The Study of the Impact of Selected Mutations in FMS-like Tyrosine Kinase III (FLT3) and Nucleophosmin (NPM1) – and HIV status on Patients with Acute Myeloid Leukemia and their Response to Induction Therapy

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Submitted in fulfilment of the academic requirements for the degree of Masters of Science in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg.

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As the candidate's supervisor I have/have not approved this dissertation for submission:

Prof E Kormuth: ..... **Date:** .....

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Acute Myeloid Leukemia (AML), the most common form of acute leukemia in adults, is only curable in approximately 30% of all cases. Despite prognostic risk stratification using sub-typing and cytogenetic analysis to direct therapy, the mortality and relapse rate remains high. AML patients with normal karyotypes are defined as intermediate risk and are the most challenging to treat. Somatic mutations may be the key in refining prognostic stratification and providing useful therapeutic targets. The FMS-like tyrosine kinase 3 (*FLT3*) and Nucleophosmin (*NPM1*) genes have common mutated forms that are associated with overall survival and response to therapy. We assessed mutations in the *FLT3* and *NPM1* genes and their levels of expression in twenty eight AML patients in the presence and absence of HIV and their response to induction therapy. Furthermore, we used a novel technique, High Resolution Melting (HRM) Analysis to detect *FLT3* Internal Tandem Duplications (ITD) and *NPM1* exon 12 mutations. Five of the patients in this study were HIV positive, three of whom did not survive post-induction therapy. Of the AML patients, 17.9% were positive for the *NPM1* mutation and 21% had mutated *FLT3*. Interestingly, the presence of the *FLT3* and *NPM1* mutations were coupled with an increase in expression levels of FLT3 and NPM1 from presentation to post-induction respectively and the loss of these mutations were coupled with a decrease in levels of expression from presentation to post-induction. However, an increase/decrease from presentation to post-induction did not necessarily denote the presence/absence of a mutation. Therefore, while mutational status of genes may generally confer mRNA levels, our results showed that there existed no definitive trend between mRNA levels of *NPM1* and *FLT3* expression and mutational status. We found that the HRM method was definitive for the simpler *NPM1* mutation however detection of the *FLT3-ITD* mutation was challenging. There isn't a clear distinction between mutated and non-mutated *FLT3* due to the formation of hetero-duplexes during analysis, making detection highly subjective and error-prone. Sequencing allowed confirmation of mutated *FLT3* and non-mutated *FLT3* which were not in all instances in concordance with HRM analysis. The prognostic value in terms of overall survival of *NPM1* and *FLT3* mutations in this study is indefinite. Furthermore, the analysis of the HIV positive AML patients revealed no clear correlation between *NPM1* and *FLT3* levels of mRNA expression and mutational status. Also, the small number of HIV positive AML patients did not allow for conclusions to be made regarding HIV status and survival when affected with AML.

PREFACE

The experimental work described in this dissertation was carried out at Inkosi Albert Luthuli Central Hospital, Durban, from January 2010 to December 2011, under the kind supervision of Dr V.L Naicker, Dr Natasha Sewpersad and Mrs Safiya Ebrahim. Further experimental work was carried out at the Hasso Plattner Research Laboratory, from September 2011 to May 2012, under the kind supervision of Mr Ravesh Singh and Dr Michelle Gordon.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1 – PLAGIARISM

I, Horacia Naidoo declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION II – PUBLICATIONS

None – preparation for publication in progress.

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TABLE OF CONTENTS

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To my family, you are my strength, my motivation and my greatest comfort! Dad, you are the greatest man of all men; Mum, you persistence and support has been invaluable and Del, thank you for never letting me take myself too seriously!

DEDICATION

To Lertasha, lost but never forgotten.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AML	Acute Myeloid Leukemia
APML	Acute Promyelocytic Leukemia
ALL	Acute Lymphoblastic Leukemia
CML	Chronic Myelogenous Leukemia
CR	Complete Remission
MRD	Minimal Residual Disease
FLT3	FMS like Tyrosine Kinase 3
NPM1	Nucleophosmin 1
HRM	High Resolution Melting
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
mRNA	Messenger RNA
cDNA	Complementary DNA
WHO	World Health Organisation
FAB	French-American-British
WBC	White Blood Cell
TLC	Total Leucocyte Count
HSC	Haematopoeitic Stem Cell
ITD	Internal Tandem Duplication
PCR	Polymerase Chain Reaction
HIV	Human Immunodeficiency Virus
PK	Protein Kinase
MDS	Myelodysplastic Syndrome
PTK	Protein Tyrosine Kinase
TKD	Tyrosine Kinase Domain
OS	Overall Survival
EFS	Event free Survival

DFS	Disease free Survival
FISH	Fluorescent In Situ Hybridisation
EL Buffer	Erythrocyte Lysis Buffer
$MgCl_2$	Magnesium Chloride
dNTP	Dideoxynucleotides
DTT	Dithiothreonine
DEPC	Diethylpyrocarbonate
DIC	Disseminated Intravascular Coagulation
HLA	Human Leukocyte Antigen
EAC	Europe Against Cancer
RE	Restriction Enzymes
ABL	c-abl oncogene 1, non-receptor tyrosine kinase
CD	Cluster of Differentiation

Chapter 1

Literature Review and Introduction

This chapter will seek to provide clarity on the scientific and medical areas that our research traverses. Extensive reading of literature with a focus on Acute Myeloid Leukemia (AML) and the somatic mutations in the following genes - FMS-like tyrosine kinase III (*FLT3*) and Nucleophosmin (*NPM1*) has largely formed the core of this chapter.

In this dissertation please note that "*FLT3* mutations" and "*NPM1* mutations" make reference to mutations in the *FLT3* and *NPM1* genes.

1.1 Acute Leukemia

1.1.1 Definition of Acute Leukemia:

Acute leukemias are malignant clonal disorders originating in haematopoietic stem cells(HSCs) characterized by the proliferation of poorly-differentiated blast cells in the bone marrow and a rapidly progressive fatal course if left untreated (Kawthalkar, 2006). Proliferating leukaemic blast cells replace normal bone marrow cells and subsequently enter into the peripheral blood. Hence there is a dysregulation of processes that control proliferation, apoptosis, self-renewal and differentiation in HSCs (Kawthalkar, 2006).

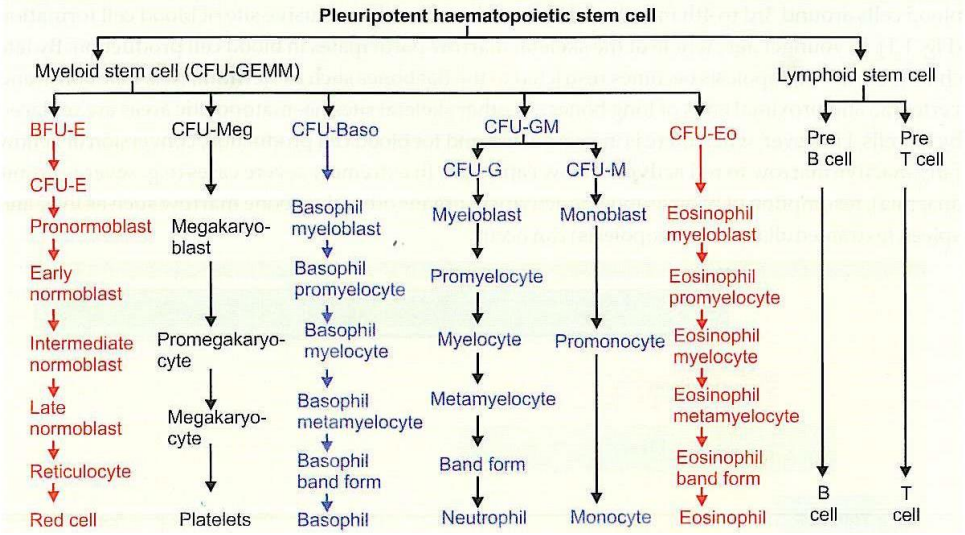

Figure 1 : Normal Haematopoiesis (Reproduced from Kawthalkar, 2006)

Evidence suggests that an acquired genetic abnormality in a HSC may be the initiating event in acute leukemias (Kawthalkar, 2006). This may lead to a dysregulation of processes that control proliferation, differentiation, apoptosis and self renewal enabling leukaemic cells to thrive.

Chromosomal translocations occurring in various leukemias play a major role in leukaemogenesis and are associated with a unique disease phenotype, responsiveness to therapy and prognosis. Different mechanisms such as the activation of a proto-oncogene and increased tyrosine kinase activity are insufficient to cause leukemia and require additional genetic mutations and inactivation of a tumour suppressor gene pathway to cause leukaemic transformation.

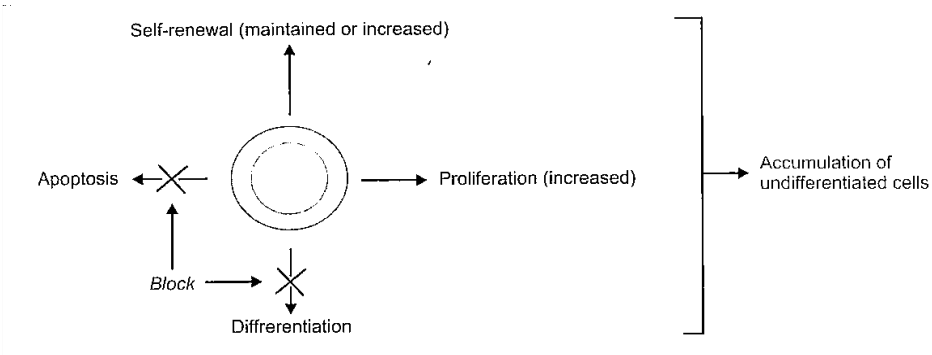

Figure 2 : A simple visualisation of the process of leukaemogenesis (Reproduced from Kawthalkar, 2006)

Risk factors that increase the predisposition of acute leukemias include: hereditary factors such as congenital disorders associated with leukemia and acquired factors such as ionizing radiation (nuclear explosions collateral damage, therapeutic irradiation and diagnostic X-rays), chemical agents (exposure to benzene, alkylating agents and topoisomerase II inhibitors), viruses (Human T lymphotropic virus type I and Epstein-Barr virus), acquired conditions (myeloproliferative disorders, myelodysplastic syndromes and aplastic anaemia) and the influence of age and sex (Kawthalkar, 2006, Bullinger et al., 2004, Appelbaum, 2007).

Symptoms are attributed to lack of red cells, white cells and platelets (bone marrow failure) and manifests itself as anaemia, infections due to neutropaenia and bleeding due to thrombocytopaenia or disseminated intravascular coagulation respectively. Organ infiltration of leukaemic blasts result in clinical features that include: organomegaly, bone pain and tenderness, central nervous system (CNS) disease, gum hypertrophy and chloromas. Symptoms escalate and can be fatal within weeks or months if left untreated (Appelbaum, 2007, Kawthalkar, 2006).

There are two major types of acute leukemia – acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). The differentiation between the two is important as treatment regimens differ accordingly. Classification can be achieved using either the FAB (French-American-British) system or the WHO (World Health Organisation) classification system. FAB classification is based on morphology, cytochemical reactions and immunophenotyping where as WHO classification is based on morphology, immunophenotype, and genetics and has clinical, prognostic and therapeutic relevance (See Table 1 and 2).

The diagnosis of acute leukemia is based on examination of peripheral blood and bone marrow. Laboratory studies include morphological examinations, cytochemistry, immunophenotyping, cytogenetic analysis and molecular genetic analysis. These methods are important in terms of treatment strategies and evaluation of the response to treatment (Basso et al., 2007, Kawthalkar, 2006). Future diagnostic tools may include microarray technology and gene expression profiling which will refine accuracy of diagnosis and sub-typing. Studies have shown that each leukemia subtype has a specific genetic fingerprint therefore a specific combination of genes can pre-empt a tailored therapy for the affected individual (Basso et al., 2007).

1.2 Acute Myeloid Leukemia (AML)

1.2.1 Definition of AML:

Acute Myeloid Leukemia (AML) represents a group of malignant haematopoietic neoplasms originating in the bone marrow and is characterised by the proliferation of blast cells of myeloid lineage (Figure 1). These myeloid neoplasms usually have maturation or proliferation abnormalities (Kawthalkar, 2006).

AML is the most common form of acute leukemia in adults and curable in about 30% of all cases (Falini et al., 2005).

1.2.2 Incidence of AML:

AML occurs in adults and children under 1 year and equally common between the sexes. AML accounts for 15-20% of childhood leukemia (Braoudaki et al., 2010).

1.2.3 Genetics of AML:

Genetic abnormalities are present in more than 90% of AMLs and are usually recurrent (Basso et al., 2007). Whilst oncogenic transformation usually involves alterations in processes of transcription involved in regulation of cell differentiation, these abnormalities alone cannot cause leukemia. The initiation of leukemia is hypothesized to be due in part, to non-random chromosomal translocations which target and deregulate genes that code for transcription factors and subsequently cause a differentiation block. AML progression is often accompanied by gain of function mutations of the signal-transducing molecules such as FLT3 and RAS (Schnittger et al., 2002).

Specific antigen expression in leukaemic cells due to the activation of various transcriptional factors allows acute leukemias to exhibit genetically distinct leukaemic subtypes. Subsequently, studies show that AML patients with cytogenetic aberrations such as a translocation from chromosome 8 to 21 [t(8;21)], t(15;17) and inversion on chromosome 16[inv(16)] compared to ALL patients with t(1;19) and t(12;21) have distinct immunophenotypic profiles which are associated with distinct clinical and prognostic features (Kawthalkar, 2006). AML cases with chromosomal aberrations comprise 30% of

all cases compared to normal karyotype (AML-NK) which comprises 40-50% (Basso et al., 2007); (Braoudaki et al., 2010).

Currently, one of the most frequent chromosomal abnormalities, t(8;21) long arm band 22 (q22;q22) is found in 5-12% of all AML cases, usually in AML M4E0. The translocation t(8;21) is found in approximately 40% of patients with AML M2 and (11q23) is common in patients with AML M4 and M5 and observed in 6-8% of primary AML and approximately 85% of secondary AML (Braoudaki et al., 2010); (Lowenberg et al., 1999).

Interestingly, AML with specific primary mutations accumulate certain secondary mutations such as AML with t(8;21) that tends to lose a sex chromosome.

1.2.4 Possible Causes of AML:

Patients who usually have no risk factors or exposures that cause AML are regarded as having primary AML. Secondary AML can arise from pre-existing disorders such as myelodysplastic syndrome, myeloproliferative diseases (chronic myeloid leukemia, polycythaemia vera, myelofibrosis), paroxysmal nocturnal haemoglobinuria, alkylating drugs, epipodophyllotoxins and radiotherapy given for treatment of neoplasms and genetic diseases with increased disposition to AML such as Down's Syndrome, Bloom's Syndrome & Fanconi's anaemia (Cotta and Tubbs, 2008).

1.2.5 Clinical Features of AML:

Common clinical features include anaemia which presents as weakness and fatigue, granulocytopaenia which presents as persistant infections and thrombocytopaenia which manifests as bleeding tendencies. These symptoms are due to bone marrow failure caused by infiltration of neoplastic cells. Other symptoms include bone pain, hepatomegaly, splenomegaly, skin infiltration, gum hypertrophy, possible lymphadenopathy and isolated masses of leukaemic cells in the bone, skin or lymph nodes (Cotta and Tubbs, 2008, Kawthalkar, 2006).

Hyperleukocytosis can result in ocular or cerebrovascular dysfunction or bleeding. Although rare at presentation, metabolic abnormalities such as hyperuricemia or hypocalcemia can occur (Lowenberg et al., 1999).

1.2.6 Diagnosis of AML:

The diagnosis of AML is confirmed by peripheral blood and/or bone marrow examination, cytochemical stains, immunophenotyping and cytogenetic analysis. These techniques allow the elucidation of the subtype of AML which has prognostic and therapeutic relevance.

Bone Marrow examinations are performed on days 10-14 or 21-28 to assess for residual leukemia after induction chemotherapy (Ravandi et al., 2010).

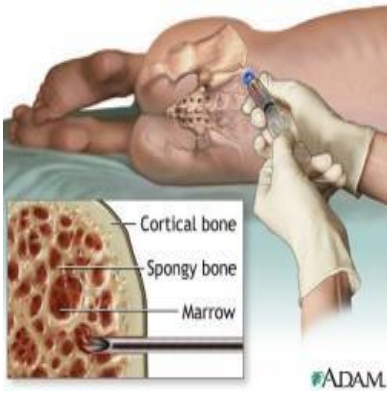

Figure 3: Lumbar puncture used to retrieve patient bone marrow (Reproduced from Appelbaum, 2007)

A peripheral blood examination can confirm AML by anaemia or a low red blood cell count, a low absolute granulocyte count, thrombocytopaenia or a low platelet count and the presence of myeloblast cells which often contain Auer rods in their cytoplasm (Figure 5). These cells have round or irregular nuclei, distinct nucleoli and little cytoplasm. Morphologic abnormalities of the neutrophils such as Pelger Huet cells may be seen. A bone marrow examination can definitively confirm the diagnosis of AML, should there be >20% myeloblasts in all nucleated cells in the bone marrow.

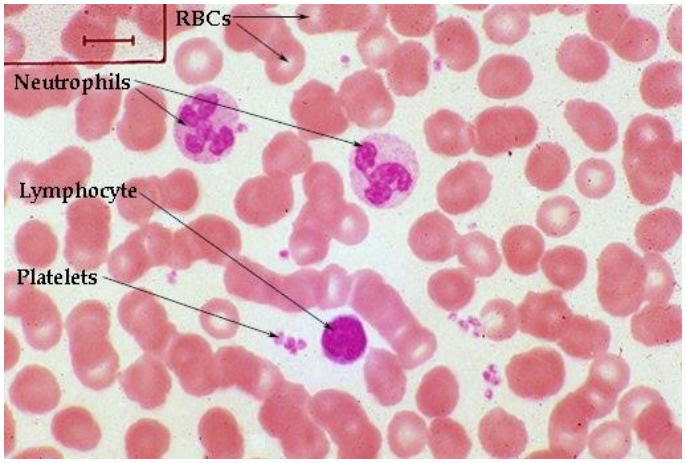

Figure 4: Healthy blood smear showing presence of red blood cells, neutrophils, lymphocytes and platelets in ideal proportions and numbers (Reproduced from Kawthalkar, 2006)

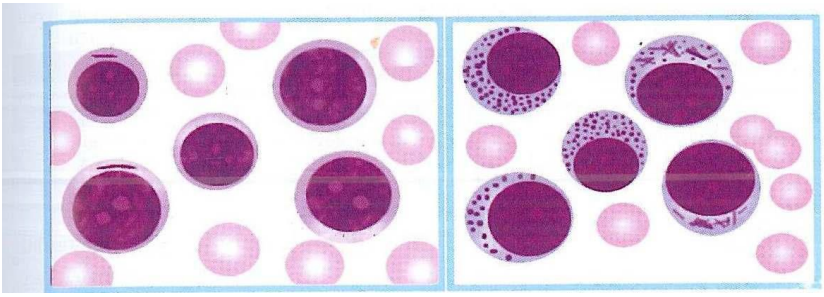

Figure 5: Representation of blood smear from a patient with AML (left) compared to a patient with APML (right). Myeloblasts are seen in both panels as well as Auer rods. (Reproduced from Kawthalkar, 2006)

Cytochemical stains are used to subtype AML and classify into French-American-British (FAB) categories (Kawthalkar, 2006). The myeloperoxidase stain will distinguish AML from ALL by showing granular positivity and will confirm AML subtypes M1, M2, M3 and M4. Alpha naphthyl acetate esterase in the nonspecific esterase reaction shows positivity in AML M4 and M5. The Periodic Acid Schiff's reaction shows granular positivity in AML M6.

Cytochemical stains however do not definitively differentiate between AML M0, M6 and M7 therefore flow cytometry is employed (Kawthalkar, 2006). Flow cytometry is also used to differentiate between AML and ALL as it enables the visualization of the morphology of cells. The method of immunophenotyping, involves identifying cell surface antigens using antigen specific antibodies. The CD (cluster of differentiation) antigen system is a nomenclature system for differentiation antigens found on the surface of white blood cells (WBC). Immunophenotyping, together with flow cytometry shows that CD 13 or CD 33 are present on AML M0 cells, glycophorin A is present on AML M6 cells and CD41 or CD61 are present on AML M7 cells.

Table 1: French-American-British (FAB) Classification of AML and Associated Genetic Abnormalities (Reproduced from Lowenberg et al., 1999)

FAB Subtype	Common Name (% of Cases)	Results of Staining			Associated Translocations and Rearrangements (% of Cases)	Genes Involved
		Myeloperoxidase	Sudan Black	Nonspecific Esterase		
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	−	−	−*	inv(3q26) and t(3;3) (1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15–20%)	+	+	−		
M2	Acute myeloblastic leukemia with maturation (25–30%)	+	+	−	t(8;21) (40%), t(6;9) (1%)	*AML1-ETO, DEK-CAN*
M3	Acute promyelocytic leukemia (5–10%)	+	+	−	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	*PML-RARα, PLZF-RARα, NPM RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23 (20%), inv(3q26) and t(3;3) (3%), t(6;9) (1%)	*MLL, DEK-CAN, EVI1*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)	+	+	+	inv(16), t(16;16) (80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2–9%)	−	−	+	11q23 (20%), t(8;16) (2%)	*MLL, MOZ-CBP*
M6	Erythroleukemia (3–5%)	+	+	−		
M7	Acute megakaryocytic leukemia (3–12%)	−	−	+†	t(1;22) (5%)	Unknown

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).

†Cells are positive for α-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate.

Studies by Brunning *et al* (2001) and Stanley *et al* (1985) have shown incidences of FAB subtypes to be in agreement with those listed above. Briefly, the following subtypes occur in AML cases in the following proportions; FAB Mo (5%), FAB M1 (10%), FAB M2 (30-45%), FAB M3 (5-8%), FAB M4 (15-25%), FAB M5 (8-14%), FAB M6 (5-6%) and FAB M7 (3-5%) (Brunning et al., 2001a); (Stanley et al., 1985); (Brunning et al., 2001b) .

Previously the diagnosis of AML has been based on cytomorphology and cytochemistry using the French–American–British (FAB) classification. Recently however, the World Health Organization (WHO) classification for AML redefines AML subtypes on the basis of histopathology, immunophenotyping, genetic aberrations and clinical characteristics.(Bao et al., 2006). Definitive subtypes are important as they warrant specific therapies.

The WHO classification of AML includes 4 major categories: AML with recurring genetic abnormalities, AML with multilineage dysplasia, AML - therapy related, and AML not otherwise categorized as shown in the table below. The first category is further divided into subcategories: (a) AML with t(8;21)(q22;q22); *AML1/ETO*, (b) AML with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q22); *CBFB/MYH11*, (c) acute promyelocytic leukemia (AML with t(15;17)(q22;q12); *PML-RARA* and variants) and (d) AML with 11q23/*MLL* abnormalities.(Schoch et al., 2003)

Table 2: World Health Organisation (WHO) Classification of AML (Adapted from Kawthalkar, 2006)

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); (AML1/ETO)
AML with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q22); (CBFβ/MYH11)
Acute promyelocytic leukemia (AML with t(15;17)(q22;q12) (PML/RARα) and variants
AML with 11q23 (MLL) abnormalities
Acute myeloid leukemia with multilineage dysplasia
Following a myelodysplastic syndrome
Without antecedent myelodysplastic syndrome
Acute myeloid leukemia and myelodysplastic syndromes, therapy related
Alkylating agent-related
Topoisomerase type II inhibitor-related
Other types
Acute myeloid leukemia not otherwise categorised
Acute myeloid leukemia minimally differentiated
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

Molecular biology approaches such as PCR based assays and fluorescence in situ hybridization (FISH) has improved diagnosis of haematological malignancies to an accuracy range of 75-92% and helped to elucidate important prognostic information (Basso et al., 2007).

1.2.7 Differential Diagnosis:

It is imperative that the diagnosis of AML is clear as it shares many diagnostic features with other medical conditions such as Leukaemoid Reaction, where WBC is high; Acute Lymphoblastic Leukemia, where clinical features include anaemia, thrombocytopaenia and neutropenia; and a blast crisis of Chronic Myelogenous Leukemia (CML), where blast cells infiltrate the bone marrow.

