CYTOGENETIC AND MOLECULAR PROFILING OF ACUTE MYELOID LEUKEMIA PATIENTS PRESENTING TO THE ADULT HEMATO-ONCOLOGY UNIT OF KENYATTA NATIONAL HOSPITAL

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I, **DR.MERCY NJERI GATUA** medical oncology fellow, department of clinical medicine and therapeutics (UON) do hereby declare that this proposal is my original work and that to the best of my knowledge it has not been presented for the award of a degree in any other university.

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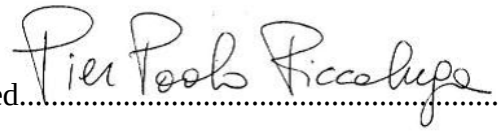
Signed  Date.....

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DEDICATION

This work is dedicated to my dear husband, Dr Peter Mwika, Sons Mwirichia and Kagunye, my parents Kagunye and Wanjiku, my siblings Kuria, Kamau, Wanjiru and Muthoni and last but not least my friend Salome. Thank you all for showing me what true love is.

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LIST OF ABBREVIATIONS

ABL Abelson Murine Leukemia Viral Oncogene Homolog 1

Abn Abnormal

ALC Absolute Lymphocyte Count

AMC Absolute Monocyte Count

AML Acute Myeloid Leukemia

ANC Absolute Neutrophil Count

APL Acute Promyelocytic Leukemia

ASX Additional Sex Combs Like

BCR Breakpoint Cluster Region

BMA Bone Marrow Aspirate

CBF Core Binding Factor

CBFB Core-Binding Factor Subunit Beta

CD Cluster of Differentiation

CEPBA CCAAT Enhancer Binding Protein Alpha

Chr Chromosome

CML Chronic Myeloid Leukemia

CN Cytogenetically Normal

CNS Central Nervous System

Del Deletion

DNA Deoxyribonucleic Acid

DNMT DNA Methyltransferases

ELN European Leukemia Net

ETS E26 transformation-specific

EVI1 Ecotropic Virus Integration Site 1 Protein Homolog

EZH2 Enhancer of Zeste 2

FISH Fluorescence In-Situ Hybridization

FLT3 Fms-Like Tyrosine Kinase 3

G-banding Giemsa Banding

HB Hemoglobin

HLA-DR Human Leukocyte Antigen – DR Isotype

HMA Hypomethylating Agents

HSC Hematopoietic Stem Cell

HSCT Hematopoietic Stem Cell Transplant

IDH Isocitrate Dehydrogenase

INV Inversion

ITD Internal Tandem Duplication

KMT2A Lysine (K)-Specific Methyltransferase 2A

KNH Kenyatta National Hospital

LDH Lactate Dehydrogenase

LFTS Liver Function Tests

LVEF Left Ventricular Ejection Fraction

MDS Myelodysplastic Syndromes

MECOM MDS1 And EVI1 Complex Locus

MFC Multiparametric Flow Cytometry

MKL1 Megakaryoblastic Leukemia 1 Gene

MLLT3	Myeloid/Lymphoid or Mixed Lineage Leukemia Translocated To, 3
MPN	Myeloproliferative Neoplasm
MPO	Myeloperoxidase
MR	Mitral Regurgitation
MRC	Myelodysplasia Related Changes
MYHII	Myosin Heavy Chain II
NGS	Next Generation Sequencing
NOS	Not Otherwise Specified
NPM1	Nucleophosmin 1
NUP	Nucleoporins
PAH	Pulmonary Arterial Hypertensions
PB	Peripheral Blasts
PBF	Peripheral Blood Film
Ph^{+ve}	Philadelphia Chromosome Positive
PLT	Platelets
PML	Promyelocytic Leukemia
PR	Pulse Rate
RARA	Retinoic Acid Receptor Alpha
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma
RBM 15	RNA Binding Motif Protein
RNA	Ribonucleic Acid
RUNX	Runt-Related Transcription Factor

SKY	Spectral Karyotyping
T	Translocation
T-AML	Therapy Related Acute Myeloid Leukemia
TET	Ten-eleven-translocation
TF	Transcription Factor
TKD	Tyrosine Kinase Domain
TR	Tricuspid Regurgitation
TS	Tumor Suppressor Gene
WCC	White Cell Count
WHO	World Health Organization

ABSTRACT

Background-Acute myeloid leukemia is an infrequent disease yet it is associated with high morbidity and mortality. It harbors a unique configuration of cytogenetic abnormalities and molecular mutations that can be detected using microscopic methods and molecular sequencing respectively. These genetic tests are core elements of diagnosis and prognostication in high-income countries. They are routinely incorporated in clinical decision making, allowing for the individualization of therapy. However, these tests are largely inaccessible to most patients in Kenya and therefore no data has been reported on this group of patients.

Objective-To determine the chromosomal abnormalities and molecular mutations of patients diagnosed with acute myeloid leukemia presenting to the adult hemato-oncology unit of Kenyatta National Hospital.

Design: A cross-sectional descriptive study.

Setting: Adult hemato-oncology unit, Kenyatta National Hospital.

Subjects: Patients with a morphological diagnosis of acute myeloid leukemia

Sampling: Consecutive sampling

Methodology- The study participants that met the inclusion criteria and consented/assented to participate were enrolled to a sample size of ten. Peripheral blood samples were collected for conventional metaphase G-banding technique and next generation sequencing methods. A study proforma was used to record the patient's social demographics, clinical and laboratory data.

Study Period: The study was done over a period of three months beginning July- September 2019

Analysis-No descriptive analysis was done due to the small sample size. Descriptive statistics are presented in tables.

Results: Ten patients underwent cytogenetic analysis and next generation sequencing using the Ampliseq for Illumina myeloid panel. Cytogenetic studies yielded no results. There were 29 mutations detected across 13 commonly mutated genes. At least one mutation was detected

for all the patients. Three patients with TP53 mutations were classifiable under the ELN adverse prognostic group.

Conclusions: The study demonstrated that patients with AML in KNH do have deleterious mutations that impart a unique genomic spectrum for each and every patient and contribute to the heterogeneity of disease outcomes among the patients. Lack of cytogenetic analysis and subsequently the results precludes us from drawing definite conclusions on the prognostic effect of these mutations.

CHAPTER ONE: INTRODUCTION

Acute Myeloid Leukemia (AML) is an infrequent disease(1). Although treatment outcomes continue to improve over time, AML is still a significant cause of mortality(2). Despite an improvement in the treatment associated mortality, chemo-resistance and post-transplant disease relapse account for one of the most challenging aspects of AML management(3). It is a heterogeneous clonal disorder that arises from a malignant myeloid stem cell that has acquired genetic and epigenetic mutations that have accumulated in a stepwise fashion. These acquired genetic alterations cause proliferative and survival advantage with reduced apoptosis leading to a buildup of abnormal, poorly differentiated neoplastic cells in the blood and bone marrow with resultant suppression of the normal hematopoietic process(3,4).

Acute myeloid leukemia cells harbor a unique configuration of cytogenetic and molecular mutations that involve critical genes that regulate the normal hematopoietic process(5). This accounts for the phenotypic heterogeneity of the disease(6). Understanding the pathobiology of AML has provided a framework for risk stratification, development of novel treatment approaches with individualization of therapy as well as detection of post treatment minimal residual disease(7–10). The urgent need for therapeutic advancement has come at the backdrop of a dismal five-year overall survival of 50% and 20 % for those below and above 60 years old respectively with traditional cytotoxic therapies(10). A landmark novel anti-leukemic agent should successfully eradicate the malignant founding clone and its sub-clones, eradicating a potential niche for recurrence(5). WHO classification of AML incorporates clinical features, morphological assessment of bone marrow specimens, cytochemical studies, immune-phenotyping, cytogenetic and molecular testing to distinguish distinct biological subgroups with clinical importance(11). The major categories included in the 2016 WHO classification include; AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, AML not otherwise specified, myeloid sarcoma and myeloid proliferations related to down syndrome (**table 1)**(11).

Cytogenetic abnormalities are analyzed using conventional metaphase G-banding techniques and fluorescence in situ hybridization (FISH) whereas molecular mutations are detected using next generation sequencing molecular methods(12,13). These technologies are able to comprehensively identify genetic lesions that are critical in the process of leukamogenesis. These genetic abnormalities are the single most powerful prognostic factors and risk stratify the patient into favorable, intermediate and adverse risk groups(**Table2)**(14). Prognostic

classification is critical in the management of AML patients, particularly in respect to establishing those with poor prognostic features who are likely to relapse or have chemo-resistant disease. It's also important for category-specific treatment(15). However, the prognostic impact of these genetic groups may change with targeted therapy(16).

In sub-Saharan Africa, acute leukemia causes high mortality(17). In a 2012 population-based study, the age-standardized rates in East Africa were 3.8 and 3.4 per 100 000 in men and women respectively(18). Latest Globocan data estimates that in Kenya, the incidence rates of leukemia are 4.8 and 4.5/100,000 in men and women respectively and is listed among the top ten causes of cancer mortality(19). Despite an increasing disease burden in sub-Saharan Africa, there is limited infrastructure and finances that deters the use of recommended genetic testing(17,20). Cytogenetic and molecular techniques, which are core elements of diagnosis in the developed world, are nonexistent in most sub-Saharan African countries(17). Significant financial challenges do exist in emerging economies, where majority of healthcare costs are personal expenditures with many falling below the poverty line. This occurs even in those emerging countries with highly skilled specialists and state of the art facilities that mirror those in developed countries(21).

Data from an ongoing prospective study at Kenyatta National Hospital (Othieno-Abinya NA, unpublished data) shows that, 30 patients are diagnosed with AML annually. The median age at diagnosis is 30 years with a male: female ratio of 1.2:1. AML diagnosis at the hemato-oncology unit of KNH is mainly through morphological assessment of bone marrow specimens with few or none of the patients undergoing karyotyping or molecular assessment. This study therefore seeks to identify the cytogenetic and molecular abnormalities found in patients' diagnosed with AML at the adult hemato-oncology unit of KNH and prognosticate them according to the EuropeanLeukemiaNet risk stratification model.

Table 1. 2016 World Health Organization Classification of Acute Myeloid Leukemia

	Classification
	AML with recurrent genetic abnormalities
	• AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1 • AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11 • APL with PML-RARA • AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A • AML with t(6;9)(p23;q34.1);DEK-NUP214 • AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM • AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1 • Provisional entity: AML with BCR-ABL1 • AML with mutated NPM1 • AML with biallelic mutations of CEBPA • Provisional entity: AML with mutated RUNX1
	AML with myelodysplasia-related changes **Therapy-related myeloid neoplasms** **AML, NOS** • AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monoblastic/monocytic leukemia • Pure erythroid leukemia • Acute megakaryoblastic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis **Myeloid sarcoma** **Myeloid proliferations related to Down syndrome** • Transient abnormal myelopoiesis (TAM) • Myeloid leukemia associated with Down syndrome

Abbreviations: **AML**: acute myeloid leukemia; **APL** acute promyelocytic leukemia; **NOS** not otherwise specified

Table 2. European Leukemia Net risk stratification by genetics

Risk category	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1); RUNX1-RUNX1T1 • inv(16)(p13.1q22) or t(16;16)(p13.1;q22), CBFB-MYH11 • Mutated NPM1 without FLT3-ITD or with FLT3-ITD low* • Biallelic mutated CEBPA
Intermediate	• Mutated NPM1 and FLT3-ITD high* • Wild-type NPM1 without FLT3-ITD or with FLT3-ITD *low (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3); MLLT3-KMT2A • Cytogenetic abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23;q34.1); DEK-NUP214 • t(v;11q23.3); KMT2A rearranged • t(9;22)(q34.1;q11.2); BCR-ABL1 • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1) • -5 or del(5q); -7; -17/abn(17p) • Complex karyotype, monosomal karyotype • Wild-type NPM1 and FLT3-ITD high† • Mutated RUNX1 • Mutated ASXL1 • Mutated TP53

*Low allelic ratio (<0.5); high allelic ratio (>0.5)

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology

Acute myeloid leukemia is the most common acute leukemia in adults whereas in children it accounts for 15-20% of the acute leukemia's(22). It has a relatively high incidence in countries with very high human development indexes. Australia and Austria have the highest incidence rates for men and women at 2.8 and 2.2 /100,000 respectively(18). There is a general male predominance for most leukemia's but more so for AML(23). It has a unimodal age distribution pattern with rates rising with increasing age(1). The median age for diagnosis in western countries is 68 years(24). This increase in incidence in the elderly parallels the increased incidence of myelodysplasia in this age group and is characterized by cytogenetic aberrations related to MDS. In low to middle income countries, AML is diagnosed at a younger age possibly due to the demographics of a younger population but environmental and genetic factors may have a role(21,25–27). At Kenyatta National Hospital, 150 patients have been diagnosed with AML over the last 5 years with an average of 30 cases per year.

2.2 Etiology

De novo AML accounts for the majority of cases, where it arises in a previously healthy individual. Secondary AML arises from exposure to ionizing radiation(28,29), benzene products(30), leukamogenic chemotherapeutic agents(31), genetic disorders(32,33) and antecedent hematological disorders. Genetic predisposition may account for 4-10% of children and adults with MDS or AML. Familial MDS/AML may arise from a background of bone marrow failure syndromes or inheritable germ line mutations in several genes with MDS/AML as the principle diagnosis. Familial non-syndromic MDS/AML disorders arise from mutations in genes coding for transcription factors i.e. GATA2, CEPBA and RUNX1 among others. Many of these genes are also recurrently mutated in de novo AML. The patients are younger and familial clustering may be present. A high index of suspicion is needed as many of these patients may not have a family history to suggest a heritable risk(3,32–34). There is increased risk of myeloproliferative neoplasms and myelodysplasia syndromes transforming to AML(35). Diagnosis of AML requires a blast count of 20% or more in the bone marrow or peripheral blood or the presence of an extramedullary accumulation of blasts as defined by the 2016 WHO classification(11). The transformation is heralded by acquisition of new

cytogenetic and molecular mutations. Secondary AML is associated with an overall poor prognosis with lower rates of complete remission due to primary treatment resistance, older age, underlying organ dysfunction and prolonged cytopenias due to ineffective hematopoiesis(31,36).

2.3 Clinical Presentation

The clinical presentation of AML is heterogeneous with many presenting initially with non-specific signs and symptoms. However with progression of disease and suppression of hematopoiesis, patients present with features of pancytopenia to include, recurring infections, anemia and bleeding tendencies due to thrombocytopenia(1,37). Extramedullary infiltration by leukemic cells can occur in the lymph node, spleen, liver and other non- hematopoietic organs causing gingival hypertrophy, myeloid sarcoma, leukemia cutis, CNS symptoms among others. However this does not predict outcome(38). Patients present with leucopenia or leukocytosis, with a small proportion having a white cell count $>100\times10^9$/l. Majority will have concomitant anemia and thrombocytopenia(39).

2.4 Morphology of AML blasts

Bone marrow assessment using an aspirate or bone biopsy remains a cornerstone in the initial diagnosis and provides morphological description of AML blasts***(figure 1)***(40). In addition it provides information on bone marrow cellularity, % of blast infiltration, dysplastic changes and residual hematopoietic activity. It is routinely incorporated in the assessment of remission status during and after induction therapy(41,42). A blast count of $\geq$20% is required for diagnosis expect for AML with T(8;21), inv(16)/T(16;16) and acute promyelocytic leukemia(11).

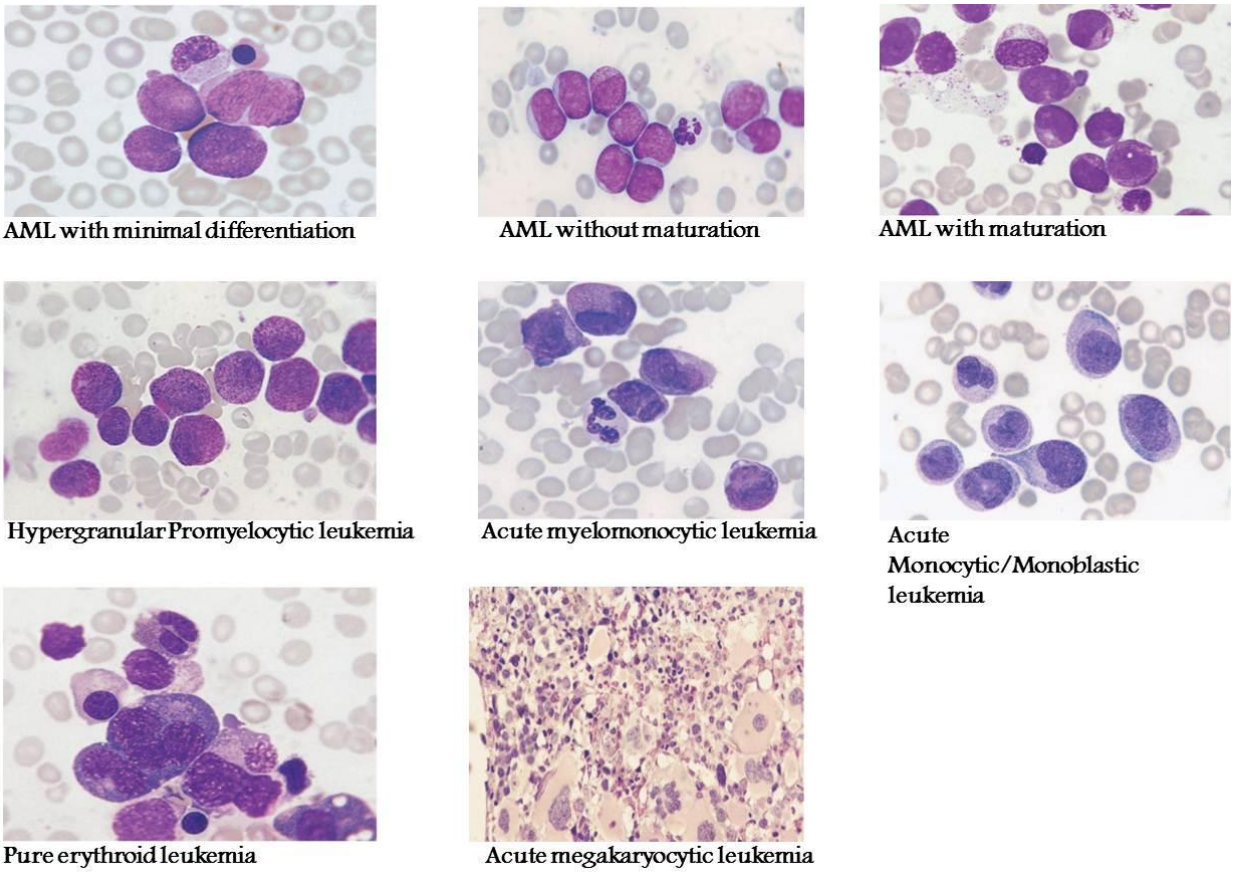

Figure 1. Morphological Spectrum of Acute Myeloid Leukemia cells.

