted to the principal investigator and the supervisors. However, any information pertinent to the patient's wellbeing was communicated to the attending medical team

Ethical approval was sought from the University of Zambia Biomedical Research Ethics Committee and granted on 20th March 2015. Ethical clearance number: Assurance No. FW A00000338. Permission to conduct the study at UTH was obtained from the: UTH medical superintendent and was granted on 28th January 2015. Permission to use equipment and facilities in the Department of Pathology and Microbiology in the UTH was obtained from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital.

3.10 Limitations

1. For immunophenotyping the study was limited to acute lymphoblastic Leukaemia patients who came in for diagnosis or follow up and consecutively conveniently sampled thus having a small sample size.
2. Some record files were not completely filed in.
3. The study did not provide any data pertaining to incidence and prevalence of Leukaemia in Zambia.
4. The study did not have a follow up programme to assess the prognostic significance of phenotypic markers of ALL subtypes. A follow up would have provided the response to treatment of the patients involved.
5. We also did not do molecular studies which go hand in hand with immunophenotyping to determine the prognosis of ALL subtypes

CHAPTER 4: RESULTS

A total of 72 Leukaemia patients' records files and laboratory results were analyzed for age and sex demographics, and frequency distribution of the different types of Leukaemia. The different types of leukemia analyzed were chronic myeloid Leukaemia (CML), acute lymphoblastic Leukaemia (ALL), acute myeloid Leukaemia (AML), chronic lymphocytic Leukaemia (CLL), and other types of Leukaemia.

While a total of 14 bone marrow and/or blood samples were analyzed by Flow Cytometric immunophenotyping and confirmed as ALL case. The different ALL subtypes were analyzed for age and sex demographics, and frequency distribution.

4.1 Frequency distribution of Acute and Chronic Leukaemia

Chronic leukemia's were the most common leukemia type observed in the study (69.4%), followed by Acute leukaemias (29.2%). The least was other leukaemias (1.4%) as shown in Table 1 below.

Table 1: Distribution of Acute and Chronic Leukaemias

SERIAL NO.	Leukaemia TYPE	NO.	PERCENT
1.	Chronic Leukaemia	50	69.4
2.	Acute Leukaemia	21	29.2
3.	Other Leukemia's	1	1.4

4.2 Frequency Distribution by Morphology of Leukaemia

Of the 72 Leukaemia cases, 50 (69.4%) were morphologically of myeloid form and 21 (29.2%) were lymphoid form Leukaemia

4.3 Age Distribution of Leukaemia

The predominant age group was between 20 to 49 years with a peak at 30 to 39 years old. The median age was 33 years as shown in Figure 2 below.