AML must also be differentiated from myelodysplastic syndrome (MDS) or secondary AML stemming from MDS as therapeutic strategies differ. MDS or MDS-related AML can be distinguished from AML using clinical, morphologic and genetic analysis. MDS is characterised by ineffective haematopoeisis, dysplastic maturation and cytogenetic lesions specifically the loss of the whole or part of chromosome 5 or chromosome 7, the loss of the long arm of chromosome 20 and the loss of the Y chromosome. Secondary AML that develops from MDS is confirmed by the presence of more than 30% blasts in the bone marrow (Lowenberg et al., 1999).

1.2.8 Prognostic Factors in AML:

Long term prognostic indicators include cytogenetics at diagnosis, age at diagnosis and relapse, whether an allogeneic transplant was performed during the first Complete Remission (CR) and the duration of the first CR and WBC count (Lowenberg et al., 1999b). However patients presenting with lower WBC counts at presentation correlated with a high rate of complete response (Zhu et al., 2010). Unfavourable prognoses are conferred by age $\geq$ 60yrs, elevated lactate dehydrogenase and hyperleucocytosis (TLC > 100 000/cmm) (Ravandi et al., 2010).

Cytogenetics classifies AML into three major risk groups (low, intermediate and high) based on karyotyping at diagnosis. The groups are as follows: Low – AML with balanced chromosomal aberrations such as t(8;21), t(15;17), or inv(16) and favourable clinical outcome; Intermediate – Normal karyotype AML cases and t(9;11) and High – AML with non-balanced chromosomal aberrations such as inv(3), -5/del(5q),-7, 11q23 or a complex karyotype and poor clinical outcome (Chauhan et al., 2011, Schnittger et al., 2002).

Low risk patients usually have high rates of complete remission (>85%) and low rates of relapse (30-40%) whereas high risk patients usually have a survival rate of approximately 20% at five years (Lowenberg et al., 1999); (Thiede et al., 2002).

Table 3: Unfavourable Prognostic Factors in AML Patients (Adapted from Lowenberg et al., 1999)

Factors used to Predict Response to Induction Chemotherapy	**Factors used to Predict Relapse**
Unfavourable karyotype	Unfavourable karyotype
Age>60 years	Age>60 years
Secondary AML	Delayed response to induction chemotherapy
Multidrug Resistance	Multidrug Resistance
White cell count of >20 000/mm^3	White cell count of >20 000/mm^3
Unfavourable immunophenotype	Female Sex
	Elevated lactate dehydrogenase level
	Autonomous growth of leukaemic cells

However, 40-50% of patients have normal karyotypes which denote intermediate risk therefore other prognostic factors need to be delineated (Chauhan et al., 2011, Tan et al., 2008). Molecular mutations such as the FLT3 and NPM1 mutations have shown their value as prognostic markers and can be useful molecular therapy targets especially in patients with normal karyotypes (Bullinger et al., 2004, Kawthalkar, 2006).

1.2.9 Treatment Options for AML:

Currently, AML patient therapy includes remission induction which consists of cytosine arabinoside and daunorubicin (7'&3'); post remission therapy consisting of consolidation therapy (high dose cytosine arabinoside) and intensive chemotherapy followed by HSC transplantation (autologous or allogeneic); and supportive therapy consisting of blood component replacement and management of infections (Kawthalkar, 2006).

1.2.9.1 Induction of Remission:

Remission Induction therapy aims to induce CR by eradicating leukaemic cells as well as the clinical manifestations of AML. CR is defined as the presence of less than 5% blasts in bone marrow. Daunorubicin is usually administered at doses of 40-60mg per square meter of body surface area, three times a day whilst cytarabine is administered intravenously in doses of 100-200mg per square

meter per day or by continuous infusion over 7-10 days. CR can be induced in 70-80% of patients under the age of 60 (Lowenberg et al., 1999).

1.2.9.2 Post Induction Therapy:

However, undetectable leukaemic cells may persist which can result in relapse if post remission therapy is not administered. Post Remission therapy often includes HSC transplantations which are more effective in achieving CR than conventional chemotherapy. Hence, post remission therapy options include: Allogeneic bone marrow transplantation, Autologous bone marrow transplantation and Chemotherapy.

Allogeneic bone marrow transplantation from a HLA-matched sibling can cure 50-60% of patients (Lowenberg et al., 1999). The risk of relapse for patients receiving an allogeneic bone marrow transplant is also significantly lower than patients not receiving a transplant. This is due to marrow ablative high dose cytotoxic therapy administered before the transplant as well as the graft-versus-leukemia effect which is the allogeneic effect of the graft versus residual leukemia in the host. However complications can arise in response to toxicity of treatment and mortality due to immunosuppression and graft-versus-host disease. Hence allogeneic transplants are usually performed on patients under 55 years of age (Lowenberg et al., 1999).

Autologous bone marrow transplantation studies showed a 45-55% survival rate. However relapse occurs fairly frequently due in part to residual disease in the absence the graft versus leukemia effect and the contamination of the marrow graft by leukaemic cells (Lowenberg et al., 1999).

Advanced age and certain cytogenetic abnormalities can result in patients being resistant to conventional cancer therapies. Younger patients and some older patients usually achieve CR after the first induction therapy however relapse is common and can hinder long term cure. Other factors that contribute to the risk of failure to achieve CR include therapy-induced AML or secondary AML that develops from another haematological malignancy such as myelodysplastic syndrome or myeloproliferative disorder (Ravandi et al., 2010).

Secondary AML that develops from MDS responds poorly to conventional therapy used to treat AML hence accurate diagnosis at initial presentation is vital (Lowenberg et al., 1999).

1.2.9.3 Relapse:

Relapsed AML is very challenging and should be avoided at all costs. Not only are patients with favourable cytogenetics who have been in remission for approximately 1 year, faced with a 20% chance of survival post relapse therapy (Lowenberg et al., 1999) but a significant number of post-relapse patients are resistant to chemotherapy and can undergo karyotype changes (Schnittger et al., 2002).

When patients relapse, future therapeutic decisions are made with factors such as age, duration of first remission and cytogenetic analysis. High dose cytotoxic therapy and haematopoetic stem cell transplantation are valuable options for children and young adults refractory to treatment or in their first relapse.

1.2.9.4 Older AML Patients:

The majority of patients with AML are of advanced age (>60 years). This can be problematic in terms of therapeutic options as older patients who are usually defined as high risk and can have intolerance to high dose chemotherapy. Chemotherapy can also serve to exacerbate existing medical conditions. Older patients with good organ function have a 50% chance of CR with induction chemotherapy and possibly a 20% chance of overall survival (Lowenberg et al., 1999). Favourable prognostic factors for older AML patients include: primary AML, younger than 80 years of age, good physical condition and the absence of both leukocytosis at diagnosis and cytogenetic abnormalities (Lowenberg et al., 1999).

Essentially curing AML is no easy task. Greater efficacy of treatment and long term survival lie in identifying subtypes and molecular markers and subsequently individualising patient treatment.

1.3 Acute Promyelocytic Leukemia (APML):

It is essential to differentiate between APML and AML as there are serious prognostic and therapeutic implications.

1.3.1 Definition of APML:

APML has two unique features that distinguish it from AML: the presence of an aberrant protein, PML/RARα which is due to a retinoic acid receptor mutation and the ability of APML blasts to differentiate completely when treated with All Trans Retinoic Acid (ATRA) (Grignani et al., 1994).

There exists an APML M3 Variant (M3v) (see Table 1, pg. 21 relevant to Subtype M3) which carry the t(15;17) abnormality, express the PML/RARα fusion protein and respond to ATRA treatment (Grignani et al., 1994). However these APML cases present with distinct clinical and molecular feature such as hyperleukocytosis and a greater risk of coagulopathy and mortality due to nervous system or lung haemorrhage.

Generally, APML patients are prone to fatal haemorrhage if left untreated (Lo Coco et al., 1999).

1.3.2 Genetics of APML:

A reciprocal translocation between chromosomes 15 and 17 (t(15;17)) involving the PML and RARα genes results in the formation of three abnormal proteins: PML/RARα, RARα/PML an aberrant PML

protein contributing to the clinical entity that is APML. The chromosome breakpoints are usually mapped to 15q24 and 17q21. (Lo Coco et al., 1999)

RARα belongs to the retinoic acid receptor family involved in cell development and differentiation. The PML/ RARα fusion protein may be responsible for two key APML features: the block in differentiation and sensitivity to ATRA. Promyelocytic leukaemogenesis is attributed to the fusion protein's ability to promote cell survival by inhibiting programmed cell death. (Grignani et al., 1994)

APML patients may also display other genetic aberrations such as trisomy 8 and translocations involving chromosome 17 such as t(11;17).

Although the t(15:17) is usually a hallmark of APML patients, studies show that patients with chronic myeloid leukemia(CML) in blast crisis may exhibit the t(15;17) and subsequent promyelocytic phenotype (Grignani et al., 1994).

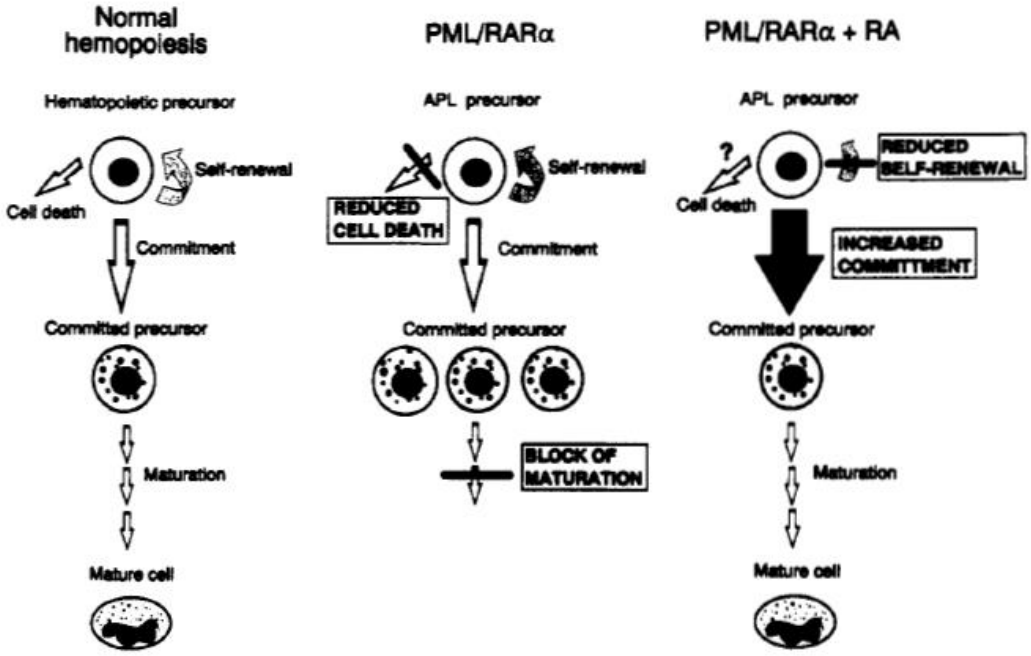

Figure 6: Schematic representation of normal haemopoeisis compared to the effect of aberrant fusion protein PML/RARα and PML/RARα combined with RA culminating in two distinct APML features: block in differentiation and high RA sensitivity respectively(Reproduced from Grignani et al., 1994)

1.3.3 Detection of APML:

Not only does APML require definitive diagnosis as treatment strategies differ to that of AML but rapid diagnosis significantly reduce the risk of fatalities.

Generally, APML has two characteristic features that aid diagnosis: blasts with a high granular content when visualised under the microscope and t(15;17). However, in the APML M3 variant, there is a distinctly lobulated nucleus and microgranules. Microscopically, APML bone marrow smears can

present with bilobular nuclei and Auer rods. The cells all stain positive for Sudan Black, myeloperoxidase and chloroacetate esterase (Grignani et al., 1994).

Newer diagnostic methods which rely on the analysis of the PML/RARα transcript include Southern Blot analysis, Fluorescent In Situ Hybridization (FISH), immunostaining with specific antibodies and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR also enables the detection of minimal residual disease (MRD) during and after treatment. RT-PCR is particularly useful as it can detect MRD and diagnose and where the other methods can be error-prone and labour-intensive, RT-PCR is efficient and specific (Lo Coco et al., 1999, Grignani et al., 1994).

1.3.4 Treatment of APML:

Patients with APML have a greater chance of achieving complete remission, long term survival and overall cure in response to induction therapy. ATRA is effective in inducing CR in 84% of all patients and 95% of APML patients with t(15;17) (Grignani et al., 1994). APML patients also have a greater sensitivity to anthracyclines such daunorubicin, idarubicin and rubidazon. Generally, ATRA combined with chemotherapy and bone marrow transplantation provides APML patients with the best chance of survival (Grignani et al., 1994).

ATRA targets the chimeric protein encoded by t(15;17) and strives to resolve the effects of coagulopathy before morphologically apparent. However, a side effect of ATRA is Retinoic Acid Syndrome which presents with fever, respiratory disease, pleural effusions, renal impairment and possible cardiac or multi-organ failure. Retinoic Acid Syndrome can be combated with steroids or standard chemotherapy (Grignani et al., 1994).

Generally, patients that fail to achieve remission are due to complications related to APML such as severe coagulation disorders and sepsis, rather than leukaemic resistance. These complications can worsen during chemotherapy and present as Disseminated Intravascular Coagulation (DIC) and excessive fibrinolysis due to the therapy induced terminal differentiation of the poorly differentiated promyelocytes cell lysis (Grignani et al., 1994); (Lo Coco et al., 1999); (Lowenberg et al., 1999); (Falini et al., 2005).

1.4 Somatic Mutations in AML:

Myeloid cells which include granulocytes and monocytes make up the dominant cellular population in bone marrow. Their proteins are responsible for the recognition, ingestion, and destruction of foreign organisms, antigen presentation, cytokine production, and other functions of the immune and inflammatory reactions.

In AML, a myeloblast accumulates genetic changes which prevent differentiation leaving the cell with diminished or no functionality. This mutation alone does not cause leukemia but when combined with

other mutations which disrupt proliferation and diminish apoptosis, the uncontrolled growth of an immature clone of cells ensues and subsequently the clinical entity of AML (Rosmarin et al., 2005, Theide et al., 2006).

AML diagnosis and prognosis depends on karyotypic analysis; however in 40-50% of AML cases, there are no chromosomal aberrations and patients are termed normal karyotype AML (NK-AML) and categorized as intermediate risk (Tan et al., 2008). Further stratification of intermediate risk and subsequent refining of prognosis, achieving CR and detecting MRD may be possible by focusing on somatic mutations (Bacher et al., 2008).

Somatic mutations responsible for impairing cellular growth, differentiation and proliferation accumulate in haematopoietic progenitor cells. Somatic mutations fall into two categories: Class I which comprises mutations that activate signal-transduction pathways and subsequently increase the proliferation and/or survival of hematopoietic progenitor cells such as fms-like tyrosine kinase 3 gene (*FLT3*) and Neuroblastoma *RAS* viral oncogene homolog (*NRAS*) mutations and Class II mutations which comprises mutations that affect transcription factors impairing haematopoietic differentiation such as Nucleophosmin gene (*NPM1*), CCAAT/enhancer binding protein α gene (*CEBPA*) and the mixed-lineage leukemia gene (*MLL*) (Schlenk et al., 2008); (Chauhan et al., 2011); (Braoudaki et al., 2010); (Chen et al., 2006); (Cotta and Tubbs, 2008).

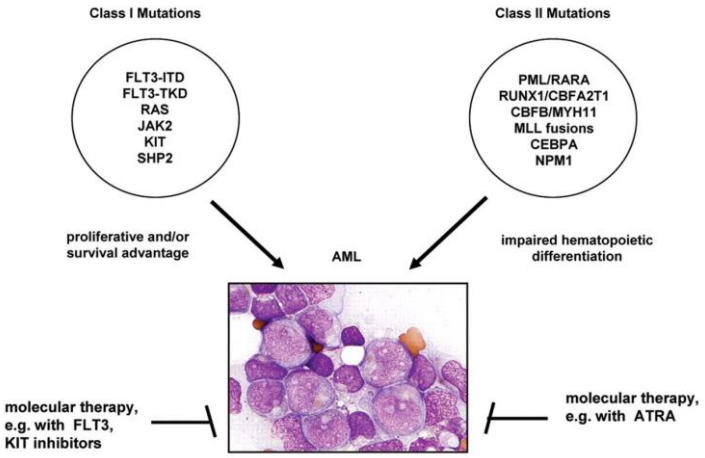

Figure 7: Schematic representation of the contributory effect of two types of mutations to the clinical entity that is AML and potential therapeutic targets (Reproduced from Dohner, 2007)

1.5 *FMS-Like Tyrosine Kinase 3 (FLT3)*

1.5.1 Definition of FLT3:

The human *FLT3* gene is located on chromosome 13q12 and contains 24 exons (OMIM: 136351; GenBank Accession No: NM_004119.2). *FLT3* encodes for a class III tyrosine kinase receptor (993

aa). The class III receptor tyrosine kinase family includes the c-kit, c-fms and platelet-derived growth factor receptors (Bacher et al., 2008).

1.5.2 Normal FLT3 Function:

FLT3 exists in two forms: a membrane-bound protein (158-160 kd) glycosylated at N-linked glycosylation sites in the extracellular domain and a non-membrane bound, unglycosylated protein (130-143 kd). Usually, the transmembrane protein is expressed by early hematopoietic and lymphoid progenitor cells including in the brain, placenta and liver and is downregulated as cells mature and differentiates. Hence, wild type *FLT3* is expressed on normal haematopoietic stem cells and is involved in receptor dimerization and autophosphorylation and activation of downstream signal transduction pathways that play an important role in cellular growth, proliferation and differentiation of hematopoietic progenitors (Chauhan et al., 2011).

1.5.3 Abnormal FLT3 Function:

Mutations in the *FLT3* gene occur in exons 14, 15 and 20 of chromosome 13q12. Although *FLT3* is important in regulating normal haematopoiesis, it is also over-expressed in many haematologic malignancies such as AML and B-lineage acute lymphocytic leukemia. *FLT3* mutations either in the form of ITDs or point mutations of tyrosine kinase domain (TKD) that result in constitutive activation of the receptor which result in increased receptor signalling and subsequent tumorigenesis (Fan et al., 2010; Kiyoi and Naoe, 2006).

The *FLT3 ITD* mutation is one of the most common AML gene mutations occurring in 20-30% of all AML patients with *FLT3 TKD* mutations occurring in 5-7% of all AML cases. Hence mutated *FLT3* with constitutive kinase activity could be an important molecular target in treating leukemia (Theide et al., 2006; Kiyoi and Naoe, 2006; Schnittger et al., 2002).

1.5.4 Structure of FLT3:

The tertiary structure of FLT3 is organized into several domains: the extracellular (N terminal) ligand binding domain composed of 5 immunoglobulin-like elements, a juxtamembrane (JM) domain, tyrosine kinase domain divided by a kinase insert domain and a C-terminal domain (Kiyoi and Naoe, 2006).

1.5.5 Specific FLT3 Mutations:

1.5.5.1 FLT3 Internal Tandem Duplications

The highly common AML mutation, the internal tandem duplication (ITD) of the region coding for the juxtamembrane (JM) domain of the FLT3 receptor, occurs in 20-30% of adult AML and in 10-16% of childhood AML (Nafea et al., 2011).

The ITD mutation, located on exons 14 and 15 of the *FLT3* gene and spanning from residues 572 to 613 usually involve duplications but can contain foreign sequences or additional nucleotides. The duplicated region is variable among individuals both in size and location. However they are in-frame mutations that result in an elongated FLT3 protein with constitutive protein tyrosine kinase (PTK) activity. This constitutive activity leads to uncontrolled proliferation of AML cells and a decrease in apoptosis. *FLT3-ITDS* are also strongly associated with the transformation of MDS to secondary AML (Bacher et al., 2008; Kiyoi and Naoe, 2006).

The expected size of a wild type *FLT3* fragment is 329bp (Kiyoi and Naoe, 2006).

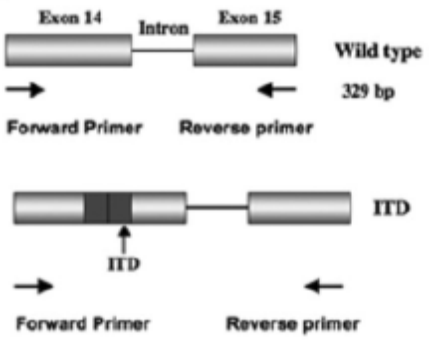

Figure 8: Comparison of Wild type *FLT3* gene to Mutant *FLT3* gene (Reproduced from Kiyoi and Naoe, 2006)

The FLT3-ITD wild type amino acid sequence is as follows:

Wild type FLT3 QFRYESQLQMVQVTGSSDNEYFYVD FREYEYDLKWEFPRENLEF (Chauhan et al., 2011)

FLT3-ITDs are less likely to occur in paediatric and lymphoblastic patients than in adult AML patients and are found predominantly in patients with normal karyotypes and t(15;17). *FLT3/ITD* mutations were found commonly in acute myelomonocytic leukemia (25.9%), AML with t(15;17) (19.2%) and in AML with 11q23/MLL abnormalities (16.6%). *FLT3-ITDs* are commonly associated with FAB subtypes M4 and M5 and show association with certain immunological CD markers such as low CD34 expression or aberrant CD7 expression (Thiede et al., 2002; Bacher et al., 2008; Chauhan et al., 2011; Schnittger et al., 2002).

1.5.5.2 FLT3 Tyrosine Kinase Domain Mutations

FLT3 Tyrosine Kinase Domain (TKD) mutations, located in exon 20 of the *FLT3* gene, are missense or point mutations that usually involve aspartic acid at position 835 but can include mutations at codons 836 and 839 to 842. They occur in the activation loop of the second tyrosine kinase domain of *FLT3*. *FLT3-TKD* mutations cause constitutive tyrosine phosphorylation and subsequent activation of the tyrosine kinase receptor (Chauhan et al., 2011; Schnittger et al., 2002).

FLT3 D835 and *I836* mutations are located in the TK2 domain and encoded by the nucleotide sequence GATATC, which forms the EcoRV restriction site. This enables the creation of two amplified products for the *FLT3* wt (68bp and 46bp) and one undigested product for the *FLT3 D835* (114bp) visualized after agarose gel electrophoresis (Kiyoi and Naoe, 2006).

The wild type *FLT3 D835* nucleotide sequence is as follows:

Wild type *FLT3* (D835) TTG GCT CGA GAT ATC ATG AGT GAT (Chauhan et al., 2011)

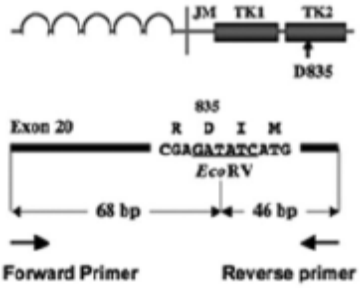

Figure 9: Representation of the D385 TKD mutation and EcoRV Restriction Site (Reproduced from Chauhan et al., 2011)

FLT3 TKD mutations occur in 5.8-7.7% of all AML cases. *FLT3*-activating loop mutations are seen most commonly in AML with maturation (25%), AML with inv(16) (20%) and in AML with t(15;17) (11.5%) (Bacher et al., 2008; Falini et al, 2005; Thiede et al., 2002).

1.5.6 Clinical Characteristics and Outcome:

Generally, *FLT3 ITDs* are associated with poor prognosis and an increased risk of relapse after conventional chemotherapy. This may be attributed to the expression of *FLT3 ITD* being able to induce factor-independent growth and resistance to radiation-induced apoptosis. Further studies have found that *FLT3 ITD* has a strong association with poor overall, disease-free and event-free survival. Therapy with *FLT3* inhibitors may have the potential to significantly improve patient prognosis (Fan et al., 2010; Chauhan et al., 2011; Kiyoi and Naoe, 2006; Schnittger et al., 2002).

AML patients with *FLT3 ITD* mutations also tend to have significantly higher WBC counts. Hence the leukocytosis may be attributed to cellular proliferation due to constitutive action of the receptor tyrosine kinase (Chauhan et al., 2011; Schnittger et al., 2002).

D835/I836 mutations however were found to be an insignificant prognostic factor conferring neither a negative or positive effect on patient outcome. There was also no association between *FLT3* TKD mutations and sex or age (Bacher et al., 2008).

However there are detractors to the usefulness of *FLT3* mutations as prognostic markers and studies conducted by Ravandi et al, have suggested that no clear association exists between *FLT3* mutational status and induction of CR in patients (Ravandi et al., 2010).