2.5 Immunephenotyping

Immunephenotyping through multiparametric flow cytometry (MFC) or immunohistochemistry is used to characterize the leukemic blast lineage. It aides in classification according to maturation stage and is routinely used to monitor minimal residual disease post therapy(14). Myeloid blasts will express CD34 and CD117. Other expressed markers include CD13, CD15, CD33 and MPO with monocytic lineage suggested by expression of CD4, CD14, bright CD33 and CD64. Erythroid lineage is determined by expression of CD36, CD71 and CD235a whereas blasts with megakaryocytic differentiation will express CD41 and CD61. A correlation of the immunephenotypic expression with AML morphological subtypes has been noted(43,44).

2.6 Cytogenetics

The introduction of chromosomal banding techniques led to an understanding of the pathogenic role played by chromosomal aberrations in AML pathogenesis(45). Although the chromosomal landscape of AML is highly heterogeneous, recurrent chromosomal abnormalities with clear

pathogenic roles in the initiation of leukamogenesis have been profiled. Chromosomal abnormalities could either be structural or numerical. Balanced structural chromosomal rearrangements include reciprocal translocations, inversions and insertions, whereas unbalanced aberrations include deletions, isochromosome and unbalanced translocations. Numerical abnormalities include monosomies and trisomies(46). Mapping of genes, associated with these chromosomal abnormalities has enabled the classification of AML into different subtypes with differing prognostic outcomes. Balanced chromosomal rearrangements, result in the formation of aberrant chimeric fusion genes involved in the transcription of hematopoietic transcription factors, epigenetic regulators and components of the nuclear pore(4,47). These aberrant chimeric fusion genes lead to impaired differentiation and self-renewal of the hematopoietic stem cell/early progenitor cell and have been shown to be an early initiating event for leukamogenesis, mainly in the younger cohort(48). Unbalanced translocations, numerical aberrations and complex karyotypes are associated with older age and secondary AML(49). Studies show that 50-60% of AML patients will have one or more chromosomal abnormalities(50).

2.6.1 Standard conventional metaphase karyotyping

Karyotyping involves detecting structural and numerical chromosomal abnormalities using a microscope. Chromosomes are isolated from leukemic blasts obtained from bone marrow specimens or even peripheral blood when more than 10% blasts are in circulation. The cells are cultured in specific conditions for 24-48hours to increase their mitotic index. Colcemid, a blocking agent is then added to the culture medium to arrest the cells in the metaphase stage, a phase in which the chromosomes are most condensed and clearly visible. A hypotonic solution and a preservative are used to preserve the cells in a swollen state subsequently to which they are stained using giemsa stain (G-banding) and characteristically create light and dark banding patterns which are visible under a microscope(51,52). An adequate number of cells in metaphase, preferably 20 or more should be analyzed to detect any abnormality. Reporting of clonal abnormalities should be done according to the international system for human cytogenetic nomenclature (ISCN)(53).

Conventional metaphase karyotyping is not without its challenges. Low mitotic index with inadequate cells in metaphase, fuzzy unreadable chromosomes and detection of numerical chromosomal abnormalities can pose a challenge leading to inadequate chromosomal analysis. For those with an inadequate cytogenetic analysis, targeted fluorescence in situ hybridization

(FISH), and spectral karyotyping (SKY) can be carried out to detect the involved chimeric fusion genes and other aberrations(12,51). Success rates of conventional metaphase karyotyping ranges between 73-98%(46). For karyotyping to be successful, the bone marrow specimen or peripheral blood sample should be collected in the right quantity in a heparinized vacutainer and arrive in the laboratory of analysis within 24-48 hours(52). This is because karyotyping relies on spontaneously dividing cells. However in countries without capacity for cytogenetic analysis, a simple process of cryopreservation of the cells can be carried out prior to transport albeit at a cost of lower yield rate of adequate cells in metaphase for analysis(54).

2.6.2 Cytogenetic abnormalities with favorable genetic risks.

t(8:21) (q22; q22) and t(16;16)(p13.1;q22) or inv(16)(p13.1q22) are collectively known as CBF leukemia's and constitute 15% of the adult denovo acute myeloid leukemia cases and 25-30% of pediatric cases and have a favorable prognosis(55). Although they have similarities in prognosis, there does exist morphological and clinical differences that can be explained by their differing secondary co-operating mutations that lead to the development of AML(55–57). Despite being classified in the good prognostic group, 40% of patients will relapse. Co-operating mutations associated with t(8;21) include mutations in the tyrosine kinase pathways, chromatin modifier mutations and cohesin complex mutations in 65%, 42% and 18% of the patients respectively(1,55,58).

Acute promyelocytic leukemia results from a balanced translocation of t(15;17)(q24.1;q21.2)(11). Cryptic rearrangements resulting in the PML-RARA fusion gene in the absence of the typical translocation have been noted in a few cases(59). APL accounts for 10-15% of adult cases and is highly lethal if not quickly recognized with treatment started based on clinical suspicion(44,60). FLT3 mutations occur in 45% of APL patients though its prognostic relevance has not been defined. Schnittger et al notes that a FLT3-ITD/Wild-type mutational load of >0.5 leads to poorer survival outcomes(61).

2.6.3 Cytogenetic abnormalities with intermediate genetic risks.

Balanced translocations at chromosome 11q23 involve the KMT2A gene.(62,63) t(9;11) resulting in MLLT3-KMT2A gene rearrangements account for 4-5% of adult de novo AML cases(63). MLLT3-KMT2A fusion protein on its own does not induce leukamogenesis. Co-operating mutations involving signaling pathways have been established in multiple studies. Such potent genetic co-operativity portends an aggressive disease with overall poor prognosis.

2.6.4 Cytogenetic abnormalities with adverse genetic risks.

AML cases harboring t(6;9)(p23;q34.1) are few at less than 1% and have a characteristic clinical and immunephenotypic expression. There is increased frequency of co-operating mutations with FLT3-ITD associated mutations. It occurs in young patients and has an overall poor prognosis(64,65).

t(3;3)/inv (3;3)(q21;q26), occurs in < 1% of the denovo AML cases but can occur in association with blast phase of CML or AML with myelodysplasia related changes(66,67). Co-operating mutations in signaling pathways are frequent as well as karyotype abnormalities involving monosomy 7 or del (7q) and 11q23 rearrangements(45,67). Co-mutations involving myeloid transcription factors and genes encoding epigenetic modifiers have also been recorded(68). It has a unique phenotypic expression of dysmegakaryopoiesis(66).

De novo BCR-ABL acute myeloid leukemia occurs in < 2% of the AML cases(69,70). In those harboring this fusion gene, 22-55% will have del (-7q). There is presence of co-mutations with CBF leukemia's, PML-RARA gene, NPM1 mutations and genes involved in the signaling pathways(69,71). Although classified in the unfavorable risk group in the ELN risk stratification(14), it's outcomes have been noted to be dependent mainly on the co-occurring cytogenetic aberrations rather than the positivity of BCR-ABL(72).

t(1;22)(p13;q13) results in the RBM15-MKL1 fusion protein that inhibits myeloid and megakaryocytic differentiation via NOTCH activation among other pathways(73). It preferentially occurs in infants and young children, though few adults with this translocation have been reported(74). It represents less than 1% of all AML cases(75,76).

Numerical chromosomal aberrations result from gain or loss of whole or partial chromosomes and include monosomy 5 and 7, trisomy 8, del (5q) or del (7q) among others. Trisomy 8 will be detected in 10-15% of patients either as a sole abnormality or in co-mutation with other clonal chromosomal abnormalities and is the commonest numerical abnormality observed(77). Complex karyotype will be detected in 10-20% of the AML patients. Complex karyotype is defined as three or more unrelated chromosomal aberrations, excluding chromosomal abnormalities in the good and intermediate genetic risks(14). Others might harbor a monosomal karyotype which is defined as two or more autosomal monosomies or one monosomy with a structural chromosomal abnormality excluding CBF-AML(14). Numerical and unbalanced translocations are often associated with older age, therapy and myelodysplasia-

related AML(49). Other poor prognostic chromosomal aberrations include -17/abn (17p) which are associated with abnormalities of TP53 suppressor gene. They have a frequency of 5-8% in adult de novo AML cases but increase in frequency in those with therapy related cases(78).

Table 3. Frequency of chromosomal abnormalities in Acute Myeloid Leukemia

Prognostic Risk	Chromosomal abnormality	Frequency
Translocations with favorable risks	• t(8;21)(q22;q22.1) • inv(16)/t(16:16)(p13.1;q22) • APL with PML-RARA	• 10% • 5% • 10%
Translocations with intermediate risk	• t(9;11)(p21.3;q23.3)	• 4%
Translocations with adverse risk	• t(6;9)(p23;q34.1 • inv(3)/t(3;3)(q21.3;q26.2) • t(1;22)(p13.3;q13.3 • BCR-ABL	• <1% • <1% • <1% • <2%
Unbalanced translocations with adverse risks	• +8 • -5/-7 • Complex karyotype • -17/abn(17p)	• 10% • 20% • 10-20% • 5%

2.7 Molecular Genetics

50% of acute myeloid leukemia patients will have no chromosomal abnormalities detected on karyotyping(50,79). However, this group of patients will have differing treatment outcomes signifying differing tumor biology. In earlier studies before advent of next generation sequencing, mutations were categorized into two classes. Category I gene mutations leading to activation of signaling and proliferation pathways causing uncontrolled hematopoiesis and category II mutations involving transcription factors leading to impaired differentiation and aberrant self-renewal of the hematopoietic stem cell(4,80). With the advent of NGS, the scope of the molecular landscape of AML was widened especially in the cytogenetically normal cohort where more than 98% harbor molecular mutations(50). With the use of these techniques, the spectrum, frequency, distinct patterns of co-operativity and exclusivity as well as clonal evolution of mutations in AML have been understood allowing refinement of the prognostic markers **(table 2 and 4).**

2.7.1 Next Generation Sequencing

The discovery and use of advanced molecular technology like next generation sequencing has provided a cost effective means of identifying recurrently mutated genes in AML with a quick turn-around time(4,13,81). Next generation sequencing utilizes different technologies that allow automated DNA extraction from blood samples enabling massive parallel sequencing of millions of DNA strands, high throughput sequencing and automated data analysis(13). Whole genome sequencing offers the possibility of sequencing all genetic mutations present in the whole genome of an individual. Ley et al sequenced the whole genome of an AML patient. In addition to identifying previously known NPM1 and FLT3-ITD mutations, he identified 8 novel mutations that had not been documented previously(82). Multiple other studies carried out identified novel mutations that validated NGS as a tool that can be used to identify the driver mutations in most AML patients(13). Other techniques utilized in studies include whole exome sequencing, transcriptome sequencing and targeted sequencing. These techniques have paved way for the identification of multiple genes as previously described that are involved in the pathogenesis of AML lending support to the previously used prognostic model of classifying AML patients, more so in those with no recognizable cytogenetic aberration(81,83,84).

The cancer genome atlas research network, using integrative techniques of the NGS, contributed further to the knowledge of molecular drivers of pathogenesis after they sequenced 200 adult patients with de novo AML. Significantly mutated genes were classified into distinct biological entities. Majority of the cases had mutations in signaling genes (59%), 44% in DNA methylation genes, 30% in chromatin modifier genes, 27% in nucleophosmin encoding gene, 22% in myeloid transcription factor genes, 18% in transcription factor fusions genes, 16% in tumor suppressor genes, 14% cohesion complex genes and 13% in spliceosome complex genes. Grouping mutations into these biological entities revealed important patterns of co-operation and mutual exclusivity that exist to drive the phenotypic expression of disease and established a catalogue of gene mutations that future studies could benchmark on (***figure 2)***(81).

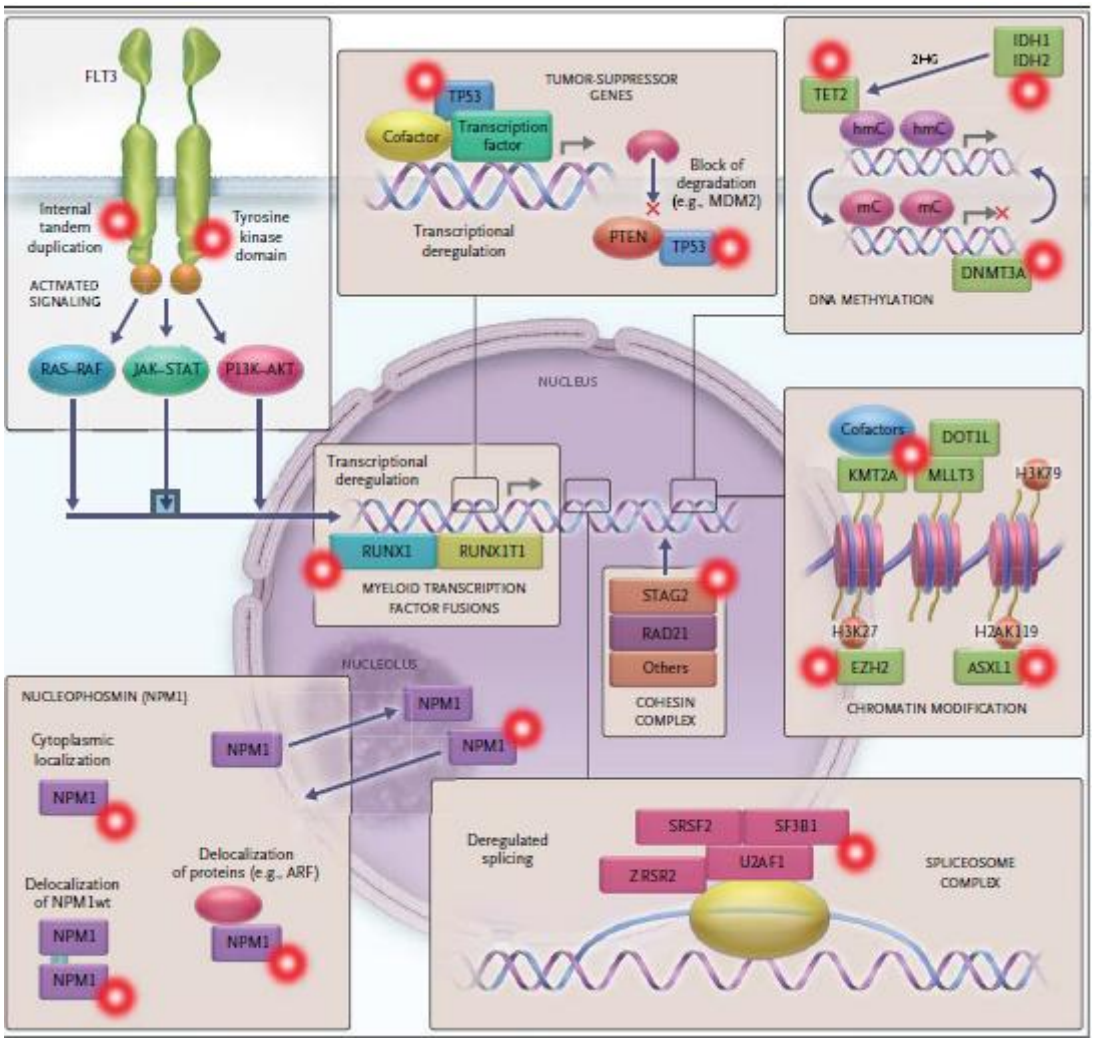

Figure 2. Nine commonly mutated gene groups in acute myeloid leukemia as reported by cancer genome atlas research network.

2.7.2 Molecular mutations with prognostic relevance

2.7.2.1 Nucleophosmin (NPM1)

NPM1 mutation results in up regulation of genes that are involved in the early stages of hematopoietic development and expansion and is associated with a normal karyotype and FLT3-ITD mutations as shown by Alcalay et al(85). It is a commonly mutated gene in AML with a reported prevalence of 30% for all cases and a higher frequency of 50-60% for those with a normal karyotype(86). Patients with a normal karyotype and NPM1 gene mutation without FLT3-ITD mutations or with a low mutational load of FLT3-ITD represent a cohort of patients with favorable prognostic features(14). Co-occurrence of NPM1, FLT3, DNMT3A has been noted and could signify a novel subtype of AML(81).

