

**CHARACTERIZATION OF
CHROMOSOMAL ABNORMALITIES IN
ACUTE MYELOID LEUKAEMIA PATIENTS
AT THE UNIVERSITY TEACHING
HOSPITAL, LUSAKA, ZAMBIA**

By

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DECLARATION

I, **KACHINDA WEZI** this 21st day of May 2018, declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references.

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CERTIFICATION OF APPROVAL

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ABSTRACT

Acute myeloid leukaemia (AML) is a highly malignant clonal hematopoietic disease caused by both inherited and acquired genetic alterations. Current AML classification and prognostic systems incorporate genetic information but are limited to known abnormalities that have previously been identified with the use of cytogenetics, array comparative genomic hybridization (CGH), gene-expression profiling, and the resequencing of candidate genes. At diagnosis, most patients with AML harbour at least 1 chromosome aberration in their marrow blasts. With the targeted cytogenetic therapy, 30% of the patients achieve long-term cure. At University Teaching Hospital(UTH) however, the current diagnostic approach of acute leukaemia involves mainly cytomorphology and occasional flow cytometry. The cytomorphological blast characterization is not enough to provide a critical determination of prognosis and developing a treatment plan. Most of the AML patients at UTH die within few months after diagnosis despite being put on chemotherapy.

To characterize AML according to WHO 2008 revised classification in patients at the University Teaching Hospital.

This was a descriptive cross-sectional study conducted to characterize acute myeloid leukaemia (AML) according to WHO 2008 revised classification in patients at the UTH. Patients with AML were simultaneously analyzed for the presence of 4 genetic abnormalities, PML/RAR α for t(15;17), AML1/ETO for t(8;21), CBF β /MYH11 for inv(16)/t(16;16) and rearrangements of the MLL gene for 11q23 abnormalities. AML was classified using the new World Health Organization (WHO) classification for haematologic malignancies. The techniques used were standardized according to the recommendations of the European BIOMED-1 Concerted Action.

The overall frequency of leukemia displaying one of the four recurrent cytogenetic translocations were 13 cases (46.5%) of which PML/RAR α transcript was present in six(6) patients (21.4%) (3 were bcr1, 1 bcr2 and 2 bcr3). The AML1/ETO fusion transcript was detected in only one(1) case (3.6%) with M2 morphology, but other cases with M2 morphology were negative. CBF β /MYH11 transcript was present in 2 cases (7.1%) and some of them displaying M4Eo morphology. Finally, 4 cases (14.3%) showed rearrangements of the MLL gene. By contrast, the frequency of AML not otherwise characterized which was 15 cases (53.6%) increased with age (13% for 6-35years age group, 20% for 36-65years age group and 67% for above 66years age group). Our results differ from those reported from the United States and North/Central Europe, particularly regarding the incidence of t(15;17) and t(8;21) translocations. In Zambia the frequency of t(15;17) is higher while that of t(8;21) is lower. This supports the view that geographic variations in tumor-associated aberrations in hematologic malignancies exist.

Our study showed that chromosomal alteration PML/RAR t(15,17) which was 21.4% ,was the commonest, whereas AML1/ETO t(8,21) which was 3.6%,was the least common among patients presenting at UTH, Lusaka, Zambia. Our study showed that

chromosomal aberration detected in our patients make them less responsive to cytotoxic drugs. The use of molecular technique at point of diagnosis would assist in identifying AML with better prognosis by administering appropriate treatment. The results support the existence of chromosomal abnormalities of AML in our Zambian patients. Awareness of these chromosomal abnormalities and morphology could contribute to the design of cost-effective screening strategies, adapted by our National Health systems according to the prevalence of locally detected genetic aberrations.

Key Words: Acute myeloid leukaemia (AML)

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DEDICATION

This work is dedicated to my loving wife Tinkani Kachinda-Zulu and our son
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ACRONYMS AND ABBREVIATIONS

AML	- Acute myeloid leukaemia
APL	- Acute Promyelocytic Leukaemia
ATRA	- All-Trans Retinoic Acid
BM	- Bone Marrow
CR	- Complete Remission
CGH	- Comparative Genomic Hybridization
FISH	- Fluorescence In Situ Hybridization
FAB	- French-American-British
FLT3	-FMS-related tyrosine kinase 3
mRNA	- Messenger Ribonucleic Acid
NPM1	-Nucleophosmin 1
PB	-Peripheral Blood
PCR	-Polymerase Chain Reaction
RT-PCR	- Reverse Transcriptase–Polymerase Chain Reaction
SNP	-Single-nucleotide-polymorphism
UNZA	-University of Zambia
UNZABREC	- University of Zambia Biomedical Research Ethics Committee
UTH	-University Teaching Hospital
WHO	- World Health Organisation
HDAC	- Histone Deacetylase
WBC	-White Blood Cell

CHAPTER 1: INTRODUCTION

1.1 Background

Acute myeloid leukaemia (AML) is a highly malignant clonal hematopoietic disease caused by both inherited and acquired genetic alterations (Song et al, 1999). Current AML classification and prognostic systems incorporate genetic information but are limited to known abnormalities that have previously been identified with the use of cytogenetics, array comparative genomic hybridization (CGH), gene-expression profiling, and the resequencing of candidate genes. There are distinct differences between sub-Saharan Africa and the industrialized countries as to the age and gender distributions of the Leukaemias.

AML is diagnosed with equal frequency as ALL in children in tropical African countries, and is especially common in boys aged 5 to 14 years, who present often with chloromas, usually arising in the orbit (AF Fleming et al,1999).The karyotyping of AML cells remains the most powerful predictor of the outcome in patients with AML and is routinely used by clinicians (Byrd JC et al,2002, Grimwade D et al,2001,).As an adjunct to cytogenetic studies, small subcytogenetic amplifications and deletions can be identified with the use of genomic methods, such as single-nucleotide-polymorphism (SNP) array and array CGH platforms. However, these techniques remain investigational, and studies suggest that there are few recurrent acquired copy-number alterations in each AML genome. Gene-expression profiling has identified patients with known chromosomal lesions and genetic mutations and subgroups of patients with normal cytogenetic profiles who have variable clinical outcomes (Bullinger L et al,2004 and Valk PJ et al,2004).

Expression profiling has yielded single-gene predictors of outcome that are currently being evaluated for clinical use. Candidate-gene resequencing studies have also identified recurrent mutations in several genes — for example, genes encoding FMS-related tyrosine kinase 3 (FLT3) and nucleophosmin 1 (NPM1) — that can help to stratify patients with normal cytogenetic profiles according to risk and to identify

patients for targeted therapy (e.g., those with mutated FLT3). However, the revised classification systems are imperfect, suggesting that important genetic factors for the pathogenesis of AML remain to be discovered (Elaine R et al, 2009). We believe that the data generated by this research, will allow us to propose as a starting point for future clinical studies the molecular-risk classification in our country.

1.2 Statement of the problem

The development of acute myeloid leukaemia (AML) is associated with accumulation of acquired genetic alterations and epigenetic changes in hematopoietic progenitor cells that alter normal mechanisms of cell growth, proliferation, and differentiation. Thus, this causes neutropenia, anaemia, and thrombocytopenia. The World Health Organisation recommends the use of morphology, cytochemistry, flow cytometry and molecular methods for the detection of genetic derangements in the diagnosis of AML. Employment of these techniques in the diagnosis of AML ensures

1. Proper diagnosis of the malignancy
2. Proper classification of the leukaemia
3. Institution of the appropriate treatment protocol
4. Establishment of baseline characteristics of the leukaemia that can be used to monitor the success of treatment, the presence of minimal residual disease or the early detection of relapse.

At diagnosis, most patients with AML harbour at least 1 chromosome aberration in their marrow blasts. Numerous recurrent structural and numeric cytogenetic aberrations have been identified and many of them not only are diagnostic markers for specific AML subtypes but also constitute independent prognostic factors for attainment of complete remission (CR), relapse risk, and overall survival (OS) (MRO´ZEK *et al*, 2007). With the targeted cytogenetic therapy, 30% of the patients achieve long-term cure. At UTH however, the current diagnostic approach of acute leukaemia involves mainly cell morphology. This diagnosis is defined by presence of >20% blast cells in peripheral blood or bone marrow at clinical presentation. With the exception of Acute promyelocytic leukaemia (APL) in which additional tests for Disseminated intravascular coagulopathy (DIC) such as prothrombin time, Thrombin time and Activated partial thromboplastin time. These tests for DIC are positive in APL. Thrombocytopenia and DIC are characteristic of promyelocytic variant of AML (APL). The cytomorphological blast characterization is not enough to provide a critical determination of prognosis and developing a treatment plan. Use of morphology alone implies that the leukaemia cannot be correctly identified

and classified and renders the physicians unable to offer the appropriate management. The lack of appropriate management for AML is reflected by the high mortality. Between January 2011 to December 2014, there were 48 cases of childhood AML diagnosed at UTH and of these 45% (10) had already died at the time the leukaemia survival data was being reviewed (Personal communication with Consultant Paediatric Oncologist). This high mortality in childhood AML reflects, amongst other factors, this include poor diagnostic capabilities available at UTH and Blood bank support which is suboptimal. AML continues to be one of the major fatal myeloid leukaemias in Zambia and indication for admission and referral to UTH; however, the management of AML at UTH is mostly based on general standard chemotherapy for AML with exception on promyelocytic leukaemia (APL) which has different treatment plan. Most of the AML patients at the UTH die within few months after diagnosis despite being put on chemotherapy. Cytogenetic analysis is not done despite the cytogenetic abnormalities being the major predictors of favourable, intermediate or adverse prognosis.