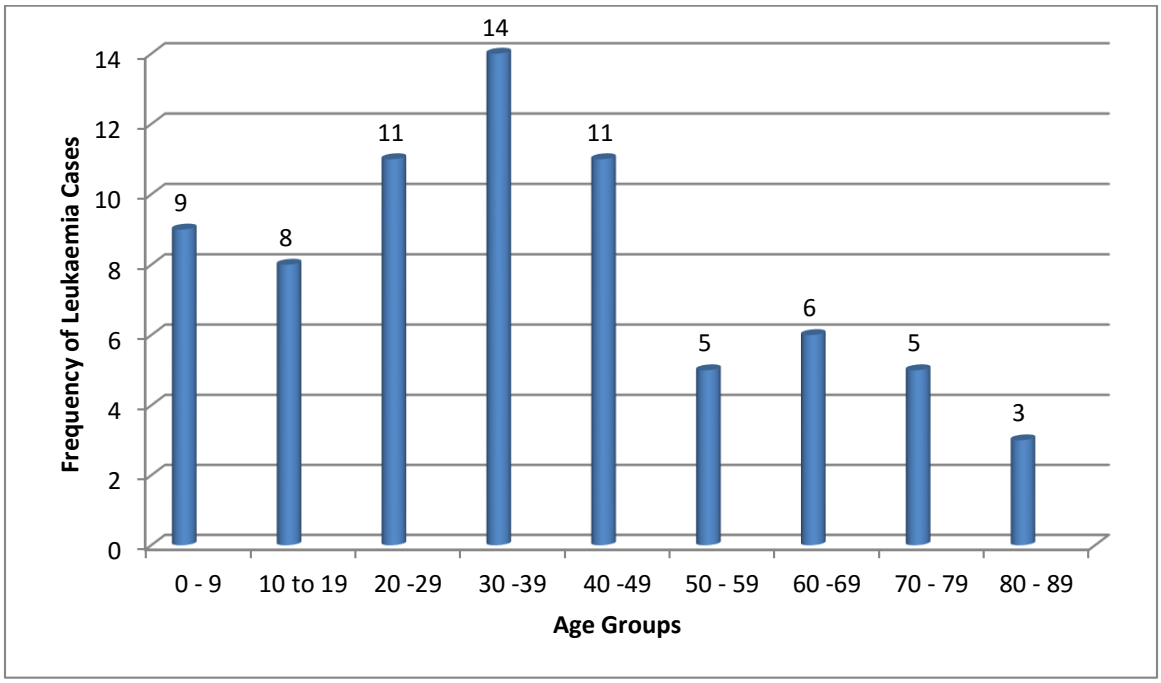

Figure 2: Age group Distribution of Leukaemia at UTH

4.4 Gender distribution of Leukemia

There was a male predominance in occurrence of leukaemia with a male to female ratio of 1.3: 1.as shown in figure 3 below

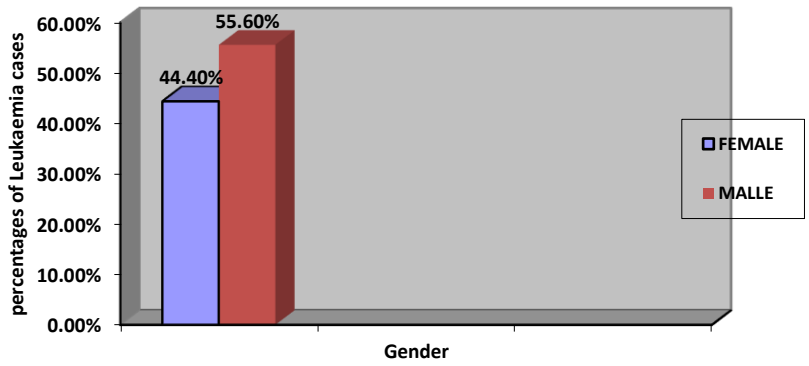

Figure 3: Percent Leukaemia distribution in males and females

4.5 Frequency Distribution of Leukaemia types

The most frequent Leukaemia type at UTH was CML followed by ALL. The least was other types of leukaemia as shown in figure 4 below.

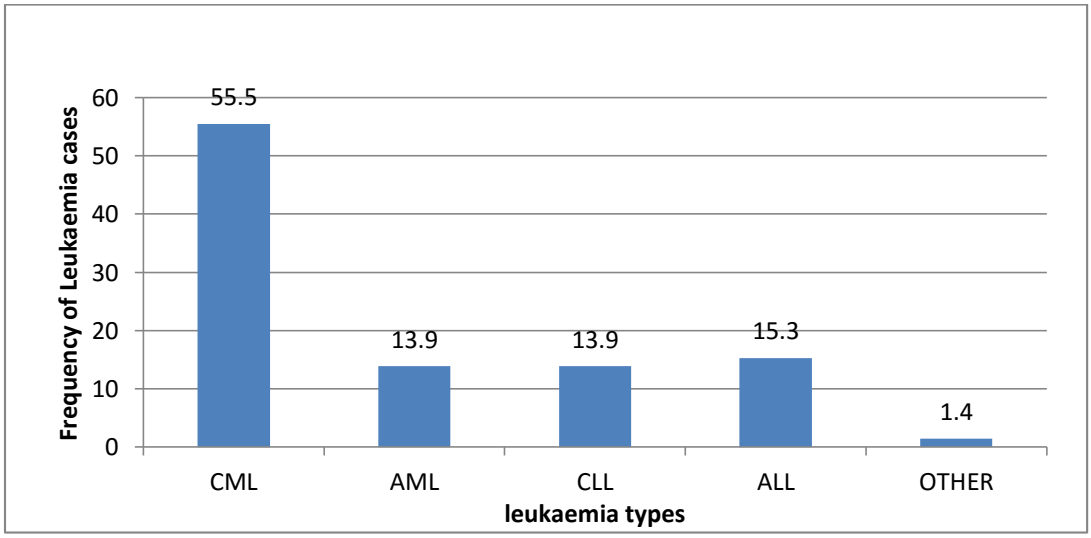

Figure 4: Percent frequency of Leukaemia types at University teaching Hospital (UTH)