2.7.2.2 Tumor Suppressor Genes

Tumor suppressor genes have an inhibitory role in cellular proliferation and act to protect the cell from genetic alterations(87). TP53 mutations are the most prevalent TSG mutations in cancers. 5-10% of de novo AML cases will harbor this mutation, though the frequency maybe higher as shown by Kadia et al(88). They are more prevalent in therapy associated AML, complex karyotype and aneuploidies. Their presence infers poor overall survival despite allogeneic transplant and predicts a very adverse risk group(88).

2.7.2.3 FMS-like receptor tyrosine kinase-3 (FLT3)

FMS-like receptor tyrosine kinase-3 (FLT3) mutations lead to activation of sustained proliferative signals(89). Internal tandem duplications (ITD) of FLT3 occur in 24% of cytogenetically normal AML cases. These mutations confer a high risk of relapse and occur at a higher frequency in the elderly patients(90,91). Prognosis associated with FLT3-ITD mutations is dependent on the mutant to wild type allele ratio with worse outcomes associated with a high mutant allele ratio(91). In 7% of patients, point mutations occur in the activating loop of the tyrosine kinase domain at codon 835 and 836 but the prognostic significance is not well defined(92,93). However, co-operation with other mutations are needed for AML to develop. Co-occurrence of FLT3 mutations with CBF leukemia's and leukemia's with 11q23 gene rearrangements portends a poorer prognosis(55). FLT3 mutations can co-exist with CEBPA mutations more so with the monoallelic variant. However it does not seem to have a prognostic influence on this set of patients(94).

2.7.2.4 Myeloid Transcription Factors

CCAAT/enhancer binding protein-alpha (CEBPA) is a transcriptional factor that is required for myeloid maturation. In patients with a normal karyotype, 10% will harbor the biallelic CEBPA mutations and have a better outcome compared to those with wild type/monoallelic mutations(95).

Runt related transcription factor 1 (RUNX1), is crucial for definitive hematopoiesis of all myeloid lineages(96–98). When mutated, it induces transcriptional repression and epigenetic silencing. Somatic mutations of RUNX occur in denovo AML with normal and non-complex karyotype as well as AML associated with familial syndromes(34). Mutations also occur in MDS/AML overlap, MDS and T-AML(96). The frequencies of RUNX1 mutation is higher in

older people and those with trisomy 13(97,98). AML with RUNX1 mutations are associated with poor prognosis and poor response to chemotherapy as shown by Gaidzik et al(96,98).

2.7.2.5 ASXL1 (Additional sex combs-like gene 1)

ASXL1 (additional sex combs-like gene 1), is a member of the polycomb group of proteins with mutations occurring as early events in the process of leukamogenesis. The polycomb group of proteins are a group of repressive protein complexes that epigenetically repress the expression of target genes(99). Mutations in this group of genes lead to either gain of function especially in those with complex karyotype or loss of function inhibiting differentiation of the progenitor cells. They occur in 10.8% of de novo AML patients with higher frequencies seen in the elderly population and those with secondary AML. They are promiscuous and can occur across different AML types. Their existence confers a poor prognosis as shown by kakosaiou et al(100).

Table 4. Molecular Mutations included in the ELN risk stratification and their prevalence

Mutations	Biological entity	Prognostic effect	Effect of Mutations	Prevalence	Co-mutations
NPM1	Nucleolar Phosphoprotein	Favorable in wild type/low allelic FLT3 mutations	Aberrant localization to the cytosol	50-60%-CN 30%-All cases	FLT3-ITD DNMT3A
FLT3-ITD	Class III Receptor tyrosine kinase	Unfavorable Mutant: wild type allele ratio >0.5	Increased activation of downstream effectors	28-34%	NPM1 DNMT3A
Biallelic CEBPA	Transcription Factor	Favorable with biallelic mutations	Arrest of maturation at myeloblastic phase	10%-CN	GATA2
RUNX1	Transcription factor	Unfavorable	Transcriptional repression and epigenetic silencing	7.2% -CN	FLT3-ITD Epigenetic regulators Spliceosome complex factors
TP53	Tumor suppressor gene	Unfavorable	Genomic instability	5-10%-de novo. 70-80% complex karyotype.	Complex Karyotype. Aneuploidies
ASXL1	Chromatin modifier	Unfavorable	Aberrant self -renewal	10.8%	

2.8 Genomic Landscape of AML patients in Africa

Kappala et al in South Africa using a microarray-based assessment of molecular variables on AML patients noted that 5.7% had inv(6)/t(16;16)(p13;q22), 11.3% had t(8;21)(q22;q22) , 3.8% had t(15;17)(q24;q21), 1.9% had double mutant CEBPA, 9.4% had NPM1-ABD mutations and 18.9% had a high expression of EVI1. NPM1 mutations were mutated at a lower frequency whereas EVII over expression occurred at a higher frequency compared to world data, which would indicate age and racial differences(26). Lower prevalence of NPM1 and FLT3-ITD mutations compared to world data were documented by Marshall et al looking at a South African cohort with de novo AML(27). Awad et al investigated the prevalence of FLT3-ITD mutations of 346 patients with AML in Egypt, 9.2% had t(8;21), 2.3% had inv(16) and <1% had both t(9;11) and inv(3)(101). FLT3-ITD mutations were present in 18.5% of the total population mirroring the lower frequency of FLT3 mutation in African studies(101). Shamaa et al in Egypt noted a frequency of 34.6% and 28.8% in FLT3-ITD and NPM1 mutations respectively in a cohort of AML patients with a normal karyotype, similar to western studies(102). DNMT3A and $IDH1^{R132}$ are frequently mutated in AML patients in Egypt whereas TET2 overexpression is not a frequent finding(103–105). The cytogenetic and molecular patterns of acute myeloid leukemia patients in Africa are difficult to elucidate and compare due to lack of large scale studies and differing study designs. In India, a country with similar demographics, a large scale analysis of the cytogenetic profile of patients with de novo acute myeloid leukemia showed that 15% had t(8;21), 9% had t(15;17), 8% had 8+, 6% had -7/del 7q, 5% had KMT2A rearrangements, 4.4% had inv(16)/t(16/16), 3% had -5/del 5q, 2% has -17/abn 17p and 1.5% had inv(3) in order of decreasing frequency(106). Other studies from India show that FLT3-ITD, CEBPA and NPM1 mutations occur in 22.3%, 8.3% and 8% of patients respectively, a frequency that's lower than that reported in western data(107,108).

2.9 Statement of problem and Justification

WHO emphasizes genetic testing of all patients with acute myeloid leukemia. It is an essential test that's expensive and out of reach for many of the patients accessing treatment at the adult hemato-oncology unit of Kenyatta National Hospital. The median age of our AML patients is 30 years. This represents a cohort of young patients with good performance status, who would benefit from risk-stratified treatment. The challenge experienced by the health care provider in this center is that they are not able to prognosticate the patients and therefore do not offer individualized treatment. Currently, there is no data in Kenya describing the chromosomal abnormalities and molecular mutations of AML patients presenting to KNH.

Data obtained from this study has provided insight into the molecular mutations that occur among acute myeloid leukemia patients in the largest public referral hospital in Kenya. In addition, this study provides impetus for a larger prospective study of the same cohort of patients whereby, the information gathered can aide in the development of a risk stratified treatment approach.

2.10 Research Question and Objectives

The research question was to determine the genetic mutations identified in Acute Myeloid Leukemia patients presenting to the adult hemato-oncology unit of Kenyatta National Hospital**.** Our main objectives were to determine their chromosomal abnormalities and molecular mutations through conventional metaphase karyotyping and next generation sequencing methods respectively, with a secondary objective of prognosticating them according to the 2017 EuropeanLeukemiaNet risk groups.

CHAPTER THREE

METHODOLOGY

3.1 Study Design

This was a descriptive cross-sectional study, conducted at Kenyatta National Hospital (KNH). KNH is the biggest public referral hospital in Nairobi, Kenya and is the teaching hospital for the University of Nairobi. The study was specifically conducted at the adult hemato-oncology unit located on the 8th floor of the hospital and admits patients who are 13 years and above.

The patients were selected consecutively until the desired sample size of ten patients was achieved. The sample population comprised of patients with acute myeloid leukemia based on morphological description and/or immunephenotyping using multiparametric flow cytometry or immunohistochemistry where necessary. The patients were 13 years and above. Those patients who refused to assent or consent were excluded from the study.

The dependent variables were cytogenetic abnormality, molecular mutations and genetic risk group. The independent variables were age, gender, total leucocyte count, hemoglobin level, platelet count, % of bone marrow blasts, history of prior chemotherapy or radiotherapy and prior hematological disorder.

3.2 Sample Size Calculation

The total number of participants for this study was ten. The formula for calculating the sample size for a simple random sample without replacement is as shown below:

$$n = \left(\frac{z}{m}\right)^2 p(1-p)$$

Where,

- z is the z value (1.96 for 95% confidence level);
- m is the margin of error (0.10 = + or – 10%); and
- p is the estimated value for the proportion of the sample that have Acute Myeloid Leukemia (0.5 for 50% based on no knowledge of the prevalence)

Using our factors for the population, and solving for the sample size equation, we found:

$$n = \left(\frac{1.96}{0.10}\right)^2 0.5\,(1 - 0.5) = 384$$

In order for this study to be done within a period of 3 months there were approximately 10 patients (average of 3 patients every month based on historical data available that had 150 patients over a 5-year period). This summed up to N= 10 patients (population size of patients expected to be seen in the 3 month-period). Since our sample (n=384) was way much greater relative to the population of patients that would be available within the 3 month study period and meeting the study criteria, we took the Finite Population Correction (FPC) into account as shown below:

$$n' = \frac{n}{1 + \frac{n}{N}}$$

Where,

n is the sample size based on the unadjusted calculation as above, and

N is population size.

Working out the adjusted sample size *n'* using the formula above, we found a minimum sample size of 10 would be adequate for this study:

$$n' = \frac{384}{1 + \frac{384}{10}} = \mathbf{10}\ patients$$

3.3 Screening and Recruitment Flow Chart

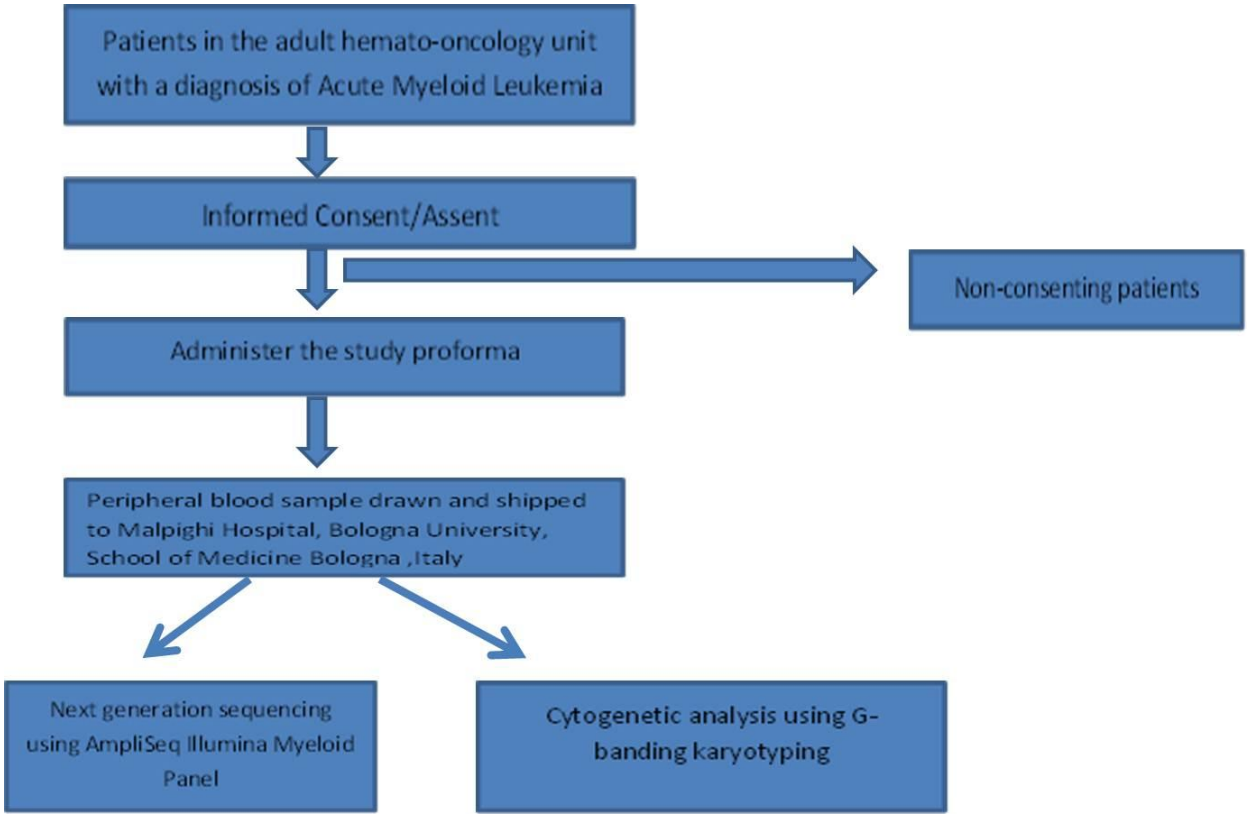

Figure 3. Recruitment flow chart

3.4 Data Collection

3.4.1 Clinical and Laboratory data

Patients admitted to the adult hemato-oncology unit with a morphological diagnosis of acute myeloid leukemia were informed of the study and a consent/assent obtained in either English/Kiswahili, the national languages of Kenya **(Appendix I, III, IV, VI)**. In case of a minor, consent/assent was obtained from a parent or guardian. For those who consented/assented, clinical and laboratory data were collected through a study proforma **(Appendix VII).**

3.4.2 Laboratory Methods

3.4.2.1 Sample Collection

The procedure of blood collection was explained to the patient using a standard written procedure consent form. Consent to ship the samples abroad was also obtained **(Appendix II**

and V). Once consent or assent was obtained, the patient was requested to sit in a comfortable position or lie in a supine position and the cubital fossa of either arm located. The skin at the venipuncture site was cleaned using a sterile swab soaked with 10% povidine iodine. Once the skin had dried, an 18 gauge needle affixed onto a 10cc gauge syringe was used to draw 10cc of blood. 5cc of the blood drawn was collected into a lithium heparinized vacutainer (green top) and 5cc was collected into an EDTA vacutainer (lavender top). Once the procedure was completed, pressure was applied to the puncture site to minimize bleeding and discomfort. Once bleeding had stopped, a sterile dressing was used to cover the puncture site to minimize risk of further bleeding or infection. Patient was provided with information on how to take care of the venipuncture site or alert a health care provider in the rare event of a complication.

3.4.2.2 Sample Transportation and Processing

Blood samples were drawn and stored in room temperature. The designated courier was contacted to pick the sample on the same day. The blood samples in EDTA for molecular analysis were transported using blue ice packs for cold shipment. The blood samples in lithium heparinized vacutainer were transported on room temperature. The courier delivered the samples to the hematopathology section, department of experimental diagnostics and experimental medicine, S.Orsola Malpighi hospital, Bologna University, School of Medicine in Italy. The transit time from specimen collection, courier pick up and arrival at the laboratory took a total of 4 days. A material transfer agreement form between both universities was obtained in addition to obtaining approval for shipment from the Ministry of Health **(Appendix X and XI).**

3.4.2.3 Cytogenetic Analysis

Cell culture: The cells were cultured using a protocol for harvesting chromosomes from whole blood**.** An aliquot of 0.25mls of fresh whole blood was collected in 10mls of RPMI media containing L-glutamine (20% fetal bovine serum, 1% Penicillin/ streptomycin, 1% fungizone, and 1% PHA) and incubated for 48 hours at 37 °C with 5% CO2. Cell counting prior to harvesting of cells revealed that the final seeding densities for each of the samples were less than 1×10^6/ul below the optimum of $1\text{-}3\times10^6$/ul for adequate metaphases. Harvesting was done according to standard protocol and all 10 samples had no evaluable metaphases(51,52).

3.4.2.4 Molecular Analysis

Molecular analysis for the ten samples was performed using Illumina next generation sequencing methods. Automated DNA/RNA extraction and amplification for library preparation was done. Analysis utilized the AmpliSeq for illumina myeloid panel. It's a targeted panel that investigates 74 genes associated with myeloid cancers **(Table 5)**(109)**.** The final phase involved data cleaning by a bioinformatics technician.