While many questions still remain unanswered, we believe that the data generated by this research, will allow us to propose as a starting point for future clinical studies the molecular-risk classification in our country.

This study, set out to characterize AML according to WHO 2008 revised classification based on cytomorphology and chromosomal abnormalities in patients presenting at The University Teaching Hospital (UTH), Lusaka, Zambia.

1.3 Justification of the study

It is clear that AML is very heterogeneous at the molecular level. Hence, it was inevitable that the 2008 revised World Health Organization (WHO) classification of AML had to consider recurrent molecular genetic rearrangements. Moreover, since 2 or more genetic alterations are present simultaneously in many patients, it is important to devise a prioritized schema that stratifies patients to risk-adapted therapies using information on all known prognostic markers (MRO'ZEK *et al* 2007). Although these leukaemias are recognized as unique entities, it is important to realize that additional genetic abnormalities may coexist and influence their biologic and clinical behaviour, including response to therapy and the overall survival.

The University Teaching Hospital has over the years invested in equipment that can be used for clinical molecular diagnostics. The hospital has several types of thermocyclers that can be used for the detection of genetic derangements. With the availability of the equipment there is capacity for the introduction of molecular diagnostics of AML. The correct molecular diagnosis and classification of leukaemia is cardinal for the selection of appropriate treatment protocols, which play an important role in the effectiveness of therapy. With optimal application of this technique in the diagnosis of acute leukaemia, treatment strategies can be more specifically directed and new therapeutic approaches can be evaluated more effectively. This is achievable based on the knowledge of the specific genetic derangement targeted at and this is only determined by the use of molecular analysis such as PCR. This study will show the possibility of use of molecular diagnostics in the diagnosis of AML and will offer the attending physicians the information need to select the appropriate treatment protocols. Furthermore, with this study, the molecular signatures of the leukaemia will be known and this will enable better patient management in the case of detection of minimal residual disease and in the gauging of the success of therapy. In addition, gene mutations are increasingly being recognized as important diagnostic and prognostic markers in myeloid neoplasms.

1.4 Research question

What are the chromosomal abnormalities in AML patients seen at the University Teaching Hospital according to WHO 2008 revised classification?

1.5 Objectives

1.5.1 General objective

To characterize acute myeloid leukaemia (AML) according to WHO 2008 revised classification in patients seen at the University Teaching Hospital.

1.5.2 Specific objectives

1.To characterize acute myeloid leukaemia (AML) according to WHO 2008

2.To determine the prevalence and phenotypes of AML according to cytomorphology subgroups.

3.To describe any genetic mutation of chromosomal abnormalities associated with AML in patients at The UTH.

CHAPTER 2: LITERATURE REVIEW

2.1 Epidemiology of Acute Myeloid Leukaemia

AML accounts for approximately 25% of all leukaemias diagnosed in adults, and the median age at diagnosis is 67 years (Thomas et al 2009). The incidence of AML in the <65 years' age group is 1.8 cases per 100,000 patients, and the incidence in the >65 years' age group is 17.9 cases per 100,000 patients (Jemal et al 2010). The incidence of AML is expected to increase in the future in line with the aging population, and along with its precursor myelodysplasia, AML prevalence appears to be increasing, particularly in the population older than 60 years of age, and represents the most common type of acute leukaemia in adults (Chandra et al 2011). The incidence of Acute Leukaemia (AL) subtypes varies according to geographical distribution and more predominant in developing countries (Sanddhya *et al* 2016). There are distinct differences between SubSaharan Africa and the industrialized countries as to the age and gender distributions of the Leukaemias. AML is diagnosed with equal frequency as Acute Lymphoblastic Leukaemia (ALL) in children in tropical Africa, and is especially common in boys aged 5 to 14 years, who present often with choroidomas, usually arising in the orbit (AF Fleming et al, 1999). From the few reports of leukaemias in Zambians it may be concluded that age and gender distributions of leukaemias in Zambia resemble those of other subSaharan tropical African countries.

In Zambia, a review of the record at the UTH- Children Oncology ward shows that 73 acute leukaemia patients have been admitted for treatment since 2012. There were 48 acute myeloid leukaemia, 22 of these cases were acute lymphoblastic leukaemia (but 10 are already dead) and 3 unclassified acute leukaemia. The genetic lesions affecting these patients are not known.

2.2 Aetiology and Pathogenesis

Acute myeloid leukemia (AML) is a clonal hematopoietic disorder resulting from genetic alterations in normal hematopoietic stem cells (Lowenberg et al 1999). These alterations disrupt normal differentiation and/or cause excessive proliferation of abnormal immature leukemic cells known as blasts (Estey et al 2006). As the disease progresses, blast cells accumulate in the bone marrow, blood, and organs and interfere with the production of normal blood cells. This leads to fatal infection, bleeding, or organ infiltration in the absence of treatment within 1 year of diagnosis (Shipley et al 2009). AML is characterized by more than 20% blasts in bone marrow. AML can arise *de novo* or secondarily either due to the progression of other diseases or due to treatment with cytotoxic agents (referred to as therapy-related AML). Up to 10% to 15% of patients with AML develop the disorder after treatment with cytotoxic chemotherapy (usually for a solid cancer). There are 2 main types of therapy-related AML. The “classic” alkylating-agent type has a latency period of 5 to 7 years and is often associated with abnormalities of chromosomes 5 and/or 7 (Leone et al 2001).

Development of AML has been correlated with exposure to a variety of environmental agents, most likely due to links between exposure history and cytogenetic abnormalities (West et al 2000). Radiation, benzene inhalation, alcohol use, smoking, dyes, and herbicide and pesticide exposure have all been implicated as potential risk factors for the development of AML (Crane et al 1996). Survivors of the atomic bombs in Japan had an increased incidence of myeloid leukaemias that peaked approximately 5 to 7 years following exposure (Prestone et al 1994). Therapeutic radiation also increases AML risk, particularly if given with alkylating agents such as cyclophosphamide, melphalan, and nitrogen mustard.

Exposure to agents, such as etoposide and teniposide, that inhibit the DNA repair enzyme topoisomerase II is associated with secondary AML with a shorter latency period, usually 1 to 3 years, with rearrangements at chromosome 11q23 (Felix et al 1998). Drugs, such as chloramphenicol, phenylbutazone, chloroquine, and methoxypsoralen, can induce marrow damage that may later evolve into AML

(Appelbaum et al 2004). Secondary AML may also occur because of progression of myelodysplastic syndrome (MDS) or chronic bone marrow stem cell disorders, such as polycythemia vera, chronic myeloid leukemia, primary thrombocytosis, or paroxysmal nocturnal hemoglobinuria (Pedersen et al 2002). Secondary AML has a particularly poor prognosis and is not considered to be curable, with the exception of secondary acute promyelocytic leukemia (APL) (Licht et al 2006). This is largely due to the high percentage of secondary AML associated with multidrug resistance (MDR) mechanisms: up to 70% of secondary AML patients show overexpression of P-glycoprotein (Pgp) or other MDR mechanisms (Sztokowski et al 2010).

2.3 Classification of AML

The two systems commonly used in the classification of AML are the French-American-British (FAB) system and the World Health Organization (WHO) system. The FAB system is based on morphology and cytochemistry and recognizes 8 subtypes of AML (**Bennett et al 1999**). FAB Classification

- M0 -- Undifferentiated AML
- M1 -- AML without maturation
- M2 -- AML with maturation
- M3 -- Acute Promyelocytic Leukemia
- M4 -- Acute Myelomonocytic Leukemia
- M5 -- Acute Monocytic Leukemia
- M6 -- Erythroleukemia (DiGuglielmo's)
- M7 -- Megakaryoblastic Leukemia

The WHO classification was introduced to include newer prognostic factors, such as molecular markers and chromosome translocations, and lowered the blast minimum criterion to 20%, thus including many cases classified as high-grade MDS in the FAB system (Harris et al 1999). The WHO classification system identifies 4 AML subgroups: 1) AML with recurrent genetic abnormalities, 2) AML with multilineage dysplasia, 3) therapy-related AML and MDS, and 4) those that do not fall into any of these groups. This system created a minimum of 17 subclasses of AML, allowing physicians to identify subgroups of patients who might benefit from specific treatment strategies. Recently, a revised classification has been published as part of the fourth edition of the WHO monograph series (Vardiman et al 2008). The aim of the revision was to incorporate new scientific and clinical information to refine diagnostic criteria for previously described neoplasms and to introduce newly recognized disease entities.