4.6 Gender Distribution of Leukaemia types

CML and ALL had more males than females as shown in Figure 5

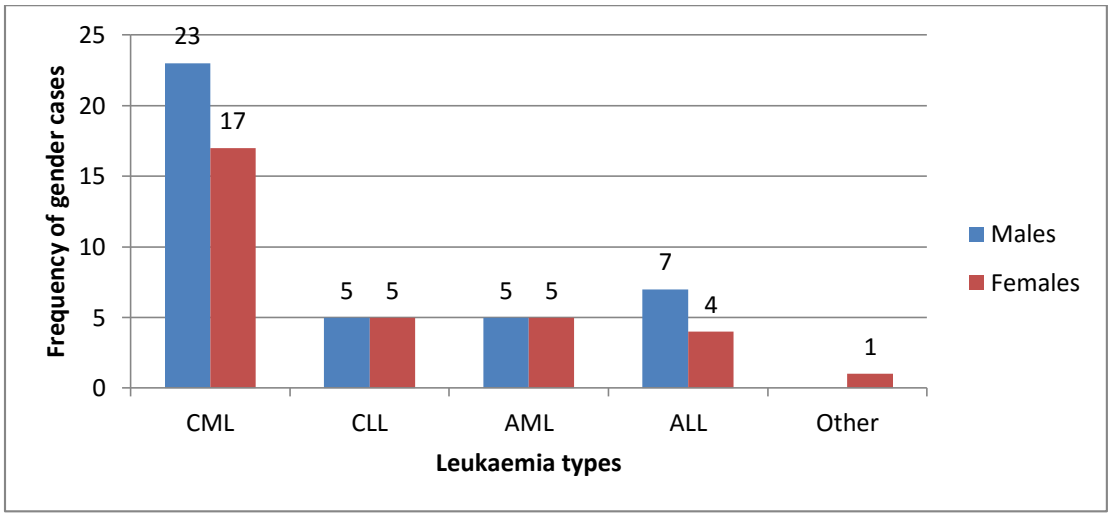

Figure 5: Gender distribution of Leukaemia types

4.7 Immunophenotypic characterization of Acute lymphoblastic Leukaemia

The B cell lineage and T cell Lineage ALL were occurring in equal proportion at 6 each while 2 cases were of Mixed lineage. Among the B cell lineage ALL the Common ALL was predominant while among the T cell Lineage the Mature T cell ALL was predominant. T cell lineage ALL was occurring more in children. More males than females were affected as shown in Table 2

Table 2: Immunophenotypic characteristics of Acute lymphoblastic leukaemia

Immunophenotypic characterization	**No.**	**Percent**	**Adults (>15 yrs.)**	**Children (≤ 15 yrs.)**	**Age range (Years)**	**Median**
Common ALL (B Cell) (TdT+/-,CD19+,CD20+, CD79a, CD10+)	4	28.6	2	2	8 – 24	15.5
Mature B –ALL (TdT-, Lambda+ or Kappa+)	2	14.3	1	1	9 -36	22.5
Precursor T Cell ALL (TdT+, CD34+, CD2+, CD3+, CD5+, CD7+)	1	7.1	0	1	15	15
Mature T Cell ALL (TdT- , CD2+, CD3+, CD5+, CD7+)	5	35.7	2	3	3 – 84	14.5
Mixed Lineage ALL (CD2+,CD3+,CD5+,CD7+,CD10+, CD19+, CD20+)	2	14.3	1	1	7 – 19	13
TOTALS	14	100	6	8	3 – 84	15