Table 5. Panel of genes tested

Hot spot genes	**Full genes**	**Fusion driver genes**	**Gene Expressions**
ABL1	ASXL	ABL	BAALC
BRAF	BCOR	BCL2	MECOM
CBL	CALR	BRAF	MYC
CSF3R	CEPBA	ALK	SMC1A
DNMT3A	ETV6	CCND1	WT1
FLT3	EZH2	CREBBP	
GATA2	IKZF1	EGFR	
HRAS	NF1	ETV6	
GATA2	PHF6	FGFR2	
IDH1	PRPF8	FGFR1	
IDH2	RB1	FUS	
JAK2	RUNX1	HMGA2	
KIT	SH2B3	JAK2	
K-RAS	STAG2	KMT2A	
MPL	TET2	MECOM	
MYD88	TP53	MET	
NPM1	ZRSR2	MLLTI0	
N-RAS		MYBL1	
PTPN11		MYH11	
SETBP1		NTRK3	
SRSF2		NUP214	
U2AF1		PDGFRA	
WT1		PDGFRB	
		RARA	
		RBM15	
		RUNX1	
		TCF3	
		TFE3	

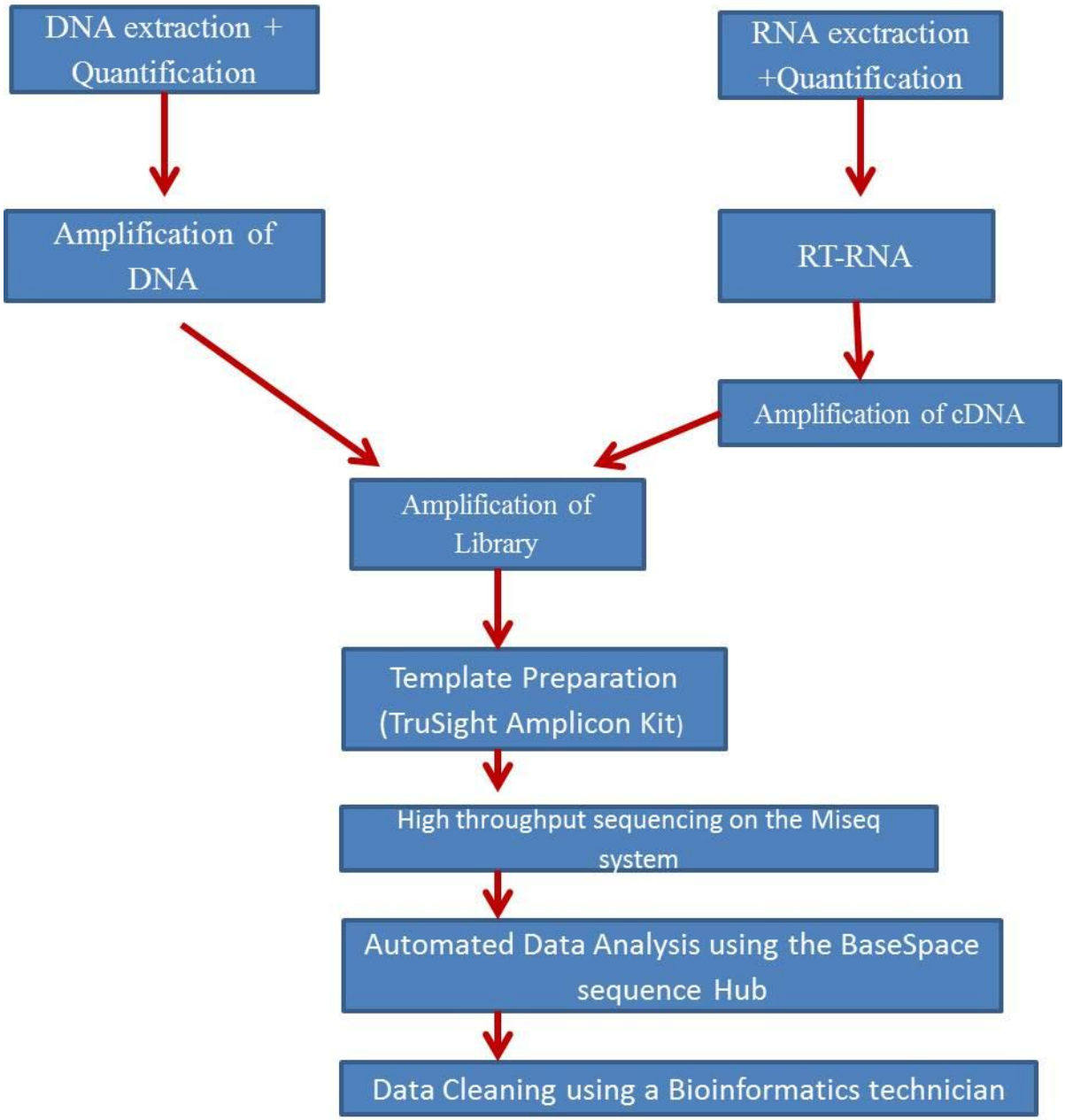

Figure 4. Work flow for Next generation sequencing

3.5 Data Management

Data analysis was done by the principal investigator with the help of a statistician and the supervisors. Data was entered and cleaned in Microsoft Excel. Descriptive analysis using frequencies (percentages) for categorical variables, means (standard deviations) and medians (interquartile ranges) as appropriate for continuous variables was not done due to the small sample size. In addition, no formal bivariate differences were analyzed due to the small sample size. Descriptive statistics were presented in tables.

3.6 Data Protection and Study Dissemination plan

All the raw data collected during the study has been kept safe and will only be accessible to the principal investigator. Data bears a unique code (MG) for each participant.

Results of the genetic testing were relayed to the patients and filed in their respective hospital records. The final results of the study will be submitted to the University of Nairobi in form of a thesis required for the completion of the fellowship. The findings will also be shared in scientific forums and published in a peer reviewed journal.

3.7 Ethical Consideration

Institutional consent was obtained from the Department of Clinical Medicine and Therapeutics, University of Nairobi (UON) and Ethics and Research Committee of KNH. Written informed consent or assents were obtained from all patients and guardians. Enrollment was voluntary. Failure to give consent did not jeopardize patient care in anyway. Confidentiality and privacy was observed throughout the study. Request for shipment of the samples for cytogenetic and molecular tests, was obtained from the Ministry of Health subject to the fulfillment of the requirements of KNH-ERC. Material Transfer Agreement between the two institutions was obtained.

All procedures were done after a written informed consent from the patient or guardian. The procedure was done under aseptic conditions with all the necessary precautions taken. Pain management was emphasized during and after the procedure as per standard protocols to ensure patient was as comfortable as possible. All samples were collected in the right specimen bottle with proper labeling and transported according to the specimen transportation and packaging procedures of hematopathology section, department of experimental diagnostics and experimental medicine, S. Orsola Malpighi hospital, Bologna University, School of Medicine, Italy.

3.8 Quality Control

Kenyatta National Hospital/University of Nairobi Hematology Lab is ISO-9001:2015 certified. The Hematopathology Section, Department of Experimental, Diagnostic, and Specialty Medicine, S. Orsola-Malpighi Hospital, Bologna University School of Medicine, Bologna, Italy has carried out many genetic studies and has several publications in peer reviewed journals.

CHAPTER FOUR

RESULTS

Fifteen patients with a morphological diagnosis of AML were seen in the department of hemato-oncology unit during the study period. Ten patients were enrolled into the study consecutively. Three patients died before enrollment, 1 declined enrollment and 1 started treatment before enrollment.

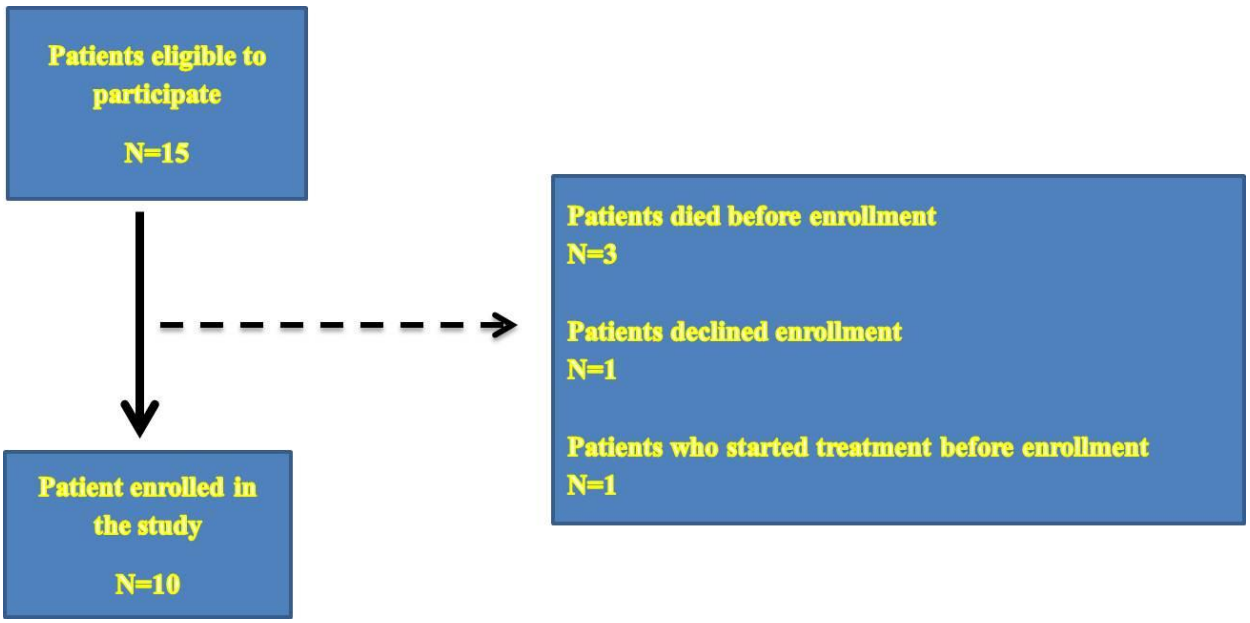

Figure 5. Flow chart of enrollment

4.1 Demographic, Clinical and Laboratory characteristics of the patients

The study's 10 participants comprised of 5 females and 5 males with an age range of 13-70 years. One patient had a previous diagnosis of aplastic anemia **(table 6).**

Table 6. Patient characteristics

Case	Age Years	Gender	% of Marrow Blast	WCC /×10^9/l	HB g/dl	Platelets /×10^9/l	Prior Cytotoxic therapy or Radiotherapy	Antecedent Hematological Disorder
MG1	**19**	**M**	**30**	**20.92**	**6.8**	**17**	**NONE**	**NONE**
MG2	**22**	**F**	**80**	**10.59**	**3.8**	**58**	**NONE**	**NONE**
MG3	**13**	**F**	**62**	**0.77**	**7.3**	**46**	**NONE**	**NONE**
MG4	**50**	**F**	**82**	**44.8**	**8.2**	**20**	**NONE**	**NONE**
MG5	**36**	**M**	**40**	**2.86**	**5.8**	**11**	**NONE**	**APLASTIC ANEMIA**
MG6	**34**	**F**	**44**	**2.63**	**4.3**	**6**	**NONE**	**NONE**
MG7	**60**	**M**	**75**	**2.60**	**3.1**	**44**	**NONE**	**NONE**
MG8	**53**	**M**	**70**	**36**	**10.8**	**53**	**NONE**	**NONE**
MG9	**13**	**M**	**60**	**3.49**	**6.9**	**16**	**NONE**	**NONE**
MG10	**70**	**F**	**30**	**1.59**	**7.9**	**48**	**NONE**	**NONE**

4.2 Molecular Mutations using Next Generation Sequencing methods

Among the 74 analyzed genes, 29 deleterious mutations across 13 genes commonly mutated in AML were detected. Of these, 14 were missense mutations, 13 were frame-shift mutations, 3 were nonsense mutations, and 1 was a fusion gene. Mutations in two genes not commonly mutated in AML were also detected. All patients had at least one mutation that has previously been described with majority having 2 mutations each (**Table 7)**. TP53, WT1, N-RAS, STAG2 and mono-allelic CEBPA mutations were detected among three patients. DNMT3A, IDH2 and PTPNII were detected in two patients. PML-RARA, NF1, GATA2, FLT3-TKD and RUNX1 were each detected in one patient. ETV6 and IKZF, mutations that are not prevalent in AML were also detected in one patient each. Detailed report of the mutations detected is provided in **(Appendix IX)**. Pairwise mutations to assess for co-mutations could not be undertaken due to the small sample size.

Table 7. Mutational landscape according to the categories of related genes

	Mutations	MG1	MG2	MG3	MG4	MG5	MG6	MG7	MG8	MG9	MG10
TRANSCRIPTION FUSION GENE	PML-RARA			■							
TUMOR SUPPRESSOR GENES	NF1						■				
	TP53						■			■	■
	WT1	■							■	■	
DNA METHYLATION	DNMT3A		■		■						
	IDH2				■			■			
ACTIVATED SIGNALING GENES	FLT3-TKD				■						
	NRAS	■							■	■	
	PTPNII					■				■	
MYELOID TRANSCRIPTION FACTORS	GATA2									■	
	RUNX1					■					
	CEBPA		■	■					■		
COHESIN	STAG2						■		■		■
ETS transcription factors	ETV6						■				
IKZF1	IKZF1									■	
Total number of mutations	15	2	2	2	3	2	4	1	4	5	2

■ Presence of mutation

4.3 Description of Clinical and Molecular Abnormalities per Patient

4.3.1: Case 1: MG1

MG1 was a 19 year old man who presented to hospital with a one month history of severe dizziness, palpitations, global headache, general malaise, weight loss and epistaxis. His past medical history was unremarkable. He was the first born in a family of four siblings and both his parents are small scale traders. He had just completed high school and was waiting to join college. There were no identifiable risk factors. On first presentation, he was febrile with a temperature reading of 38^0C, BP reading of 112/58mmHg and PR of 81b/minute. On examination, the positive findings were pallor, right lower cervical lymphadenopathy, petechie on the palate, epigastria tenderness and massive splenomegaly. Investigation included a total blood count that showed a (WCC) of 20.92×10^9/l, (ANC) of 6.52×10^9/l, (ALC) of 5.52×10^9/l, (AMC) 8.33×10^9/l, (HB) 6.8g/dl and (PLT) 17×10^9/l (post 5 packed cells and 3 platelets). His first BMA was not representative for comprehensive evaluation but had suggested a myeloproliferative neoplasm in blast transformation. BCR-ABL was negative for the 210kd transcript. PBF showed blasts of 30% some with Auer rods. Trephine biopsy showed a hypercellular marrow with a diffuse infiltrate of immature cells with open chromatin and prominent nucleoli. IHC stained positive for CD33, CD117 and MPO, while it was negative for CD 34, PAX5, TdT, CD10 and CD20. CD 3 and CD 5 were positive in a reactive pattern (**Images not available**). Flow cytometry was not done. Uric acid was 442umol/l (normal 214-450), LDH 450IU (normal 109-245), U/E/C and LFT were normal. Sputum analysis was negative for AAFB but positive for candida albicans that was deemed not to be pathogenic. CT scan chest showed multiple hilar and mediastinal adenopathy. Abdominal ultrasound showed massive splenomegaly of 26cm length, liver span of 16.9 and multiple para-aortic adenopathy. Targeted sequencing identified two mutations affecting NRAS (c.181C>A p.Q61K) and WT1 (c.1141_1142insCCTTGTACGGT/ c.1142_1143insCTTGTACGGTC p.A382fs) genes.

4.3.2 Case 2: MG2

MG2, a 22 year old female presented to hospital with complains of dizziness, hotness of body, night sweats, dry cough, occipital headache and weight loss over the last 7 months. She initially presented with dizziness and fatigue which she attributed to the effects of a manual evacuation she had undergone for an incomplete abortion. She developed palpitations, bilateral pedal edema, persistent cough with no chest pain and a severe recurring headache. Patient received packed cells on several occasions due to recurrent anemia. She has no major comorbidities.

She is married with two children and works as a casual laborer. On examination, she was febrile at 38^0 Celsius; BP was 106/48mmgh with a tachycardia of 133b /minute. She had severe pallor and bilateral inguinal lymph nodes. Total blood count showed (WCC) 10.59 × 109/l, (ANC) 7.19×109 /l, (LYM) 2.35×109 /l, (MON) 0.50×109 /l, (HB) 3.8 g/dl and (PLT) 58×10^9/l. Flow cytometry was not done. PBF revealed 73% type I and II myeloblasts. BMA showed a hypercellular marrow with predominant 80% blasts without maturation (AML: FAB-M1) ***(fig6)***. Targeted sequencing identified CEBPA (c.389G>T p.G130V) and DNMT3A (c.2645G>A; p. R882H) mutations.

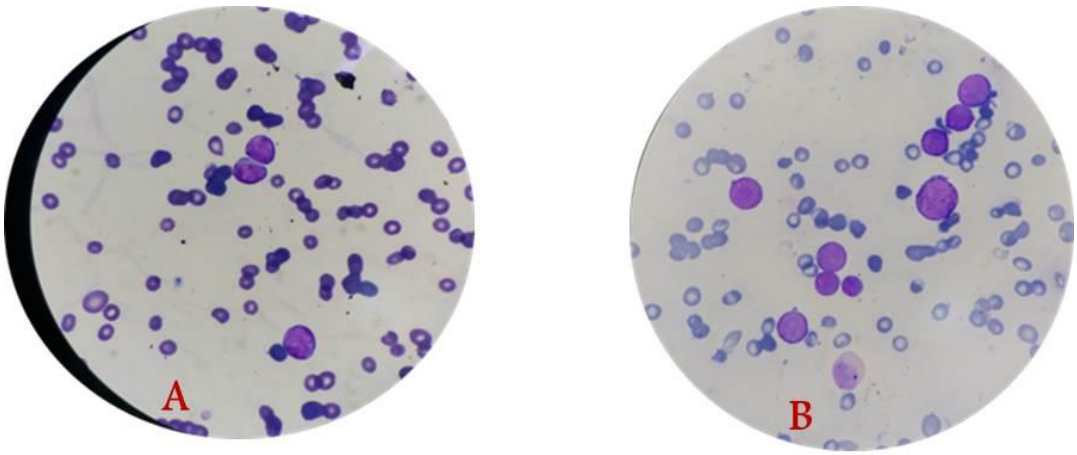

Figure 6. PBF and BMA images for MG2: (PBF (A) type I and II myeloblasts (B) blasts without maturation (FAB M1).