WHO 2008 revised classification of AML and related neoplasms.

1. AML with recurrent genetic abnormalities:

- AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1*
- AML with inv(16) (p13.1q22) or t(16;16)(p13.1;q22); *CBEF-MYH11*
- Acute promyelocytic leukaemia (APL) with t(15;17) (q22;q12); *PML-RARA*
- AML with t(9;11) (p22;q23); *MLLT3-MLL*
- AML with t(6;9) (p23;q34); *DEK-NUP214*
- AML with inv(3) (q21q26.2) or t(3;3)(q21;q26.2); *RPNI-EVII*
- AML (megakaryoblastic) with t(1;22) (p13;q13); *RBM15-MKLI*
- Provisional entity: AML with mutated NPM1
- Provisional entity: AML with mutated CEBPA

2. Acute myeloid leukaemia with myelodysplasia-related changes

3. Therapy-related myeloid neoplasms

4. Acute myeloid leukaemia, not otherwise specified

- Acute myeloid leukaemia with minimal differentiation
- Acute myeloid leukaemia without maturation
- Acute myeloid leukaemia with maturation
- Acute myelomonocytic leukaemia
- Acute monoblastic/monocytic leukaemia
- Acute erythroid leukaemia
 - Pure erythroid leukaemia
 - Erythroleukaemia, erythroid/myeloid
- Acute megakaryoblastic leukaemia
- Acute basophilic leukaemia
- Acute panmyelosis with myelofibrosis

5. Myeloid sarcoma

AML is also classified as "cytogenetically normal" based on the type of genetic changes involved in its development. Cytogenetically normal refers to the fact that this form of acute myeloid leukaemia is not associated with large chromosomal abnormalities. About half of people with AML have this form of the condition; the other half has genetic changes that alter large regions of certain chromosomes. Mutations in a large number of genes have been found in people with cytogenetically normal-acute myeloid leukaemia (CN-AML); the most commonly affected genes are NPM1, FLT3, DNMT3A, CEBPA, IDH1, and IDH2. The proteins produced from these genes have different functions in the cell. Most are involved in regulating processes such as the growth and division (proliferation), maturation (differentiation), or survival of cells.

2.4 Genetic Abnormalities associated with AML

Acute myeloid leukaemia (AML) is clinically, cytogenetically, and molecularly heterogeneous (Gilliland et al, 2004). About 30% of cases carry recurrent chromosomal abnormalities that identify leukaemia entities with distinct clinical and prognostic features (Jaffe et al, 2001), and 10–15% of AMLs have non-random chromosomal abnormalities (Amani et al, 2011). The recent WHO classification reflects the fact that an increasing number of acute leukaemia can be categorized based upon their underlying cytogenetic or molecular genetic abnormalities, and that these genetic changes form clinico-pathologic-genetic entities (Vardiman et al, 2009). AML is characterized by a high degree of heterogeneity with respect to chromosome abnormalities, gene mutations, and changes in expression of multiple genes and microRNAs. Cytogenetic abnormalities can be detected in approximately 50% to 60% of newly diagnosed AML patients (Martens et al 2010). The majority of AML cases are associated with nonrandom chromosomal translocations that often result in gene arrangements. Cytogenetics is the most important prognostic factor for predicting remission rate, relapse, and overall survival (Martens et al 2010). Several chromosomal abnormalities such as monosomies or deletions of part or all of chromosomes 5 or 7 ($-5/-7$ AML) and trisomy 8 are common in AML (Byrd et al 2002). The chromosomal abnormalities also include the long arm of chromosome 11 (11q); balanced translocations between chromosomes 15 and 17 ($t(15;17)$); chromosomes 8 and 21 ($t(8;21)$); others such as ($q22;q22$), ($q31;q22$), and $t(9;11)$; and inversion such as $inv(16)$ (Mrózek et al 2009). The most frequent chromosomal aberrations and their corresponding fusion genes in AML. The translocation in $t(15;17)$ is always associated with APL and leads to the expression of PML-RAR α oncofusion gene in hematopoietic myeloid cells (Melnick et al 1999). Generally, patients with APL $t(15;17)$ phenotype represent a unique group characterized by distinct biological features and good prognosis, particularly when all-trans retinoic acid (ATRA) is used as part of remission induction.

The subgroup “AML with recurrent genetic abnormalities” comprises several primary AML entities. “AML with $t(8;21)$ ($q22;q22$);RUNX1-RUNX1T1” and

“AML with *inv(16)(p13.1q22)* or *t(16;16)(p13.1;q22);CBFB-MYH11*” are considered as AML regardless of bone marrow blast counts (DOHNER *et al*, 2010).

Many of the gene rearrangements involve a locus encoding a transcriptional activator, leading to expression of a fusion protein that retains the DNA-binding motifs of the wild-type protein. Moreover, in many instances, the fusion partner is a transcriptional protein that is capable of interacting with a corepressor complex. A commonly accepted paradigm is that through aberrant recruitment of a corepressor to a locus of active transcription, the fusion protein alters expression of target genes necessary for myeloid development, thus laying the groundwork for leukemic transformation (Mitelman *et al* 2007). Potential targeting of this interaction has become a major focus for the development of novel therapeutics. ATRA serves as a prototype: by altering corepressor interaction with the APL fusion protein, ATRA effectively induces remission and has become a mainstay of treatment of this previously fatal disease (Licht *et al* 2006). However, to date, APL represents both the most curable and the best-studied subtype of AML, while molecular data on other fusion proteins are limited or absent. Still, the work on PML-RAR α has inspired the molecular analysis of many other AML-associated oncofusion proteins, especially AML1-ETO, CBF β -MYH11, and MLL fusions.

Oncofusion Proteins Associated with AML

A total of 749 chromosomal aberrations have been catalogued in AML ((Mitelman *et al* 2010). The frequencies of the 4 most common translocations are between 3% and 10%, while for others, the prevalence is significantly smaller. The most frequent oncofusion proteins, PML-RAR α , AML1-ETO, CBF β -MYH11, and MLL fusions, are described below.

t(15;17), PML-RAR α

The *t(15;17)* translocation is found in approximately 95% of APLs, a specific subtype of AML. The translocation results in the expression of the PML-RAR α oncofusion gene in hematopoietic myeloid cells (Licht *et al* 2006). The PML-RAR α oncofusion

protein acts as a transcriptional repressor that interferes with gene expression programs involved in differentiation, apoptosis, and self-renewal.

t(8;21), AML1-ETO

Approximately 10% of AML cases carry the t(8;21) translocation, which involves the AML1 (RUNX1) and ETO genes, and express the resulting AML1-ETO fusion protein (Cameron et al 2004). AML1 is a DNA-binding transcription factor crucial for hematopoietic differentiation (de Bruijn et al 2004), while ETO is a protein harboring transcriptional repressor activities (Davis et al 2003). The fusion protein AML1-ETO is suggested to function as a transcriptional repressor that blocks AML1-dependent transactivation in various promoter reporter assays, suggesting it may function as a dominant-negative regulator of wild-type AML1 (Meyers et al 1995) (Frank et al 1995).

inv(16), CBF β -MYH11

inv(16) is found in approximately 8% of AML cases. inv(16) fuses the first 165 amino acids of core binding factor β (CBF β) to the C-terminal coiled-coil region of a smooth muscle myosin heavy chain (MYH11) (Lutterbach et al 2000). CBF β -MYH11 fusion protein is suggested to cooperate with AML1 to repress transcription (Lutterbach et al 1999).

11q23, MLL Rearrangements

Mixed lineage leukemia (MLL) is implicated in at least 10% of acute leukaemia of various types. In general, the prognosis is poor for patients harboring MLL translocations (Eguchi et al 2005). In these patients, the MLL protein fuses to 1 of >50 identified partner genes, resulting in an MLL fusion protein that acts as a potent oncogene (Krivtsov et al 2007). The amino-terminal portion of MLL serves as a targeting unit to direct MLL oncoprotein complexes to their target loci through DNA binding, whereas the fusion partner portion serves as an effector unit that causes sustained transactivation.

Gene Mutations in AML

Approximately 40% to 50% of patients with AML have a normal karyotype and represent the largest subset of AML (Mrózek et al 2007). All such cases of cytogenetically normal AML are currently categorized in the intermediate-risk group; yet, this group is quite heterogeneous, and not all patients in this subset have the same response to treatment. This is likely a result of the large variability in gene mutations and gene expression in this population. These alterations appear to fall into 2 broadly defined complementation groups. One group (class I) comprises mutations that activate signal transduction pathways and thereby increase the proliferation or survival, or both, of hematopoietic progenitor cells. The other complementation group (class II) comprises mutations that affect transcription factors or components of the cell cycle machinery and cause impaired differentiation.

Class I Mutations

Mutations in KIT, FLT3, and NRAS fall into the class I mutations.