4. Antigenic Expression of ALL immunophenotypes

Antigenic Expression of B cell lineage ALL

Positive antigenic markers of CD19 and/ or CD79a, and/or CD20, and/or CD10, and /or lambda, and/or TdT, and/or CD34 were diagnostic markers for a B cell lineage ALL. The C ALL was diagnosed when CD10 was positive in addition to CD19 and /or CD79a, and/or CD20. Mature B All was diagnosed when TdT was negative, and/or lambda was positive in addition to CD19, CD79a, and/or CD20 (Table 3)

Antigenic Expression of T cell lineage ALL

Positive antigenic markers of CD5, CD7 and/or CD3, and/or CD2, and/or CD4 and/or TdT and /or CD34 were diagnostic markers for T cell lineage ALL. Precursor T cell ALL was diagnosed when TdT was positive in addition to CD2, CD3, CD4, CD5 and CD7.Mature T cell ALL was diagnosed when TdT was negative and positive for CD5, CD7, and/or CD3, and/or CD2, and/or CD4 (Table 3)

Antigenic Expression of Mixed cell lineage ALL

Positive antigenic markers of CD3, CD5, CD7, CD4, CD19, CD20 were diagnostic markers for a Mixed lineage ALL (Table 3)

Table3: Diagnostic antigens expressed by ALL immunophenotypes

	Common ALL (CD10+) NT/+ (%)	**Mature B –ALL NT/+ (%)**	**Precursor T Cell ALL NT/+ (%)**	**Mature T Cell ALL NT/+(%)**	**Mixed Lineage ALL NT/+ (%)**
CD Marker					
CD3			1/1(100)	5/4 (80)	2/2 (100)
CD79a	4/3 (75)	2/2 (100)			
Lambda		2/1 (50)			
CD5			1/1 (100)	5/5(100)	2/2 (100)
CD10	4/4 (100)		1/1(100)		
CD19	4/4 (100)	2/2(100)			2/2 (100)
TdT	4/1 (25)	2/0(0)	1/1(100)	5/0 (0)	
CD2			1/1(100)	5/4 (80)	2/1 (50)
CD7			1/1 (100)	5/5(100)	2/2 (100)
CD20	4/3 (75)	2/1 (50)			2/2 (100)
CD4			1/1(100)	5/2 (40)	2/2 (100)
CD45	4/4 (100)	2/1 (50)	1/1(100)	5/4 (80)	2/2 (100)

CHAPTER 5: DISCUSSION

In most developing nations, Zambia inclusive, identification of Leukaemia is on the basis of morphology. Even with experience, morphologic examination can separate only about 70% to 80% of acute leukemia's as ALL or AML, (Reddy and Perkins, 2004). Data of 72 Leukaemia patients collected from laboratory results register and UTH statistics Centre of morphologically diagnosed Leukaemia was analyzed for frequency and demographics. Flow Cytometric Immunophenotyping a WHO recommended diagnostic and lineage assignment tool for Leukaemia was used to diagnose and lineage assign 14 ALL patients' using their blood or bone marrow samples. This study provides information on the distribution of Leukaemia types and Acute Lymphoblastic Leukaemia Immunophenotypes at UTH in both adults and Children using the immunophenotyping technic by flow cytometry.

5.1 Frequency and demographical distribution of Leukaemia

In our findings most cases of Leukaemia occurred more in adults (83.3%) than in children (18.9%). Pearson's Chi-Square test statistic was 2.192 which is less than the Critical value of 3.84 (P value = 0.05). Therefore there is enough evidence of the relationship of leukaemia and age, occurring more in adults. This is in agreement with the pattern reported globally were more cases are occurring in adults. In Kenya, Kasili reported that Leukaemia was less in Children (28%) than in Adults (Kasili, 1985). In UK Leukaemia was strongly related to age, with the highest incidence rates being in older people aged 70 and over at 52% in 2013 (Cancer ResearchUK, 2011). In our study the Leukaemia cases were occurring in all age groups but more common in the age group 30 to 39 years. This is similar to a study in Nigeria who found that there was a higher prevalence of Leukaemia among the middle aged. The median age for all Leukaemia types in our study was 33, which was in contrast to that observed in USA which observed 66 years (Facts-spring, 2014). Our findings could be attributed to the youthful population in Zambia due to a low life expectancy compared to those in USA and other developed nations.