1.5.7 FLT3 Detection:

Methods of detection of *FLT3* mutations include: PCR, high resolution melting (HRM) analysis, sequencing and digestion with restriction enzymes (RE) followed by visualization using agarose gel electrophoresis.

The *FLT3* ITD PCR product would be indicated by a 326 bp band however Chauhan et al, 2008 found that any longer, additional bands greater in size than 133bp would be considered *FLT3* ITD positive. Studies by Schnittger et al (2002) conclude that the *FLT3* ITD mutations vary in size from 3-400 bp.

The *FLT3* D835 and I836 mutations are detected by a fragment 114 bp in size due to a lack of digestion at the mutated EcoRV restriction site. The wild type allele will be visualized as two fragments, 68 and 46 bp in size.

(Ansari-Lari et al., 2004, Sallmyr et al., 2008, Bao et al., 2006, Cotta and Tubbs, 2008, Quentmeier et al., 2003, Theide et al., 2006; Chauhan et al., 2011)

1.6 *Nucleophosmin (NPM1)*

1.6.1 Definition of NPM1:

The *NPM1* gene, located on chromosome 5q35 contains 12 exons (Fig. 11 pg. 35), encodes for 2 isoforms of a nucleocytoplasmic shuttling protein that constantly exchanges between nucleus and cytoplasm (OMIM: 164040; GenBank Accession No: NM_002526.6). Subsequently *NPM1* plays an important role in ribosomal protein assembly, initiation of centrosome duplication, and protein folding.

The NPM protein is localized in the nucleus and nucleolus of normal haematopoeitic cells whereas the mutated NPM protein is found in the cell cytoplasm.

1.6.2 Normal NPM1 Function

Nucleophosmin is a multifunctional protein, 38 kD in size, that exchanges between the nucleus/nucleolus and cytoplasm. NPM1 contains several domains responsible for specific cellular functions and include: ribosome processing and biosynthesis; control of centrosome duplication during the cell cycle; regulation of the function of tumour-suppressor transcription factors and the regulation and stability of several nuclear proteins. The phosphoprotein has an important tumour suppressor function namely the regulation of the ARF-p53 tumour suppressor pathway (Chen et al., 2006; Braoudaki et al., 2010; Kiyoi and Naoe, 2006).

The NPM gene encodes specific fusion proteins that may contribute to oncogenesis if mutated (Falini et al, 2005; Nafea et al., 2011).

Generally the NPM family is involved in cellular processes which include genome stability, ribosome assembly, DNA duplication and transcriptional regulation (Boonthimat et al., 2008).

1.6.3 Abnormal NPM1 Function

Currently, *NPM1* mutations are reported as being the one of the most common mutations occurring in AML-NK patients in European countries (Boonthimat et al., 2008).

NPM1 mutations in AML patients usually occur in exon 12 of the *NPM1* gene on chromosome 5q35 and result in a frame shift in the region encoding the C-terminal of the NPM protein, which disrupts the nucleolar localization signal. The subsequent elongated protein is retained in the cytoplasm and is a feature found in approximately 35% of AML patients (Chen et al., 2006). Although most *NPM1* mutations involve exon 12 (residues 288 and/or 290), mutations can also occur in exons 9 and 11. These mutations generate an abnormal nucleolar localization signal or a leucine-rich nuclear export signal (NES) motif which results in the abnormal cytoplasmic localization of the NPM protein. Furthermore, the mutated *NPM* affects the p53 pathway which can induce genetic instability(Chen et al., 2006 ; Braoudaki et al., 2010; Kiyoi and Naoe, 2006; Falini et al, 2005).

Reciprocal chromosomal translocation results in a fusion protein that retains the N-terminus of NPM as well as the NPM-retinoic acid receptor-α in APML and NPM-myeloid leukemia factor 1 in AML. *NPM1* gene mutations involving the C-terminus at exon 12 are specific for AML as they are not found in secondary AML or other haematological malignancies (Chen et al., 2006; Zhu et al., 2010).

Generally *NPM1* mutations result in 6 sequence variants (Types A-F). Mutation A, occurring in about 70% of all cases in exon 12, consists of a TCTG tetranucleotide duplication from positions 956-959 which results in a replacement of the last 7 amino acids in the NPM protein with 11 different residues. Mutations B – D contain 4bp insertions at position 960 resulting in the same frame shift as Mutation A. Mutations E and F contain deletions at positions 965-969 and insertions of different 9bp sequences resulting in a distinct 9 amino acids C-terminal. Mutations A-F show mutations in at least one of the tryptophan residues (positions 288 and 290) and share the same ending amino acid residues (VSLRK). (Falini et al, 2005).

Type of Mutation	GenBank Accession No.	Sequence	Predicted Protein
None (wild type)	NM_002520	GATCTCTG....GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLWQWRKSL
Mutation A	AY740634	GATCTCTGTCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation B	AY740635	GATCTCTGCATGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCMAVEEVSLRK
Mutation C	AY740636	GATCTCTGCGTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCVAVEEVSLRK
Mutation D	AY740637	GATCTCTGCCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation E	AY740638	GATCTCTG....GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG	-DLWQSLAQVSLRK
Mutation F	AY740639	GATCTCTG....GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG	-DLWQSLEKVSLRK

Figure 10: The wild-type NPM sequence (nucleotides 952-989) compared to 8 common *NPM* mutations (Types A-F), their GenBank Accession numbers and predicted proteins. (Nucleotide insertions are indicated in red, wild type residues indicated in yellow, mutated residues indicated in grey and the VSLRK common amino acid sequence in green).(Reproduced from Falini et al., 2005)

Recent studies indicate that mutated *NPM1* may contribute to the oncogenesis of other types of leukemia due to the gene being involved in several tumour associated chromosomal translocations. *NPM1* mutations also may cause AML cells to acquire additional genetic mutations (Braoudaki et al., 2010; Kiyoi and Naoe, 2006).

NPM1 mutations occur in approximately 30-50% of adult AML-NK cases (Nafea et al., 2011). These mutations occurred more frequently in the older versus the younger adult age groups. However *NPM1* gene mutations have been detected in both adult and paediatric cases with variable prevalence and prognostic significance. Generally, *NPM1* mutations were not detected in the presence of recurrent cytogenetic abnormalities which include: t(15;17), t(8;21), inv (16), t(16;16), 11q23 or complex karyotype. Also, secondary or therapy-related AML rarely express mutated *NPM1* (Braoudaki et al., 2010; Boonthimat et al., 2008; Nafea et al., 2011; Chen et al., 2006).

1.6.4 Structure of NPM1:

The protein structure includes: a nuclear export signal, an oligomerization domain, a histone binding domain, a DNA/RNA binding domain, nuclear localization signals, and a nucleolar localization signal.

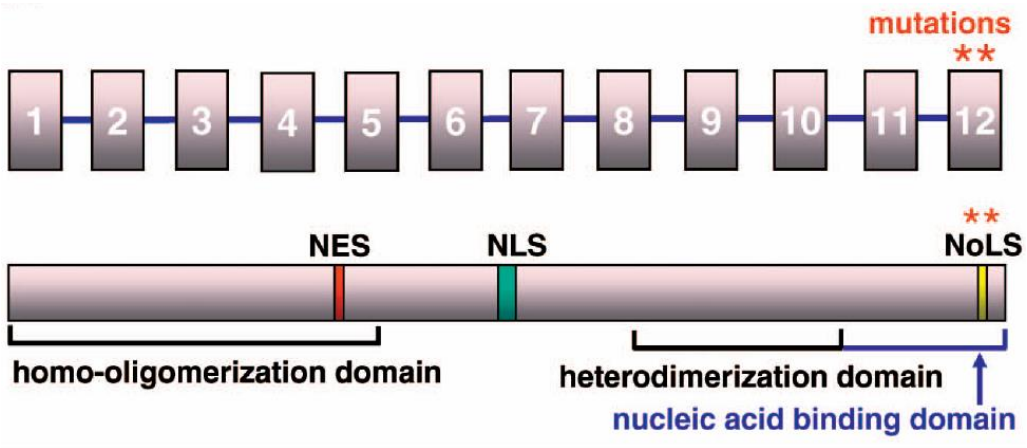

Figure 11: Schematic representation of the normal nucleophosmin (*NPM1*) gene and protein and the point of mutation (NES, nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal) (Reproduced from Chen et al., 2006)

Wild type *NPM1*

GATCTCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG (Braoudaki et al., 2010)

The nucleotide sequence of human nucleophosmin cDNA has a coding sequence equivalent to a protein of 294 amino acids.

1.6.5 Clinical Characteristics and Outcome:

Clinically, AML patients with *NPM1* mutations present with marked extramedullary involvement including gingival hyperplasia and lymphadenopathy.

NPM1 gene mutations occur more frequently in women than men and have been associated with older age (Theide et al., 2006).

NPM1 mutations strongly correlate with higher WBC, blast cell counts and platelet counts (Theide et al., 2006; Boonthimat et al., 2008).

NPM1 mutations occur in all FAB categories except FAB M3 which is reserved for APML. These mutations occur in approximately the following frequencies: 20% of M2, 40-50% of M4, 40-50% of M5a and 90% of M5b cases (Chen et al., 2006; Theide et al., 2006). However, Falini et al (2005) found more specifically that cytoplasmic NPM, specific to primary AML, was found in all leukaemic cells except M3, M4eo and M7 (Falini et al., 2005).

Studies concur that *NPM1*-mutated AML is characterized by the absence of haematopoietic stem cell markers CD34 and CD133 and the presence of myeloid antigen markers CD13 and CD33. However Zhu et al (2010) found that the absence of CD43 and CD133 in patients with AML only applied to those with cytoplasmic localization of the NPM protein but were present if the protein was found in the nucleus (Falini et al., 2005; Boonthimat et al., 2008; Zhu et al., 2010).

Generally, NK-AML patients with mutated *NPM1* have better disease-free and overall survival compared to patients with the absence of the mutation as they respond better to induction therapy. However if patients with *NPM1* mutations have recurrent genetic abnormalities, their response to therapy is significantly affected. (Zhu et al., 2010; Nafea et al., 2011; Falini et al, 2005)

1.6.6 Detection of NPM1:

Methods for detecting *NPM1* mutations include: PCR assays to amplify genomic DNA at exon 12 followed by either direct sequencing or sequencing following cloning; PCR assay to amplify genomic DNA at exon 12 using a fluorescently-labelled forward primer followed by capillary electrophoresis which will show *NPM* mutations as having a 297 bp wild type peak and additional longer peaks and immunofluorescence or alkaline phosphatase monoclonal anti-alkaline phosphatase using peripheral blood or bone marrow smears or immunohistochemical stain on fixed paraffin-embedded bone marrow samples to detect cytoplasmic NPM (Cotta & Tubbs, 2008).

1.7 FLT3 and NPM1 in Combination:

NPM1 mutations are highly associated with *FLT3* mutations; approximately 60% of AML patients with *NPM1* mutations also have *FLT3* mutations. There is a high frequency of *FLT3* ITD mutations and *NPM1* mutations occurring in AML patients concurrently. However, the combination of the *NPM1* mutation and the *FLT3* ITD mutation in a patient often was an unfavourable prognostic factor in terms of overall and event-free survival. This could be attributed to the combination of a class I and class II mutations which allow for the ability to proliferate and impaired differentiation respectively. Conversely, a patient with mutated *NPM1* and no *FLT3* ITD mutation has a generally favourable response and associated with CR after induction therapy (Kiyoi and Naoe, 2006; Zhu et al., 2010; Braoudaki et al., 2010; Chen et al., 2006; Schlenk et al., 2008; Theide et al., 2006).

However some studies suggest a negative impact of the *FLT3* ITD mutation regardless of the *NPM1* mutational status (Boonthimat et al., 2008).

The wealth of information generated from these studies of the use of *NPM1* and *FLT3*-ITD as prognostic markers has resulted in possibly formulating aggressive treatment plans for unfavourable AML.

1.8 ***HIV and Haematopoiesis***

1.8.1 Definition of HIV:

The human immunodeficiency virus (HIV) infects mainly the haematopoietic and immune systems. After infection, the immune system is initially activated then suppressed due to direct viral infection of cells, inhibitory effects of HIV proteins, an altered microenvironment with cytokine imbalance and increased apoptosis of both infected and non-infected cells.

1.8.2 Symptoms Attributed to HIV:

The haematopoietic system is the primary target of HIV infection thus manifesting in anaemia, thrombocytopenia, lymphocytopenia, neutropenia and/or monocytopenia. These manifestations can be either due to chemotherapy for HIV and related conditions, opportunistic infections or from direct effects of the virus on various components of the blood and bone marrow.

Isolated thrombocytopenia is usually the first sign of HIV-1 infection with subsequent anaemia and granulocytopenia. The low platelet counts and elevated levels of anti-platelet antibodies are typical of idiopathic thrombocytopenic purpura (ITP). Infected patients are also prone to autoimmune disorders and immune dysregulation which ultimately is responsible for ineffective haematopoiesis (Panoskaltsis and Abboud, 1999).

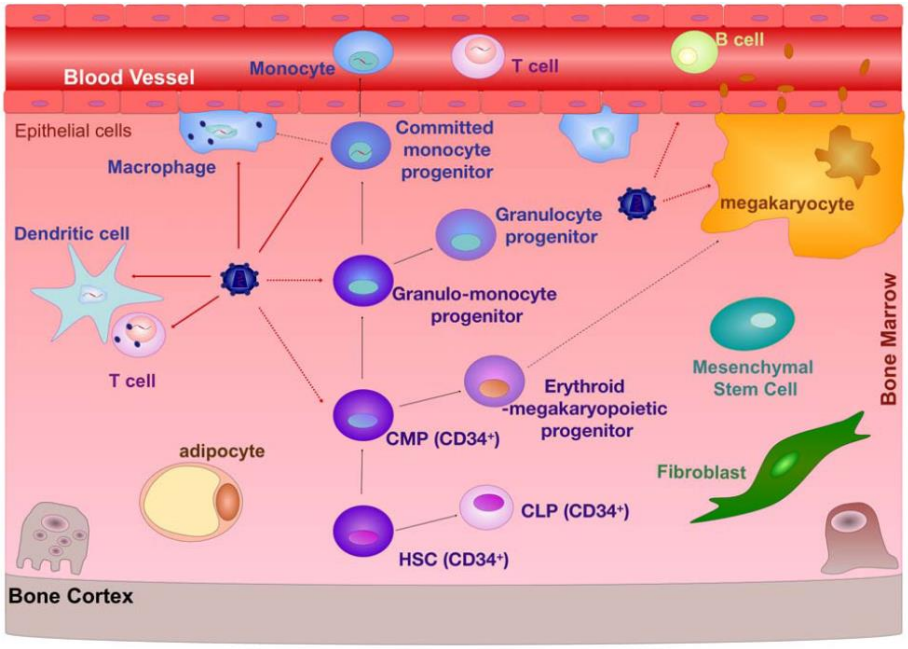

Figure 12 : Haemopoeitic cells susceptible to HIV infection (Reproduced from Alexaki and Wigdahl, 2008)

1.8.3 Detection of HIV:

Usually peripheral blood counts are analysed for absolute CD4 counts however the gold standard for detecting HIV is the use of HIV-1 Western Blot or ELISA assays.

Following a thorough understanding of the various concepts covered so far the thinking and motivation for undertaking the research will follow.

1.9 Motivation for the Project

Acute myeloid leukemia, a common form of leukemia, affects thousands of people each year yet only 30% of those treated achieve a long-term cure. Although FAB and WHO sub-typing and recurring chromosomal abnormalities allow for patients to be risk stratified and treated with greater efficacy, there are approximately 50% of all AML patients with a normal karyotype and therefore intermediate risk which make specific treatment difficult.

Therefore molecular mutations may allow for the development of highly specific risk-stratified treatment plans for patients with normal karyotypes. This would enable more intensive treatments to be offered to high risk patients and save low risk patients with favourable prognoses from unnecessary toxicity. For instance, molecular mutations of *NPM1* may be prognostically favourable whereas mutations of *FLT3* ITD are prognostically unfavourable.

Currently at IALCH, the induction therapy is standard to all AML and APML patients followed by consolidation therapy that is ideally directed by cytogenetics. The lack of timeous cytogenetics analyses result in a heavier reliance on targeted FISH and molecular results to base treatment which can be time-consuming and prone to error.

The introduction of rapid and accurate testing for the detection of *NPM1* and *FLT3* mutations as a routine clinical analysis may allow for targeted treatment of patients and subsequently increased disease-free and long term survival.

A significant number of patients admitted to IALCH are infected with HIV due to the high incidence of HIV in the population in KwaZulu-Natal (Lehohla, 2011). Hence there exist patients with AML who are also HIV positive which can complicate response to treatment as well as long term survival. This study will also attempt to elucidate the effect of HIV status on patients with AML.

1.10 Specific Aims of the Project

Hence the specific aims of the project are as follows:

1. To detect mutations in *NPM1* exon 12 and *FLT3* ITD using High Resolution Melting Analysis in AML patients

2. To compare the mRNA levels expressed by *FLT3* and *NPM1* in patients with acute myeloid leukemia (AML) in the presence and absence of HIV infection and their prognostic impact

3. To assess the response of AML patients with and without HIV to induction therapy

We hypothesize that the differential expressions of specific mutations in *FLT3* and *NPM1*, and HIV status may have an impact on prognosis, severity and response to induction treatment in patients diagnosed with AML.

We further hypothesize that the increased expression of FLT3 and NPM1 correlates with the presence of a corresponding single nucleotide polymorphism and increased expression of FLT3 has an additive effect on the expression of NPM1.

With our reason for the undertaking of this study and specific aims in mind, the next chapter will facilitate an understanding of how we achieved these aims by explaining the protocols.

Chapter 2

Methods

This chapter will provide a comprehensive account of the methods we employed in achieving our aims and to an extent, our reason for utilising them.

2.1 Patients and Samples:

Patients, either newly diagnosed or relapsed, with AML were identified at Inkosi Albert Luthuli Central Hospital. (Informed consents were obtained for the patients in the study and analyses of results were conducted in accordance with the UKZN Biomedical Research Ethics Committee guidelines (Ref No: BF261/09)).

Peripheral blood samples and/or bone marrow as part of the clinical investigation were sent for routine laboratory analysis which included cytochemical stains, immunophenotyping and flow cytometry. These tests are useful in sub-typing AML according to FAB classification. Cytogenetic analyses were conducted after short term culture and karyotyping according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005) on most of the samples. Patient samples were also analysed in the Department of Virology to determine HIV status. White cell counts and absolute blast counts at presentation and post induction were obtained using manual differentiation and flow cytometry. Data from these routine tests as previously discussed were all recorded to create a comprehensive dataset on each patient.

Ideally patients (< 60 years) are stratified into risk groups based on cytogenetic analysis : high risk - -5/del(5q), -7/del(7q), hypodiploid karyotypes (except 45, X, -Y or –X), inv (3q), abn 12p, abn 11q, +11, +13, +21, +22, t(6;9), t(9;22), t(9;11), t(3;3), multiple aberrations; intermediate risk – normal karyotype and low risk – t(8;21) and t(8;21) combined with other aberrations.(Thiede et al., 2002). However, at IALCH risk stratification of patients based on cytogenetic results direct consolidation therapy rather than induction therapy due to the lag time for cytogenetic analyses.

AML/APML patients that were newly diagnosed or relapsed and about to undergo induction chemotherapy were included in the study. Any patients presenting with any other haematological malignancies and at a stage post-induction chemotherapy were excluded from the study.

2.2 Routine Treatment:

There are 2 protocols for induction therapy that are routinely used which consist of: Protocol 1: 3 days of daunorubicin (60mg/m²) and 7 days of cytarabine (200mg/m²) Protocol 2: 3 days of daunorubicin (90mg/m²) with a continuous infusion of cytarabine (100mg/m²) – The longer infusion has a greater efficacy. CR is achieved when there are morphologically less than

5% blasts present in a patient with AML after treatment; whereas for APML, CR is based on PCR results after completion of induction treatment.

Residual blood samples were analysed for the presence of *FLT3* and *NPM1* mutations and their level of expression at diagnosis and again after induction therapy (day 28-35) to determine their worth as prognostic and therapeutic markers.

2.3 Standardisation of Primers:

Before patient samples were run, normal *FLT3* and *NPM1* gene expression were required to establish a standard curve to enable accurate comparison between the wild type gene and the presence of mutations. Therefore, normal peripheral blood samples were obtained from seven healthy donors and the isolated DNA and cDNA were used to optimise *FLT3* and *NPM1* primers (Kuchenbauer et al., 2005, Schnittger et al., 2009, Tan et al., 2008). Standard curves were produced on the Lightcycler 480 (Roche Diagnostics, Penzberg, Germany) using SyBr Green, a DNA intercalating dye. The experimental runs were analysed in relation to the standard curves allowing for a clear distinction if mutations were present. The seven healthy controls created a range of healthy expression that patient samples could be compared to.

2.4 Controls and Housekeeping Genes:

The negative control was PCR grade nuclease free water and the normal controls were seven healthy samples without HIV or AML. c-abl oncogene 1 non-receptor tyrosine kinase (*ABL*) was chosen as a suitable housekeeping gene based on current literature(Weisser et al., 2004) which has shown it to have a high PCR efficiency in quantitative AML analysis. Results were depicted as a ratio of *NPM1* or *FLT3* to *ABL* per μg of cDNA (Kuchenbauer et al., 2005, Schnittger et al., 2009).

2.5 Detection of FLT3 and NPM1 levels of expression:

2.5.1 RNA Isolation:

RNA was extracted using the standard phenol/chloroform method (Chomczynski and Sacchi, 1987) followed by ethanol precipitation. The RNA extraction was modified with only one EL Buffer washing step instead of two, to increase yield of RNA particularly in patients with low white blood cell count.

Briefly, EL Buffer (Qiagen, France) was added to peripheral blood and/or bone marrow aspirate and placed on ice for 15 minutes. After vortexing, the tubes were centrifuged (Jouan) for 10 minutes at 4˚C. The resulting supernatant was decanted and discarded. EL Buffer (kept on ice) was added to the pellet, the contents were vortexed (Heidolph) and centrifuged again for 10 min at 4 C. The supernatant was decanted and the pellet retained.

1ml of TRIzol Reagent (Invitrogen, USA) was added to the pellet and vortexed. The contents were then disrupted using a 20 gauge needle and syringe. 300ul Chloroform/1ml Trizol was added to each sample and inverted till the contents appeared milky pink in colour. The samples were put on ice for 20 minutes.

Following centrifugation (10 000rpm, 4 ˚C, 15 min), the aqueous layer was removed while leaving the interphase (protein) and lower phase (DNA) intact. The protein and DNA were stored at -80˚C for long step DNA isolation if necessary. 2-propanol was added to the aqueous layer in a separate tube and the contents inverted vigorously. The samples were put on ice for 30 minutes.

After centrifugation (12 000rpm, 4˚C, 10 min), the supernatant was decanted and 75% ethanol added to wash the pellet then dislodged with vortexing. The samples were centrifuged (8000rpm, 4˚C, 5 min), supernatant removed and 75% ethanol added to wash the pellet. After a final centrifugation (8000rpm, 4˚C, 5 min) and removal of supernatant, the pellets were air dried under a fume hood. PCR grade, nuclease free water was added to the pellets and the contents placed on the heating block at 55˚C for 10 minutes. The concentration of RNA was then quantified using a nanodrop (ThermoScientific ND-1000, Ver 3.6.0).

2.5.2 cDNA Synthesis:

RNA was converted to complementary DNA (cDNA) using the standardised Europe Against Cancer (EAC) Reverse Transcription protocol (van der Velden et al., 2003) and the relevant Ipsogen Kit (Ipsogen, France). 2ug of RNA, 2ul of Random Hexamer (Promega, USA, 500ug/ml) and DEPC treated water to give a total of 9ul was renatured on a run for 10 minutes at 70˚C using the GeneAmp PCR System 9700 (Applied Biosystems, Ver 3.12) and then immediately cooled on ice for 5 minutes. The RNA isolate was centrifuged briefly at 10 000 rpm and kept on ice. The EAC mastermix or RT premix was prepared with the following components: 5X Expand reverse transcriptase buffer (first strand), 5mM $MgCl_2$, dNTP (10mM each), 100mM DTT, 40U/ul RNAse Inhibitor, and Superscript III Reverse Transcriptase (Invitrogen, USA, 200U/ul). Prior to preparation, all the components were centrifuged and put on ice and volumes of each component were determined based on the number of RNA samples converted. Generally, for 1 sample, the following volumes were used: 4ul of 5x RT Buffer, 1ul of 5mM $MgCl_2$, 1ul of dNTP, 2ul of DTT, 0.5ul of RNase Inhibitor and 0.5ul of Superscript RT III. Post preparation of the mastermix, 11ul of EAC mastermix was added to RNA isolates and made up to a total volume of 20ul. These samples were centrifuged at 10000rpm and then run on the thermal cycler at 25˚C for 10 minutes, 42˚C for 45 minutes, 99˚C for 3 minutes and then cooled to 4˚C. The resulting cDNAs were centrifuged at 10 000rpm and kept on ice until needed.