KIT mutations

Although patients with AML and inv(16) and t(8;21) in general have a more favorable prognosis, there remains a significant failure rate, and the long-term disease-free survival rate is approximately 60%. Studies have shown that activating KIT mutations in approximately 30% to 40% of patients with inv(16) are associated with higher incidence of relapse and significantly lower survival. In those with t(8;21), the incidence of KIT mutations appears to be variable (Patscka et al 2006).

FLT3 mutations

Fms-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase that plays a key role in cell survival, proliferation, and differentiation of hematopoietic stem cells (Stirewalt et al 2003). It is frequently overexpressed in acute leukaemia. FLT3 mutations occur in approximately 30% of AML patients and confer a poor prognosis. The 2 major types of mutations that occur are internal tandem duplication (ITD)

mutations of the juxtamembrane region and point mutations in the tyrosine kinase domain (TKD), which frequently involve aspartic acid 835 of the kinase domain. Both mutations result in constitutive activation of the receptor's tyrosine kinase activity in the absence of ligand (Small et al 2008). The incidence of FLT3 mutations also increases with age, but the FLT3 ITD mutations have less prognostic impact in patients >60 years of age possibly because other adverse prognostic factors are more prevalent.

RAS mutations

Mutations in NRAS and KRAS occur in approximately 10% and 5% of AML patients, respectively. IRASS mutations occur only rarely in conjunction with FLT3 mutations and do not appear to have a significant impact on AML survival (Tyner et al 2009).

Class II Mutations

In addition, mutations in MLL, brain and acute leukemia gene (BAAL), Wilms tumor gene (WT-1), CCAAT/enhancer-binding protein α (CEBP α), and nucleoplasmin 1 (NPM1) have also been observed in AML patients (Nerlov et al 2004). Recently, mutations in DNA methyltransferase gene *DNMT3A* have been identified in one third of patients with *de novo* AML with intermediate-risk cytogenetics (Ley et al 2010). *DNMT3A* represents 1 of 3 human genes that encodes DNA methyltransferase that catalyzes the addition of methyl groups to cytosine within CpG dinucleotide, resulting in repression of nearby genes. Genomes with *DNMT3A* mutations commonly harbored additional mutations in *FLT3*, *NPM1*, and *IDH1*. The presence of any *DNMT3A* mutation, either alone or in combination with *FLT3* ITD mutation, is associated with significantly shorter overall survival (OS) (Ley et al 2010).

2.5 Methods for Detection of Genetic Lesions

Current standards for acute myeloid leukaemia (AML) diagnosis integrate the study of cell morphology, immunophenotype and genetics/cytogenetics as detailed in the

2008 World Health Organisation (WHO) classification of lymphoid neoplasms (Vardiman *et al*, 2009). The innovated technologies, such as gene-expression analysis, comparative genetic hybridization (CGH) and Small Nuclear Polymorphisms (SNP) arrays, still remaining at the experimental level, but have the potential to hold a place in routine clinical evaluation and management of the disease (Tatyana *et al*, 2011).

Morphological Characteristics

A morphological bone marrow assessment represents the first step in the diagnostic pathway for the primary diagnosis of AML and for the differentiation from acute lymphoblastic leukaemia (ALL), since AML, by definition, always presents with bone marrow involvement (Lai *et al*, 2000; Vardiman *et al*, 2009). From the morphological point of view, there are no reproducible criteria to distinguish between B- and T-lineage AML.

Immunophenotyping

Immunophenotyping is now usually performed largely by flow cytometry using anticoagulated whole blood or bone marrow samples in which red cells have been selectively lysed. In this technique, antibodies labelled with different fluorochromes recognise the pattern and intensity of expression of different antigens on the surface of normal and leukaemic cells. Normal cells each have a characteristic profile but malignant cells often express an aberrant phenotype that can be useful in allowing their detection (Hoffbrand, 2006). However, flow cytometry requires a high degree of expertise and a thorough understanding of technical details, e.g, characteristics of fluorochromes, gating strategies, compensation, etc, for meaningful analysis (Rishu and Surender, 2013).

Cytogenetics/ Florescent *In Situ* Hybridization Studies

Cytogenetics and Florescent *In Situ* Hybridization (FISH) studies play an important role in the diagnosis of haematological neoplasms. They also help in deciding management of patients as well as to predict prognosis, response to treatment and

disease progression (Rishu and Surender, 2013). FISH involves fluorescently labelled probe binding to specific chromosome sequences that is visualized under fluorescent microscope. The structural microscopic or submicroscopic cryptic lesions, as well as different numeric chromosomal changes can be identified depending on a probe design (Wolff *et al*, 2007).

The FISH technique is comprehensive, reliable and available for clinics approach, but it requires expensive basic equipment and does not allow the detection of all targets of interest simultaneously, and is not sensitive enough for effective monitoring of minimal residual disease (MRD) (Manuel, 2003).

Expression Microarray Analysis

Array-based gene-expression profiling (GEP) combines synthesis of complementary DNA (cDNA) from the entire mRNA transcriptome with DNA array technology to evaluate the entire transcriptome of samples. GEP is used to determine the level at which genes are expressed in samples compared with controls rather than evaluating changes in copy number or sequence of the nuclear DNA, as it is in the case of PCR and FISH (Mrozek *et al*, 2013). Sample cDNA is generated from mRNA, labelled with fluorochromes, and subsequently hybridised to chips spotted with probes corresponding to known transcripts. Fluorescent signals are captured and analysed. The intensity of the signal at each spot corresponds to the amount of cDNA and, thus, mRNA or expression of the gene targeted by the spot on the probe (Mrozek *et al*, 2013).

Although expression arrays analyse gene expression, and thus could potentially identify highly differentially expressed genes as target entry points for biological studies, for the most part expression profiling has been used for classification of leukaemias, rather than the identification of genes whose aberrant expression may be important in leukemic transformation (Armstrong *et al*, 2003).

Array-Based Comparative Genomic Hybridization

Array based comparative genomic hybridisation (a-CGH) is a recently developed technology that combines the ability to screen the entire genome like conventional comparative genomic hybridization (CGH) with the ability to detect small variations in DNA copy number. This broad-spectrum, high-resolution technique uses cloned DNA fragments of relatively large size (100–200 kb), or, more recently, long (60–75 nucleotides) oligonucleotides spotted onto a solid matrix. The resolution of this technique is determined by the size of and distance between the clones used to construct the array. Test sample and control DNA are labelled with different fluorochromes and then hybridised to the DNA on the array (Oostlander *et al*, 2004). A-CGH augments traditional karyotyping in the detection of deletions or amplifications smaller than those that can be found by banding techniques, and allows analysis of samples for which metaphase chromosomes are not available (Kuchinskaya *et al*, 2008).

Single Nucleotide Polymorphism Array Analysis

Single nucleotide polymorphism (SNP) arrays are oligonucleotide arrays with probes specific for regions flanking SNPs. When labelled genomic DNA from an individual is hybridized to the array, the DNA will bind with greater frequency to the probes that correspond to that individual's SNPs, and those regions of the chip will fluoresce with greater intensity. (Mullighan, *et al*, 2009). In the recent development, using these types of chips, researchers have been able to identify new leukaemia associated genes by identifying small regions of acquired deletions, amplifications, and uniparental disomy in acute leukaemias and associated some of these genetic aberrations with high-risk leukaemias (Kuiper *et al*, 2007; Mullighan, *et al*, 2008).

Next Generation Sequencing

Nucleic acid sequencing is a method for determining the exact order of nucleotides present in a given DNA or RNA molecule and massively parallel sequencing

technology such as Next Generation Sequencing (NGS) facilitates high-throughput sequencing, which allows an entire genome to be sequenced in less than one day (Grada and Weinbrecht, 2013).

The biggest challenge for the genetics laboratory when creating large amounts of sequence data by NGS as indicated by the National Genetics and Genomics Education Centre is the vast quantity of computer data that is produced. This can be as much as 600 gigabytes in a single run that require management and storage in a safe and secure manner as well the responsibility of reviewing and scientifically interpreting the data produced to translate into useful information that can be passed back to the clinician and patient. This is the responsibility and role of Clinical Scientists and technologists working closely with Clinical Bioinformaticians in the laboratory.

Multiplex Reverse Transcription Polymerase Chain Reaction

Most translocations are best detected by reverse transcription-polymerase chain reaction (RT-PCR) in the routine diagnostic setting. (Haferlach *et al.*, 2005).

Standard RT-PCR assays for the individual detection of the prognostically significant fusion transcripts in AML is labour-intensive, material-demanding and not practically feasible. In addition, several primer sets are required for each translocation because of breakpoint diversity in these fusion genes, further increasing the number of reactions that need to be performed. Multiplex protocol has therefore been developed to diminish time and labour intensity of procedure.

The multiplex RT-PCR assay is usually multi-stage: the reaction is followed by a series of identifying PCR reactions with primers specific for individual translocations. The hybridisation step increases specificity of the assay and at the same time reduces associated costs and amount of patient material required (Pallisgaard *et al.*, 1998). This assay has been employed in the detection of 29 translocations/ chromosomal aberrations simultaneously, including more than 80 mRNA breakpoint or splice variants (Pallisgaard *et al.*, 1998).