4.3.3 Case 3: MG3

MG3 is a 13 year old girl who presented to hospital with a one month history of epistaxis, gum bleeding, swollen gums, fever, easy fatigability, dizziness and palpitations. She had recently developed a dental abscess that was bleeding profusely. She also presented with a 2 week history of an upper lip ulcer that was about 2×3cm in size with irregular margins and was tender to palpation. While admitted she developed bilateral vitreous hemorrhages and bilateral acute otitis media. She is the second born in a family of three siblings. BP was 119/77mmhg, PR 89 b/min with a temperature reading of 38.5^0 c. Total blood count: (WCC) 0.77 ×109/l, (ANC) 0.06 ×109/l, (ALC) 0.67×109/l, (AMC) 0.04×109/l, (HB) 7.3g/dl and (PLT) 46×109/l. Her first BMA evaluation was not conclusive due to hypocellularity. PBF showed marked pancytopenia with 8% blasts. Repeat BMA showed 62% blasts as promyelocytes ***(fig7)***. U/E/C, LFT, Uric acid and LDH were all within normal ranges. INR was 0.97, fibrinogen 63.7mg/dl (normal 169-515mg/dl) and d-dimer 27.04 µg/ml (normal 0.00-0.50µg/ml). Targeted sequencing identified PML-RARA fusion gene and CEBPA (c.694_695insG p. V232fs) mutation.

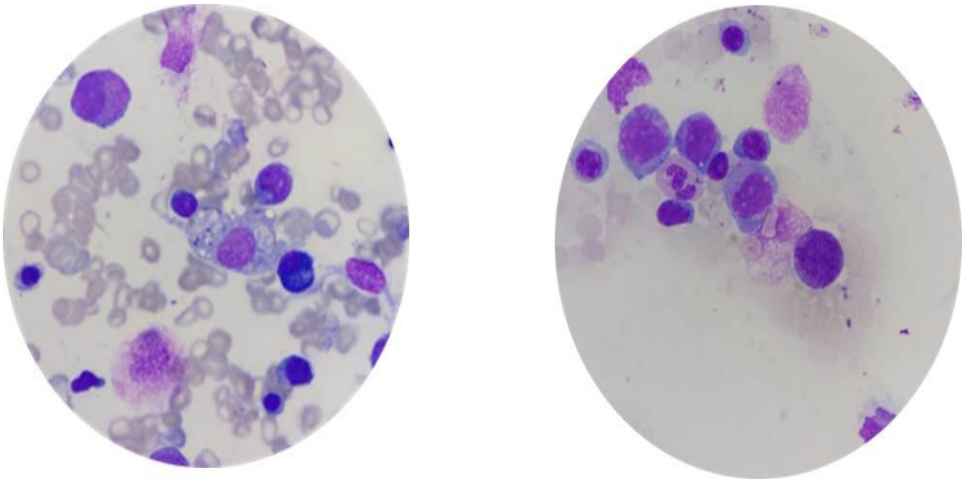

Figure 7: BMA Images for MG 3: Both show predominance of promyelocytes

4.3.4 Case 4: MG4

MG4, a 50 year old peri-menopausal lady presented to hospital with complains of persistent heavy per vaginal bleeding for the past 3 months. She also complained of having a one month history of hematuria, dysuria and straining when passing urine. Her main co-morbidities are hypertension of which she is taking amlodipine and enalapril. She is also on 100mcg of L-thyroxine for hypothyroidism. She is known to have asthma on bronchodilators as needed. Her main risk factor was prolonged exposure to pesticides.. She is married and has three children.

On examination, the main findings were pallor and a 14 week pelvic mass. Pelvic ultrasound revealed bulky uterus with multiple sub-serous fibroids and a prominent cervix with no definite mass. A pap smear done was positive for high grade squamous intraepithelial lesion (HGSIL) and she subsequently underwent LEEP. Biopsy results showed cervical intraepithelial neoplasia (CIN). She was admitted to hospital to undergo hysterectomy and bilateral salpingo-oophorectomy. Total blood count done as part of pre-surgical work up revealed (WCC) of 44.8× 10^9/l, (ANC) of 3.14×10^9/l, (ALC) 5.29×10^9/l, (AMC) 33.44×10^9/l, (HB) 8.2g/dl and (PLT) of 20×10^9/l. PBF showed atypical monocytoid cells with high N:C ratio, scanty basophilic cytoplasm, open chromatin pattern and prominent nucleoli accounting for 82% of the nucleated cells. BMAT was markedly hypercellular with diffuse infiltration by blasts ***(fig8)***. IHC was positive for CD 34, CD 117 and MPO and negative for PAX5 and CD3. Urine M/C/S grew escherichia coli sensitive to Amikacin and Meropenem only. Echocardiogram showed Type 1 left ventricular diastolic dysfunction, Trace MR, mild TR/PAH of 41mmHg and LVEF of 62%. TFTS were normal. U/E/C and LFTs were normal, uric acid value of 544umol/l (normal 120-430) and LDH value of 2039 IU/L (normal 120-250). Targeted sequencing identified IDH2 (c.515G>A p.R172K) and DNMT3A (c.2645G>A p.R882H) missense mutations with FLT3 (c.1815T>C, p. =).

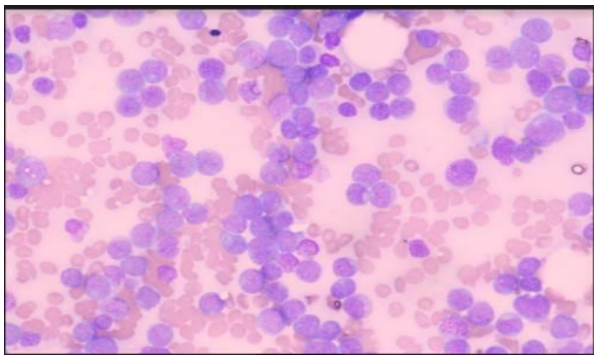

Figure 8: BMA image for MG4: Increased cellularity with blast infiltration

4.3.5 Case 5: MG 5

MG 5 was a 36 year old man who had been managed for recurrent anemia since 2013. A trephine biopsy done in 2014 had shown fibrous replacement of the marrow with scanty nucleated cells and an occasional megakaryocyte with no stainable iron stores. A diagnosis of aplastic anemia was made. He was put on oxymethalone 50mg TID and received countless packed cells. 6 years later, he presented with refractory pancytopenia and subsequently he was referred to our hemato-oncology unit. On presentation to the unit, his main symptoms were

dizziness, easy fatigability, palpitations, headache and epistaxis. There were no other risk factors identified. He was married with three children and worked as a small scale trader. On examination BP was 146/75mmhg and PR 86b/min. His main findings were pallor and a hemic murmur. Total blood count: (WCC) 2.86×10^9/l, (ANC) 0.37×10^9/l, (ALC) 2.10×10^9/l, (HB) 5.8g/dl and (PLT) 11×10^9/l. PBF showed marked pancytopenia with rare circulating blasts. Trephine biopsy showed marrow cellularity of 50% with presence of primitive cells consistent with blasts which were positive for PAX5, CD34, CD 117 and TdT and were negative for CD19, CD 22, CD 79a and CD10 **(images not available).** Blasts were 30-40%. Targeted sequencing identified RUNX1 (c.592G>A p. D198N) and PTPN1 (c.182A>G p.D61G) molecular mutations.

4.3.6 Case 6: MG 6

MG 6 was a 34 year old lady who presented to hospital with generalized ill-defined abdominal pain and diarrhea. 2 weeks prior to admission she had developed a transient dry cough and had used over the counter medication. She reported occasional headache and easy fatigability that had not been of major concern to her. She had no significant past medical history. A lifetime nonsmoker, who never took alcohol and she had no identifiable risks factors. She was married, had 2 children and was a business lady. On examination she was noted to be febrile, T 38.3^0c with BP 96/56mmhg, PR 102 b/min. Total blood count showed (WCC) 2.63×109/l, (ANC)0.63×109/l , (ALC)1.2×109/l, (AMC)5.34×109/l, (HB) 4.3g/dl, (PLT)6×109/l. U/E/CR and LFT were normal Uric acid 234umol/l (normal 120-430) and LDH 560IU/L(120-250). Stool analysis, blood cultures and CXR were non-revealing. PBF had 32% blasts. BMA showed increased cellularity, 44% blasts and 56% abnormal erythropoiesis series characterized by megaloblastoid forms (FAB M6) ***(Fig 9)*** . Targeted sequencing identified mutations in TP53 (c.743G>A p.R248Q), NF1 (c.7618_7619insT p.T2540fs), STAG2 (c.2359_2360insT p.A787fs), STAG2 (c.2359_2360insTT p.A787fs) and ETV6 c.403_404insT p.(H135fs).

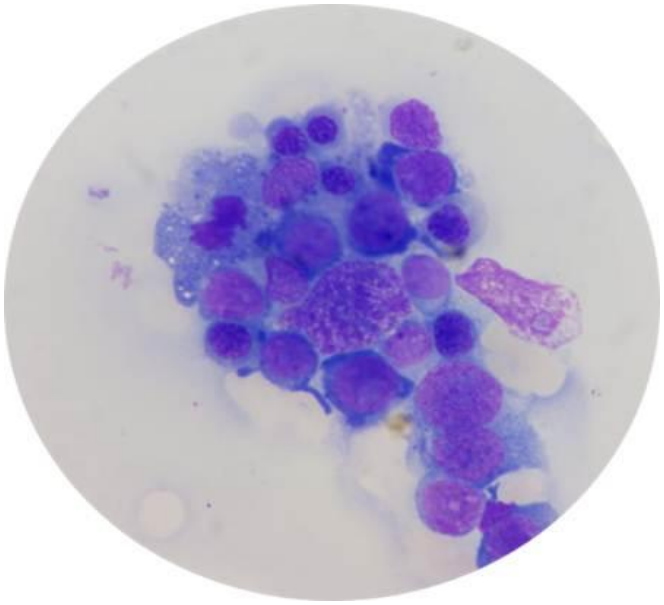

Figure 9: BMA image for MG 6: Abnormal erythropoiesis series exhibited by megaloblastoid forms (FAB M6)

4.3.7 Case 7: MG 7

MG 7, a 60 year man presented to hospital with a one month history of persistent back pain, joint weakness, chest pain, non-productive cough, painful swallowing, chest pain and abdominal discomfort. He also had 2 episodes of syncope. His past medical history was significant for hypertension which he was taking amlodipine and enalapril. He was married with 3 children with no family history of cancer. He was a chef in a primary school in his county. He was known to take alcohol and smoke with 3 pack years. On examination, his main findings were mild pallor, mucositis and gingival hypertrophy. Total blood count showed (WCC) 2.6×109/l, (ANC) 0.58×109/l, (ALC) 1.80×109/l, (AMC) 0.18×109/l, (HB) 3.4 g/dl and (PLT) 213×109/l. Bone marrow aspiration revealed blasts of 75% with underlying myelodysplasia (AML-MRC) ***(fig10)***. Creatinine value was 95umol/l (normal 60-120), Uric acid 232umol/l (normal 214-458), LDH 137IU/l (109-245) and normal LFTS. Chest X-ray was normal and ECHO showed a LVEF of 64% with mild MR and moderate TR/PAH of 53mmHg. Targeted sequencing revealed IDH2 (c.515G>A p.R172K) as the only mutation.

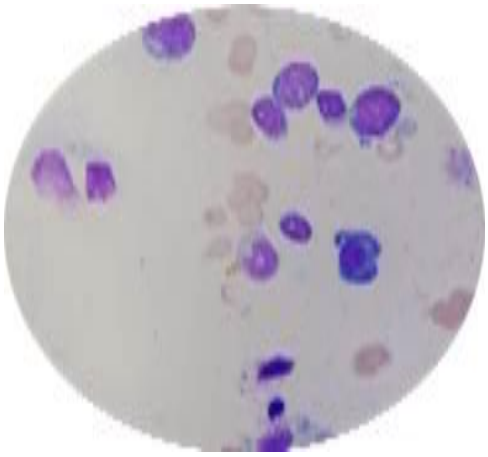

Figure 10: BMA image for MG 7: Blasts with monocytoid appearance and myeloblast characteristics with underlying myelodysplasia AML-MRC

4.3.8 Case 8: MG 8

MG 8 was a 53 year old man. He presented to hospital with a 5 month history of general malaise and was noted to be anemic. He was given 5 units of packed cells and discharged home. Two months later he presented again with similar complains but now had fever and anorexia. He was admitted to hospital given an additional 5 pints of packed cells and other supportive treatment and referred to our unit after a BMA done was reported as acute myeloid leukemia. He had no comorbidities. He was known to take alcohol but was a lifetime non-smoker. His maternal aunt died from breast cancer. He was married with three children and worked as a telephone operator in a government office. On examination, his main findings were small discrete cervical lymph nodes and a hepatomegaly, 2 cm below costal margin. Total blood count (WCC) of 36×10^9/l, (ANC) of 26.6×10^9/l, (ALC) 10.3×10^9/l, (AMC) 5.7×10^9/l, (HB) 10.6g/dl (after transfusion) and (PLT) of 53×10^9/l. PBF showed blasts of 67%. BMA was hypercellular with predominance of myeloblasts at 70% (FAB M2) **(fig11)**. ECHO showed LVEF of 72% with type I left ventricular diastolic dysfunction and mild right sided pleural effusion. Chest X-ray was normal. Targeted sequencing revealed CEBPA (c.539_540insC p.Y181fs), NRAS (c.35G>A p.G12D), STAG2 (c.2359_2360insT p.A787fs) and WT1 (c.1137_1138insA p.R380fs).

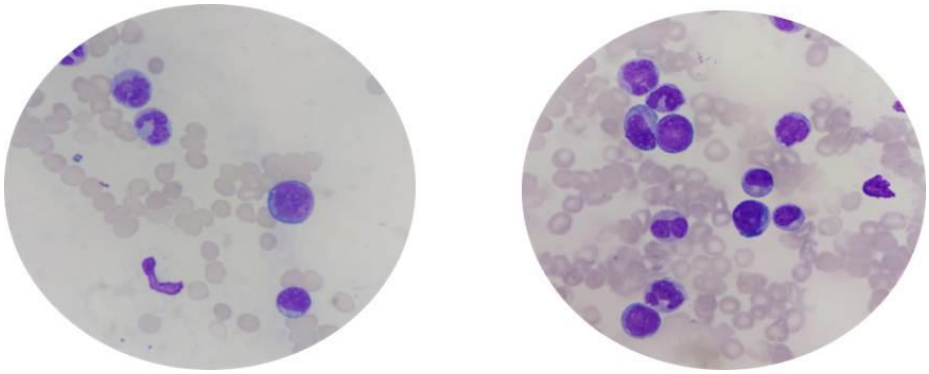

Figure 11: BMA image for MG 8: Hypercellular marrow with predominance of myeloblasts (FAB-M2)

4.3.9 Case 9: MG 9

MG 9 is a 13 year old boy who presented to hospital with a one month history of general malaise, easy fatigability, palpitations and generalized arthralgia. He complained of recent onset chills, rigors and recurrent mouth ulcers. He had no prior medical conditions. His mother was deceased during infancy and he was under the care of a maternal grandmother and aunt. Father was blind since childhood. On examination, he had a temperature reading of 39.6^0 Celsius, BP reading of 110/67mmHg with a tachycardia of 110 b/min. His main positive findings were pallor, multiple mouth ulcers and purulent tonsillitis. Total blood count values; (WCC) 3.49×10^9/l, (ANC) 0.9×10^9/l, (ALC) 2.04×10^9/l, (HB) 6.9g/dl, (PLT) 16×10^9/l. PBF showed 69% blasts. BMA and Trephine biopsy was reported as hypercellular with markedly hyperplastic erythropoiesis, majority being erythroblasts (M6 variant-acute erythroblastic leukemia). IHC was positive for CD 117, MPO, and CD 235 and negative for CD34, CD 79, CD 3 and CD 20. 60% were blasts ***(fig12)***. Targeted sequencing identified TP53 (c.215_216insG p.V73fs), NRAS (c.35G>A p.G12D), WT1 (c.1142C>A p.S381*), PTPN11 (c.227A>G p.E76G), GATA2 (c.953C>T p.A318V) and IKZF1 (c.963C>G p.Y321*).

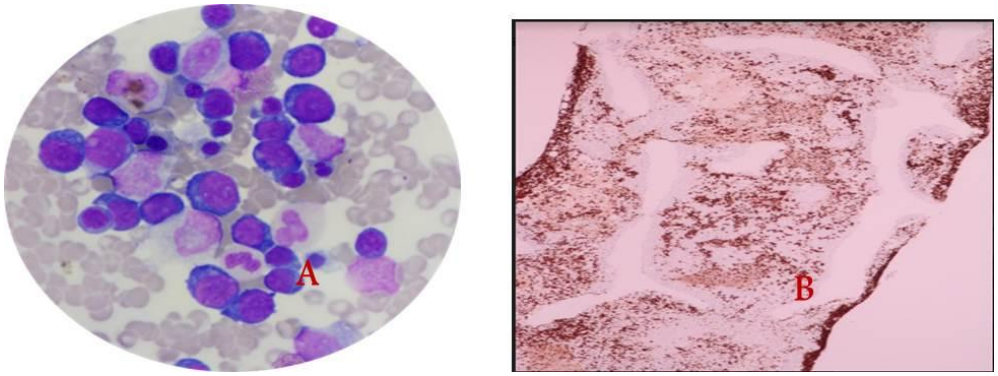

Figure 12: BMA and Trephine Images for MG9: (A) Marked erythroid hyperplasia, left shifted with erythroblasts seen (FAB M6) (B) CD 235 positivity)

4.3.10 Case 10: MG 10

MG 10 is a 70 year old lady who presented to hospital with a long standing history of easy fatigability, joint pains and weight loss. She had presented to hospital due to recent onset easy bruisability and spontaneous epistaxis. She had no known co-morbidities and was previously active for her age. The main risk factor identified was prolonged history of sniffing tobacco. She was a widow with eight living children. On examination, her main findings were frailty and severe pallor. Total blood count showed (WCC) 1.59×109/l, (ANC) 0.34×109/l, (ALC) 1.19×109/l, (AMC) 0.04×109/l, (HB) 7.9g/dl and (PLT) 48×109/l. PBF showed rare circulating blasts, nRBC and leucopenia. BMA revealed a hypocellular marrow with 30% blasts ***(fig 13).*** Targeted next generation sequencing identified TP53 (c.377A>G p. Y126C) and STAG2 (c.2245_3425insGG p.V346fs).