2.5.3 Real Time PCR RNA Quantitation:

Although the presence of *FLT3 ITD* and *NPM1* mutations are significant, whether these mutations occur in combination, and the levels at which they are expressed, are also of some consequence. Hence, the expression levels of the *FLT3 ITD* and *NPM1* mutations were analysed by isolating RNA, converting RNA to the more stable cDNA and then using RT-PCR quantitation:

The PCR primers and cycling conditions used for *FLT3* and *NPM1* real-time quantitative PCR was validated and is shown in table 4. *ABL* was used as the housekeeping gene as previously discussed. Briefly, each PCR reaction consisted of 0.5pmol/ul of each primer, 5ul of Fast Start SYBR Green I (Roche,France), 1ug cDNA and PCR grade water to 10ul. Reactions were run in duplicate on a Roche Lightcycler 480 version 1.5 with an initial incubation followed by amplification consisting of denaturation, annealing and extension for 45 cycles. Detection of the fluorescent products was carried out at the end of the 72˚C extension period (Tan et al., 2008, van Dongen et al., 1999, Beillard et al., 2003).

Amplification specificity was confirmed by subjecting PCR products to a melting curve analysis and sequencing respectively.

Table 4: *FLT3*, *NPM1* and *ABL* Primers used for Expression Assay and their Cycling Conditions

Gene	Sequence 5'-3'	Cycling conditions: denaturation, annealing and extension
FLT3	F: 5'-TGCAGAACTGCCTATTCCTAACTGA-3'	(95 ˚C, 5min), 45×((95˚C,15s), (60˚C, 15s), (72˚C, 15s))
	R: 5'-TTCCATAAGCTGTTGCGTTCATCAC-3'	
NPM1	F:5'-TGATGTCTATGAAGTGTTGTGGTTCC-3'	(95 ˚C, 5min), 45×((95˚C,15s), (60˚C, 10s), (72˚C, 10s))
	R:5'-CTCTGCATTATAAAAAGGACAGCCAG-3'	
ABL	F: 5'- CCTTCAGCGGCCAGTAGC-3'	(95 ˚C, 5min), 45×((95˚C,15s), (60˚C, 15s), (72˚C, 15s))
	R: 5'-GGACACAGGCCCATGGTAC -3'	

2.6 Detection of FLT3 ITD and NPM1 mutations using High Resolution Melting Analysis:

2.6.1 Extraction of DNA:

Genomic DNA was extracted using the Qiagen MiniAmp DNA extraction Kit according to manufacturer's instructions (Qiagen DNA Blood Kit, Qiagen, France).

Some DNA concentrations and or purities after isolation using the Qiagen MiniAmp DNA Extraction Kit were unsatisfactory and as a result the Trizol Long Step Method (Chomczynski, 1993) was utilised. Post isolation of RNA using the trizol method, the resulting trizol pellet was used to isolate DNA. Briefly, DNA was precipitated by adding 300ul of 100% Ethanol per 1ml of Trizol used for phase separation during RNA isolation. After vortexing, samples were incubated at 15-30 ˚C for 3 minutes and then centrifuged at 12 000 rpm for 5 minutes at room temperature. After removing the ethanol supernatant, the resulting DNA pellet was washed twice with 0.1M sodium citrate dehydrate solution (Appendix D- Reagent Preparation). 1ml of 0.1M sodium citrate dehydrate solution per 1ml Trizol was used. After vortexing, the samples were incubated in wash solution at 15-30˚C for 30 minutes with periodic mixing and centrifuged at 12 000 rpm for 5 minutes at room temperature. After the washes, the DNA was resuspended in 1ml of 75% Ethanol per 1ml TRIZOL and incubated at 15-30˚C for 20 minutes with periodic mixing. After centrifugation at 12 000 rpm for 5 minutes at room temperature, the supernatant was removed and the pellets dried under the fume hood for 5 minutes. The DNA was redissolved in 100ul of DEPC-treated water and placed on the heating block at 55˚C for 10 minutes. After centrifugation at 12 000 rpm for 10 minutes at room temperature, the supernatant is transferred to a labelled eppendorf and quantified as pure DNA whilst the pellets can be stored at -80˚C.

2.6.2 PCR and High Resolution Melting Analysis:

FLT3 internal tandem duplications and *NPM1* exon 12 mutations were detected by isolating DNA and subsequent PCR and High Resolution Melting Analysis:

The Lightcycler 480, a 96/384 well capacity RT-PCR machine was used for the PCR and melting analysis. Samples were tested in duplicate and seven normal controls for the *FLT3* and *NPM1* genes were included in each run. Run conditions were similar to those described by Tan AYC et al (2008). Briefly, 10ng of DNA was amplified in a mixture containing 400nM of primers, as shown in Table 2, 4mM (*NPM1*) or 4.5mM (*FLT3*) $MgCl_2$ and Lightcycler 480 High Resolution Melting Master. Although this experiment was conducted based on the work done by Tan AYC et al (2008), $MgCl_2$ concentration was optimised for both *NPM1* and *FLT3* detection and 4.5mM of $MgCl_2$ was found to be optimal for the *FLT3* detection experiment compared to 3mM of $MgCl_2$ as previously prescribed. At the end of the run, the analysis was performed using the Lightcycler 480 software (Ver. 1.5.0.39). The melting curves were normalised and temperature shifted to allow direct comparison of samples. Difference plots were generated by using a negative control as the baseline and fluorescence of the other samples were plotted relative to this baseline. For the *FLT3* difference plot, the temperature sliders were set at 77˚C- 78˚C and 83˚C - 84˚C with the threshold at zero and the sensitivity set at 0.15. For the *NPM1* difference plot, the temperature sliders were set at 72˚C- 73˚C and 79˚C - 80˚C with the threshold at 4 and the sensitivity set at 0.15. Mutations were indicated by significant differences in fluorescence.

Table 5: *FLT3* and *NPM1* Primers used for High Resolution Melt Analysis and the PCR cycling conditions

Gene	Sequence 5'-3'	Cycling conditions: denaturation, annealing and extension
FLT3	F: 5'-TGCAGAACTGCCTATTCCTAACTGA -3'	(95˚C, 5 min), 60×((95˚C,30s), (65˚C, 30s), (72˚C,30s)) + ((95˚C,1min), (40˚C,1min), (65˚C, 1s), (95˚C, contin)) + (40˚C, 30s)
	R: 5'- TTCCATAAGCTGTTGCGTTCATCAC-3'	
NPM1	F: 5'-TGATGTCTATGAAGTGTTGTGGTTCC -3'	(95˚C, 5 min), 45×((95˚C,10s), (65˚C, 10s), (72˚C,10s)) + 1×((95˚C,1min), (40˚C,1min), (65˚C,1s), (95˚C, contin)) + (40˚C, 30s)
	R: 5'-CTCTGCATTATAAAAAGGACAGCCAG -3'	

2.7 Verification of the presence of FLT3 ITD and NPM1 mutations using direct sequencing:

Essentially, this method documents the use of the Big Dye Terminator v3.1 Cycle Sequencing Kit to prepare template DNA for sequencing, actual sequencing reactions and the purification of the resulting products.

2.7.1 Template Preparation for Sequencing:

2.7.1.1 Visualization of Specific PCR product using Agarose Gel Electrophoresis:

2% Agarose gels were loaded with 8ul of PCR product (amplified DNA) mixed with 2ul of Gel Loading Buffer to enable elucidation of specific bands that were later excised using a SYNGENE Transilluminator. The gels were electrophoresed at 100V, 50mA for 1-1.5 hours and using GEL RED dye (Invitrogen).

2.7.1.2 Purification of PCR product:

Purification of the PCR product is performed according to the ILLUSTRA GFX PCR DNA and Gel Band Purification Kit Handbook instructions. Briefly, 500ul of Capture Buffer Type 3 was added to excised gel bands and incubated at 60°C for 15-30 minutes. The tubes were mixed by inversion every 3 minutes. Once dissolved, the yellow/pale orange mixture was added to a GFX Microspin column and collection tube, incubated at room temperature for 1 minute then centrifuged for 30s at 16000 × g (13000 rpm). The flow through was discarded and the column was placed back in the collection tube. 500ul of Wash Buffer Type 1 was added and the tube centrifuged for 30s at 16 000 × g. The collection tube was then discarded and the column transferred to a clean 1.5ml DNase-free microcentrifuge tube. 20ul of Elution buffer type 6 was then added to the column and incubated for 1 minute at room

temperature. The column and collection tubes were then centrifuged for 1 minute at 16000 × g. The flow through was retained and the purified DNA was stored at -20°C until required.

2.7.1.3 Quantification of PCR product:

A 2% agarose gel was prepared. 5ul of purified PCR product was mixed with 1ul of Gel Loading Buffer (GLB) containing Gel Red and loaded onto submerged agarose gel. Lane 1 was reserved for 2ul of Low DNA Mass Ladder (Invitrogen, Canada) mixed with 1ul of GLB. The gel was electrophoresed at 10V/cm until the loading dye had migrated at least 5cm into the gel. The gel was examined using the SynGene Gel Box and the concentrations of the PCR products were deduced. According to the gel, the PCR products were diluted to approximately 10ng per sequencing reaction in preparation for the sequencing reaction. After dilution, the samples were vortexed for 3-5 seconds and then centrifuged for 5-10 seconds and stored at -20°C until needed.

2.7.2 Big Dye Terminator (v3.1) Cycle Sequencing Reaction:

2.7.2.1 Sequencing Reaction:

Master mixes were prepared for the samples to be sequenced in the following manner:

REAGENT	QUANTITY FOR 1 REACTION
Terminator Ready Reaction Mix	0.4ul
5X Sequencing Buffer	2.0ul
Primer (1.6pmol)	2ul
Deionised Water	4.6ul
TOTAL VOLUME	9ul

The master mix was mixed thoroughly and centrifuged and aliquoted onto a plate. 1ul of 10ng/ul diluted template was then added to the reaction and the plate was centrifuged. The plate was placed in a thermal cycler and run at the following conditions:

Initial Denaturation for 1 cycle at 96°C for 1 minute followed by 35 cycles of DNA denaturation (96°C, 10s); Primer Annealing (50°C, 5s) and Primer Extension (60°C, 4 min) and then final cooling at 4°C till required for purification.

2.7.3 Purification of Sequencing Products:

2.7.3.1 Plate Cleanup:

1ul of 125mM EDTA (pH 8) was added to each well and mixed well with pipette tips. 1ul of 3M NaOAc (pH 5.2) combined with 25ul of 100% Ethanol was then added to each well. The plate including each well was sealed using adhesive foil and vortexed briefly. The plate was then centrifuged (3000 × g, 20 min). This was followed by the centrifugation of the inverted plate at 150 × g for 1 minute. 35ul of 70% cold ethanol was added immediately to each well and the plate was covered and then centrifuged (3000 × g, 5 min). The plate was then inverted onto an absorbent towel and centrifuged (150 × g, 1 min). The samples were then dried in a thermal cycler at 50°C for 1 minute. The plate was then sealed with an adhesive cover and stored in the dark at -20°C till needed.

Analysis of the sequencing products were performed on the ABI 3130xl Genetic Analyzer. The output was analysed using BioEdit Sequence Alignment Editor (Version 5.0.9) and Clustal W Alignment (Version 1.8). *FLT3* consensus sequences were compared to a reference sequence – NM_004119.2 and *NPM1* consensus sequences were compared to a reference sequence – NM_002520.6 obtained from Entrez PubMed.

2.8 Statistical Considerations:

Two groups within the study cohort emerged; patients with AML who were HIV positive (Group 1) and patients with AML who were HIV negative (Group 2). Clinical features and laboratory data were compiled on each patient at diagnosis and after induction therapy. Comparisons were subsequently made between the two groups to establish clinical and molecular impact of the mutations, their levels of expression and HIV status on AML patients.

Comparisons between FLT3 and NPM1 expression data were made using Graph Pad Prism 5 and unpaired t-tests for paired patient samples (presentation and post-induction). The data was transformed and 1-way ANOVA tests were used for analysis.

Patient white blood cell counts' were analysed using Graph Pad Prism 5 and unpaired t-tests.

Patients 10,11 and 25 had to be excluded from the "paired sample" dataset as their presentation samples were of questionable quality either due to laboratory experimental error or error while harvesting sample.

The next chapter will focus on the data we procured from experimental work as well as from clinician and patient input. The results will be presented in relation to the systematic way the methods, as discussed above, are presented.

Chapter 3

Results

This chapter will reveal the results we obtained from experimental work and clinician input. These results are ordered in relation to the methods discussed in the previous chapter.

3.1 Patient Demographics and Clinical Diagnosis

There were 28 patients in the study. The study cohort made up 2 groups - AML patients with HIV (Group 1) and AML patients without HIV (Group 2). There were 5 patients in group 1 and 23 patients in group 2. Table 6 below shows age, gender, race and disease variables for the participants in the study.

Table 6: Demographics and Clinical Diagnosis of Patients Involved in this Study

Patient Number	Age	Sex	Race	HIV Status	FAB	Prior Disease if any
1	34	Female	African	Negative	AML M4	No prior disease
2	37	Male	Indian	Negative	APML M3	No prior disease
3	66	Male	Caucasian	Negative	AML Unknown	CML with blast crisis
4	43	Female	African	Negative	AML M7	CML with blast crisis
5	44	Male	African	Negative	AML Unknown	CML with blast crisis
6	25	Female	African	Positive	AML M4/M5	No prior disease
7	50	Female	African	Negative	AML M7	CML with blast crisis
8	33	Female	Indian	Negative	AML M4/M5	No prior disease
9	19	Female	African	Negative	AML M7	No prior disease
10	36	Male	Indian	Negative	AML M5	No prior disease
11	15	Female	African	Negative	APML M3	No prior disease
12	39	Female	African	Positive	AML M5	No prior disease
13	47	Female	Indian	Negative	AML M2	No prior disease
14	16	Female	African	Negative	AML M4	No prior disease
15	55	Male	African	Negative	AML M4/M5	No prior disease
16	24	Female	African	Positive	APML M3	No prior disease
17	19	Female	African	Negative	AML M7	No prior disease
18	23	Male	African	Negative	AML M2	MDS
19	22	Female	African	Negative	AML M4	No prior disease
20	20	Male	African	Negative	AML Unknown	No prior disease
21	14	Male	African	Negative	AML M4	No prior disease
22	33	Male	Indian	Negative	APML M3	No prior disease
23	22	Female	African	Positive	AML M4	No prior disease
24	13	Female	African	Negative	APML M3	No prior disease
25	17	Female	African	Negative	AML M4	No prior disease
26	34	Male	African	Positive	AML M4	No prior disease
27	41	Male	African	Negative	APML M3	No prior disease
28	17	Male	African	Negative	AML M4	No prior disease

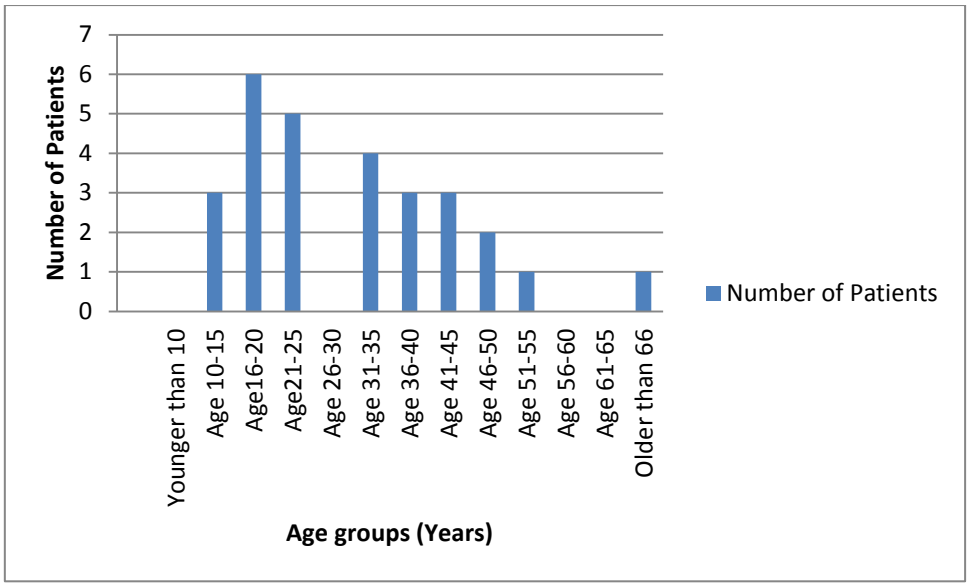

Figure 13: Bar graph illustrating Patient Age Group Distribution

The age of patients in the study ranged from 13-66 years (Fig. 13 & Table 6). The median age was 30 years.

There were 12 males and 16 females - 42.9% versus 57.1% of the total cohort (Table 6).

In the study cohort – the racial split is as follows: 22 Africans, 5 Indian, 1 Caucasian and 0 Coloured/Mixed Race - 78.6% versus 17.9% versus 3.5% versus 0% (Table 6).

There were 5 HIV positive patients and 23 HIV negative patients in the study cohort -17.9% versus 82.1% (Table 6).

The FAB subtypes are shown in Figure 14 below.

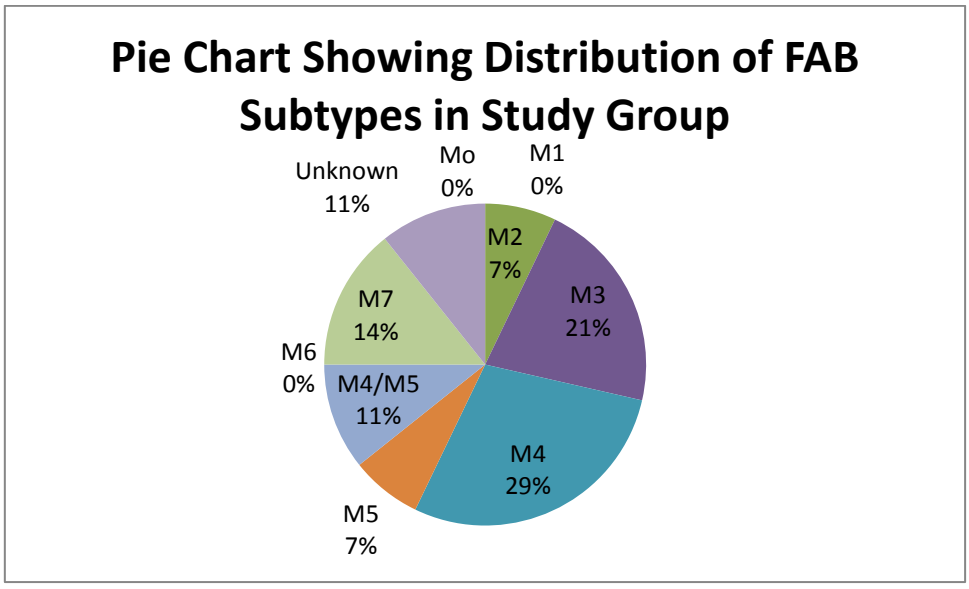

Figure 14: Pie Chart Showing Distribution of FAB Subtypes in Study Group

In this study group, there were no patients with FAB subtypes Mo and M1; 2 patients with M2; 6 patients with APML M3; 8 patients with M4; 2 patients with M5; 3 patients with M4/M5; 0 patients with M6; 4 patients with M7 and 3 patients with FAB subtypes unknown (Table 7).

The most common FAB subtype is M4 (proportion of 0.29 of cohort). The least common FAB subtype is Mo, M1 and M6 (0 patients in cohort). It appears as if the most common subtypes would be the M4, M5, M4/M5 which account for 0.46 of the cohort cumulatively (13 patients in study) (Fig.14).

In this study, there was 1 patient with MDS which transformed into secondary AML and 4 patients with blastic CML that transformed into AML. The majority of patients in the study had primary AML as they had no prior disease pre-disposing them to AML (Table 6).

3.2 Patient Prognostic Risk Stratification

Table 7 shown below contains information pertinent to prognostic risk and patient survival after induction therapy.

Table 7: Patient Prognostic Risk based on Diagnosis, FAB Subtype, Cytogenetics and Outcome

Patient Number	Clinical Diagnosis	FAB Subtype	Cytogenetics	Risk of Morbidity/Mortality	Conclusion after Induction Therapy
1	AML of ambiguous lineage	AML M4	Normal Karyotype	Intermediate	Deceased
2	APML	APML M3	Normal Karyotype	Intermediate	Deceased
3	Blastic CML with AML transformation	AML Unknown	Normal Karyotype	Intermediate	Deceased
4	Blastic CML with AML transformation	AML M7	Normal Karyotype	Intermediate	Alive
5	Blastic CML with AML transformation	AML Unknown	Normal Karyotype	Intermediate	Deceased
6	AML with a Monocytic Component	AML M4/M5	11q23 Positive	High	Deceased
7	Blastic CML with AML transformation; AML with Basophilic Component	AML M7	Normal Karyotype	Intermediate	Deceased
8	AML	AML M4/M5	11q23 Positive	High	Deceased
9	AML	AML M7	Normal Karyotype	Intermediate	Alive
10	AML	AML M5	Normal Karyotype	Intermediate	Alive
11	AML	APML M3	Normal Karyotype	Intermediate	Alive
12	AML	AML M5	Normal Karyotype	Intermediate	Alive
13	AML	AML M2	8.21 Positive	Low	Alive
14	AML	AML M4	Normal Karyotype	Intermediate	Alive
15	AML mixed phenotype btw B-ALL and myeloid	AML M4/M5	MLL rearrangement; 11q23 Positive	High	Deceased
16	APML	APML M3	Normal Karyotype	Intermediate	Alive
17	AML	AML M7	Normal Karyotype	Intermediate	Defaulted on treatment
18	Prior MDS transformed to secondary AML	AML M2	Trisomy 8	High	Deceased
19	AML with a Monocytic Component	AML M4	Normal Karyotype	Intermediate	Deceased
20	AML	AML Unknown	Normal Karyotype	Intermediate	Deceased
21	AML	AML M4	Normal Karyotype	Intermediate	Alive
22	APML	APML M3	t12.17 Positive, additional chr 17 abnormalities	High	Alive
23	AML	AML M4	Normal Karyotype	Intermediate	Deceased
24	APML	APML M3	Normal Karyotype	Intermediate	Alive
25	AML	AML M4	8, 21 Positive	Low	Deceased
26	AML	AML M4	11q23 Positive	High	Deceased
27	APML	APML M3	Positive PML-RARA (BCR1)	Low	Alive
28	AML	AML M4	8,21 Positive	Low	Alive

There were 28 patients at the beginning of the study, after induction only 13 were alive with 14 dying and one defaulting on treatment (Table 7).

The prognostic risk stratification indicated that there were six high risk patients (0.21 of the cohort), only one patient deemed high risk survived post therapy. Whereas, there were four low risk patients (0.14 of the cohort) and three survived post therapy. There were 18 intermediate risk patients (0.64 of the cohort), the most common type of prognostic risk. Of these 18 patients, eight died before the end of therapy and nine survived till the end of post-induction. One intermediate risk patient defaulted on treatment and their survival is unknown.

There were five HIV positive patients in the study (Patients 6, 12, 16, 23 and 26), of which the two that were high risk did not survive post induction therapy (Patient 6 & 26). From the three other HIV

positive patients deemed intermediate risks, two survived post-induction (Patients 12 &16). (Tables 6 & 7)

There were 23 HIV negative patients in the study, 11 of which survived induction therapy and 11 that did not survive. One patient, that was HIV negative, defaulted on treatment and her survival is unknown (Patient 17). (Tables 6 & 7)

Patients' samples and data were collected at two time points – presentation and post-induction; these occurred at similar intervals owing to the schedules imposed by the oncology clinic at IALCH – 28 days between the first and second time point.