The molecular laboratories of the BIOMED-1 Concerted Action, in an extensive collaborative studies by the 10 laboratories over a period of 4 years, have resulted in a

standardized RT-PCR protocol and PCR primer sets for the detection of nine well-defined chromosomal aberrations (van Dongen *et al*, 1999). It is a fast, accurate and sensitive method compared to other molecular methods such as FISH, and it is relatively affordable in countries with limited economic resources (Ariffin *et al* 2003).

The integration of results of several techniques, i.e. gene expression profiling (GEP), SNP array analysis, and currently next-generation sequencing (NGS), have permitted a better definition of the molecular scenario of AML and the identification of a constellation of novel mutations (Chiaretti *et al*, 2014).

Molecular techniques can contribute to establishing the correct diagnosis, prognostic stratification and predicting and assessing response to treatment in haematological malignancy (Rishu and Surender, 2013). In Zambia, this has not been done despite recommendations from the World Health Organisation to utilise these techniques.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design, site and period

This was a descriptive cross-sectional study. It was done at The University Teaching Hospital (UTH), Lusaka, Zambia. This site was purposely chosen owing to the fact that, the UTH is the largest hospital in Zambia and offers both inpatient and outpatient care and is a centre for specialist referrals from across the country. Furthermore, the site was used because of the availability of the facilities and equipment needed to conduct this study.

3.2 Target Population

Patients with Onco-haematological diseases being managed at the Onco-Haematology Clinic of the University Teaching Hospital in Lusaka.

3.3 Sampling frame

The study population was all patients with Onco-haematological disease being managed at the Onco-Haematology Clinic of the University Teaching Hospital in Lusaka.

3.4 Sample size

Prevalence of AML at The UTH is unknown. Since the prevalence is unknown, a conservative estimate of 50% was used. In order to estimate the prevalence within 5% (or 0.05) and considering 95% confidence level, a convenient sample size of 28 was analysed, as shown by the calculation. Then every k^{th} case will be selected (Castillo *et al*, 2009).

Applying correction for finite population size formula (Dell *et al*, 2002), the new sample size was:

$$\text{New ss} = \frac{n}{1 + \frac{n-1}{\text{pop}}}$$

Where, ss= sample size; pop = population,

$$\text{We calculate the new } n = \frac{n}{1 + \frac{n-1}{N}}, = \frac{400}{1 + \frac{400-1}{48}} = 43$$

n= sample size 400 as calculated above, N=Total number of AML cases from January 2011 to December 2014.

Therefore, the minimum sample size (n) was 43.

A convenient sample size of 28 was used in this study based on the leukaemia survival record reviewed at the oncology ward that had a cohort of 48 acute myeloid leukaemia patients as at December 2014. Of this cohort, some were lost to follow up and others had died. Our convenient sample size was based on the availability of cases within the study period.

3.5 Inclusion Criteria

All available confirmed cases of AML both newly diagnosed patients based on morphology and those already on treatment for AML bearing in mind that genetic abnormalities can still be detected in the early induction & consolidation phases of treatment at The UTH were enrolled in our study. This included both male and female patients of all age groups diagnosed with or suspected to have AML.

3.6 Exclusion Criteria

Patients with Onco-haematological diseases other than AML were excluded.

2.7 Materials and Methods

Identification of AML patients

Participants were recruited at Onco-haematology Clinic during the haemato-oncology clinic days. As the participants were being seen, the attending clinician would inform and explain the study to them, and also provide each participant with the study information sheet. Patients that are referred to the clinic were also included in the study. Parents/guardians whose children/wards are enrolled in this study were duly informed on the benefits of this study to their children/wards. Patients that agreed to be part of the study were required to sign the consent form, and assigned a serial number. Thereafter, the patient's demographic data, medical and drug history was collected and compiled using a questionnaire. The data collected was summarized in a frequency table. The variables of interest included; age, sex, location of residence and clinical manifestations at diagnosis.

Specimen Collection, Preparation, Analysis and Storage

Blood samples (4ml) were collected from the consenting research participants via venepuncture from the ante-cubital vein using the Evacuated Tube System (ETS) into EDTA containers for determining full blood count, peripheral smear examination and the PCR analysis. Samples were well mixed to avoid clotting. As shown in figure one.

Complete Blood Count

A well-mixed blood sample was processed on the Sysmex XT 2000i and 4000i machines (Sysmex Corporation, Kobe, Japan). Whole blood was stored at 2 to 8°C for 4 days in case the tests were not run the same day. EDTA samples were analysed

using either Sysmex XT 4000i or 2000i. The machine counts the numbers and types of different cells (WBC, RBC, platelets) within the blood. The results were authorised and printed via the laboratory information management system (LIMS).

Peripheral Smear Procedure

A drop of blood was placed on one end of a clean glass slide and holding a spreader at 45°C, a single layer of cells was made for easy visualisation. The film was then air dried. The smear was labelled on the frosted end of the slide using an HB pencil. The film was fixed in absolute methanol for 5 minutes and then transferred to a jar containing May-Grunewald stain freshly diluted with an equal volume of Soresen's buffer. It was then allowed to stain for 15 minutes and then transferred to a jar containing Giemsa's stain freshly diluted with 9 volumes of the buffer. The slides were allowed to stain for 15 minutes and then transferred to a jar of buffer for 5 minutes. Slides were then allowed to drain dry in an upright position and later examined under oil immersion for malignant cells to determine the phenotype.

Bone Marrow Aspirate Smear Procedure

The bone marrow aspirate sample for the study were obtained from that which was meant for routine clinical work-up. A drop of bone marrow aspirate with particles was placed on one end of a clean glass slide and holding a spreader at 45°C, a single layer of cells was made for easy visualization. The film was then air dried. The smear was labelled on the frosted end of the slide using an HB pencil. The film was fixed in absolute methanol for 5 minutes and then transferred to a jar containing May-Grunewald stain freshly diluted with an equal volume of Soresen's buffer. It was allowed to stain for 15 minutes and then transferred to a jar containing Giemsa's stain freshly diluted with 9 volumes of the buffer. The slides were allowed to stain for 15 minutes and then transferred to a jar of buffer for 5 minutes. Slides were then allowed to drain dry in an upright position and later microscopic examination under oil immersion for malignant cells to determine the cytomorphological phenotype.

Bone Marrow Cell Collection

We Pipetted the solution containing cells into a 50 ml conical tube (for optimal results the strain cell material was strained through a cell strainer to remove any large particles).

To maximize the number of cells collected, the cell strainer was rinsed in a Petri dish with extra *Phosphate Buffered* Saline (PBS). This was centrifuged at low speed at 1,400 rpm (400 x g) for 5 minutes at room temperature. The supernatant was discarded without disturbing the pellet. Centrifuge again at 400 x g for 1 minute and carefully remove all traces of remaining supernatant.

Cell Lysis

The lysis of cells was done by resuspending the cell pellet with 300 µl of Solution G1 (RBC Lysis Solution) and transferring it into a clean 2.0 ml microcentrifuge tube, then incubating for 1 minute at room temperature, inverting gently two times during this incubation. Centrifuge for 30 seconds at 13,000x g removing 280 µl of supernatant afterwards using a pipette tip leaving behind 20 µl of supernatant. The vortex was used to mix by vibrating the resuspended pellet completely. Thereafter, Checking Solution G2 (Cell Lysis Solution) was added. If precipitated, heat to 55-65°C for 5 minutes to dissolve and adding 300 µl of Solution G2. This was followed by Pipetting up and down to lyse the cells. This was done with caution to avoid excessive pipetting as it could lead to sheared genomic DNA.

NOTE: Samples were stable at room temperature in Solution G2 for extended periods; up to 8 months. Sample should be at room temperature before proceeding.

About 1.5 µl of Proteinase K Solution (20mg/ml) will be added and followed by incubation at 55°C for 15-30 minutes with inversion at least once during the incubation.

Protein Precipitation

The protein precipitation was done by adding 100 µl of Solution G3 (Protein Precipitation Solution) and vortex to mix. Then centrifugation at 13,000-16,000x g for 5 minutes.

DNA Precipitation

The clear supernatant was removed and transferred to a clean 2 ml microcentrifuge tube. Thereafter, add 300 µl of 100% Isopropanol, mix the sample by inverting 15 times and incubating at room temperature for 3 minutes. Then Centrifugation at 13,000 x g for 5 minutes and carefully discard the supernatant. A small DNA pellet should be visible. We added 300 µl of 70% Ethanol and Invert tubes 5 times to wash the pellet. It was then centrifuged at 13,000 x g for 1 minute. Lastly, inverted and drained the tube on a clean absorbent paper and air dried it for 1-2 minutes.

DNA Hydration

The DNA hydration was done by adding 50 µl of Solution G4 (DNA Hydration Solution). Then, incubating at 65°C for 1 hour. DNA samples were incubated at room temperature overnight. We tapped the tube at least twice during the incubation. Thereafter, Centrifuged the tube containing DNA sample for 20 seconds. The Genomic DNA in the tube was now ready to be used for any application.