We observed a male predominance of all types of Leukaemia (M: F ratio 1.3: 1). Pearson Chi – Square test statistics was 1.831 which is less than the critical value of 3.841 (P value = 0.05). Therefore there is enough statistical evidence of the relationship of leukaemia and gender, occurring more in males. This was similar to those observed in other studies. In the UK, in 2013

60% of Leukaemia cases occurred in Men and 40% in females (CancerResearchUK, 2011). In Kenya a report on Leukaemia and Lymphoma in Kenya found that the Male to Female ratio was 1.47: 1 (Fleming, 1993). All the Leukaemia types found a higher male count proportional to females except Acute myeloid Leukaemia and Chronic lymphocytic Leukaemia which had an equal representation of both sexes. AML low frequency in males was as reported in sub-Sahara Africa as it occurred at about equal gender frequency (Fleming, 1993). A study done in Nigeria found that CLL occurred more in females (60%) than in males (Babatunde et al., 2008). And This was in contrast to a study in Pakistan which found the M: F ratio of 1.4: 1 (Ahmad et al., 2015).

In our study the most frequent Leukaemia type was CML followed by ALL. This was in contrast to the study in USA where the most frequent type of leukaemia reported were AML (36%) and CLL (30%), (Facts Leukaemia and lymphoma society, 2014). Our findings are also at variance with a Nigerian study that found CLL and ALL to be more frequent at 35.2% each followed by CML at 19.6% and AML at 10.0% of all Leukaemia (NIA et al, 2010). . However our findings on frequency of ALL are similar to those in the USA occurring at 11.5% (Facts-spring, 2014). Another diverse finding was in Pakistan where the most frequent leukaemias were AML which was at 32.8%, followed by CML 23.8%, (Ahmad et al., 2015).

In our study chronic leukemia's (69.4%) were more compared to acute leukemia's (29.2%). This was similar as to a study in Nigeria which found Chronic Leukaemias at 63.7% were more prevalent than acute Leukaemias at 36.7% (NIA et al, 2010). This was however in contrast to studies in India and Pakistan which found acute leukemias at 59.4% and 55.2% respectively and chronic Leukaemia at 40.6% and 44.8% respectively (Bera and Chauhan, 2016) (Ahmad et al., 2015).

We found that myeloid morphological forms were more common than lymphoid morphological forms. This was similar to other studies such as in Kenya and Pakistan which found more myeloid leukemias than Lymphoid leukemias (Kasili, 1985) (Ahmad et al., 2015).

5.2 Immunophenotyping of Acute lymphoblastic leukaemia

Immunophenotyping was able to diagnose and further subtyped ALL into three groups namely ; B cell , T cell and Mixed lineage according to McGregor et al., 2012 and Alves et al,2012.
We observed 6 (42.9%) B lineage ALL and 6 (42.9%) T Cell lineage ALL. These findings are in contrast with a study in America in which 77% were B lineage ALL and 23% were T lineage ALL (Thalhammer-Scherrer et al., 2002). A study in Saudi Arabia had reported a lower incidence of T cell Lineage at 6.2% and a higher B lineage ALL 93.8% (Al-SheikhIman, 1999) and in Brazil Alves et al., 2012 T cell lineage was reported at 43.6% similar to our findings .

Subtyping of the B cell revealed a predominance of C ALL at 66.7%. This is similar to other studies such as in Saudi Arabia and USA who found C ALL B cell subtypes at 68.9% and 48.9% respectively (Al-SheikhIman, 1999, Thalhammer-Scherrer et al., 2002).