3.3 Patient White Blood Cell Counts at Presentation and Post-Induction

Table 8 shows patient WBC counts at presentation and after induction therapy.

Table 8 : Patient White Blood Cell Counts at Presentation and Post-Induction

Patient Number	WBC @ Presentation ($\times 10^9$)	WBC @ Post-Induction ($\times 10^9$)
1	28.78	80.76
2	63.21	17.15
3	65.39	9.2
4	60.49	3.5
5	78.2	0.08
6	16.43	0.93
7	97.77	7
8	15.92	3.57
9	6.31	4.83
10	40.79	11.29
11	119.55	9.43
12	18.94	no info
13	4.34	3.15
14	16.76	6.48
15	15.09	2.19
16	0.89	3.23
17	4.76	5.5
18	4.91	61.68
19	135.41	1.29
20	73.25	92.59
21	1.57	5.14
22	73.12	3.5
23	10.58	0.45
24	1.52	1.07
25	48.68	2.45
26	34.22	35.82
27	75.37	2.87
28	34.86	7.92

Table 8 shows that the highest WBC at presentation was 135.41 × 10^9 cells/L (Patient 19). The lowest WBC at presentation was 0.89 × 10^9 cells/L (Patient 16). The median WBC for patients in this cohort at presentation was 40.97 × 10^9 cells/L.

The highest WBC at post induction was 92.59 × 10^9 cells/L (Patient 20). The lowest WBC at post induction was 0.08 × 10^9 cells/L (Patient 5). The median WBC for patients in this cohort at post induction was 14.19 × 10^9 cells/L.

Patient 12 did not have WBC count at post induction on hospital record.

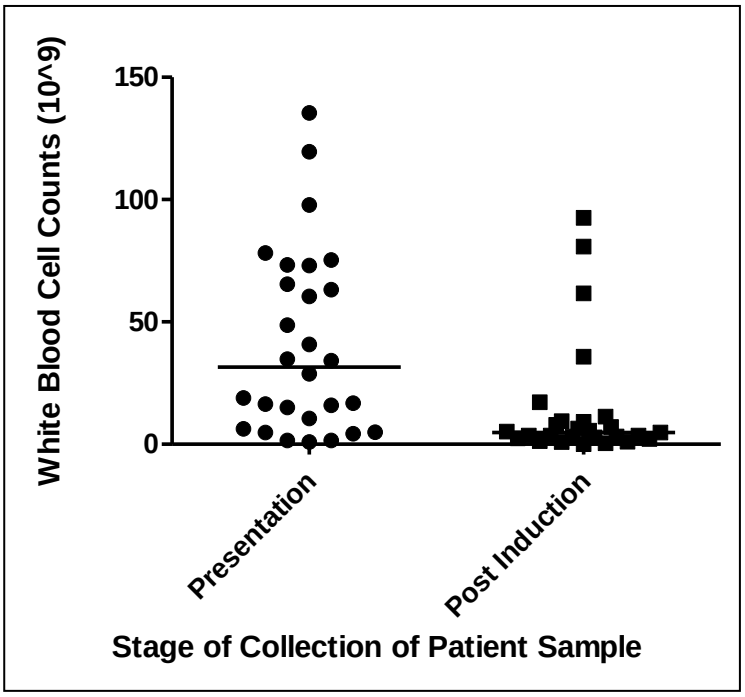

Figure 15: Comparison of Patient White Blood Cell Counts (10^9) at Presentation and Post-Induction ($p<0.05$ therefore differences are statistically significant)

According to the scatter plot graph above, WBC counts are generally higher at presentation than post-induction. From the patients with persistent high WBC counts (Patients 1, 2, 10, 20 and 26), only 1 patient (Patient 10) survived to the end of post-induction therapy. Patients with low WBC counts at both presentation and post-induction (Patients 16 & 24) survived post-induction. Patient 18 had normal WBC counts at presentation and high WBC counts at post-induction and did not survive the end of therapy.

Table 9 details the statistical analysis using GraphPad Prism 5 and non-parametric t-tests. The deduction is that the means of WBC counts at presentation and post-induction were significantly different.

Table 9: Statistical Analysis of Patient White Blood Cell Counts at Presentation and Post-Induction using Graph Pad Prism 5

Unpaired t test	
P value	0.003
Means significantly different (P<0.05)	Yes
One or Two Tailed P value	Two tailed
t ratio (t)	3.106
Degrees of Freedom (df)	53
F test to compare variances	
F ratio (F)	2.367
Degree of freedom for numerator of F (Dfn)	27
Degree of freedom for denominator of F (Dfd)	26
P value	0.031
Variances significantly different	Yes

Two sets of values were compared- white blood cell counts before and after treatment. The unpaired t-test and F test to compare variances determine if the WBC counts had significant differences from presentation to post-induction ie. If there was an effect. The parameters listed allow for reproducibility.

3.4 FLT3 and NPM1 Levels of Expression

Table 10: Levels of FLT3 and NPM1 Expression (Mutational and Normal) at Presentation and Post-Induction determined by the Expression Assay conducted on AML patient samples

		PRESENTATION					POST INDUCTION				
Patient Number	**Disease Specifics**	**ABL Mean Conc**	**FLT3 Mean Conc**	**NPM1 Mean Conc**	**FLT3/ABL Expression**	**NPM1/ABL Expression**	**ABL Mean Conc**	**FLT3 Mean Conc**	**NPM1 Mean Conc**	**FLT3/ABL Expression**	**NPM1/ABL Expression**
1	AML M4	11820.5010	324.4845	710.8258	0.0275	0.0601					
2	APML M3	6830.0513	641.3872	806.0690	0.0939	0.1180					
3	Blastic CML with AML transformation	10008.2124	1227.0015	4419.3535	0.1226	0.4416					
4	Blastic CML with AML M7 transformation	637.8645	51.8623	112.4112	0.0813	0.1762	814.4758	46.8948	72.3969	0.0576	0.0889
5	Blastic CML with AML transformation	17005.1045	305.1639	544.2112	0.0179	0.0320					
6	AML M4/M5	243.7564	418.2500	109.7868	1.7159	0.4504					
7	Blastic CML with AML M7 transformation	29.2337	0.1213	0.5792	0.0041	0.0198	590.9198	4.1164	6.0628	0.0070	0.0103
8	AML M4/M5	1717.4128	118.4798	29.3770	0.0690	0.0171	643.6939	57.3262	105.7323	0.0891	0.1643
9	AML M7	215291.6432	196.8635	107.2359	0.0009	0.0005	68829.2042	116.3083	92.0320	0.0017	0.0013
10	AML M5										
11	APML M3										
12	AML M5	3092.8136	81.8304	89.2695	0.0265	0.0289	650.0717	381.2308	671.0124	0.5864	1.0322
13	AML M2	4098.7213	148.9082	403.1185	0.0363	0.0984	711.4540	261.6053	307.5771	0.3677	0.4323
14	AML M4	5176.0042	247.1850	318.8188	0.0478	0.0616	664.8373	1119.8621	1501.3801	1.6844	2.2583
15	AML mixed phenotype (B-ALL/myeloid); AML M4/M5	3034.3427	1191.1281	2343.0851	0.3925	0.7722					
16	APML M3	1179.6840	177.0205	232.8655	0.1501	0.1974	3293.9899	332.9646	612.6008	0.1011	0.1860
17	AML M7	528.2537	140.7596	169.7952	0.2665	0.3214	1724.0979	1778.5917	3794.3943	1.0316	2.2008
18	MDS transformed to AML M2	9835.9242	818.8908	432.6317	0.0833	0.0440	8013.5941	701.4998	655.7820	0.0875	0.0818
19	AML M4	3660.4883	225.1667	1586.8200	0.0615	0.4335					
20	AML Unknown	6819.3626	718.7786	5455.1512	0.1054	0.8000					
21	AML M4	3627.8197	1143.3815	1589.6714	0.3152	0.4382	597.2728	242.4667	318.8420	0.4060	0.5338
22	APML M3	7224.0850	524.0725	280.8797	0.0725	0.0389	3380.2184	559.7775	806.2116	0.1656	0.2385
23	AML M4	3697.4861	189.2845	111.0899	0.0512	0.0300	5622.2929	200.6774	470.9772	0.0357	0.0838
24	APML M3	5072.8008	171.6483	424.7794	0.0338	0.0837	1438.2826	143.5732	128.1158	0.0998	0.0891

25	AML M4										
26	AML M4	3121.7181	86.3371	4005.1350	0.0277	1.2830					
27	APML M3	1378.8602	111.2566	96.2225	0.0807	0.0698	1713.1326	852.1916	747.7487	0.4974	0.4365

Patients with missing expression values were deceased before reaching the post- induction therapy phase.

Patients 10, 11 and 25 were excluded from statistical analysis due to their anomalous values.

Table 10 shows that the average expression for FLT3 at presentation was 0.1625 and 0.3323 at post-induction. The highest FLT3 expression values at presentation is 1.7159 (Patient 6) and at post-induction is 1.6844 (Patient 14). The lowest FLT3 expression values at presentation is 0.0009 (Patient 9) and at post-induction is 0.0017 (Patient 9).

The average expression for NPM1 at presentation was 0.2510 and 0.5114 at post–induction. The highest NPM1 expression values at presentation is 1.2830 (Patient 26) and at post-induction is 2.2583 (Patient 14). The lowest NPM1 expression values at presentation is 0.0005 (Patient 9) and at post-induction is 0.0013 (Patient 9).

Table 11 shows mRNA levels of expression for FLT3 and NPM1 for healthy volunteers.

Table 11: Levels of FLT3 and NPM1 Expression for Healthy Controls

Control	ABL Mean Conc	FLT3 Mean Conc	NPM1 Mean Conc	FLT3/ABL Expression	NPM1/ABL Expression
1	202.0778	206.9645	201.0803	1.0242	0.9951
2	1014.4869	36.7590	100.4865	0.0362	0.0991
3	1022.6422	92.6707	191.3589	0.0906	0.1871
4	223.8643	41.3588	72.9943	0.1847	0.3261
5	153.6688	88.8531	84.9443	0.5782	0.5528
6	626.6277	240.8025	300.1055	0.3843	0.4789
7	504.8754	44.0327	135.1657	0.0872	0.2677

The average FLT3 expression for the healthy donors was 0.3408 and the average NPM1 expression for healthy donors was 0.4152.

3.4.1 FLT3 Expression and Statistical Analysis

Table 12 shows FLT3 mRNA levels of expression for patients at both presentation and after induction therapy.

Table 12: Paired (Presentation and Post-Induction) Patient Samples showing FLT3 Levels of Expression

Samples	FLT3/ABL	Samples	FLT3/ABL
S4 Pre	0.0813	S4 Post	0.0576
S7 Pre	0.0041	S7 Post	0.0070
S8 Pre	0.0690	S8 Post	0.0891
S9 Pre	0.0009	S9 Post	0.0017
S12 Pre	0.0265	S12 Post	0.5864
S13 Pre	0.0363	S13 Post	0.3677
S14 Pre	0.0478	S14 Post	1.6844
S16 Pre	0.1501	S16 Post	0.1011
S17 Pre	0.2665	S17 Post	1.0316
S18 Pre	0.0833	S18 Post	0.0875
S21 Pre	0.3152	S21 Post	0.4060
S22 Pre	0.0725	S22 Post	0.1656
S23 Pre	0.0512	S23 Post	0.0357
S24 Pre	0.0338	S24 Post	0.0998
S27 Pre	0.0807	S27 Post	0.4974
S28 Pre	0.1775	S28 Post	0.0988

Anomalies were excluded from the dataset (Patients 10, 11 & 25).

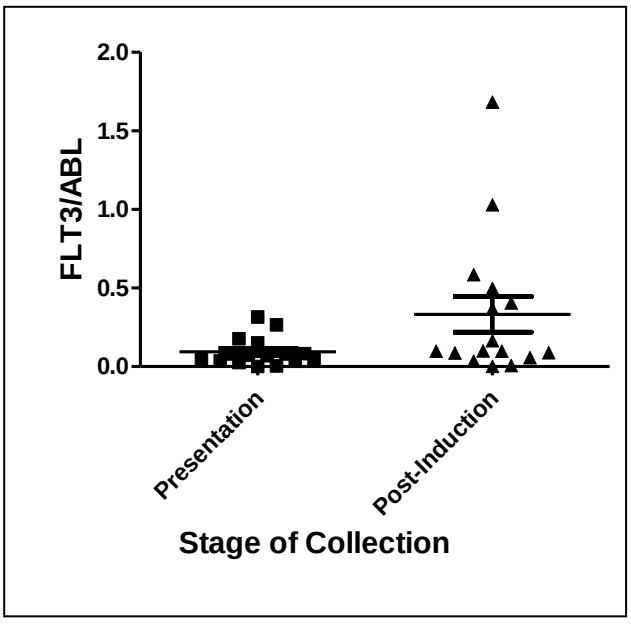

Figure 16: FLT3 Levels of Expression for Patient Paired Samples ($p<0.05$ therefore statistically significant; Graph Pad Prism 5)

According to the scatter – plot graph above, the FLT3 post induction expression values have greater variance than the presentation FLT3 expression values. The highest FLT3 post induction values are conferred to 3 patients (Patients 14, 17 & 12) of which 2 are alive and 1 unknown (Tables 12 & 7).

Table 13 shows the differences between FLT3 expression values at presentation and post-induction are statistically significant as determined by an unpaired t-test (p value = 0.0482).

Table 13: Statistical Analysis of FLT3 Expression Levels for Paired Patient Samples (Presentation and Post-Induction) using Graph Pad Prism 5

Unpaired t test	
P value	0.0482
Means significantly different (P<0.05)	Yes
One or Two Tailed P value	Two tailed
t ratio (t)	2.06
Degrees of Freedom (df)	30
F test to compare variances	
F ratio (F)	25.33
Degree of freedom for numerator of F (Dfn)	15
Degree of freedom for denominator of F (Dfd)	15
P value	<0.0001
Variances significantly different	Yes

Two sets of values were compared- FLT3 levels of expression before and after treatment. The unpaired t-test and F test to compare variances determine if the levels of FLT expression had significant differences from presentation to post-induction ie. If there was an effect. The parameters listed allow for reproducibility.

3.4.2 NPM1 Expression and Statistical Analysis

Table 14 show mRNA levels of expression for NPM1 in patients at presentation and after induction therapy.

Table 14: Paired (Presentation and Post-Induction) Patient Samples showing NPM1 Levels of Expression

Samples	NPM1/ABL	Samples	NPM1/ABL
S4 Pre	0.1762	S4 Post	0.0889
S7 Pre	0.0198	S7 Post	0.0103
S8 Pre	0.0171	S8 Post	0.1643
S9 Pre	0.0005	S9 Post	0.0013
S12 Pre	0.0289	S12 Post	1.0322
S13 Pre	0.0984	S13 Post	0.4323
S14 Pre	0.0616	S14 Post	2.2583
S16 Pre	0.1974	S16 Post	0.1860
S17 Pre	0.3214	S17 Post	2.2008
S18 Pre	0.0440	S18 Post	0.0818
S21 Pre	0.4382	S21 Post	0.5338
S22 Pre	0.0389	S22 Post	0.2385
S23 Pre	0.0300	S23 Post	0.0838
S24 Pre	0.0837	S24 Post	0.0891
S27 Pre	0.0698	S27 Post	0.4365
S28 Pre	0.2581	S28 Post	0.3441

Anomalies were excluded from the dataset (Patients 10, 11 & 25).

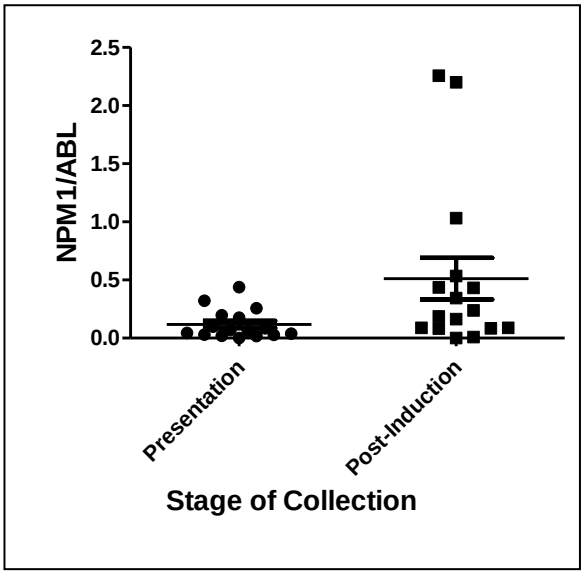

Figure 17: NPM1 Levels of Expression for Patient Paired Samples ($p<0.05$ therefore statistically significant; Graph Pad Prism 5)

According to the scatter – plot graph above, the NPM1 post induction expression values have greater variance than the presentation NPM1 expression values. The highest NPM1 post induction values are conferred to 3 patients (Patients 14, 17 & 12) of which 2 are alive and 1 unknown (Table 14 & 7).

Table 15 shows that the differences between NPM1 expression values at presentation and post-induction are statistically significant as determined by an unpaired t-test (0.0392).

Table 15: Statistical Analysis of NPM1 Expression Levels for Paired Patient Samples (Presentation and Post-Induction) using Graph Pad Prism 5

Unpaired t test	
P value	0.0482
Means significantly different (P<0.05)	Yes
One or Two Tailed P value	Two tailed
t ratio (t)	2.157
Degrees of Freedom (df)	30
F test to compare variances	
F ratio (F)	32.13
Degree of freedom for numerator of F (Dfn)	15
Degree of freedom for denominator of F (Dfd)	15
P value	<0.0001
Variances significantly different	Yes

Two sets of values were compared- NPM1 levels of expression before and after treatment. The unpaired t-test and F test to compare variances determine if the levels of NPM expression had significant differences from presentation to post-induction ie. If there was an effect. The parameters listed allow for reproducibility.

3.5 High Resolution Melting Analysis, Agarose Gels and Sequencing

3.5.1 NPM1 Results

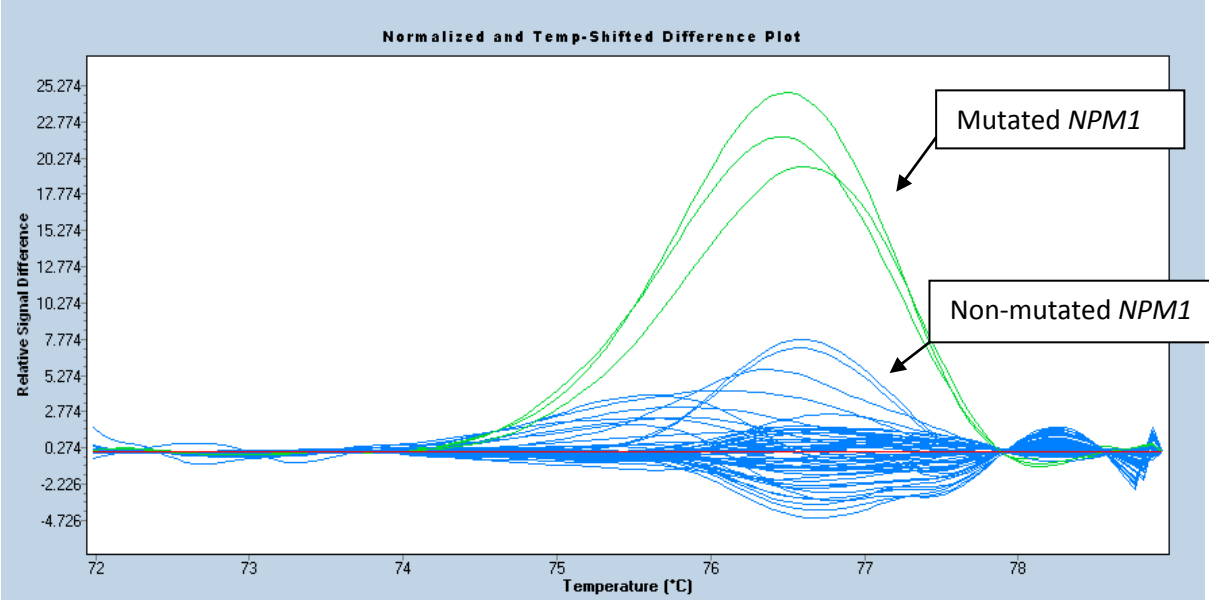

Figure 18: Normalized and Temperature Shifted Difference Plot for Detection of *NPM1* mutations derived from High Resolution Melt Analysis of Patient Samples

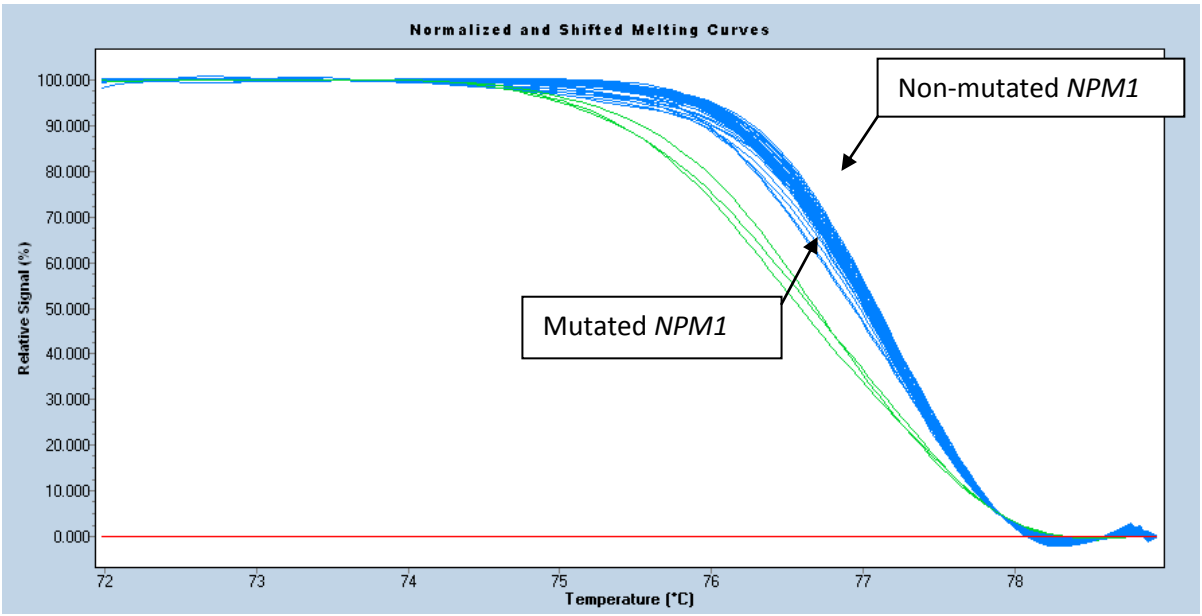

Figure 19: Normalized and Shifted Melting Curves Enabling Detection of *NPM1* mutations derived from High Resolution Melt Analysis of Patient Samples

As seen above, the normalized and temperature shifted difference plot (Fig. 18) as well as the normalized melting curve (Fig. 19) rendered two groups – the normal group representing non-mutated *NPM1* indicated in blue and the affected group representing mutated *NPM1* indicated in green. Figure 18 shows a single peak in green compared to a large blue peak followed by a smaller peak towards the end of the melt.

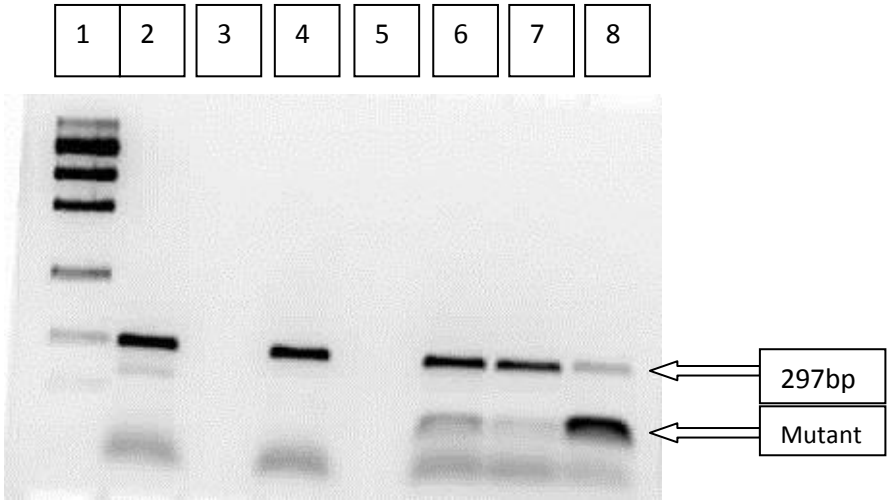

Figure 20: Patients positive for the *NPM1* mutation visualized on a 2% agarose gel (100V, 50mA) using PCR products after HRM detection for NPM1 (Lane 1- Low DNA Mass Ladder (1kb); Lane 2 – Patient 7; Lane 4 – Patient 24 PI; Lane 6 – Patient 28 PI; Lane 7 – Patient 6; Lane 8 – Patient 20)

Figure 20 shows 5 patients positive for the *NPM1* mutation as determined by HRM analysis appearing as multiple bands on the gel. Patients 7 at presentation, Patient 28 at post-induction, Patient 6 at presentation and Patient 20 at presentation have 3 bands on the gel. Patient 20 at presentation (Lane 8) has 3 bands on the gel, the first being feint and the second band being most pronounced. Patient 24 at post-induction however has 2 bands on the agarose gel. All these patients appear in the mutated group as determined by HRM analysis (Figs. 18 & 19). All the bands were sequenced in lanes 2-8.