NOTE: DNA can be stored at 4°C. For long term storage, store at -20°C or -80°C.

We used the UltraClean™ Bone marrow DNA Isolation Kit.

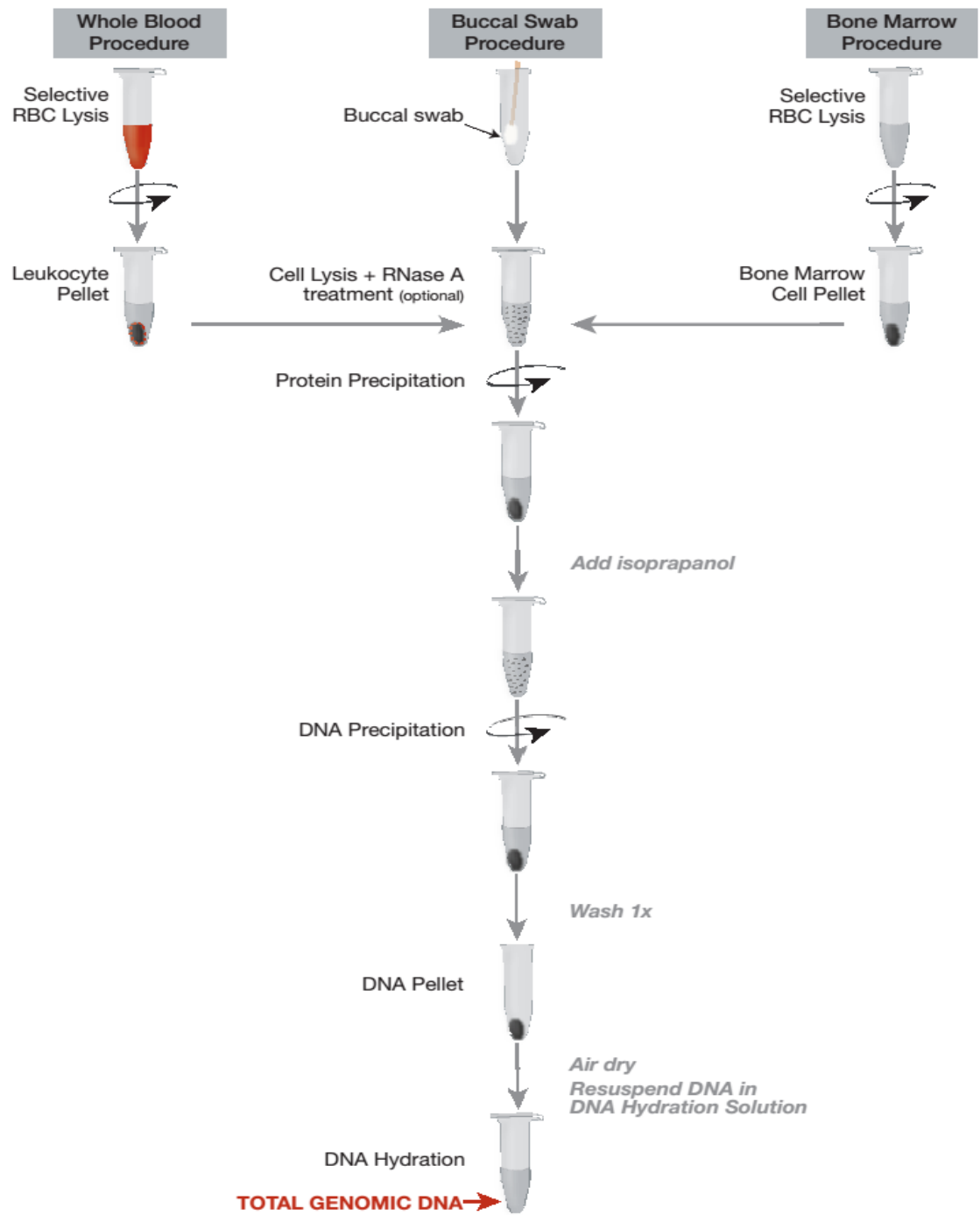


Figure 1: Summary of Specimen Collection, Preparation, Analysis and Storage

DNA Estimation

The DNA concentration was estimated by ultraviolet spectroscopy at 260nm. A DNA sample with an optical density (OD) of 1 at 260nm corresponds to a DNA concentration of 50µg/ml of double-stranded DNA. The purity of the DNA was determined by a DNA/protein absorbance ratio of 260nm/280nm. The DNA was stored at -20°C until required.

AML Detection

DNA Extracted from bone marrow as described above, was used and AML gene sequences was targeted for t(8;21), t(16;16) and t(15;17) by PCR amplification using the following AML primers: NK and NPM11/FLT3 ITD-;NK and CEBPA1/1. These primers would amplify a t (16; 16) and t (8; 21). The primer needed to amplify t (15; 17) is still under consideration i.e. not yet available. PCR was performed in a 100µl reaction containing 1 x UITm a reaction buffer, 1mM each primer, 40mM dNTPs, 1.5mM MgCl₂ and 3 units of DNA polymerase for 40 cycles. Each cycle consisted of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 20 seconds and primer extension at 72°C for 45 seconds. After all cycles are completed, a final elongation step at 72°C for 7 minutes was done. Amplification products were subjected to electrophoresis on a 1.5% agarose gel.

AML Subtyping

PCR was performed on DNA extracted from bone marrow tissue described above. PCR was carried out by using AML primers indicated in the fore-going section. DNA fragments were extracted from bone marrow for direct use in nucleotide sequencing reactions. Detection of t (8; 21) and t (16; 16) was performed by using standard PCR assays specific primers: NK and NPM11/FLT3 ITD-; NK and CEBPA1/1 for AML in two sets of amplification tubes. After denaturation of the template DNA at 94°C for 5 minutes, the PCR was performed for 25 cycles. Each cycle consisted of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 20 seconds and primer extension at 72°C for 45 seconds. After all cycles are complete, a final elongation step at 72°C for 7 minutes was done. Ten microliters of the PCR product was electrophoresed in 2% agarose gel containing ethidium bromide. The products of the type-specific primers were visualized under ultra violet

light. A DNA marker was used as a molecular size marker. A translocation specific band would show the test is positive for a translocation. The target translocations are shown in Table 1. The identity of this translocation was established by the PCR.

Table 1: Chromosomal alteration targets.

Chromosomal Alterations	Genes Involved	Primer Sequence (5'-3')
t(8;21)(q22;q22)	RUNX1	AGCCGAGTAGTTTTTCATCATTGC
	RUNX1T1	TCTCCTATCTCGGGTGAAATGTC
t(15;17)(q24;q21)	PML	GTGCGCCAGGTGGTAGCTC
	RARA	AAGCCCTTGCAGCCCTCAC
inv(16) (p13;q22)	CBFB	TTTGAAGGCTCCCATGATTCTG
	MYH11	AGGTCCCCTTCCAGCTTCTTCT

Statistical Analysis

A descriptive analysis technique was used to analyse the data using Graph pad software version 6.0. Demographic characteristics of AML patients and responses to questions was organised into categorical or numerical data. The Chi-square and Fischer exact test was used to correlate the clinical and laboratory features. A *p*-value of <0.05 was taken as statistically significant.

CHAPTER 4: RESULTS

The distribution of the 28 cases that were finally included in the study, according to the FAB classification was as follows: M0 in two (2) cases, M1 in four (4), M2 in five(5), M3 in seven(7) (6 hypergranular and 1 microgranular), M4 in five(5), M5 in four(4), M6 in one(1) and M7 zero(0) case. There were 17 males and 11 females, with a median age of 63 years (range 6-90). Four patients were aged <14 years. The relative frequencies of each group according to the FAB system and WHO classification within the 28 consecutive valid cases are shown in Table 2. Six (6) patients (21.5%) were found to have the PML/RAR- α fusion transcript (three with type bcr1, one bcr2 and two bcr3) that identifies the presence of t(15;17). Of these, 5 (83%) corresponded to cases identified as having a M3 morphology (4 hypergranular, 1 microgranular), while there was one case initially diagnosed as having an M0 morphology. The presence of the AML1/ETO fusion transcript which results from the t(8;21) was detected in only one case (3.6%). This case displayed a M2 morphology, while the remaining other cases with such morphology were negative. The presence of the CBF- β /MYH11 fusion transcript was detected in two cases (7.1%). The CBF- β /MYH11 positive cases was identified as a FAB-M4, but without a sufficient number of eosinophils to be classified as M4Eo. The MLL gene was rearranged by Southern blot analysis in four cases (15.4%). These included one M1, one M2, one M4 and one M5. The most frequent chromosome/molecular rearrangements, that is, t(15;17)(q22;q12 ~ 21)/ PML-RARA characteristic of acute promyelocytic leukemia (APL), AML with 11q23 abnormalities (MLL), inv(16)(p13q22)/t(16;16)(p13;q22)/CBF β -MYH11 characteristic of core-binding factor (CBF) AML and t(8;21)(q22;q22)/RUNX1-RUNX1T1 and, confer favourable clinical outcome when patients receive optimal treatment, that is, regimens that include high-dose cytarabine for CBF AML and all- trans -retinoic acid and/or arsenic trioxide for APL. Recently, mutations in such genes as KIT in CBF AML and FLT3 in APL have been correlated with clinical features and/or outcome of patients with these AML subtypes, and microarray gene expression profiling has been successfully used for diagnostic purposes and to provide biologic insights. As far as the age distribution was concerned, the study sample was representative of the population of our country.