Subtyping of the T cell lineage ALL observed two subtypes the Mature T Cell ALL at 83.3% and the Precursor T Cell ALL at 16.7%. The low frequency of Precursor T Cell was also observed in the American study that found 14.3% and the Mature T Cell ALL frequency also at 14.3 %(Thalhammer-Scherrer et al., 2002.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Despite of the increasing importance of molecular and genetic features in the sub-classification of leukemias, morphologic and Immunophenotypic analysis remains the main modality to diagnose leukaemia for initial evaluation. Therefore in Zambia with the availability of Flow Cytometers in Hospitals immunophenotyping should be performed as a routine diagnostic tool for acute Leukaemias

This study showed that Overall leukemia's are more common in males than in females and often occur between 20 to 44 years of age with a peak at 30 to 39 years. ALL was more commonly observed in children whereas both CML and CLL were mostly more observed in adults. CML was the commonest Leukaemia type at UTH and that the distribution of B cell lineage ALL and T cell lineage ALL in patients at UTH was equal. C ALL was the most common B cell lineage ALL while Mature T cell ALL was the most common T cell lineage ALL.

These findings helps us to compare with other geographical regions thereby having a base on which to elucidate the common factors that can cause similar leukaemias or Leukaemia sub types.

6.2 Recommendations

Studies like ours that determine patterns of Leukaemia demographics and frequencies can help in elucidating the causal factors of leukemia and identify high risk groups when further studies are done. In Zambia diagnosis of Leukaemia is only by Morphological examination of Blood and Bone marrow smears. Use of Flow cytometry will help in diagnosis and subtyping of Leukaemia subtypes hence support an optimal risk-oriented therapy and thus increase the curability rate.

It is therefore recommended that:

1. Further studies on causal factors of Leukaemia be encouraged in Zambia
2. Immunophenotyping of leukemia's be routinely performed at UTH.
3. Molecular studies of leukemia's be routinely performed to identify Leukaemia subtypes and their prognosis in conjunction with immunophenotyping

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APPENDICES

Appendix 1: information sheet

My name is Jonas Zimba studying for a Master of Science degree (MSc.) in Pathology (Haematology) at the University of Zambia- Ridgeway Campus. I am carrying out a research as a requirement for fulfillment of my Master's degree.

About the study:

The study will use immunophenotyping to diagnose and characterize the biology of acute lymphoblastic leukaemia (ALL). At the moment diagnosis of ALL is done by smear microscopy examination despite having flow cytometers in Zambian public and private hospitals which can be used to diagnose ALL and other leukaemias. Immunophenotyping is the WHO recommended diagnostic and lineage assignment tool for leukaemia.

Correct diagnosis and Lineage assignment of ALL is helpful as different lineages may have different clinical features and prognosis. Therefore lineages which are aggressive clinically can effectively be managed. This descriptive study will provide a foundation in elucidating the etiology of ALL by other further investigative strategies.

Participating in the study

You have been invited to participate in this study because you/your child was previously diagnosed with ALL or with an unknown acute leukaemia by blood or bone marrow smear morphological examination and are being managed at the haematology clinic OR You /your child is a newly suspected acute lymphoblastic leukaemia individuals. After being assessed by the clinician you meet the study inclusion criteria and as such the study questionnaire will be administered to you.

You can decide whether you want to join this study or not. You are free to say yes or no. If you decide to take part in the study, you/your childs clinic files will be reviewed and you will be asked a few questions and also you/your child will be requested to give 8mls of blood and/or bone marrow aspirate collected routinely by the clinician. If you/your child was previously diagnosed with ALL or with an unknown acute leukaemia by blood or bone marrow smear morphological

examination the blood and/or bone marrow collected is needed to characterise ALL or to distinguish the ALL from AML by immunophenotyping . If you/your child a newly suspected acute lymphoblastic leukaemia individuals the blood and/or bone marrow collected is needed to diagnose ALL by smear microscopy and immunophenotyping and to characterize ALL . The study requires as many people as possible to participate.

Benefits for participation:

There are no costs to you/your child for being in this study and the study may not benefit you directly. However, the study results generated in this study through your participation will provide valuable information which can be used to improve the health of individuals in future and this information will be made available to you through your/your childs clinician .. The results from the study may identify patients who may benefit from aggressive clinical monitoring before and after commencing .