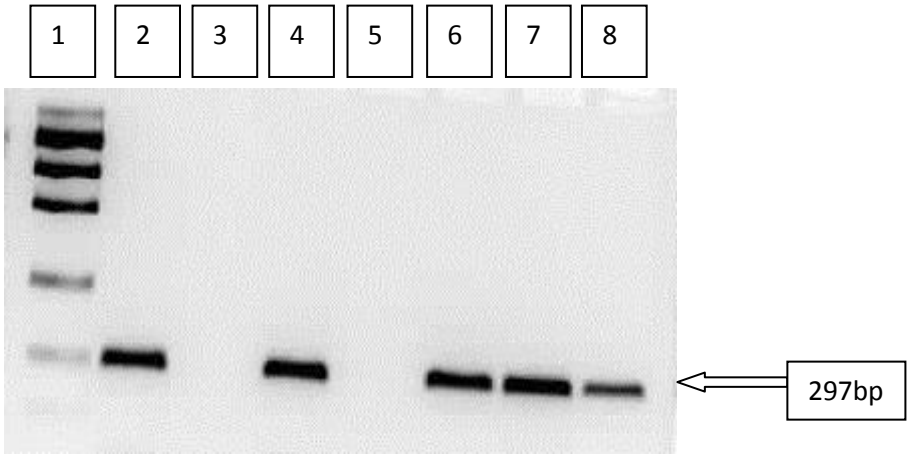

Figure 21: Patients with non-mutated *NPM1* visualized on a 2% agarose gel (100V, 50mA) using PCR products after HRM detection for *NPM1* (Lane 1- Low DNA Mass Ladder (1kb); Lane 2 - Patient 21; Lane 4 – Patient 10; Lane 6 – Patient 16; Lane 7 – Patient 22; Lane 8 – Patient 25)

Figure 21 shows 5 patients negative for the *NPM1* mutation as determined by HRM analysis appearing as a single band on the gel. Patients 21, 10, 16, 22 and 25, all at presentation, were from the non-mutated group determined by HRM analysis (Figs. 18 & 19).

3.5.2 FLT3 Results

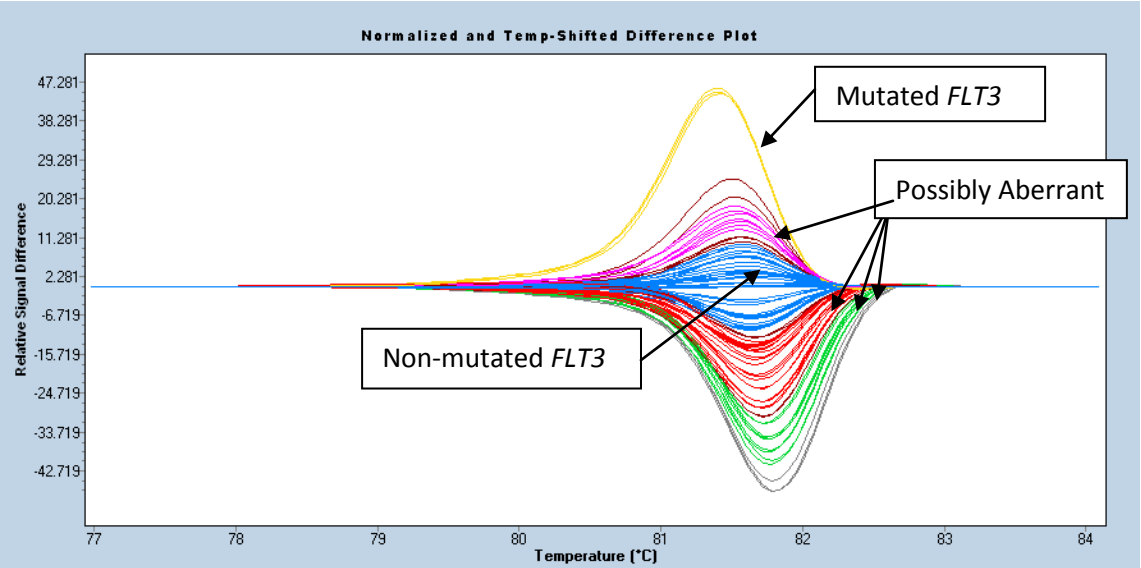

Figure 22: Normalized and Temperature Shifted Difference Plot for Detection of *FLT3* mutations derived from High Resolution Melt Analysis of Patient Samples

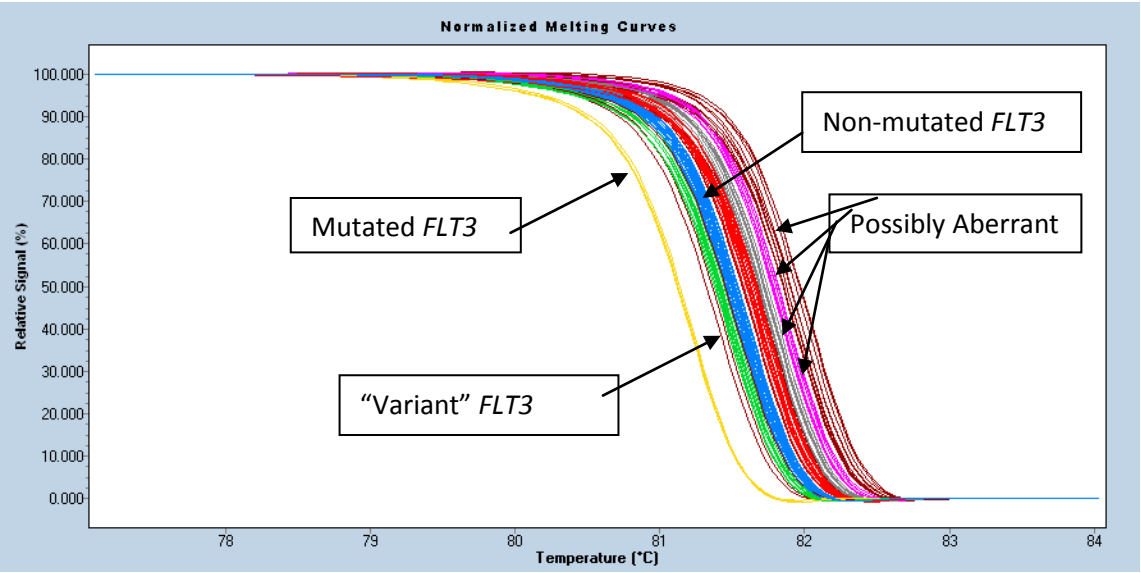

Figure 23: Normalized and Shifted Melting Curves Enabling Detection of *FLT3* mutations derived from High Resolution Melt Analysis of Patient Samples

According to the normalized and temperature shifted difference plot (Fig.22) and normalized melting curve (Fig.23) seen above, gene scanning for the *FLT3* mutation resulted in 7 groups (indicated in various colours). Mutated *FLT3* is indicated in yellow and normal or non-mutated *FLT3* is indicated in blue. However there are patients with possible *FLT3* aberrations indicated in green, red, grey, purple and maroon. There was also a possibly "variant" group which could indicate a novel *FLT3* mutation indicated in brown.

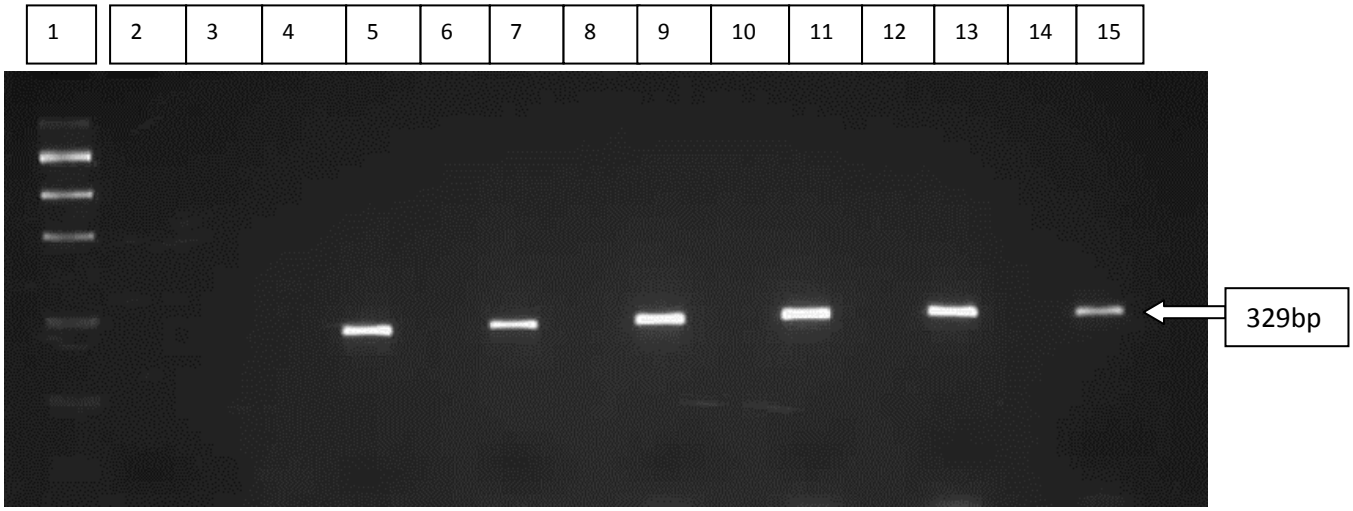

Figure 24: Patients with mutated *FLT3-ITD* and suspected *FLT3* mutations visualized on a 2% agarose gel (100V, 50mA) using PCR products after HRM detection for *FLT3* (Lane 1- Low DNA Mass Ladder (1kb); Lane 2 – Patient 25 PI; Lane 3 – Patient 28; Lane 5 – Patient 1; Lane 7 – Patient 19; Lane 9 – Patient 25; Lane 11 – Patient 4; Lane 13 – Patient 4 PI; Lane 15 – Patient 7)

Figure 24 shows mutated *FLT3* (Patient 25 at post-induction, Patient 28 at presentation, Patient 1 at presentation and Patient 19 at presentation) as determined by HRM analysis. Patient 25 at presentation (Lane 9) had a "variant" mutation detected by HRM. Patients 4 at presentation and post-induction and Patient 7 at presentation have possibly mutated *FLT3*. Patient 25 at post-induction and Patient 28 at presentation, both with mutated *FLT3* had no discernible bands on the gel and appear blank in Lanes 2 and 3.

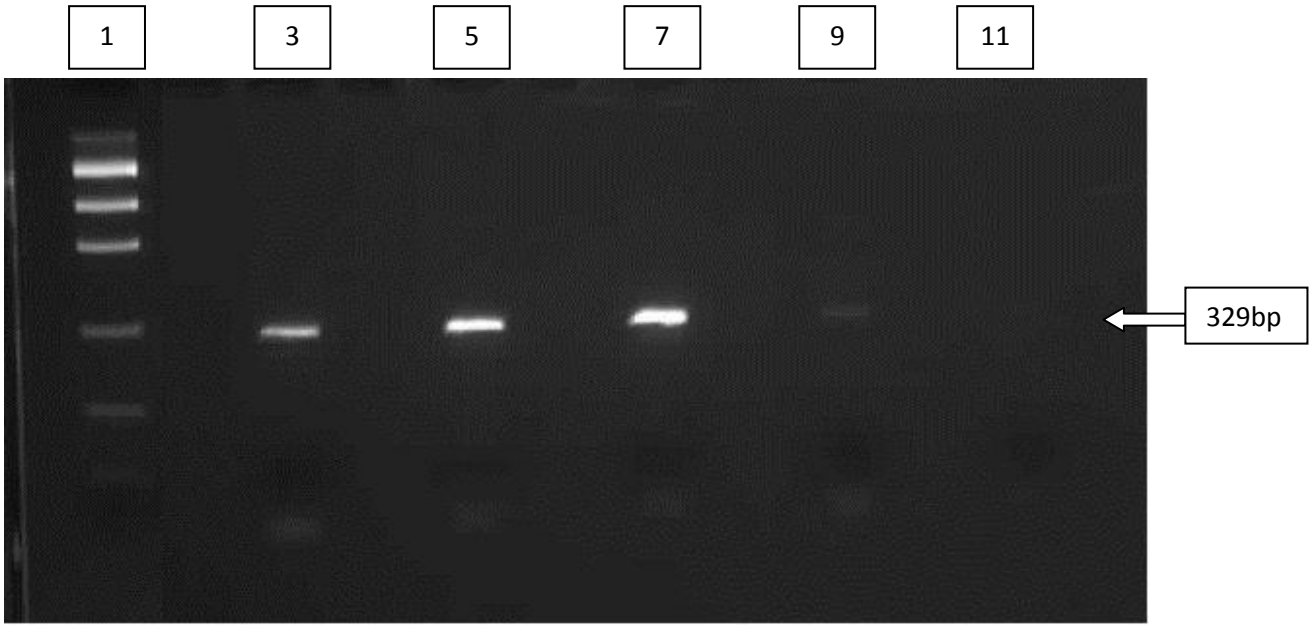

Figure 25: Patients with mutated, possibly mutated and non-mutated *FLT3* visualized on a 2% agarose gel (100V, 50mA) using PCR products after HRM detection for *FLT3* (Lane 1- Low DNA Mass Ladder (1kb); Lane 3 – Patient 26; Lane 5 – Patient 10; Lane 7 – Patient 11; Lane 9 – Patient 25 PI; Lane 11 – Patient 28)

Figure 25 show possibly mutated *FLT3* (Patient 26 at presentation), mutated *FLT3* (Patient 25 at post-induction and Patient 28 at presentation) and non-mutated *FLT3* (Patient 10 at presentation and Patient 11 at presentation). Patient 25 post-induction's sample appeared as a very feint band (Lane 9) and Patient 28 at presentation's sample had no discernible band (Lane 11). All visible bands are single bands and have migrated an equal distance.

Table 16 shows patients that had mutated *FLT3* and *NPM1* at presentation.

Table 16: *FLT3* and *NPM1* Mutations Detected at Presentation

	Presentation		
Patient Number	**HRM - *NPM1***	**HRM - *FLT3***	**Seq - *FLT3***
1	Normal	ABERRANT	ABERRANT
2	Normal	Normal	Normal
3	Normal	Normal	Normal
4	Normal	POSSIBLY ABERRANT	Normal
5	Normal	Normal	Normal
6	ABERRANT	Normal	Normal
7	ABERRANT	POSSIBLY ABERRANT	Normal
8	Normal	Normal	Normal
9	Normal	POSSIBLY ABERRANT	Normal
10	Normal	POSSIBLY ABERRANT	Normal
11	Normal	Normal	Normal
12	Normal	POSSIBLY ABERRANT	Normal
13	Normal	Normal	Normal
14	Inconclusive	Inconclusive	-
15	Inconclusive	Inconclusive	-
16	Normal	POSSIBLY ABERRANT	ABERRANT
17	Normal	Normal	Normal
18	Normal	Normal	Normal
19	Normal	ABERRANT	Normal
20	ABERRANT	Normal	Normal
21	Normal	POSSIBLY ABERRANT	Normal
22	Normal	POSSIBLY ABERRANT	Normal
23	Normal	POSSIBLY ABERRANT	Normal
24	Normal	POSSIBLY ABERRANT	Normal
25	Normal	VARIANT	Normal
26	Normal	POSSIBLY ABERRANT	Normal
27	Normal	POSSIBLY ABERRANT	Normal
28	Normal	ABERRANT	ABERRANT

According to the HRM analysis, at presentation, there were 3 patients positive for the *NPM1* mutation (Patients 6,7 and 20) and 3 patients positive for the *FLT3* mutation (Patients 1, 19 and 28). However,

HRM rendered 1 patient (Patient 25) a "variant" which could indicate a novel *FLT3* mutation and 12 patients "possibly aberrant" for the *FLT3* gene (Patients 4,7,9,10,12,16,21,22,23,24,26 and 27). Patients 14 and 15 were excluded due to degraded DNA samples. (Table 16)

Sequencing conducted on the samples after HRM analysis confirmed that Patients 1, 16 and 28 were positive for the *FLT3* mutation (NM_004119.2). Patient 25 with a suspected "variant" mutation had non-mutated or wild-type *FLT3* as well as Patient 19 which HRM analysis ruled as positive (Table 16).

Table 17 show patients with mutated *FLT3* and *NPM1*after induction therapy.

Table 17: *FLT3* and *NPM1* Mutations Detected at Post-Induction

	Post-Induction		
Patient Number	**HRM - *NPM1***	**HRM - *FLT3***	**Seq - *FLT3***
1			
2			
3			
4	Normal	POSSIBLY ABERRANT	Normal
5			
6			
7	Normal	Normal	Normal
8	Normal	POSSIBLY ABERRANT	Normal
9	Normal	POSSIBLY ABERRANT	Normal
10	Normal	POSSIBLY ABERRANT	Normal
11	Normal	POSSIBLY ABERRANT	ABERRANT
12	Inconclusive	Inconclusive	-
13	Normal	POSSIBLY ABERRANT	ABERRANT
14	Normal	POSSIBLY ABERRANT	Normal
15			
16	Normal	Normal	Normal
17	Normal	Normal	Normal
18	Normal	POSSIBLY ABERRANT	Normal
19			
20			
21	Normal	Normal	Normal
22	Normal	Normal	Normal
23	Normal	POSSIBLY ABERRANT	Normal
24	ABERRANT	Normal	Normal
25	Normal	ABERRANT	ABERRANT
26			
27	Normal	POSSIBLY ABERRANT	Normal
28	ABERRANT	Normal	Normal

According to the HRM analysis at post-induction, there were 2 patients positive for the *NPM1* mutation (Patients 24 and 28) and 1 patient positive for the *FLT3* mutation (Patient 25). However, there are 10 patients that may be "possibly aberrant" for the *FLT3* mutation (Patients 4, 8, 9,10,11,13,14,18,23 and 27). Patient 12 was excluded due to degraded DNA samples. 9 patients (Patients 1, 2,3,5,6, 15, 19, 20 and 26) have no post-induction mutational status due to early demise before post-induction (Table 17).

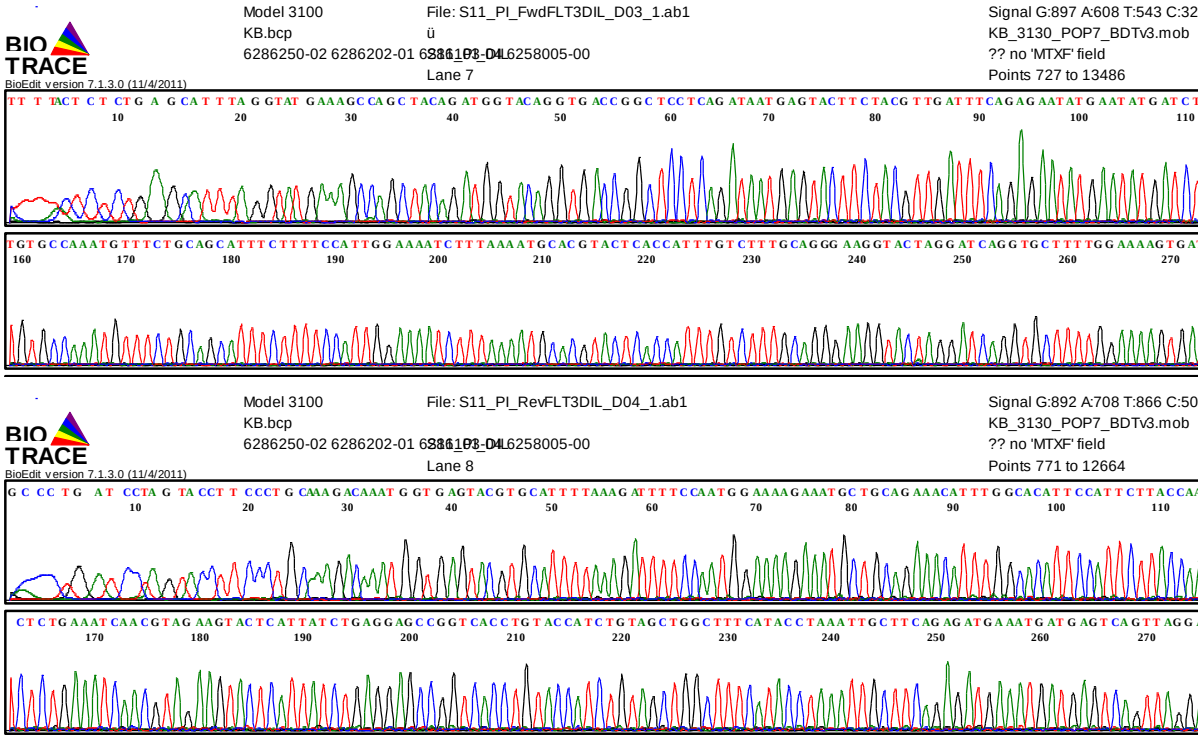

Figure 26: Chromatograms (Forward and Reverse Primers) after sequencing for *FLT3* mutation was performed on Patient Sample 11 Post-Induction before consensus sequences were created for alignments

After sequencing was conducted, there were 3 patients positive for mutated *FLT3* (Patients 11, 13 and 25) and the rest of the patients had non-mutated *FLT3*.

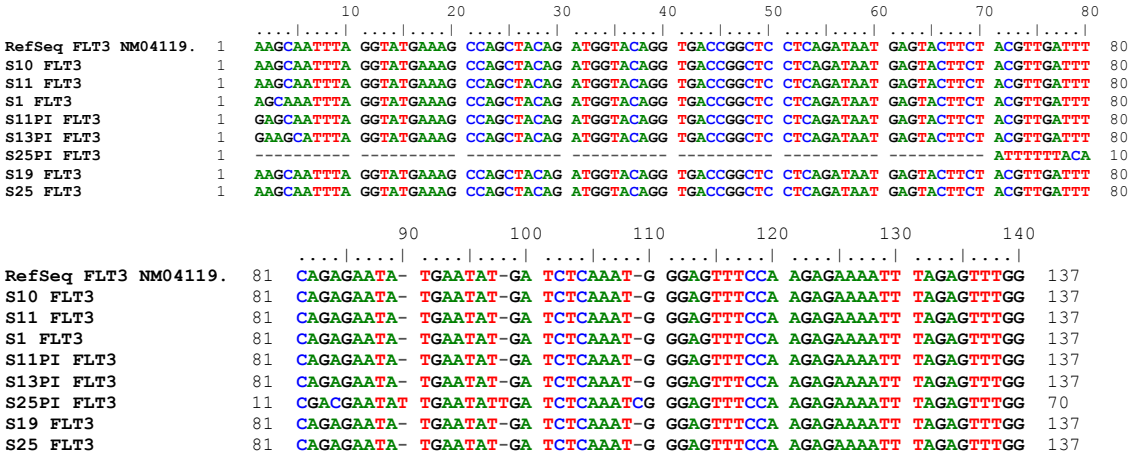

Figure 27: Comparison of the *FLT3* Reference Sequence (NM_004119.2) to non-mutated and mutated DNA sequences from a region of interest after alignment (BioEdit)

The figure above shows the *FLT3* reference sequence compared to 2 non-mutated patient samples (Patients 10 and 11 both at presentation represented by S10 and S11) and their 100% sequence similarity. Patient 1 at presentation and Patients 11, 13 and 25 all at post-induction, show either single nucleotide polymorphisms (S11 PI and S13PI) or regions of altered nucleotides (S1 and S25PI). Patient 19 at presentation (S19) and Patient 25 at presentation (S25) have non-mutated *FLT3* which differs from HRM results discussed previously in this chapter.