Table 2. Relative frequencies of the different AML subtypes in patients

Acute myeloid leukemias	cases	
	n = 28	%
<i>AML with recurrent cytogenetic translocations</i>	13	46.5%
AML with t(15;17) PML/RAR-□□	6	21.4%
AML with t(8;21) AML1/ETO	1	3.6%
AML with inv(16) or t(16;16) CBF-□/MYH11	2	7.1%
AML with 11q23 abnormalities (MLL)	4	14.3%
<i>AML not otherwise characterized</i>	15	53.6%
AML minimally differentiated	1	3.6%
AML without maturation	3	10.7%
AML with maturation	5	17.9%
Acute myelomonocytic leukemia	2	7.1%
Acute monocytic leukemia	3	10.7%
Acute erythroid leukemia	1	3.6%
Acute megakaryocytic leukemia	0	0.0%
Acute basophilic leukemia	0	0.0%
Acute pan-myelosis with myelofibrosis	0	0.0%

Table 3. Distribution of chromosomal translocations in AML according to patients' age.

Acute myeloid leukemias	6-35 years	36-65 years	>66 years	Total
AML with t(15;176) PML/RAR-⟨	4 (67%)	1 (17%)	1 (17%)	6 (21.4%)
AML with t(8;21) AML1/ETO	0 (0%)	0 (0%)	1 (100%)	1 (3.6%)
AML with inv(16) or t(16;16) CBF-®/MYH11	1 (50%)	1 (50%)	0 (0%)	2 (7.1%)
AML with 11q23 abnormalities (MLL)	1 (25%)	1 (25%)	2 (50%)	4 (14.3%)
AML not otherwise characterized	2 (13%)	3 (20%)	10 (67%)	15 (53.6%)
Total	8 (29%)	6 (21%)	14 (50%)	28

AML high in > 66 and < 35years.

t(15,17) PML/RAR was the commonest chromosomal translocation in our patients mostly below 35years of age and AML1/ETO t(8,21) the least common.

CHAPTER 5: DISCUSSION

In our study we analyzed, a number of AML patient representatives of the population from our country, the chromosomal abnormalities in AML patients at the University Teaching Hospital according to WHO 2008 revised classification. Two important prerequisites of this study were:

i) the patients were recruited from a national referral hospital as based on AML cases and

ii) in all cases the four genetic abnormalities were simultaneously explored, using well-standardized molecular techniques according to BIOMED-1 protocols.

Our data show that in Zambia the PML/RARA t(15;17) is more frequent and the AML1/ETO t(8;21) is rarer than in other countries (USA and North-Central Europe). The PML/RAR t(15;17) and AML1/ETO t(8;21) in USA was found to be equal at 10% (Kumar et al,2011). The frequency of these alterations may differ according to geographic distribution.

The PML-RARA protein binds to corepressor/histone deacetylase (HDAC) complexes with higher affinity than the wildtype RARA, leading to aberrant chromatin acetylation and alterations of chromatin conformation inhibiting the normal transcription of genes regulated by RARA. This blocks cell differentiation and leads to the accumulation of abnormal promyelocytes (Jing et al,2004). Notably, therapeutic doses of all- trans -retinoic acid (ATRA) change conformation of the PML-RARA protein and release corepressor/HDAC complexes resulting in transcriptional activation of downstream target genes. Additionally, both ATRA and arsenic trioxide, a compound also used to treat APL, induce proteolysis of PML-RARA protein resulting in granulocytic differentiation of the leukemic blasts (Jing et al,2004). While more APL patients carry t(15;17) or its complex variants, some of the patients harbor an insertion of chromosomal material from 17q with the RARA gene into 15q22, the PML gene locus (Grimwade et al, 2000). Most of these insertions are cryptic, associated with a normal karyotype, and detectable only using reverse transcription – polymerase chain reaction (RT – PCR) and/or fluorescence in situ

hybridization (FISH). In about one-third of APL patients with t(15;17), this translocation is accompanied by at least one secondary aberration, most commonly +8 or a partial trisomy of 8q (Hernández et al,2001). The presence of secondary chromosome abnormalities does not impact on prognosis of APL patients treated with chemotherapy and ATRA (Sanz et al,2005). The rearrangements of PML/RAR t(15;17) is very strongly correlated with characteristic marrow morphology in which abnormal promyelocytes predominate (FAB M3). The diagnosis should always be supported by results of cytogenetic, FISH, and/or RT – PCR analyses. RT – PCR determination of the particular PML-RARA isoform in each case is important for disease monitoring because the probability of relapse is increased in patients who after 3 – 4 cycles of ATRA and chemotherapy are still positive for the presence of the PML-RARA transcript, whereas persistent negative RT – PCR results correlate with long-standing remissions in most, but not all, patients (Lo-Coco et al,2006). The historically very poor prognosis of APL patients with t(15;17)/ PML-RARA , with a median survival of 2 weeks in the 1960s, has become favorable with the use of therapies containing anthracyclines, ATRA, and/or arsenic trioxide, with a cure rate exceeding 80% in recent studies (Frankfurt et al,2006). Recently, gemtuzumab ozogamicin, which is a monoclonal antibody directed against CD33, an antigen present on leukemic blasts of almost all patients with APL, has been shown to be effective in producing molecular remissions in both newly diagnosed and relapsed patients (Lo Coco et al, 2006). In one study, a combination of gemtuzumab, ATRA and arsenic trioxide was successfully used to induce prolonged second CRs in APL patients who had experienced first hematologic recurrence (Aribi et al, 2007).

Translocation (8;21) is the first reciprocal translocation in AML that was identified using banding techniques (Rowley et al, 1973). Both the standard t(8;21) and its relatively rare variants, that is, complex translocations involving three or four different chromosomes that consistently affect bands 8q22 and 21q22 or the insertions ins(8;21)(q22;q22q22) or ins(21;8)(q22;q22q22) (Mrózek et al, 2001), disrupt the RUNX1 gene that encodes subunit alpha of core-binding factor (CBF), and lead to the creation of a chimeric gene RUNX1-RUNX1T1 (AML1-ETO) (Miyoshi et al, 1993). The presence of t(8;21)/ RUNX1-RUNX1T1 is associated with

AML with maturation in the neutrophil lineage (FAB M2). It has been suggested that characteristic pink-colored cytoplasm of neutrophils and an increased number of eosinophil precursors without abnormalities typical for AML with inv(16)/t(16;16) morphologically distinguish patients with t(8;21) from other patients with AML M2 who do not harbor t(8;21)/RUNX1-RUNX1T1 (Nakamura et al, 1997). The prognosis of patients with inv(16)/t(16;16) and those with t(8;21) is relatively favorable (Bloomfield et al, 1994). Because of these similarities in response to treatment and of involvement of subunits of CBF at the molecular level, many clinical trials and reports have combined patients with inv(16)/t(16;16) with those with t(8;21) into one, favorable risk prognostic category of AML(Mrózek et al, 2008). Nevertheless, despite similarities, patients with t(8;21) differ from those with inv(16)/t(16;16) with respect to many pretreatment features. In univariable analyses, neither relapse risk nor overall survival (OS) differed significantly between t(8;21) and inv(16)/t(16;16) groups (Schlenk et al, 2004). However, after adjusting for age, log(WBC), and log(platelets), the OS of t(8;21) patients was significantly shorter than OS of those with inv(16)/t(16;16) (Marcucci et al, 2005). The difference may be in part explained by a dissimilar response to salvage treatment because t(8;21) patients had a significantly shorter survival after relapse than inv(16)/t(16;16) patients in three large, independent studies (Appelbaum et al, 2006).