Problems with the study:

The problem that participants will experience in this study is some pain when bone marrow aspirate and blood are being drawn even though these are done routinely in diagnosis of leukaemia. You will also feel loss of confidenciality as you/your childs personal medical records are reviewed. However, every effort will be made to reduce the pain that you feel as bone marrow aspirate and blood are collected and also to keep your/your childs personal medical records confidential.

Contact Details: In case you have any more questions about this study at any time, you can call any of the numbers below.

1. **Jonas Zimba**

   The Researcher

   Contact number: 0977- 222351 Email

   Address: jonaszmb@yahoo.co.uk

2. **Dr. H. Mantina**

   Supervisor

   Contact Number: 0974021971

   Email Address: hmantina@uthlabs.gov.zm

3. **Mrs. Theresa Chisoso**

   The Secretary

   University of Zambia Biomedical Research Ethics Committee (UNZABREC):

   Contact Number: 260-1-256067 Email Address: unzarec@zamtel.zm

Appendix 2: Informed consent form

Consent to participate in the study

Study title: **Flow Cytometric Immunophenotypic characterization of acute ymphoblastic leukaemia presenting at University Teaching Hospital (UTH), Lusaka, Zambia**

By signing my name below, I ……..…… Confirm the following

- I have read (or has been read to me) this entire consent document and all of my questions have been answered adequately.
- The study's purpose, procedures, risks and possible benefits have been explained to me.
- I agree to let the study team use and share the health information and other information gathered for this study. I voluntarily agree to participate in this research study and I agree that my /my chids medical records can be reviewed and small amount of blood and /or bone marrow aspirate be collected if necessary.

Participant signature …… Date……..

Thumb print if participant can't sign…….

Statement by the researcher/person taking consent:

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the research procedure. I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Person taking the consent…… Date……

Signature of Person taking the consent …….. Date…….

NOTE: The participant will be provided with a signed and dated copy of this consent form. It will help him/her remember what was discussed .

Appendix 3**:** Questionnaire

THE UNIVERSITY OF ZAMBIA

PATHOLOGY AND MICROBIOLOGY DEPARTMENT

This questionnaire is administered for;

A Study on Flow Cytometric Immunophenotypic Characterization of acute lymphoblastic leukaemia presenting at University Teaching Hospital (UTH) , Lusaka , Zambia

Date……..... Study ID.....

Specimen serial number ……

Demographic data:

Sex…….....

Age…….....

Race ----------

Occupation……

Physical address……

……

History of Leukaemia in family yes/no ……..

Any previous haematological disease of participant yes/no …….

Participant on chemotherapy for ALL/ unknown acute leukaemia yes/no……..

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
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E-mail: unzarec@unza.zm

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

20th March, 2015.

Our Ref: 001-01-15.

Mr. Jonas Zimba,
Ministry of Health,
Kabwe Mine Hospital,
P.O Box 80145,
Kabwe.

Dear Mr. Zimba,

RE: RESUBMITTED RESEARCH PROPOSAL: **"FLOW CYTOMETRIC IMMUNOPHENOTYPIC CHARACTERIZATION OF ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL) PRESENTING AT UNIVERSITY TEACHING HOSPITAL (UTH), LUSAKA, ZAMBIA" (REF. No. 001-01-15)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 13th March, 2015. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

M.M.Mbewe (mrs)
CHAIRPERSON

Date of approval: 20th March, 2015. **Date of expiry:** 19th March, 2016.

THE UNIVERSITY OF ZAMBIA

SCHOOL OF MEDICINE

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17th December, 2014

Mr. Jonas Zimba
Department of Pathology & Microbiology
School of Medicine
UNZA
LUSAKA

Dear Mr. Zimba,

RE: GRADUATE PROPOSAL PRESENTATION FORUM

Having assessed your dissertation entitled **"Flow Cytometric Immunophenotypic Characterization of Acute Lymphoblastic Leukaemia (ALL presenting at University Teaching Hospital (UTH), Lusaka, Zambia"**. We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. M. Makusa
ACTING ASSISTANT DEAN, POSTGRADUATE
CC: HOD, Pathology &Microbiology

UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE
17 DEC 2014
ASSISTANT DEAN (PGT)
P.O. BOX 50110, LUSAKA