Sequencing was conducted on *NPM1* samples to confirm results from HRM analysis using the GenBank Reference Sequence NM_002520.6. Sequencing analysis of PCR products were in accordance with HRM detection. Patients 6, 7 and 20 were positive for mutated *NPM1* at presentation and Patients 24 and 28 were positive for mutated *NPM1* at post-induction.

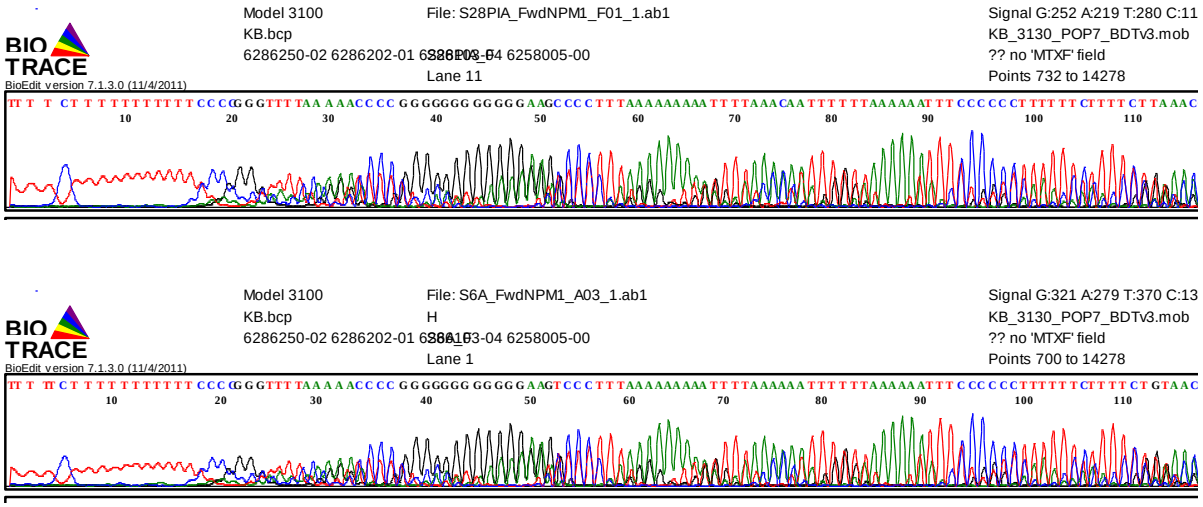

Figure 28: Chromatograms after sequencing for *NPM1* mutation was performed on Patient Samples' 28 Post-Induction and 6 at Presentation before consensus sequences were created for alignments

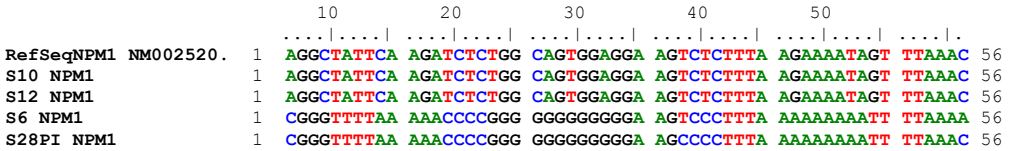

Figure 29: Comparison of *NPM1* Reference Sequence (NM_002520.6) to non-mutated and mutated DNA sequences from a region of interest after alignment (BioEdit)

This figure shows a region of sequence alignment of the *NPM1* reference sequence compared to 2 normal/non-mutated *NPM1* patient samples (Patients 10 and 12 both at presentation) and to 2 mutated *NPM1* patient samples (Patients 6 at presentation and 28 at post-induction). The non-mutated samples have 100% sequence similarity whereas the mutated samples differ as seen above.

3.5.3 Cumulative Results

Table 18 gives an overview of the somatic mutations detected, patient prognostic risk at diagnosis and the outcome post-induction therapy.

Table 18: Cumulative Results of the Impact of *FLT3* and *NPM1* Mutational Status on Response to Therapy and Prognosis

			Presentation		**Post-Induction**	
Patient Number	**Prognostic Risk**	**Outcome after Therapy**	***FLT3* Mutational Status**	***NPM1* Mutational Status**	***FLT3* Mutational Status**	***NPM1* Mutational Status**
1	Intermediate	Deceased	ABERRANT	Normal		
2	Intermediate	Deceased	Normal	Normal		
3	Intermediate	Deceased	Normal	Normal		
4	Intermediate	Alive	Normal	Normal	Normal	Normal
5	Intermediate	Deceased	Normal	Normal		
6	High	Deceased	Normal	ABERRANT		
7	Intermediate	Deceased	Normal	ABERRANT	Normal	Normal
8	High	Deceased	Normal	Normal	Normal	Normal
9	Intermediate	Alive	Normal	Normal	Normal	Normal
10	Intermediate	Alive	Normal	Normal	Normal	Normal
11	Intermediate	Alive	Normal	Normal	ABERRANT	Normal
12	Intermediate	Alive	Normal	Normal	Inconclusive	Inconclusive
13	Low	Alive	Normal	Normal	ABERRANT	Normal
14	Intermediate	Alive	Inconclusive	Inconclusive	Normal	Normal
15	High	Deceased	Inconclusive	Inconclusive		
16	Intermediate	Alive	ABERRANT	Normal	Normal	Normal
17	Intermediate	Unknown	Normal	Normal	Normal	Normal
18	High	Deceased	Normal	Normal	Normal	Normal
19	Intermediate	Deceased	Normal	Normal		
20	Intermediate	Deceased	Normal	ABERRANT		
21	Intermediate	Alive	Normal	Normal	Normal	Normal
22	High	Alive	Normal	Normal	Normal	Normal
23	Intermediate	Deceased	Normal	Normal	Normal	Normal
24	Intermediate	Alive	Normal	Normal	Normal	ABERRANT
25	Low	Deceased	Normal	Normal	ABERRANT	Normal
26	High	Deceased	Normal	Normal		
27	Low	Alive	Normal	Normal	Normal	Normal
28	Low	Alive	ABERRANT	Normal	Normal	ABERRANT

At presentation there were 3 patients positive for mutated *FLT3* – Patient 1 with intermediate prognostic risk, Patient 16 with intermediate risk and Patient 28 with low risk. Patient 1 was deceased before post-induction and Patients 16 and 28 survived therapy. Post-induction, Patient 16 and Patient 28 were negative for mutated *FLT3*. At the stage after post-induction therapy, 3 patients were positive for the mutated *FLT3* – Patient 11 with intermediate risk and Patients 13 and 25 both with low risk. Patient 25 was deceased soon after therapy and Patients 11 and 13 survived therapy.

In terms of the *NPM1* mutation, at presentation there were 3 patients positive for mutated *NPM1* – Patient 6 with high prognostic risk and Patients 7 and 20 with intermediate risk. Patient 6 and 20 did not survive to receive post-induction therapy while Patient 7 survived post-induction therapy and then tested negative for mutated *NPM1*. At post-induction, there were 2 patients positive for mutated *NPM1* – Patient 24 with intermediate prognostic risk who survived post-induction therapy and Patient 28 with low prognostic risk who also survived therapy.

The next chapter will elucidate the meaning of the results stated in this chapter, conclusions derived from the study and recommendations for future work.

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Chapter 4

Discussion and Conclusion

This chapter will discuss the results in this study and provide possible reasons as to their manifestation. The results, the protocols used in achieving them and their relation to current literature will be discussed as well as a reference back to the aims envisaged for this study. This chapter will culminate in conclusions derived from the study and make future recommendations.

4.1 Patient Demographics

Patient demographics are essential to see if the study cohort is representative of the South African or more specifically the population in KwaZulu-Natal. A census conducted by Statistics South Africa in the middle of 2011 provided valuable insights(Lehohla, 2011). There exist 4 racial groups in South Africa – African, those of Indian/Asian descent, Caucasian/White and Coloured/Mixed Race. The racial groups in South Africa are split as follows: 79.5% are African, 9% Coloured, 9% Caucasian and 2.5% Indian. The gender split is as follows: approximately 52% are female and 48% are male. Interestingly, approximately 31.3% of people in SA are younger than 15 years with 7.7% representing those aged 60 years and older (Lehohla, 2011).

Comparatively, the study cohort had a racial split as follows: 78.6% are African, 17.9% are Indian, 3.6% are Caucasian and 0% are Coloured (Table 6). While the African population is adequately represented in the study cohort when compared to census counts, the other population groups are not. However, it is important to note the high incidence of Indian patients with AML. The study cohort had a gender split of 42.9% males and 57.1% females (Table 6) which is in approximate agreement with census figures and literature which states that AML is equally common between the sexes (Braoudaki et al., 2010). In terms of patient age, patients in the cohort were aged between 13-66 years and the median age was 30 years (Table 6). The age group with the most patients was the 16-20 year age group (21.4%). In SA, approximately 10% of the population fulfil this age group so this may be significant. AML has been shown to be equally prevalent in adults as well as children younger than 1 year old however this study could not include paediatric samples as per the constraints in the Ethical Approval (Ref 261/09 – Appendix A). Differences in patient demographics observed in this study compared to South African census counts and literature could be attributed to several factors: the small study cohort has reduced statistical significance, patients from lower income households generally attend government hospitals and KwaZulu-Natal has a large Indian population, with Durban having the largest Indian population outside India, as well as a significantly young population compared to the other 9 provinces in South Africa (Lehohla, 2011). KwaZulu-Natal also has a very high African population compared to the other provinces.

4.2 Patient Clinical Diagnosis and Sub-typing

The diagnosis of AML must be precise as well as the differentiation between primary AML and secondary AML as this affects prognostic stratification and therapeutic decisions respectively. Thus accurate AML diagnosis is heavily dependent on thorough laboratory testing, FAB classification and if possible WHO classification as well as investigating patient prior disease. Although highly recommended, IALCH does not currently prescribe to AML patient classification using the WHO classification and uses the FAB mode of classification instead. WHO classification relies heavily on cytogenetic analysis, which is an area in early development at IALCH whereas flow cytometry and cytochemical stains are used routinely enabling easy FAB classification. In this study, there were 5 patients with prior disease (4 with Blastic CML and 1 with MDS) who transformed into secondary AML (Table 6), the majority of patients however had primary AML (82% of the cohort). The most common FAB subtypes observed in this study were FAB M4 (29%) and FAB M3 (21%) however the literature indicates that FAB M2 is the most common subtype occurring at 25-45% of all AML cases. FAB M4 generally occurs at 15-25% of all cases which is in agreement with findings in this study. The FAB M3 subtype however is fairly uncommon occurring at 5-10% of all AML cases but was observed in 21% of patients in this study (Table 6 & Fig. 14). (Brunning et al., 2001a); (Brunning et al., 2001b); (Lowenberg et al., 1999). 11% of patients in this study had an unknown FAB subtype due to samples not being processed adequately for various reasons or clinicians not requesting sub-typing on samples.

4.3 The impact of HIV on Patients with AML

Recent statistics indicate that in South Africa, there are 5.38 million HIV positive individuals and approximately 16.6% of the adult population aged between 15-49 years are HIV positive (Lehohla, 2011). In this study, there was 17.9% HIV positive patients which is largely in agreement with census figures despite the small study cohort (Table 6). In this study, of the HIV positive patients, there were 40% (2 out of 5) deemed high risk and 60% (3 out of 5) deemed intermediate risk (Tables 6 & 7). The high risk patients did not survive post-induction therapy and 67% (2 out of 3) of the intermediate risk patients survived post-induction (Tables 6 & 7). However the small size of this group cannot allow for definite conclusions to be made with regards to AML patients with HIV and their survival.

Furthermore, the levels of expression of NPM1 and FLT3 and presence of mutated forms were investigated in patients with HIV. Patient 6, who did not survive to post-induction, had the highest FLT3 level of expression but had non-mutated FLT3 yet had approximately twice the average level of NPM1 expression and had mutated NPM1. Patient 12, who survived post-induction therapy, had low FLT3 and NPM1 expression at presentation and normal FLT3 and non-mutated NPM1 respectively. Patient 12 also had low FLT3 and excessively high NPM1 expression at post-induction however the mutational status could not be ascertained using HRM or sequencing due to a degraded DNA sample.

Patient 16, who survived post-induction therapy, had less than average levels of expression for both FLT3 and NPM1 at presentation and low levels of expression for both FLT3 and NPM1 at post-induction. Patient 16 had non-mutated NPM1 at presentation and post-induction and mutated FLT3 at presentation and normal FLT3 at post-induction. Patient 23, who died after post-induction therapy, had low levels of NPM1 expression at presentation and post-induction and had non-mutated NPM1 at both stages. Patient 23 also had low levels of FLT3 expression at both presentation and post induction and non-mutated FLT3 at both stages. Patient 26, who did not survive to post-induction therapy, had a low level of FLT3 expression and non-mutated FLT3 at presentation and the highest NPM1 expression and non-mutated NPM1 at presentation (Tables 6, 7, 10, 16 &17).

The analysis of this group (HIV positive patients with AML) with regard to FLT3 and NPM1 levels of expression and mutational status has resulted in no clear correlation between levels of expression and mutational status. Less than average levels of NPM1 expression can mean mutated NPM1whereas low levels of NPM1 expression can also result in non-mutated NPM1 being detected. Surprisingly, the high levels of NPM1 expression did not necessarily denote a NPM1 mutation (Patient 26). This is true of the FLT3 levels of expression too – low levels of FLT3 expression can indicate non-mutated (Patient 23) or mutated (Patient 16) status. Our results showed that, FLT3 and NPM1 levels of expression followed a similar trend with both levels being low, high or close to the average with the exception of Patient 26, who had low FLT3 expression and very high NPM1 expression.

4.4 White Blood Cell Counts of AML Patients

A healthy person will have a white blood cell count in the range of 4.3-10.8 $\times 10^9$ cells/L. Generally higher WBC counts at diagnosis indicate poor prognosis. Studies by Zhu et al (2010) found that patients presenting with lower WBC counts at presentation correlated with a high rate of complete response. This was in agreement with findings in this study (Tables 7 & 8) where of the 14 patients who were deceased post induction therapy, 12 of those patients had excessively high WBC counts at diagnosis. Also WBC counts were generally higher at presentation compared to post-induction and these differences were found to be statistically significant (Table 9 & Fig. 15).

Previous studies have found that *NPM1* mutations strongly correlate with higher WBC counts (Theide et al., 2006; Boonthimat et al., 2008). In this study, there were 5 patients positive for *NPM1* mutations (Patient 6 at presentation, Patient 7 at presentation, Patient 20 at presentation and Patient 24 and 28 both at post-induction) – 4 of these patients (Patients 6, 7, 20 and 28) presented with very high white blood cell counts (Tables 8 & 16).

In this study, there were 6 patients with mutated *FLT3* (Patients 1, 16 and 28 all at presentation and Patients 11, 13 and 25 all at post-induction) – 3 of these patients had low WBC counts (Patients 16, 13 and 25); 2 had high WBC counts (Patients 1 and 28) and Patient 11 had a normal WBC count.

Literature unequivocally states that mutated *FLT3* leads to constitutively activated FLT3 protein that causes proliferation, inhibits apoptosis and suppresses differentiation of leukemic cells ultimately resulting in higher WBC counts however the findings in this study provide relative neutrality to link between higher WBC counts and mutated *FLT3* (Chauhan et al., 2011; Schnittger et al., 2002).

4.5 Patient Prognostic Risk Stratification

In order to guide therapeutic decisions, it is important to use certain patient demographics, clinical and laboratory examinations and subsequent accurate diagnosis and sub-typing. Generally, patients are stratified into low, intermediate and high risk groups which have influences on treatment type and long term survival. Prognostic factors directed by current literature (Ravandi et al., 2010; Schnittger et al., 2002; Lowenberg et al., 1999) allowed the patients in this study to be stratified into low (14%), intermediate (64%) and high (21%) (Table 7). In terms of survival post-induction therapy, of the low risk patients, there was a 75% survival rate (3 out of 4 patients) and of the high risk patients, there was a 16% survival rate (1 out of 6 patients) hence the prognostic risk stratification is fairly accurate. However, this precision is not true of the intermediate group. The intermediate group, largely due to patients' normal karyotype status, can be challenging in terms of prognostic risk stratification. Of the 18 patients that were grouped in the intermediate risk category, eight died (44%) and nine survived (50%) and the survival of one intermediate patient is currently unknown as the patient defaulted on treatment (Table 7). Hence, the intermediate risk group is in dire need of further refinement in determining patient prognosis.

Consultations with clinicians regarding patient management revealed that the majority of patients' treatment plans are not directed by risk stratification due to either FAB or WHO classification as cytogenetic laboratory investigation would often take too long before patients could be treated. As a result, clinicians would resort to confirming the diagnosis of AML or APML before commencing patient treatment. Patient treatment would then be amended if necessary after receiving cytogenetic and molecular test results. Ultimately, clinicians need reproducible detection tests with a fast turnaround time and prognostic markers of use to effectively direct patient treatment.

Consequently, the primary aim of this study was to find the use of *NPM1* and *FLT3 ITD* gene mutations as prognostic markers. Previous studies have found conflicting evidence regarding the prognostic value of molecular aberrations like *NPM1* and *FLT3 ITD*s. The investigation of these somatic mutations began by looking at the levels of expression followed by the detection of single nucleotide polymorphisms (SNPs) using a novel technique, High Resolution Melting (HRM) analysis, and ultimately confirmed by sequencing. Levels of expression were depicted as a ratio of NPM1 or FLT3 to ABL per ug of cDNA. *ABL* was chosen as a suitable housekeeping gene due to its consistent expression regardless of leukemia or tissue type as well as its high PCR efficiency in quantitative AML analysis (Weisser et al., 2004).

4.6 FLT3 and NPM1 Levels of Expression

Initially FLT3 and NPM1 levels of expression were analysed in all patients in the study at presentation (Table 10). 3 patients were excluded from statistical analysis (Patients 10, 11 and 25) due to their anomalous values. The highest FLT3 expression value at presentation was 1.7159 (Patient 6) who was deceased after induction therapy. The lowest FLT3 expression value at post-induction was 0.0009 (Patient 9) who survived post-induction therapy. The highest NPM1 expression value at presentation was 1.2830 (Patient 26) who was deceased before induction therapy. The lowest NPM1 expression value at presentation was 0.0005 (Patient 9) who survived induction therapy.

FLT3 and NPM1 levels of expression were analysed in all patients at post-induction (Table 10) except those excluded due to anomalous values (Patients 10, 11 and 25). The highest FLT3 expression value at post-induction was 1.6844 (Patient 14) who survived induction therapy. The lowest FLT3 expression value at post-induction was 0.0017 (Patient 9) who survived induction therapy. The highest NPM1 expression value at post-induction was 2.2583 (Patient 14) who survived induction therapy and the lowest NPM1 expression value at post-induction was 0.0013 (Patient 9) who survived after induction therapy.

Patient 9 had the lowest expression values for both FLT3 and NPM1 at presentation and post-induction and survived after induction therapy (Tables 12 & 14). However other factors contributing to this patient's survival could also be normal karyotype and therefore intermediate risk, no prior disease and young age (19 years) so low levels of expression of these potentially oncogenic genes may not necessarily confer a survival advantage however it is worth noting. Similarly, patients 6 and 26 who had the highest FLT3 and NPM1 expression levels at presentation respectively did not survive induction therapy (Tables 7 & 10). Patient 6, although not of advanced age and without prior disease was 11q23 positive and stratified as high risk which could have contributed to demise post-induction (Table 7). Patient 26 was also 11q23 positive, HIV positive and deemed high risk which could have ultimately contributed to early demise (before induction therapy). Although again, the high levels of FLT3 and NPM1 expression are note-worthy.

NPM1 and FLT3 levels of expression for all the patients at presentation and post-induction were compared to the average levels of expression for the entire cohort and subsequently referred to as low, high or approximately average. The average NPM1 expression at presentation and post-induction was 0.2510 and 0.5114 respectively (Table 10). The average FLT3 expression at presentation and post-induction was 0.1625 and 0.3325 respectively (Table 10). Generally, the FLT3 expression and NPM1 expression were either both high or both low either at presentation or post-induction. The exceptions to this trend were Patients 19, 20, 26 all at presentation and Patient 27 at post-induction. Patients 19, 20 and 26, all of which did not survive to post-induction, had low FLT3 expression and high NPM1 expression. Patient 19 had non-mutated FLT3, Patient 20 had mutated NPM1 and Patient 26 had non-

mutated FLT3. Patient 27 at post-induction shows an inverse relationship between levels of FLT3 and NPM1 expression too, but the patient survived post-induction therapy. An inverse relationship between levels of expression between NPM1 and FLT3 may indicate presence of somatic mutations and possibly poor survival.

NPM1 and FLT3 levels of expression and their relation to mutational status were analysed. Patients 6, 7 and 20 at presentation and Patients 24 and 28 at post-induction were all positive for NPM1 mutations (Tables 16 & 17). If the levels of expression are analysed at the time of the mutation occurring (presentation or post-induction), there is no trend – Patients 6 and 20 at presentation have high NPM1 expression and Patient 7 at presentation together with Patients 24 and 28 at post-induction have low levels of NPM1 expression when compared to the average cohort's expression levels yet all of these patients are positive for the NPM1 mutation (Table 10). However, with the exception of Patients 6 and 20 who had no post-induction samples, there was a tentative trend where a change in mutational status from non-mutated to mutated NPM1 would correlate with an increase in individual NPM1 expression and vice-versa. For example, Patients 24 and 28 attain mutated NPM1 at post-induction and their expression levels from presentation to post-induction increase from 0.0837 to 0.0891 and 0.2581 to 0.3441 respectively (Table 10). Another important finding was that high levels of NPM1 expression did not denote mutated NPM1 – Patient 26 had the highest NPM1 expression (1.2830) at presentation and Patient 14 had the highest NPM1 expression (2.2583) at post-induction however they were both negative for NPM1 mutations at presentation and post-induction (Tables 10; 16 & 17).

Patients 1, 16 and 28 at presentation and Patients 11, 13 and 25 at post-induction were all positive for FLT3 mutations (Tables 16 & 17). Similar to the levels of expression for mutated NPM1, the patients with mutated FLT3 did not have levels of expression that would correlate to mutational status. Patient 1 at presentation had a high FLT3 expression, Patients 16 and 28 at presentation and Patient 13 at post-induction had average levels of FLT3 expression. Patient 1 at presentation had no post-induction sample due to early demise and Patients 11 and 25 at post-induction had anomalous expression values and so could not be further analysed for expression trends. When Patient 16 was analysed, it emerged that the expression level from presentation to post-induction decreased (0.1501 to 0.1011) and was accompanied by mutated FLT3 at presentation to non-mutated FLT3 at post-induction. Patients 13 and 28 follow this trend too – a decrease in expression was coupled with an absence of the FLT3 mutation and the converse of this trend was true too, where an increase in expression was coupled with the presence of the FLT3 mutation (Tables 10; 16 & 17).

If a larger cohort of AML patients were available to study, their levels of FLT3 and NPM1 expression at presentation may have correlations to long term survival. However at this stage this expression assay cannot definitively confer mutational status on patient samples.

FLT3 and NPM1 levels of expression were analysed in 16 patients and termed paired samples as these patients contributed samples at both presentation/diagnosis and at post-induction (Tables 12 & 14). 3 paired patient samples (Patients 10, 11 and 25) were excluded from statistical tests as they exhibited anomalous values due to experimental error. Unpaired samples or patients contributing only presentation samples were also excluded from the statistical analysis. FLT3 expression values had greater variance at post-induction than presentation and the differences in expression between presentation and post-induction were statistically significant (Table 13 & Fig.16). NPM1 expression values also had a greater variance at post-induction than presentation and the differences in expression between presentation and post-induction were also statistically significant (Table 15 & Fig.17). The small variances in expression values for FLT3 and NPM1 at presentation could be due to all patients being affected with the onset of AML and subsequent impaired haematopoiesis. However the greater variances in expression values for both FLT3 and NPM1 at post-induction could be attributed to patients being at different stages of convalescence – ranging from complete remission to relapse.