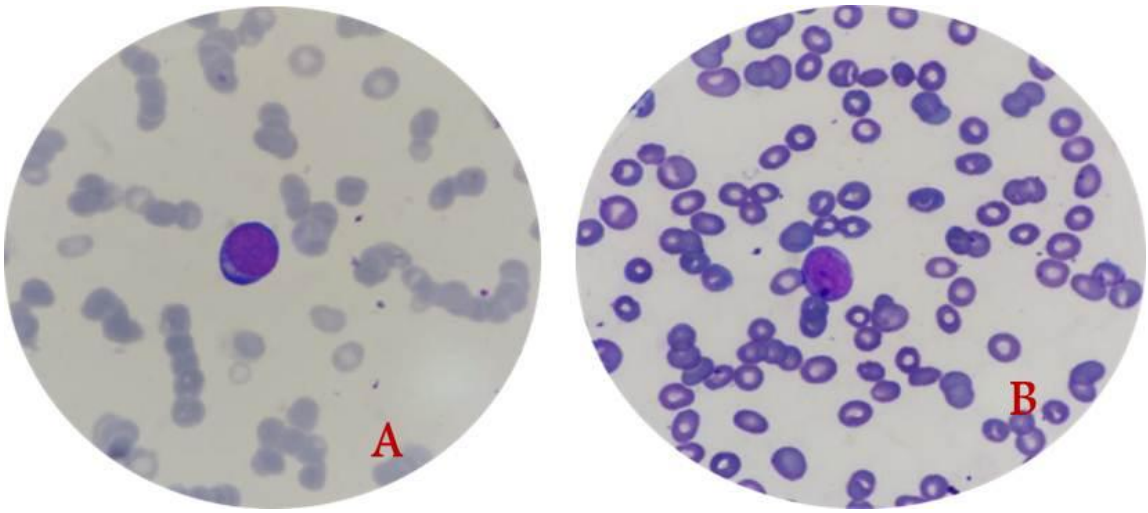

Figure 13: PBF and BMA images for MG10: (A) Nucleated RBC and (B) Myeloblast

CHAPTER FIVE

DISCUSSION

This study was undertaken to determine the chromosomal abnormalities and molecular mutations of acute myeloid leukemia patients presenting to the hemato-oncology unit of Kenyatta National Hospital. It also sought to prognosticate patients according to the 2017 EuropeanLeukemiaNet Cytogenetic and Molecular risk stratification. Ten patients participated in the study. There were no reportable data on the cytogenetic arm and therefore the ELN cytogenetic and molecular risk stratification could not be reported on.

5.1 Conventional Cytogenetic

Cytogenetic studies are routinely carried out on bone marrow specimens as they provide higher yield with few failure rates. However, peripheral blood is an alternative source in the presence of adequate circulating blasts and on occasions where it's not possible to obtain an aspirate(110). In this study patients already had a bone marrow aspirate or trephine biopsy done prior to enrollment in the study. Bone marrow specimens provide enough metaphases unlike peripheral blood even though the chromosomal abnormalities detected do not differ between the two specimen(111). Cytogenetic analysis by karyotyping requires that the sample be delivered to a skilled lab within 24-48hours(52). In low income countries, few countries have such laboratories and the samples have to be shipped abroad which increases the failure rate of achieving adequate metaphases. FISH test, can be used for detecting targeted known abnormalities(12).

The reported rate of unsuccessful karyotyping in hematological malignancies is 10-20%(46,47,112,113). Unsuccessful karyotyping will occur in occasions where there is reduced cell viability resulting in too few metaphases for analysis, inadequate chromosome banding as well as the cytobiology of the leukemic cell. Bruno et al, in a retrospective review showed that out of a cohort of 1,623 patients, 14% had unsuccessful karyotype. These patients were older and had a poor prognosis similar to those with unfavorable risk factors(114). Santos et al had failed cytogenetic studies in 13.5% of the acute myeloid patients. Low cellularity and a peripheral blood sample were predictive of a failure(115). A retrospective review of a Pakistani Cohort of AML patient could not report cytogenetic abnormalities in 33 patients (10%) due to inadequate metaphases(112). Li et al demonstrated that postal transit of peripheral blood to the lab decreased the yield of metaphases required for a successful analysis(111). Peripheral blood

with circulating blasts of $>0.1\times10^9$cells/l predicts for successful cytogenetic studies using peripheral blood as the primary source(110). These studies underline the importance of obtaining the appropriate specimen for cytogenetic analysis and expediting the specimen handling process.

In this study we used peripheral blood. Specimen handling during transit and the customs clearance process increased transit time to an average of 4 days which could have contributed to the low mitotic index seen during the cell culturing process. This challenge is not unique to our center. Chaufaille et al, reported on the utility of an inexpensive, feasible and reliable method of using cryopreserved bone marrows cells albeit with the caveat of a slightly higher chance of failure rate compared to fresh samples(113).

5.2 Next Generation Sequencing

This study provided information on the molecular landscape of ten acute myeloid leukemia patients in Kenyatta National Hospital, utilizing targeted sequencing for recurrently mutated genes in myeloid neoplasms. In our study, at least one mutation was identified in all patients similar to what is reported in literature(116). The molecular spectrum of each patient is discussed.

5.2.1: Case 1: MG1

MG1 had two mutations detected in the NRAS, an activated signaling gene and WT1, a tumor suppressor gene. N-RAS (Q61K) is a point mutation that substitutes glutamine for lysine. This amino acid change abrogates the GTPase activity on the N-RAS proto-oncogene resulting in a perpetual state of activation. The resultant accelerated cell growth and proliferation occurs due to unregulated activation of the intracellular signaling pathways. Single point mutations occurring at codon 16 are the commonest occurring in 10% of AML patients with 0.5% of these being Q61K(117). N-RAS mutations occur more prevalently in patients with T (16:16), inv (16), inv (3) or T (3:3) where mutations in codon 61 predominate, though this cannot relate to MG1 as cytogenetic studies failed. It occurs equally in de novo and secondary AML cases and its prognostic significance has not been well defined(67,118). Mutations in the N-RAS gene appear as a late acquisition in the clonal evolution of AML(84). In a case report, it was noted that N-RAS (Q61K) was the pathogenic mutation transforming an MDS case to AML(119). This mutation is not unique to AML and has been described in other hematological and non-hematological malignancies(117). Trials targeting N-RAS with Farnesyl transferase inhibitors

have not been successful so far. Patients with this mutation benefit from consolidative therapies utilizing high dose cytarabine.

Two frameshift insertion mutations of WT1 were also detected. These mutations alter the DNA binding domain of WT1 gene. This oncogenic mutation has been described previously in 4 pediatric AML cases(120). Among AML patients, 10% will have mutations in the hotspot genes commonly clustered at exon 7. The resultant dysfunctional protein lacks the ability to bind to its DNA domain losing its ability to regulate multiple genes involved in controlling cellular functions(121). Its prognostic impact is yet to be defined. Even though this study was not assessing co-mutations, there seems to be a synergistic association between WT1 mutations and mutations involving the activation signaling genes(i.e. N-RAS) with a higher prevalence in younger patients as shown by Hsin-An Hou et al(122). Both alterations contribute to leukamogenesis by inducing proliferation in progenitor cells incapable of differentiating and undergoing apoptosis(123).

MG1 also presented with significant cervical lymphadenopathy and massive splenomegaly. Extramedullary infiltration by leukemia blasts leading to lymphadenopathy and hepatosplenomegaly is not uncommon in AML(39). However massive splenomegaly is not common. In this case, it could represent a clonal evolution from an unidentified myeloproliferative neoplasm. This patient did not harbor any mutations that could explain his massive splenomegaly as the common mutations associated with MPN were not detected.

While admitted, MG1 received multiple packed cells and platelet concentrates. He had been treated severally for recurrent nosocomial infections. He succumbed to the disease as he awaited induction with the 7+3 AML induction protocol.

In conclusion, MG1 had two mutations whose prognostic relevance is still not yet defined and no targeted therapy has been developed.

5.2.2 Case 2: MG2

MG2 had two mutations affecting CEBPA gene, a myeloid transcription factor and DNMT3A, an epigenetic modifier through its role of regulating DNA methylation. CEBPA (G130V) is a monoallelic missense mutation occurring on the N-terminal region of the CEBPA gene. CEBPA gene has a Carboxyl and N-terminal domain that codes for a transcription factor tasked with the emergence, differentiation and terminal maturation of the myeloid cells. Mutations affecting the carboxyl domain result in disruption of the DNA binding interface; whereas mutations affecting the N-terminal result in a truncated isoform that inhibits the wild type normal allele. This mutation has been identified by Santhi et al, in an Indian cohort of AML

patients(124). CEBPA mutations are associated with good clinical outcomes but only in those with the biallelic mutation (mutation affecting both the N and carboxyl terminal domains). CEBPA mutations cluster among AML cases with a morphological FAB-M1, M2 and M3(125).

DNMT3A is an epigenetic modifier whose mutations results in a hypermethylated DNA state. DNMT3A (c.2645G>A; p. R882H) is the prototype mutation found in AML cases, and is one of the most frequently mutated genes in AML(84). Though its prevalence increases with age, it has been described in younger AML patients and is significantly co-mutated with NPM1 and FLT3-ITD, mutations that were not detected in MG3. In this cohort of patients, it has no prognostic relevance(126). It is thought that acquisition of this early mutational event occurs in the founding clone and is detected during relapse(84). Co-mutations with monoallelic CEBPA mutations have been described and confer no prognostic relevance(127).

While admitted MG2, was stabilized on antibiotics, packed red blood cells and platelet transfusions. She has repeatedly developed recurrent infections varying from cellulitis, septic arthritis, bartholin abscess and septic wounds at cannula sites. She also has had recurrent gum bleeding, ecchymosis at cannula sites and menorrhagia. She awaits induction with the standard 7+3 AML protocol.

In conclusion, MG2 has 2 mutations whose prognostic relevance is yet to be defined. Currently there are no targeted therapies that have been developed for these mutational events.

5.2.3 Case 3: MG3

MG3 had PML-RARA, a transcriptional fusion gene and CEBPA(V232fs) mutations detected. PML-RARA fusion gene has long been identified as the main oncogenic driver of acute promyelocytic leukemia, a type of AML occurring in 10% of the AML cohort(128). However, it is recognized that APL is a heterogeneous disease with differing outcomes and is not exempt from sharing co-operating mutations. Historically, FLT3, RAS and WT1 mutations are known to co-operate with PML-RARA(116). Ibáñez and Riva et al characterized the molecular landscape of acute promyelocytic leukemia and identified other gene mutations that co-operate with the PML-RARA gene to induce leukamogenesis(129). None were found in MG2.
A point mutation resulting in a mono allelic CEBPA mutation was also identified. CEPBA is a transcription factor that regulates myeloid differentiation with mutations linked more to the M0-M3 FAB classification(125). In addition, there is documented mutual exclusivity with

transcription factors(116). The relevance of this co-mutation could not be established as literature search did not yield any co-existence of PML-RARA fusion gene and CEPBA mutations. In a study by Long Su et al, none of the patients with PML-RARA harbored either mono-allelic or bi-allelic mutations of CEBPA(130). Recently, novel CEBPA target genes were identified that regulate APL differentiation suggesting that not all co-operating mutations have been mapped(125).

In conclusion, MG3 had acute promyelocytic leukemia, a distinct type of acute myeloid leukemia with a tendency for severe hemorrhage as evidenced by her clinical presentation. Currently APL is the most curable form of AML, thanks to targeted therapy. Achieving cure with the combined use of all-trans retinoic acid (ATRA) and arsenic trioxide in the absence of cytotoxic therapy is a model to be enumerated by future targeted AML therapies(128).

5.2.4 Case 4: MG4

MG4 had three mutations detected in the DNMT3A (R882H), IDH2 (R172K) and FLT3 gene. DNMT3A and IDH 2 are epigenetic modifiers that regulate DNA methylation status. When mutated, they result in a hyper methylated state inhibiting the process of hematopoietic differentiation. They constitute the most prevalent mutations in AML and are clustered more in those with normal karyotype and those in the intermediate risk group(131). Their prevalence increases with age and they occur early in the clonal evolution of AML and exhibit co-mutations with FLT3 and NPM1 gene(84). Among AML patients, 22% will exhibit DNMT3A mutations that occur at residue R882H, near the carboxyl terminus of the DNMT3A protein ***(Fig14)***. Studies looking at their prognostic significance are not conclusive; however most studies show a trend towards inferior survival and increased risk of failure to achieve complete remission with standard therapy(131). Elderly and unfit patients show improved response to hypomethylating agents. In the younger population, the addition of HMA to standard chemotherapy in those harboring this mutation is under active research(132). $IDH2^{R172K}$ is a novel mutation that is present in 8-19% of AML patients. It results in the replacement of arginine for alanine in the 172 codon altering the active domain of the gene. It is associated with older age, higher white cell count and higher platelet count, characteristics that are similar in those with DNMT3A mutations. Patient's in the intermediate risk group harboring this mutation have a poor prognosis(133,134).

FLT3 mutations are commonly mutated in AML patients and in association with DNMT3A mutations(116). FLT3-ITD mutations confer a poor prognosis especially in those with a high mutational load. On the other hand, the prognostic relevance of the TKD mutations is not well

defined. FLT3 (c.1815T>C, p. =) is a nonsense mutation that results in a stop codon resulting in formation of a dysfunctional protein interfering with the tyrosine kinase domain. Interference with the TKD confers resistance to inactivation of the receptor causing the receptor to be in a perpetual active state with increased intracellular signaling. Targeted therapies inhibiting the FLT3 pathway have been engineered and are in clinical use(135).

In conclusion, MG4 is a patient with multiple co-morbidities precluding the use of standard chemotherapy. Despite the fact that none of the mutations detected are prognostically recognized in the ELN risk groups, multiple therapies targeting these mutations have been identified, signifying the utility of NGS in clinical practice. In our current setting the presence of DNMT3A mutations implies that she will greatly benefit from hypomethylating agents with improved outcome(132).

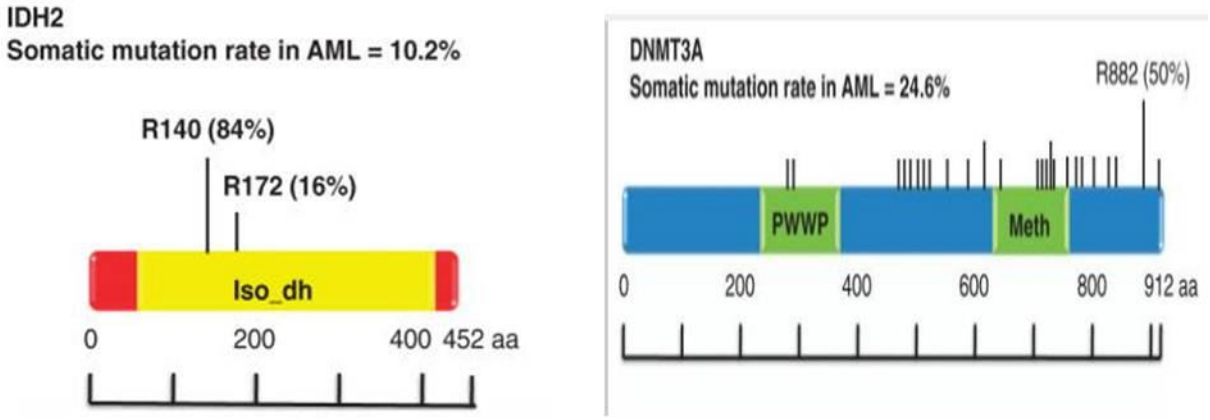

Figure 14 : Location and frequency of DNMT3A and IDH2 mutations as determined by the Cancer Genome Atlas

5.2.5 Case 5: MG5

MG 5 had two mutations detected in the RUNX1 gene, a myeloid transcription factor and PTPNII an activated signaling gene. RUNX1 (c.592G>A p. D198N) is a missense mutation that results in a truncated protein that abrogates its ability to bind to its DNA domain to effect its regulatory activity on the process of hematopoiesis. RUNX 1 mutations are identified in 10-16% of AML patients(136). It clusters more in the elderly patients, males and those with previous antecedent hematological disorder. Patients with RUNX 1 mutations are likely to have cytopenias, normal karyotype, induction related deaths and an overall poor response to

chemotherapy especially in the younger cohort. On the other hand, elderly patients are more likely to have good treatment response to HMA(98,137). In addition, it's more prevalent in those with minimal differentiation (FAB MO) and patients harboring trisomy 8. RUNX1 somatic mutations are not only prevalent in acute myeloid leukemia but also in other hematological malignancies. This is closely related to its critical role in the generation and differentiation of the hematopoietic stem cell(136). Co-mutations associated with RUNX1 mutation include activated signaling genes among then PTPNII. The co-existence of these two groups of genes is thought to be synergistic with perturbations of signaling pathways resulting in increased proliferation of the immature blasts. Although this co-existence is of no prognostic significance, generally mutations in RUNX1 are more likely to have poor outcomes especially in the young patients(137).