Although the cases were also analyzed by cytogenetics, there were a substantial number of cases lacking these data due to inappropriate storage or transportation, or due to the lack of metaphases or clonal cytogenetic abnormalities after the analysis. So, no epidemiological data can be added based on conventional cytogenetics, although we can say that no differences were observed between the two techniques when cytogenetics was available. Using cytogenetic and morphologic data it was reported that the incidence of M3/t(15;17) positive AML was between 5% and 11% in United States and North-Central Europe(Head et al, 1995). More recent data confirm that the frequency is around 10% (Grimwade et al, 1998), as it is in Australia and Japan (Nakase et al, 2000). In a British series including 1,612 AML patients and based on cytogenetic data, the incidence of t(15;17) is 13%(Grimwade et al, 1998).However, there are some data suggesting that the frequency of the t(15;17) can

be higher in some countries and/or specific populations. Thus, this frequency increases to 17% in Italian children and seems to be over 20% in countries such as Brazil, Mexico, Peru and Spain (Ruiz-Argüelles et al, 1998). Even more significantly, this frequency has been shown to be as high as 37.5% in patients of Latin origin from Los Angeles (Douer et al,1996). However, as mentioned above, these findings are mainly based on morphologic and cytogenetic data, so some variations could be expected upon using molecular analysis. Our study confirms that AML with t(15;17) represents one quarter of all *de novo* AML, which actually means that it is the most frequent type of acute leukemia in our population. Moreover, the incidence is greater in younger patients, which makes this epidemiological data even more valuable, since such patients can take advantage of curative treatments and quick screening tests for the disease. The second observation of note in our study is the extremely low frequency of t(8;21) AML1/ETO found: only 3.6%, even when we included specially referred patients . So far, we have only seen few cases in all AMLs already screened in the haematology laboratory for this genetic abnormality. The initial reports showed a global frequency of t(8;21) of around 15%, increasing within those cases with FAB-M2 morphology. However, marked geographic variations have been shown. Thus, the reported incidence of t(8;21) within the FAB M2 subtype ranges from 58-88% in Asian patients, 19-54% in other European countries and 12-27% in USA .In addition, the most recent data from European countries based on cytogenetic or molecular studies have shown that the frequency of this aberration is around 8% of all AMLs(Grimwade et al, 1998). These data indicate that, according to the frequency detected in our study (3.6% of all AMLs; 6% of all FAB M2 morphologies), this genetic abnormality is more unusual in Zambia than in other countries, although apparently the incidence of t(8;21) is higher in young AML patients in other countries. Nevertheless, this factor is not strong enough to justify the great variation that has been obtained compared to previous reports and moreover, our analysis according to age subgroups did not show any difference. Finally, the frequencies of the CBF- β /MYH11 fusion gene and MLL rearrangements (7.1% and 14.3%, respectively) coincide with data reported in the literature (Grimwade et al, 1998).

Upon dividing into three age groups (6-35, 35-65 and >60 years), we observed that the overall frequency of leukemia displaying one of the four recurrent cytogenetic translocations was much higher in the youngest age group. By contrast, the frequency of AML not otherwise characterized increased with age (13%, 20% and 67%) ($p < 0.05$, Table 4). It should be noted that this difference was mainly due to the higher incidence of PML/RAR α positive cases among young AML patients, while AML with 11q23 abnormalities (MLL) abnormalities were more frequent in the advanced age group.

CHAPTER 6: CONCLUSION

6.1 Conclusion

Our study showed that chromosomal alteration PML/RAR t(15,17) which was 21.4% ,was the commonest, whereas AML1/ETO t(8,21) which was 3.6%,was the least common among patients presenting at UTH, Lusaka, Zambia.The use of molecular technique at point of diagnosis would assist in identifying AML with better prognosis by administering appropriate treatment. The results support the existence of chromosomal abnormalities of AML in our Zambian patients. Awareness of these chromosomal abnormalities and morphology could contribute to the design of cost-effective screening strategies, adapted by our National Health systems according to the prevalence of locally detected genetic aberrations. Acquired genetic alterations such as balanced and unbalanced chromosome aberrations and submicroscopic gene mutations and changes in gene expression strongly affect pre-treatment features and prognosis of patients with acute myeloid leukemia (AML).

6.2 Strengths and Weaknesses

One of the strengths of this study is its novelty; no work has been published on the characterization of acute myeloid leukaemia (AML) according to WHO 2008 revised classification in patients at the University Teaching Hospital, Lusaka, Zambia, using cytogenetics.

The weakness is that the study sample size is small to make generalised inferences, however, it has yielded some very important information which could be used to do further studies. Other studies have used similar small sample sizes too, due to high costs for the molecular cytogenetic studies and availabilities of AML cases.

6.3 Future works

Cytogenetic studies with use of Fluorescent in situ hybridisation (FISH) and Polymerase chain reaction would give better insight into the chromosomal abnormalities of the AML we have in our country.

6.4 Recommendation

These data underscore the value of genetic testing for common translocations for diagnosis, prognostication, and, increasingly, selecting therapy in acute leukemia. In addition, this information must be taken into account during the development of therapeutic protocols, since the data reflect the distribution of some leukemias, such as promyelocytic leukemias, with specific therapeutic strategies. We recommend that a study with a much larger sample size be carried out to verify our findings as this was the first study on this topic in Zambia. A larger study not restricted to the University Teaching Hospital would need to be carried out to generate more representative results as a small sample size of 28 does not give a true picture of the AML chromosomal aberration in our country. However, the information from this study can be used as a stimulus to start pushing for the use of Cytogenetic as a routine test for AML patient.

6.5 Dissemination and utilization of findings

The findings of the study were presented to the Department of Pathology and Microbiology, School of Medicine, UNZA. Then, the results were later presented at the postgraduate seminar week. The results will be also presented to various stakeholders involved in the management of AML at various fora such as conferences.

UTH which was a study site will be given a copy of the study results report so that it can be used for further knowledge of AML. Furthermore, five copies of the research report will be printed and submitted to the following;

1. Department of Pathology and Microbiology
2. UTH and Cancer Disease Hospital
3. Ministry of Health
4. UNZA Medical Library and Main Library
5. Research

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APPENDICES

APPENDIX 1 : STUDY AREA APPROVAL

Kachinda Wezi
University of Zambia
School of Medicine
Department of Pathology and Microbiology
LUSAKA,

15TH October 2015.

The Senior Medical Superintendent
University Teaching Hospital
LUSAKA.

Dear Sir,

**RE: REQUEST FOR PERMISSION TO CONDUCT THE RESEARCH PROJECT AT
THE UNIVERSITY TEACHING HOSPITAL (UTH)**

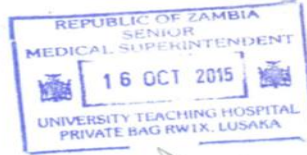
I am a student pursuing a master of science in pathology (haematology) at the University of Zambia, school of medicine.

I have written to request for permission to conduct my research at your institution titled "Characterization of acute myeloid leukaemia according to WHO 2008 revised classification based on cytomorphological and chromosomal abnormalities in patients presenting at The University Teaching Hospital, Lusaka, Zambia".

Please find attached a copy of my project proposal containing all the necessary information. The school has approved the research proposal and Ethical approval will be sought from UNZABREC upon your consideration of my request.

Yours sincerely


Kachinda Wezi.



Handwritten initials and signature

Approved


APPENDIX 2 : GRADUATE FORUM PRESENTATION



THE UNIVERSITY OF ZAMBIA

SCHOOL OF MEDICINE

Telephone : +260211252641

Telegram: UNZA, Lusaka

Telex: UNZALU ZA 44370

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P.O Box 50110

Lusaka, Zambia

21st September, 2015

Dr. Wezi Kachinda
Department of Public Health
School of Medicine
UNZA
LUSAKA

Dear Dr. Kachinda,

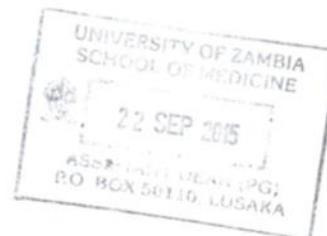
RE: GRADUATE PROPOSAL PRESENTATION FORUM

Following the presentation of your dissertation entitled "**Characterization of Acute Myeloid Leukaemia according to WHO 2008 revised Classification Based on Cytomorphological and Chromosomal Abnormalities in Patients presenting at the University Teaching Hospital, Lusaka** " your supervisor has confirmed that the necessary corrections to your research proposal have been done.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE
Cc: HOD, Pathology & Microbiology



APPENDIX 3: HAEMATOLOGY LABORATORY ATTACHMENT



THE UNIVERSITY OF ZAMBIA
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Telephone: +0211-252641
(Pre-Clinical) Ridgeway Camp
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Department of Pathology & Microbiology
P.O. Box 50110
Lusaka, Zambia

Your Ref:
Our Ref:

24th September, 2015

The Director of Laboratory Services
Department of Pathology & Microbiology
University Teaching Hospital
P.O. Box RW 1X
LUSAKA

Dear Sir,

Re: CLINICAL LABORATORY ATTACHMENT FOR MSc PG STUDENTS

This is to introduce Dr. Kachinda Wezi an MSc Pathology student in Pathology (Haematology). It is the programme requirement that MSc students will be required to learn laboratory techniques used in diagnosis of clinical specimens. It is to this effect that the School of Medicine recommends the above candidate for the clinical laboratory attachment for a period of 1 year to enable him/her learn diagnostic techniques and conduct his/her Research Project in the University Teaching Hospital laboratories.

We request you to kindly facilitate this process.

Yours faithfully,

Dr T. Kaile
p.p Marah Simakando
HOD AND COURSE COORDINATOR FOR MSc PROGRAMME