MG 5 was treated with the standard 7+3 AML induction protocol with the goal of travelling overseas for allogeneic transplant. However, 30 days post induction, patient succumbed from neutropenic sepsis.

In conclusion, the predominant mutation in MG5 was RUNX1 mutation, whose poor prognostic effect was compounded by the presence of the PTPNII mutations. However due to lack of cytogenetic results, we could not conclusively classify him according to the ELN risk groups. ELN recommends excluding the favorable risk AML subtypes, prior to classifying RUNX1 mutation as an adverse risk factor(14).

5.2.6 Case 6: MG 6

MG6 had four mutations affecting the TP53 and NF1, tumor suppressor genes, STAG2, a cohesion gene and ETV6 a gene belonging to the ETS family of transcription factors. TP53 p. (R248Q) is a missense mutation that results in a truncated protein variant, disrupting its capability of binding to its DNA domain. TP53 is the most commonly mutated gene in cancers. It's not only the guardian of the genome but has many other cellular regulatory functions(138). Among AML patients 6-10% of will harbor this mutation that is associated with significant inferior survival outcomes. It clusters among the elderly, males and in secondary AML cases(88,139). Patients are likely to have lower white cell and platelet counts, lower blast % and complex karyotype. There is a higher frequency of TP53 mutations in patients with erythroleukemia (FAB M6) and is pathogenic in the progression of polycythemia vera and essential thrombocythemia to AML(140). Patients with TP53 mutations have lower survival rates compared to their wild type counterparts regardless of mode of therapy. Curiously it co-

occurs with the rarer infrequent mutations reported in AML similar to MG6 co-mutation pattern.

Mutations affecting the STAG2 gene have also been reported in AML patients. STAG2 is a cohesion complex gene that regulates the process of mitosis. Thota et al noted that patients with STAG2 mutations were likely to be categorized in the ELN poor risk category with inferior outcomes to treatment(141).

NF1, was also noted to be mutated in this patient. NF1 is a GTPase protein that regulates the RAS-RAF-MAPK signaling pathway. Mutations in this gene results in accelerated signaling activity and confers an adverse risk to the younger cohort of patients(142). ETV6 c.403_404insT p. (H135fs) frameshift mutation was also identified. ETV6 genetic lesions are mainly described in the context of a fusion abnormality with multiple genes. They have varied roles in the regulation of the hematopoietic process. Somatic mutations affecting the single entity ETV6 gene have been described by Wang et al(143). They are rare. They are mainly frameshift mutations and they result in the loss of its regulatory domain. The prognostic relevance of these mutations has not been defined.

While admitted, MG6 developed refractory diarrhea, hypokalemia and severe persistent generalized abdominal pain. She succumbed 3 weeks after she was diagnosed with acute myeloid leukemia. Her mutational profile was that of an aggressive disease that does not respond to standard therapy and would have required allogeneic transplant post induction.

In conclusion, MG6 had adverse prognostic genetic mutations. TP53 mutations are classified under the adverse risk group and are associated with increased risk of relapse despite allogeneic transplant(144).

5.2.7 Case 7: MG7

MG7 had only one mutation affecting the IDH2 gene. AML with myelodysplasia related changes is characterized by significant morphological multilineage dysplasia, an MDS related cytogenetic abnormalities or a previous history of an MDS or an MPN. It is uniformly associated with poor outcomes. IDH mutations have been reported in 9.7% of the patients with AML with MRC. These mutations are acquired before the phenotypic expression of AML and are thought to be the pathogenic driver of the dysplastic changes(145). In patients with MDS or MPN disorders, the presence of IDH2 mutation predicts a high likelihood of transforming to AML(146). IDH mutations result in a hypermethylated DNA state interfering with multiple

epigenetic functions with resultant leukamogenic effects. The clinical significance of identifying IDH mutations is three fold. Measurable values of IDH can be used to detect minimal residue disease as well as predict risk of relapse in those who have achieved complete remission(147). In addition, targeted therapy in combination with hypomethylating agents have been approved for those harboring this mutation(148). MG7 has been started on azacitidine until disease progression or unacceptable adverse effects.

In conclusion, MG7 had IDH2 mutation as the sole genetic mutation. Despite its lack of prognostic relevance and non-inclusion in the ELN risk groups, targeted therapies against the IDH mutations are in clinical use and will become standard of care in the world over in future(144).

5.2.8 Case 8: MG8

MG8 had mutations detected in the CEBPA, N-RAS, STAG2 and WT1 genes. CEBPA Y181 is a monoallelic mutation that results in the generation of a truncated isoform that inactivates the wild type CEBPA gene inhibiting myeloid differentiation. It clusters in the morphological M1, M2 and M4 FAB subgroups. Patients with intermediate risk cytogenetic studies harboring a biallelic mutation are more likely to have a better outcome compared to those with the monoallelic variants(125,149). The effect of N-RAS, STAG2 and WT1 mutations have been described previously. As reported previously, the prognostic significance of N-RAS and WT1 have not been well defined. However a tendency of these mutations to co-exist has been reported(122). Patients with STAG2 mutations are likely to cluster in the poor prognostic group, as reported previously(141).

MG8 was managed with the standard (7+3) treatment protocol for AML. On day 21 of treatment, a repeat BMA showed that he was not in remission which is not surprising considering his mutational spectrum. During and after treatment patient developed septic shock secondary to a lower respiratory tract infection. He succumbed to the disease 9 weeks after starting induction.

In conclusion, MG8 had mutations that were not classifiable under the ELN risk group. Studies however do uniformly agree that the presence of WT1 mutations especially in those with a normal karyotype predicts poor outcome(122,150). No targeted therapies for the above mutations are in clinical use.

5.2.9 Case 9: MG9

MG9 had 5 mutations detected in TP53, WT1, GATA2, N-RAS and IKZF1 genes. The frameshift mutation identified in the TP53 gene results in the deletion of the valine protein resulting in disruption of its DNA binding domain interfering with its regulatory functions. Curiously, MG9 had a FAB classification of M6 (Erythroleukemia) similar to MG6 and both had TP53 mutations, though of differing variant effect. There is a well-documented morphological correlation of FAB M6 with TP53 mutations and this has been shown to be directly related to inhibition of TP53 regulatory pathways by overexpression of GATA 1 gene(151,152).

GATA 2 gene plays a key role in the emergence of the erythroid series of hematopoietic cells. Mutations affecting this gene are clustered on its DNA binding domain disrupting its DNA binding interface resulting in loss of activity. Presence of this mutation identifies a cohort of patients with poor outcome. Co-mutation of GATA2 with N-RAS and WT1 has been reported(153). In addition, there was a non-synonymous mutation affecting IKZF1 gene that is located on chromosome 7. Loss of regulatory function of the IKZF1 gene has mainly been described in the lymphoproliferative disorders. However, in the advent of NGS, its role in the pathogenesis of AML has been described. IKZF1 gene is a key regulator of the myeloid differentiation and mutations affecting this gene were extensively described by Jasmijn et al(154). Loss of IKZF results in up regulation of GATA1 expression which as previously described plays a role in blocking erythroid maturation. There is a preferential co-mutation of IKZF1 mutations with PTPNII and N-RAS mutations which activate the RAS-RAF signaling pathways. As previously mentioned, co-mutations involving transcription factors and signaling genes co-operate to induce increased proliferation of immature blasts. MG 9 was treated with the standard 7+3 AML induction protocol. However 15 days post induction, patient succumbed from sepsis.

Among the ten patients, MG 9 had the highest mutational load. TP53 and WT1 are associated with adverse outcomes. The presence of TP53 classifies this patient in the adverse ELN risk group(14). In the absence of treatment related mortality, patient would have benefited from an allogeneic transplant even though outcomes thereafter are still poor.

5.2.10: Case 10: MG10

MG 10 had both TP53 and STAG2 mutations. TP53 (c.377A>G p. Y126C) is a missense mutation resulting in a protein isoform that has lost its DNA binding capacity abrogating its

regulatory cellular functions. The prognostic significance has previously been described(155). TP53 mutations are associated with older age and therefore it is not surprising that it was identified in this patient. MG 10 also had a frameshift mutation resulting in loss of function of the STAG2 gene. STAG 2, a cohesion complex molecule works synergistically with epigenetic regulatory genes, which control stem cells self-renew and pluripotency ability. Its prognostic relevance has not been well defined. Thol et al reported that mutations in these genes confer a poor outcome whereas Cheng-Hong Tsai et al differed with this report(156,157). In his cohort, patients had favorable outcomes. Patient with this mutation are more likely to respond to hypomethylating agents(156). On further discussion with the family, due to frailty, age and long distance of travel, a decision was reached to offer her best supportive care.

In conclusion, MG10 had adverse prognostic genetic mutations according to the ELN risk group(14) and due to frailty and advanced age, she would not have benefited from aggressive treatment. However, recent trials do show that elderly unfit patients with TP53 mutations do achieve durable response when treated with HMA in combination with venetoclax, a BCL2 inhibitor which induces cellular apoptosis independent of the TP53 pathways(144). With the advent of NGS and discovery of novel molecular mutations, individualization of therapy in AML patients with improved outcomes is becoming a reality even amongst those deemed unfit to receive cytotoxic therapy and those whom unfavorable genetic lesions predominate.

6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusion

This study looked at the cytogenetic and molecular abnormalities among AML patients presenting to the hemato-oncology unit of Kenyatta National Hospital. There was no reportable data on the karyotype status of the patients as no cells were cultured from peripheral blood. Increased transit time to the laboratory was the most likely cause for this failure. The study demonstrated that patients with AML in KNH do have deleterious mutations that impart a unique genomic spectrum for each and every patient and contribute to the heterogeneity of disease outcome among the patients. We therefore conclude that lack of cytogenetic analysis and subsequently the results precluded us from drawing definite conclusions on the prognostic effect of these mutations. In the near future, incorporating next generation sequencing in the diagnosis of our AML patients will allow us to refine and personalize the management of patients with AML presenting to KNH.

6.2 Study Limitations

1. The study population is small and limited to one center, so this data will not be extrapolated to other acute myeloid leukemia patients presenting to other facilities in Kenya
2. The small sample size did not allow us to determine the co-mutations patterns of genetic lesions and their frequencies and therefore comparisons with other studies could not be done.
3. The lack of cytogenetic analysis results due to low mitotic index precluded us from determining the prognostic relevance of the mutations detected.

6.3 Recommendation

1. A larger prospective study is needed to profile the genetic lesions associated with AML patients presenting to Kenyatta National Hospital.
2. In our set-up with increased transit time to a specialized laboratory for cytogenetic studies, bone marrow aspirate is the ideal specimen.
3. A laboratory with expertise in cytogenetic and FISH studies needs to be set up in our country to enable timely and cost effective analysis of karyotype abnormalities.
4. Next generation sequencing modalities that integrate karyotyping and mutational screening therefore bypassing the technical difficulties of conventional cytogenetic metaphase studies would be useful in our setup.

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APPENDICES

Appendix I: Consent Explanation

1. STUDY PARTICIPANT CONSENT FORM

"Cytogenetic and Molecular profiling of acute myeloid leukemia patients presenting to the adult hemato-oncology unit of Kenyatta National Hospital."

Name	Qualification	Institution	Department	Position
Dr. Mercy Gatua	MBChB MMED-Internal Medicine	UON/KNH	Clinical medicine and Therapeutics	Medical Oncology Fellow

Purpose of the study

I, **Dr. Mercy Gatua** am undertaking this study on "Cytogenetic and Molecular profiling of acute myeloid leukemia patients presenting to the adult hemato-oncology unit of Kenyatta National Hospital".

Procedures

You are being asked to participate in this study. If you agree to participate, I will ask you to sign a consent form. There will be a series of questions that I will ask you in confidence and all your responses will be noted down. These questions will be in the form of a questionnaire.

I will request to draw 10-15 cc of blood from a vein on your arm. The blood sample that has been taken from you will undergo genetic analysis to determine what the underlying cause of your acute leukemia is.

Risks to you as a participant

There is a risk associated with drawing blood from you. You will experience minor pain from the site where blood will be drawn from. In rare cases, you may experience prolonged bleeding, prolonged discomfort or infection from the site where blood has been drawn from. However, I will minimize this risk by disinfecting the puncture site with a sterile swab and povidine disinfectant (brown in color). I will use a disposable sterile 18 cc gauge needle (Color Coded blue) to collect blood in a sterile 20cc syringe. After drawing blood, I will apply pressure to

the puncture site to minimize bleeding and discomfort. I will thereafter use a sterile pressure dressing to cover the puncture site to stop any further bleeding and minimize risk of infection.

In case of prolonged bleeding do not hesitate to call me or any other health care provider near you. I will also give you information on how to take care of the puncture site to minimize risk of infection.

Benefits

This study will provide an opportunity to determine the genetic mutations that are causing the leukemia you have been diagnosed with. This will determine the next course of treatment after you have undergone chemotherapy to get rid of the leukemia cells in your blood. This study has not been carried out before in KNH and will provide information on the underlying genetic causes of acute myeloid leukemia in patients seeking treatment at KNH.

Right to refuse.

Your participation in this research is voluntary. You are free to withdraw from the interview at any time and you shall not be discriminated upon. You are free to ask any questions and have a right to satisfactory answers before you sign the consent form.

If you agree to participate in this study you may kindly sign the consent form.

Appendix II: Consent to Ship Samples Abroad

Blood sample handling

The blood sample that has been drawn from you will be transported using a designated courier to the hemato-pathology section, department of experimental diagnostics and experimental medicine, S.Orsola Malpighi hospital, Bologna University, School of Medicine, Italy. This laboratory is equipped with the appropriate laboratory machines and personnel with the capability of carrying out the tests.

Justification for shipment of blood sample

The genetic studies to be carried out on your blood sample are highly specialized tests. There is no laboratory in Kenya currently with the capacity to carry out such tests. I have partnered with a research laboratory in Italy (mentioned above) to carry out the test on my behalf. I will seek all the necessary authorization from regulatory bodies prior to transporting your samples.

Procedure for transport and data protection

In this study, we will assign you a study subject number and your name will not appear in any records. Access to your samples will be limited to me and the laboratory personnel assisting in the processing of your tests. The results of your genetic tests will be availed to you as this will influence your subsequent management. The blood sample will be handled safely and disposed thereafter by the laboratory in Italy.

If you agree to your blood sample being shipped abroad, kindly sign the consent form below

Appendix III: Consent Certificate by Patient

I….....consent to participate in the study on "Cytogenetic and molecular profiling of acute myeloid leukemia patients presenting to the adult hemato-oncology unit of Kenyatta National Hospital". I do this with the knowledge of the purposes of the study and the procedures thereof. The purpose of the study and procedure has been explained to me clearly by DR.MERCY GATUA. I am aware that my blood samples will be shipped abroad for the genetic tests as there are no laboratories in Kenya with the capacity to carry out such tests currently. I am also aware that I can withdraw from this study without losing any benefits and quality of care of my medical condition.

Signature of patient…… Date……

Signature of witness……..……. Date……

If you have any questions during the course of the study, you may contact the following.

Dr. Mercy Gatua

Mobile number 0722-276700

In case of any ethical concerns please contact

The Chairman, KNH/UON-Ethics and Research Committee

Hospital road along Ngong Road

P.O BOX 20723, Nairobi (CODE 00202)

Telephone Number (+254-020)2726300 ext 44355

Chairperson: Professor A.N Guantai

Contact person: Esther Wanjiru Mbuba

Email: uonknh_erc@uonbi.ac.ke

Appendix IV: Maelezo ya kibali

Fomu ya Mshiriki Masomo

"Programu hi ni ya uchunguzi wa “Profaili ya Cytogenetiki na Molekuli ya wagonjwa wa leukemia wanaowasili kitengo cha watu wazima cha kemati-oncology, hospitali taifa ya Kenyatta”.

Jina	Ustahili	Taasisi	Idara	Nafasi
Dr. Mercy Gatua	MBChB MMED- Magonjwa wa Ndani	UON/KNH	Idara ya kliniki na Matibabu	Washirika wa Matibabu ya Oncology

Sababu ya utafiti

Mimi, **Dk. Mercy Gatua** ninafanya utafiti huu juu ya “Profaili ya Cytogenetiki na Molekuli ya wagonjwa wa leukemia wanaowasili kitengo cha watu wazima cha kemati-oncology, hospitali taifa ya Kenyatta”.

Taratibu

Unastahili kushiriki katika utafiti huu. Ikiwa unakubali kushiriki, nitakuomba kusaini fomu ya idhini. Kutakuwa na mfululizo ya maswali ambayo nitakuuliza kwa ujasiri na majibu yako yote yatafahamika. Maswali haya yatakuwa katika fomu ya maswali.

Nitaomba kuteka 10-15cc ya damu kutoka kwenye mshipa kwenye mkono wako. Sampuli ya damu ambayo imechukuliwa kutoka kwako itaenda kuchunguzwa zaidi ili kujua sababu ya msingi ya leukemia yako.

Hatari kwako kama mshiriki

Kuna hatari inayohusishwa na kutoa damu kwako. Utapata maumivu madogo kutoka kwenye tovuti ambayo damu itatolewa. Katika hali mbaya, unaweza kuvuja damu kwa muda mrefu, uchungu wa muda mrefu au maambukizi kutoka kwenye tovuti ambayo damu imetolewa. Hata hivyo, nitapunguza hatari hii kwa kuzuia tovuti ya kufuta kwa kutumia swab ya kuzaa na suluhisho la povidine(rangi ya kahawia).Nitatumia sindano ya kupima 18 cc ya sindano (rangi ya bluu) kukusanya damu katika sindano ya mbolea 20cc. Baada ya kuchora damu, nitatumia shinikizo kwenye tovuti ya kupamba ili kupunguza damu na uchungu. Baadaye nitatumia

shinikizo la kuzaa ili kuzuia kuvuja damu kwa muda mrefu na kupunguza hatari ya maambukizi.

Ikiwa utavuja damu kwa muda mrefu usisite kuniita au mtoa huduma mwingine wa afya karibu na wewe. Pia nitakupa habari kuhusu jinsi ya kutunza tovuti ya kupitisha ili kupunguza hatari ya maambukizi.

Faida

Utafiti huu utatoa fursa ya kuamua mabadiliko ya maumbile yanayotokana na ugonjwa wa leukemia. Hii itaamua kozi inayofuata ya matibabu baada ya kupata chemotherapy ya kuondoa seli za leukemia katika damu yako. Utafiti huu haujafanyika tena hapa hospitali taifa ya Kenyatta na utatoa ufahamu juu ya sababu za maumbile zinazosababisha leukemia katika wagonjwa wanaotafuta matibabu hospitali taifa ya Kenyatta.

Haki ya kukataa

Kushiriki kwako katika utafiti huu ni hiari yako. Wewe uko huru kujiondoa kwenye mahojiano wakati wowote bila madhara kwako. Wewe ni huru kuuliza maswali yoyote na kuwa na haki ya majibu ya kuridhisha kabla ya kusaini fomu ya ridhaa.

Ikiwa unakubali kushiriki katika somo hili unaweza saini fomu ya idhini.

Appendix V: Kibali cha kusafirisha sampuli ng'ambo

Kuhusu njisi damu itaotolewa na kuwekwa

Sampuli ya damu iliyotokana na wewe itatumwa kwa kutumia barua pepe iliyochaguliwa kwenye sehemu ya hematopatholojia, idara ya uchunguzi wa majaribio na dawa ya majaribio, Hospitali ya S.Orsola Malpighi, Chuo Kikuu cha Bologna, Shule ya Matibabu, Italia. Maabara hii ina vifaa vya maabara sahihi na wafanyakazi wenye uwezo wa kufanya vipimo.

Habari ya kusafirisha sampuli kwa inchi ya nje

Uchunguzi wa maumbile unaofanywa juu ya sampuli yako ya damu ni vipimo maalum sana. Hakuna maabara nchini Kenya sasa yenye uwezo wa kufanya vipimo hivyo. Nimeungana na maabara ya utafiti nchini Italia (yaliyotajwa hapo juu) kutekeleza mtihani kwa niaba yangu. Nitatafuta idhini zote muhimu kutoka kwa miili ya udhibiti kabla ya kusafirisha sampuli zako.

Mfiduo wa Transporti na Ukimuji wa Data

Katika somo hili, tutakupa nambari ya somo la utafiti na jina lako halitaonekana katika kumbukumbu yoyote. Upatikanaji wa sampuli zako utakuwa kwangu na wafanyakazi wa maabara wanaosaidia katika usindikaji wa vipimo vyako. Matokeo ya vipimo vya maumbile yatatumika kwako kama hii itaathiri matibabu yako yanayofuata. Sampuli ya damu itashughulikiwa na maabara ya mamlaka.

Ikiwa unakubali sampuli yako ya damu itumwe nje ya nchi, saini fomu ya kibali.

Appendix VI: Cheti ya ridhaa ya mgonjwa

Mimi .... na peana idhini ya kushiriki katika utafiti juu ya "Profaili ya Cytogenetiki na molekuli ya wagonjwa wenye leukemia wanaowasili kitengo cha watu wazima wa kemati-oncology hospitali taifa ya Kenyatta". Ninafanya hivyo kwa ujuzi wa madhumuni ya utafiti na taratibu zake. Kusudi la utafiti na utaratibu umeelezewa kwangu na **DR.Mercy Gatua**. Ninafahamu kwamba sampuli yangu ya damu itatumwa nje ya nchi kwasababu hakuna maabara katika Kenya wenye uwezo wa kufanya vipimo hivi kwa sasa. Pia ninajua kwamba ninaweza kuondokana na utafiti huu bila kupoteza faida yoyote ya ubora wa huduma ya hali yangu ya matibabu.

Saini ya mgonjwa .....Tarehe .....

Hau Kidole gumba.....

Saini ya shahidi .....Tarehe……

Ikiwa una maswali yoyote wakati wa utafiti, unaweza kuwasiliana na yafuatayo.

DKT. Mercy Gatua

Simu ya mkononi 0722-276700

Ukiwa na maswali yoyote ya maadili

Mwenyekiti, Hospitali ya Kitaifa ya Kenyatta / Chuo Kikuu cha Nairobi -Ethics and Research Committee

Sanduku La Posta 20723-00202, Nairobi)

Simu (+254-020)2726300 ext 44355

Mwenyekiti: Professor A.N Guantai

Kuwasiliana naye: Esther Wanjiru Mbuba

Email:uonknh_erc@uonbi.ac.ke

Appendix VII: Study Proforma

(TO BE FILLED BY INVESTIGATOR)

Participant's number……

Social Demographics

- Age…….
- Gender…….
- Occupation Status…….
- County of residence……
- Antecedent hematological disorder……
- History of previous cytotoxic therapy or radiotherapy exposure……..

Laboratory Parameters at first presentation

- Total Leucocyte Count/$\times 10^9$/l
- Hemoglobin level/g/dl
- Platelet Count/$\times 10^9$/l
- Bone Marrow blast %

Appendix VIII: Laboratory Methods

1. Clean the site of needle prick with a sterile swab soaked with povidine solution.
2. Use a 20 cc syringe with an 18 gauge needle to draw blood.
3. Draw 10-15 cc of blood from a peripheral vein.
4. Collect blood in the appropriate vacutainer.
5. Fill in a laboratory request form with relevant clinical data, study participants number, specimen source and time and date of collection.

Cytogenetic studies

Container: Sodium heparin vacutainer (blue top)

Specimen volume: 5-10 cc

Sample handling:

1. Invert several times to mix blood.
2. Send specimen in original tube.
3. Label specimen bottle with the study participants' number and time collected.
4. Blood sample will be kept at room temperature prior to transport. If more than 24 hours will lapse prior to arrival in the laboratory, cold ice blue packs will be used for transport

Next generation sequencing

Container: EDTA vacutainer (lavender top)

Specimen Volume: 5 mls

Sample handling:

1. Invert several times to mix blood.
2. Send specimen in original tube.
3. Label specimen using study participants' number and time collected.
4. Blood sample will be kept at room temperature prior to transport. If more than 24 hours will lapse prior to arrival in the laboratory, cold ice blue packs will be used for transport.

Appendix IX: Detailed report of driver mutations

Name	Gene:	AA coded	Locus	Allele Frequency	Variant type
MG1	NRAS	p.(Q61K)	chr1:115256530	49.35%	missense
	WT1	p.(A382fs)	chr11:32417909	25.53%	frameshift insertion
		p.(A382fs)	chr11:32417910	37.17%	Frameshift insertion
MG2	DNMT3A	p.(R882H)	chr2: 25234373	35.4%	missense
	CEBPA	p.(G130v)	Chr19:33792248	56.3%	missense
MG3	PML-RARA				
	CEBPA	p.(V232fs)	chr19:33792626	63.64%	frameshift insertion
MG4	DNMT3A	p.(R882H)	chr2:25457242	48.40%	missense
	FLT3	p.(=)	chr13:28608241	45.52%	nonsense
	IDH2	p.(R172K)	chr15:90631838	47.25%	missense
MG5	RUNX1	p.(D198N)	chr21:36231792	32.87%	missense
	PTPN11	p.(D61G)	chr12:112888166	31.35%	missense
MG6	TP53	p.(R248Q)	chr17:7577538	53.64%	missense
	STAG2	p.(A787fs)	chrX:123204999	59.34%	frameshift Insertion
	STAG2	p.(A787fs)	chrX:123204999	30.53%	frameshift Insertion
	ETV6	p.(H135fs)	chr12:12006435	5.10%	frameshift Insertion
	NFI	p.(T2540fs)	chr17:29683480	6.41%	frameshift Insertion
MG7	IDH2	p.(R172K)	chr15:90631838	6.96%	missense
MG8	NRAS	p.(G12D)	chr1:115258747	29.45%	missense
	WT1	p.(R380fs)	chr11:32417914	30.94%	frameshift insertion
	CEBPA	p.(Y181fs)	chr19:33792781	25.61%	frameshift insertion
	STAG2	p.(A787fs)	chrX:123204999	26.05%	frameshift insertion
MG9	NRAS	p.(G12D)	chr1:115258747	32.08%	missense
	GATA2	p.(A318V)	chr3:128202767	38.96%	missense
	IKZF1	p.(Y321*)	chr7:50467728	47.16%	nonsense
	WT1	p.(S381*)	chr11:32417910	44.13%	nonsense
	PTPNII	p.(E76G)	chr12:112888211	3.65%	missense
	TP53	p.(V73fs)	chr17:7579471	54.79%	Frameshift insertion
MG10	TP53	p.(Y126c)	Chr 17.7675235	32.32%	missense
	STAG2	p.(V346fs)	ChrX.125673411	15.67%	Frameshift insertion

Appendix X: Material Transfer Agreement

UON/RPE/IPMO/F2

SNo.....

UNIVERSITY OF NAIROBI

MATERIAL TRANSFER AGREEMENT FORM

(To be executed in all cases whenever tangible research materials are transferred to individuals or institutions from the University of Nairobi)

PART A: TERMS AND CONDITIONS

In response to RECIPIENT's request for the MATERIAL .....the University of Nairobi (herein referred to as the "PROVIDER") asks that the RECIPIENT and the RECIPIENT SCIENTIST/INSTITUTION agrees to the following before the RECIPIENT receives the MATERIAL:

1. The above MATERIAL is the property of the University of Nairobi
2. Where applicable, RECIPIENT agrees that it will not use the MATERIAL in human subjects, in clinical trials, or for diagnostic purposes involving human subjects without the written consent of the PROVIDER.
3. The MATERIAL will be used for teaching or not-for-profit research purposes only.
4. MATERIAL will not be used for any purpose inconsistent with this Agreement and in connection with any activity that is subject to consulting or licensing obligations to any third party.
5. Upon completion of the work for which this restricted license is granted, MATERIAL, which has not been destroyed, will be disposed of as explicitly directed by the PROVIDER.
6. The MATERIAL will not be further distributed to others without the PROVIDER's written consent. The RECIPIENT shall refer any request for the MATERIAL to the PROVIDER. To the extent supplies are available, the PROVIDER or the PROVIDER SCIENTIST agree to make the MATERIAL available, under a separate Simple Letter Agreement to other scientists for teaching or not-for-profit research purposes only.
7. The RECIPIENT agrees to acknowledge the source of the MATERIAL in any publications reporting use of it.
8. If the RECIPIENT intends to use such MATERIAL to determine if a commercializable system can be developed as a result of the RECIPIENT having received this MATERIAL whether patentable or not, RECIPIENT shall promptly notify the PROVIDER in writing of the substance of each such Intellectual Property and of the filing of any patent application thereon. RECIPIENT agrees to negotiate in good faith prior to marketing of such discovery compensation to be paid by the RECIPIENT to the PROVIDER.
9. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature. The provider makes no representations and extends no warranties of any kind, either expressed or implied. The RECIPIENT agrees to use the MATERIAL in compliance with all applicable statutes and regulations.

PART B: PROVIDER INFORMATION and AUTHORIZED SIGNATURE

Provider Scientist: .....

Provider Organization: .....

Address: .....

Signature ..... Date .....

Name of Authorized Official: .....

Title of Authorized Official .....

Signature of Authorized Official ..... Date .....

PART C: RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

Recipient Scientist: Pier Paolo Piccaluga

Recipient Organization: Department of Experimental, Diagnostic, and Diagnostic Medicine, Bologna University School of Medicine

Address: Via Massarenti 9, Institute of Hematology and Medical Oncology

Signature: Pier Paolo Piccaluga Date: May, 31st 2019

Name of Authorized Official: Pier Paolo Piccaluga

Title of Authorized Official: Professor of Pathology

Signature of Authorized Official: Pier Paolo Piccaluga Date: May, 31st 2019

Appendix XI: Approval Letter from Ministry of Health.

MINISTRY OF HEALTH
DIRECTORATE OF RESEARCH AND MONITORING & EVALUATION

Telephone: Nairobi 254-020-2717077
Email ps@health.go.ke

When replying please quote:

Ref: MOH/F/HRD/01/VOL. II

Afya House
Cathedral Road
P.O. Box 30016-00100
NAIROBI

31st May, 2019

Dr. Mercy Gatua
P.O. Box 20890 - 00202
NAIROBI

RE: APPROVAL FOR SHIPMENT OF STUDY SAMPLES TO ITALY, BOLOGNA

Your request letter dated 24th May, 2019 refers.

You are hereby requested to submit an approved proposal and a letter of Ethical Review Clearance by the KNH-UoN ERCommittee, and a no objection letter from the recipient university in Italy before we can grant a letter of approval to ship study samples.

Thank you.

Dr. Charles M. Nzioka
Ag.DIRECTOR,DIRECTORATE OF RESEARCH, MONITORING & EVALUATION

ISO 9001:2015
Certified

Appendix XII: Timeline

	2019			**2019**		
	Feb	**March**	**April-May**	**June-August**	**September**	**Oct**
Proposal Development						
Proposal Presentation						
Ethics Committee Review						
Data Collection						
Results Presentation						
Publication						

Appendix XIII: Budget

	Cost per patient in **Kshs.**	Total for 10 patients in **Kshs.**
Transport to Italy	10,000	100,000
Genetic testing	37,300	373,000
Total for tests	47,300	473,000
Miscellaneous	5,000	50,000
Gross total	52,300	520,300

Appendix XIV: KNH Ethical Approval Letter

Resub/P275/04/2019.

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300

KNH-UoN ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERCs

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9 Ext 44355, 44102
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/RR/478

21st May, 2019

KENYATTA NATIONAL HOSPITAL RECEIVED 11 JUN 2019 KNH/UoN-ERC P.O. Box 20723-00202 NAIROBI

KENYATTA NATIONAL HOSPITAL APPROVED 04 JUL 2019 KNH/UoN-ERC P. O. BOX 20723

Dr. Mercy Njeri Gatua
Reg. No. H113/10812/18
For Fellowship in Medical Oncology
Dept. of Clinical Medicine and Therapeutics
School of Medicine
College of Health Sciences
University of Nairobi

Dear Dr. Gatua,

Research Proposal: **Cytogenetic and molecular profiling of acute myeloid leukemia patients presenting to the Adult Hemato-Oncology Unit of Kenyatta National Hospital (P275/04/2019)**

This is to acknowledge receipt of your research proposal and to inform you that upon review by the KNH- UoN Ethics and Research Committee during the 243rd ERC meeting held on 8th May, 2019, the following observations and suggestions were made:

1. Take note that the abstract should have a last section on "***Expected main outcome measure(s)***".
2. Pay attention to the remarkable grammatical and typographical errors throughout the text.
3. Your literature review, largely, is unfocused! It is bloated to the extent that the reader feels cheated. Please endeavour to focus on the broad study objective.
4. Make your specific objectives "SMART" thus:
   i. *To determine chromosomal abnormalities of acute myeloid leukemia patients through conventional metaphase karyotyping.*
   ii. *To determine molecular mutations of acute myeloid leukemia patients using the next generation sequencing.*

   Secondary objective:
   To prognosticate patients with acute myeloid leukemia patients according to the 2017....
5. How would you statistically arrive at a sample of 10 cases?
6. How much blood shall be drawn on any one occasion? (Quantify this.)
7. Where is your protocol document for consent to ship samples abroad? It must include justification for such shipment.
8. Take note that you will be required to develop a Material Transfer Agreement (MTA) with the institution where the samples will be shipped. You will also be required to obtain approval from the Ministry of Health before shipment of samples. (Monday)
9. Include a section on Study Limitations and ensure to address the mitigation measures.
10. Ethical considerations: The Consent and Assent document must have a Kiswahili translation.

Recommendations

Revise and resubmit three (3) copies of the full proposal within a period of four (4) weeks with effect from the date of this letter. Include a cover letter that summarizes how you have addressed the comments and note the page number(s) where the changes have been made.

Yours sincerely,

PROF. M.L. CHINDIA
SECRETARY, KNH- UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Director, CS, KNH
The Chair, KNH- UoN ERC
The Dean, School of Medicine, UoN
The Chair, Dept. of Clinical Medicine and Therapeutics, UoN
Supervisors: Prof. N.O. Abinya (Dept. of Clinical Medicine & Therapeutics, UoN),
Prof. Pier Paolo Piccaluga (Bologna University, Italy), Dr. Matilda Ong'ondi (Hematology Dept. KNH)