Generally, both FLT3 and NPM1 levels of expression increased from presentation to post-induction however this could not be correlated with survival. Interestingly, an increase in FLT3 expression from presentation to post-induction (Patient 13) and increases in NPM1 expression from presentation to post-induction (Patients 24 and 28) were coupled with the development of the FLT3 and NPM1 mutation respectively. Similarly, the converse was found to be true, a decrease in FLT3 expression (Patient 16) and a decrease in NPM1 expression (Patient 7) were associated with a loss of the respective mutations.

4.7 Detection of NPM1 and FLT3-ITD Mutations using High Resolution Melting Analysis

The HRM analysis, a fairly novel technique, was employed to confer mutational status on patients as a result of the expression assay not being definitive. The advantage of the technique as described by Tan et al (2008) was greater efficiency, reproducibility and sensitivity. HRM has the unique ability to plot differences in fluorescence compared to a wild-type baseline whereby the wild-type adopts a null value therefore allowing mutated samples to form discernible curves. HRM relies on the use of isolated DNA, however Patients 14 and 15's samples and Patient 12's sample did not render usable DNA and so were excluded from analysis at presentation and post-induction respectively.

According to the HRM method conducted on 2 separate occasions and in duplicate, there were 3 patients positive for the *NPM1* mutation at presentation (Patients 6, 7 and 20) and 2 patients positive for the *NPM1* mutation at post-induction (Patients 24 and 28). Hence, HRM analysis enabled clear detection of *NPM1* mutations (Figs 18 & 19; Tables 16 & 17).

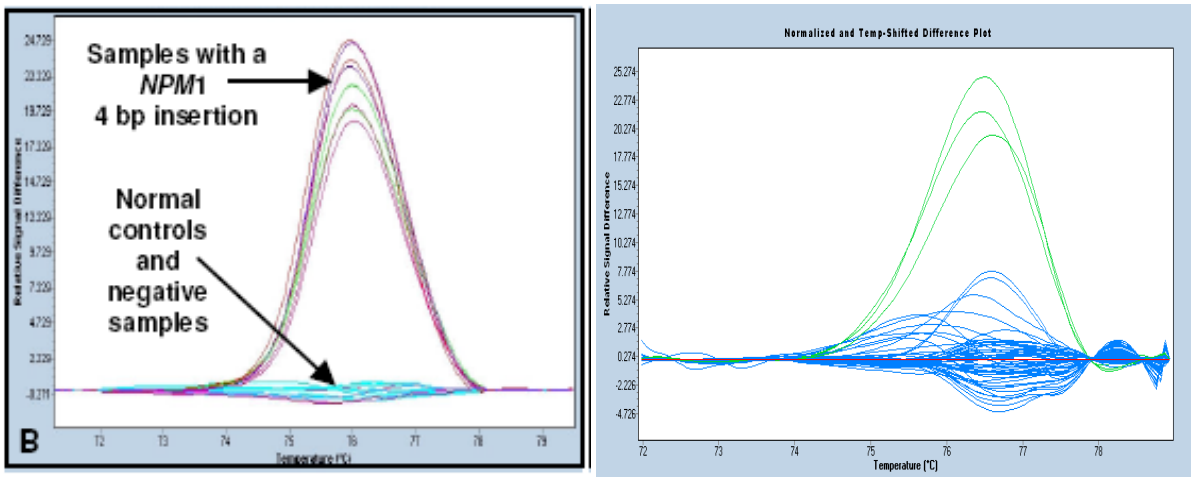

Figure 30: Comparison of normalized and temperature-shifted difference plots from a study by Tan et al (left) and on patients in this study for *NPM1* mutations (right) (Tan et al., 2008)

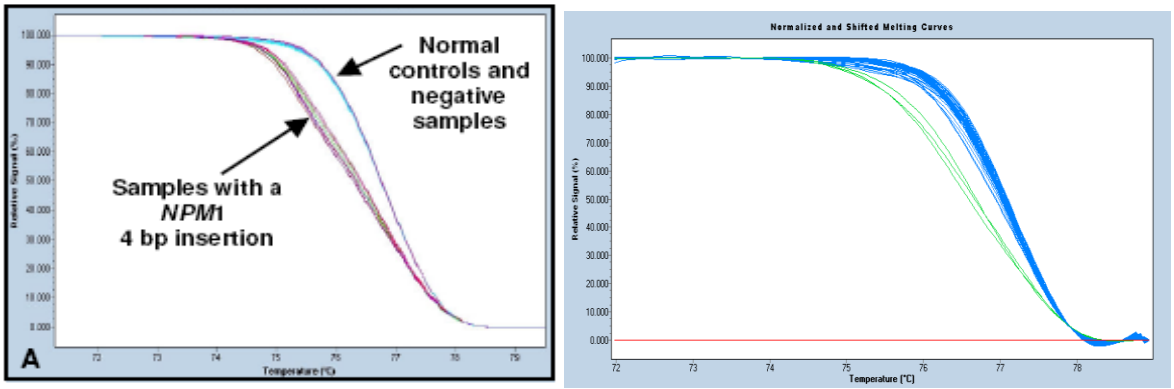

Figure 31: Comparison of normalized and shifted melting curves from a study by Tan et al (left) and on patients in this study for *NPM1* mutations (right) (Tan et al., 2008)

The HRM method of detection and specific primers were adopted from a paper by Tan et al (2008). Hence, the reproducibility of their assay should be reflected in this study's detection of *NPM1*. Figures 30 and 31 show similarity of results obtained in the study to those reported by Tan et al (2008). While the HRM method proved accurate for the detection of *NPM1* mutations, this was not true for the detection of *FLT3* mutations.

According to the HRM method conducted on 2 separate occasions and in duplicate, 3 patients were positive for the *FLT3* mutation at presentation (Patients 1, 19 and 28) (Table 16) . While HRM analysis allowed for detection of some *FLT3* mutations and non-mutated *FLT3*, in some instances a definitive conclusion was too close to judge and as such has been deemed as "Possibly Aberrant" (Figs. 22 & 23; Table 16). At presentation, there were 12 patients that could be "possibly aberrant" for the *FLT3* gene (Patients 4,7,9,10,12,16,21,22,23,24,26 and 27) and 1 patient (Patient 25) deemed a "variant" which could indicate a novel *FLT3* mutation. At post-induction, there was 1 patient positive for the *FLT3* mutation (Patient 25) and 10 patients that may be "possibly aberrant" for the *FLT3* mutation (Patients 4, 8, 9,10,11,13,14,18,23 and 27) (Table 17).

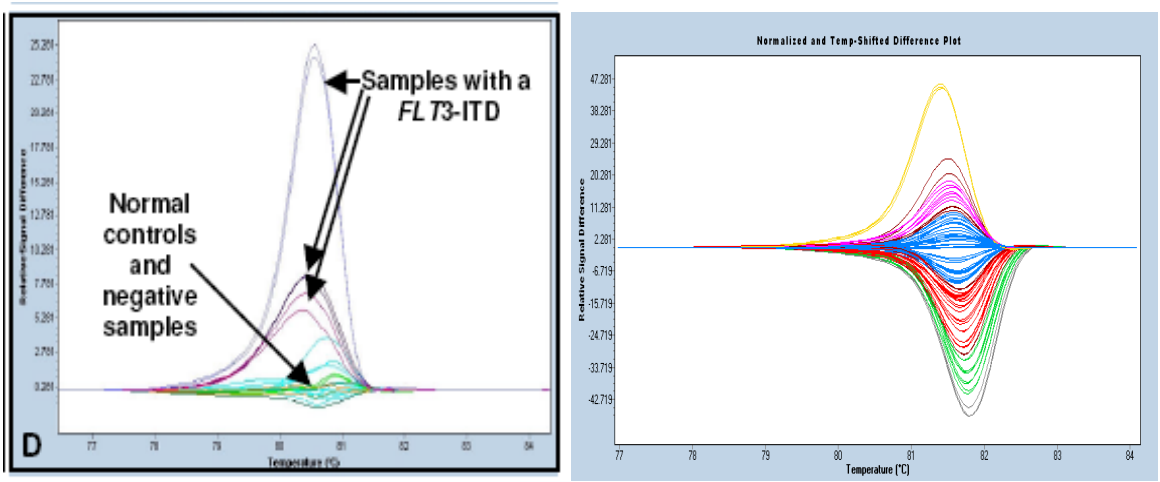

Figure 32: Comparison of normalized and temperature-shifted difference plots from a study by Tan et al (left) and on patients in this study for *FLT3* mutations (right) (Tan et al., 2008)

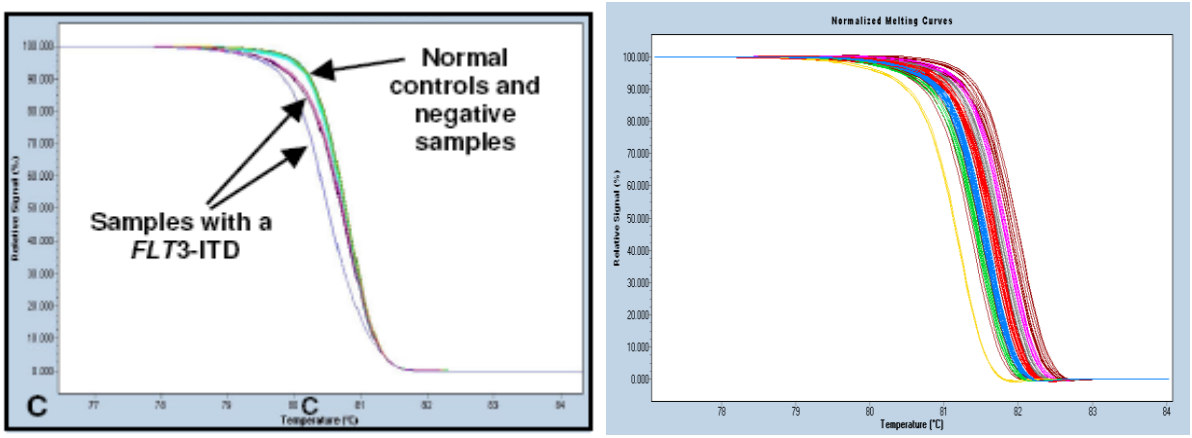

Figure 33: Comparison of normalized and shifted melting curves from a study by Tan et al (left) and on patients in this study for *FLT3* mutations (right) (Tan et al., 2008)

Figures 32 and 33 show marked differences between results obtained in this study compared to the results reported by Tan et al. There is not a clear distinction between non-mutated and mutated *FLT3* samples making detection highly subjective and subsequently prone to error.

4.8 Agarose Gel Electrophoresis of PCR products

The PCR products from the HRM plate were run on a 2% agarose gel and confirmed the presence or absence of the *NPM1* mutation. The PCR product that would be amplified by the *NPM1* primers is 186bp in size. There were 5 patients positive for the *NPM1* mutation which appeared as multiple bands after agarose gel electrophoresis (Fig. 20). These multiple bands are attributed to there being more than 1 peak visualized in Figure 18. Patient 7 at presentation, Patient 28 at post-induction, Patient 6 at presentation and Patient 20 at presentation have 3 bands which could indicate a heterozygous *NPM1* mutation. Patient 20 at presentation (Lane 8) has 3 bands on the gel, the first being feint and the second band being most pronounced which is in contrast to the other samples with

multiple bands. Patient 24 at post-induction however has 2 bands on the agarose gel which could indicate a homozygous *NPM1* mutation. All these samples were grouped as mutated *NPM1* after HRM analysis (Figs.18 & 19). Electrophoresis has shown differences in migration of the mutated *NPM1* samples and the non-mutated *NPM1* samples (Figs 20 & 21). Figure 21 shows 5 patients negative for the *NPM1* mutation as determined by HRM analysis appearing as a single band on the gel. These single bands indicate a homozygous wild-type *NPM1* gene. Patients 21, 10, 16, 22 and 25, all at presentation, were randomly chosen from the non-mutated group determined by HRM analysis.

Amplified DNA, acquired after HRM analysis for the *FLT3* mutation, was visualized using agarose gel electrophoresis. The size of the PCR products that the *FLT3* primers would amplify is 326bp. 2 gels were run comparing mutated *FLT3* with possibly mutated *FLT3* (Fig. 24) and then mutated *FLT3* with possibly mutated *FLT3* and non-mutated *FLT3* (Fig. 25). Figure 24 shows mutated *FLT3* (Patient 25 at post-induction, Patient 28 at presentation, Patient 1 at presentation and Patient 19 at presentation); Patient 25 at presentation (Lane 9) had a "variant" mutation detected by HRM; Patients 4 at presentation and post-induction and Patient 7 at presentation with possibly mutated *FLT3*. Patient 25 at post-induction and Patient 28 at presentation, both with mutated *FLT3* had no discernible bands on the gel and appear blank in Lanes 2 and 3 which could be due to low DNA concentration or experimental error (Fig. 24). All these single bands would indicate mutated *FLT3* however a subsequent gel was run with mutated *FLT3*, possibly mutated *FLT3* and non-mutated *FLT3* samples and they had no appreciable differences in migration. Figure 25 show possibly mutated *FLT3* (Patient 26 at presentation), mutated *FLT3* (Patient 25 at post-induction and Patient 28 at presentation) and non-mutated *FLT3* (Patient 10 at presentation and Patient 11 at presentation). Patient 25 post-induction's sample appeared as a very feint band (Lane 9) and Patient 28 at presentation's sample had no discernible band (Lane 11). Agarose gel electrophoresis for the visualisation of mutated *FLT3* and non-mutated *FLT3* has proven inconclusive.

After numerous *FLT3 ITD* detection experiments using HRM analysis we found that the detection of the *FLT3 ITD* is tricky if the duplication is large as it results in hetero-duplexes that don't have a simple melt. These hetero-duplexes of mutated *FLT3* are indiscernible to wild type *FLT3* and are visualized as highly similar single bands on the gel. Hence, sequencing is imperative to clearly distinguish whether the possibly aberrant group are *FLT3* mutations, a variation on non-mutated *FLT3* or a novel *FLT3* mutation as well as significant differences between mutated and non-mutated *FLT3*. The *NPM1* mutation is simpler and as a result, is perfectly suited to the HRM method of detection.

4.9 Sequencing of PCR products

As previously mentioned in Chapters 3 and 4, sequencing was carried out to confirm mutations detected by the novel HRM technique employed in this study. Sequences were analysed using BioEdit Sequence Alignment Editor (Version 5.0.9) and Clustal W Alignment (Version 1.8) which employed

the use of a Pearson format for alignments. Reference sequences were obtained from Entrez Pubmed and are as follows: NM_004119.2 for *FLT3* and NM_002520.6 for *NPM1*.

4.9.1 Sequencing of NPM1 PCR products

HRM analysis of patient samples for mutated *NPM1* rendered two groups – a non-mutated group and mutated group which were clearly discernible when visualized using difference plots and melting curves (Figs. 18 & 19). Prior to sequencing, these samples were run on agarose gels and confirmed their mutated status by multiple bands when compared to non-mutated samples which were represented by single bands (Figs. 20 & 21). Therefore sequencing, the current gold standard in mutation detection, would confirm the accuracy of the novel HRM technique in detecting HRM mutations. Figure 29 shows the alignment of the GenBank Reference *NPM1* sequence as well as two non-mutated samples (S10 and S12 – Patients 10 and 12 at presentation) and two mutated samples (S6 and S28PI – Patient 6 at presentation and Patient 28 at post-induction). The multiple alignments show approximately 56 nucleotides of each sequence where the non-mutated samples have 100% sequence similarity with the Reference Sequence whilst the mutated samples have regions of SNPs. Sequencing thus confirms the accuracy of the HRM method for *NPM1* detection.

4.9.2 Sequencing of FLT3 PCR products

HRM analysis of patient samples for mutated *FLT3* resulted in 7 groups – 2 of which were a mutated group and non-mutated group with the challenge being 4 groups being "possibly aberrant" and 1 group being a "variant" group (Figs. 22 & 23). These samples were then run on agarose gels in an attempt to visualize some appreciable differences but to no avail. All the samples on the agarose gel, which represented each of the groups created by the LC480 Gene Scanning Software, appeared as single bands that migrated an equal distance across the gel (Figs. 24 & 25). Hence, sequencing became an essential tool to figure out the apparent failings of the HRM technique in detection of *FLT3* mutations. After extensive sequencing and analysis, Patients 1 and 28 at presentation and Patient 25 at post-induction were confirmed as being correctly detected by the HRM technique. However, Patient 19 at presentation was deemed as positive for mutated *FLT3* yet sequencing confirmed it as non-mutated *FLT3* (Fig 28). Similarly aligned sequences visualized in Figure 26 show that Patient 25 at presentation was grouped as having a "possible variant" however sequencing found it to be non-mutated. There were 12 patients grouped as being "possibly aberrant" by the HRM technique yet only 1 was confirmed by sequencing to have mutated *FLT3*. Likewise, there were 10 patients grouped as being "possibly aberrant" at post-induction however sequencing only found 2 of these patients to have mutated *FLT3*. The HRM technique is therefore not suited to the detection of mutated *FLT3* as it can complicate patient management by eliciting false positives and inaccuracies in clearly discerning between affected and normal patients.

4.10 Study Limitations

While this study culminated in some interesting findings pertaining to the technique of HRM in detecting two somatic mutations and a representation of the AML patient population in KZN; there were a few limitations to findings of statistical significance. Firstly, patients were recruited at a government hospital for the predominantly low-income demographic- this resulted in an adequate representation of all population groups in the province and by virtue of that, the AML patient population. The sample size of twenty eight was also a major limitation; the small sample size made it difficult to find trends in levels of expression of the mutations and frequencies of the mutation and their prognostic impact if any. This also prevented any of the findings being statistically significant and any anomalies that were excluded further reduced the cohort size to be analysed. Furthermore, the small number of patients recruited resulted in very few patients having either/both of the somatic mutations making their prognostic significance difficult to assess. The focus of this study was to look at the association of HIV and the presence of somatic mutations found in *FLT3* and *NPM1* however there were only 5 HIV positive patients recruited in this study. The small number of HIV positive patients made it difficult to analyse the association between HIV status and the presence of somatic mutations.

Retrospectively, in terms of the methods employed to assess somatic mutations and their levels of expression in this study; it would have been advantageous to employ a well-established method of genotyping to compare findings obtained from HRM. Furthermore, the use of a positive control for HRM would have contributed to greater clarity in terms of deducing mutational status instead of the normal controls that were employed in this study.

4.11 Conclusions

This research project enabled the elucidation of valuable patient information with regard to patients with AML, prognostic risk stratification; AML associated somatic mutations and their impact if any, as well as their detection using a novel technique - HRM. This project also allowed us to study the impact of HIV on patients with AML; somatic mutation levels of expression as well as the association between WBC counts and somatic mutations.

Our first aim in this project involved the comparison of the frequencies and prognostic impact of mRNA levels of FLT3 and NPM1 in patients with AML in the presence and absence of HIV infection. We grouped our 28 patients into 2 groups – those with AML and HIV positive and those with AML and HIV negative. The HIV positive group consisted of 5 individuals; Patient 6 had high levels of FLT3 and NPM1 expression and died before post-induction therapy however Patient 23 with consistently low levels of FLT3 and NPM1 expression at presentation and post-induction also died. Patient 6, who was a high risk patient due to the 11q23 cytogenetic aberration, also tested positive for

the *NPM1* mutation which is, according to literature, a favourable prognostic factor. Patient 26 with low FLT3 expression and high NPM1 expression also suffered early demise but Patient 12 with similarly low FLT3 and high NPM1 at post-induction survived. In the HIV positive AML group, we found that levels of FLT3 or NPM1 expression were not definitive in conferring prognostic risk. The second group analysed (AML patients that were HIV negative) consisted of 20 individuals with measurable levels of FLT3 and NPM1 expression. Again, we found the levels of expression to be unable to confer prognostic risk which we measured in terms of survival. Patient 4 with consistently low levels of FLT3 and NPM1 expression at both presentation and post-induction survived treatment whereas Patient 7 with consistently low levels of FLT3 and NPM1 expression also at presentation and post-induction died after treatment. We feel these two patients were comparable as they both had secondary AML and were defined as intermediate prognostic risks. Similarly, Patient 8 with consistently low levels of FLT3 and NPM1 expression at presentation and post-induction died after treatment whereas Patient 22 with low levels of FLT3 and NPM1 expression at presentation and post-induction survived therapy. Both these patients were comparable owing to them both being at high prognostic risk. At this stage, levels of FLT3 and NPM1 expression cannot be considered in terms of prognostic stratification risk. Furthermore, we also found there to be no clear correlation between levels of expression and mutational status. For instance, low levels of FLT3 or NPM1 expression could belong to patients with mutated *FLT3* (Patients 1 & 16) or mutated NPM1 (Patient 7, 24 & 28). However we also found that high levels of FLT3 or NPM1 expression could belong to patients with mutated *FLT3* (Patients 13 & 28) or non-mutated *FLT3* (Patients 6, 14, 17, 21 & 27) or to patients with mutated *NPM1* (Patients 6 & 20) or non-mutated *NPM1* (Patients 3, 12, 14, 17, 19, 20, 21 and 26). However, there was a tentative trend where a change in mutational status from non-mutated to mutated *NPM1* or *FLT3* would correlate with an increase in individual NPM1 or FLT3 expression respectively and vice-versa. According to our results, FLT3 and NPM1 levels of expression followed a similar trend with both levels being low, high or close to the average with the exception of Patients 3, 12, 13, 19, 20, 26 and 27 who had levels of FLT3 and NPM1 expression not in concordance with each other. It is interesting that, of these exceptions, Patients 3, 19, 20 and 26 did not survive with Patients 12, 13 and 27 surviving due to having prognostically low risk leukemia. If a larger cohort of AML patients were available to study, their levels of FLT3 and NPM1 expression at presentation may have correlations to long term survival however at this stage this expression assay cannot definitively confer mutational status on patient samples.

Our next aim involved the utilisation of a novel technique, HRM for the detection of mutations in *NPM1* and *FLT3* for use in the future as an efficient, reproducible yet cost-effective routine diagnostic test. Firstly, there were 5 patients positive for the *NPM1* mutation (Patients 6, 7 & 20 at presentation and Patients 24 & 28 at post-induction); 3 of which did not survive therapy (Patients 6, 7 & 20). Patient 6 was HIV positive and at high prognostic risk and Patient 7 had prior disease; these reasons

could have contributed to early demise. Current literature indicates that the *NPM1* mutation confers a survival advantage and better response to therapy however the majority of patients with mutated *NPM1* did not survive (Nafea et al., 2011, Falini et al., 2005). However the small cohort size is a limiting factor in assigning prognostic value of *NPM1*.The HRM method of detection for mutated *NPM1* was confirmed by agarose gel electrophoresis and ultimately sequencing. While HRM analysis enabled reproducible mutated *NPM1* testing, the HRM technique was ill-suited to the detection of mutated *FLT3*. There were 6 patients positive for mutated *FLT3* (Patients 1, 16 and 28 at presentation and Patients 11, 13 and 25 at post-induction); 4 of which survived (Patients 11, 13, 16 & 28). These patients were with at low or intermediate prognostic risk which could account for their high survival rate. Definitive *FLT3* mutational status was directly derived from sequencing as HRM detection of mutated *FLT3* proved to be highly inaccurate. Patients 1, 19, 25 and 28 all at presentation and Patient 25 at post-induction were assigned as having mutated *FLT3* however after sequencing, it was found that Patients 19 and 25 at presentation had non-mutated *FLT3* – a false positive. In addition, at presentation, HRM analysis resulted several patients (Patients 4, 7, 9, 10, 12, 16, 21, 22, 23, 24, 26 and 27) and at post-induction a number of patients (Patients 4, 8, 9, 10, 11, 13, 14, 23 and 27) as either normal or mutated. The lack of clarity as to their status prompted them to be deemed "Possibly Aberrant". Sequencing allowed them to be defined as either mutant or non-mutant – only Patient 16 at presentation and Patients 11 and 13 at post-induction were confirmed as aberrant. The rest of the "Possibly Aberrant" group were all non-mutated or normal *FLT3*. All the normal or wild type *FLT3* designated by HRM were confirmed by subsequent sequencing. Retrospectively, the HRM method is ill-suited to the routine detection of *FLT3* mutations especially if there are clinical ramifications. *FLT3-ITD* mutations especially are regarded as a prognostic factor that confers resistance to treatment and poor overall survival and some clinicians may consider their presence reason for more intense treatment which can cause toxic-related mortality. Hence a false positive could be potentially detrimental to individual patient survival.

Our third and final aim involved the assessment of AML patients with and without HIV to induction therapy. In terms of HIV positive patients, with AML, we found that 3 out of the 5 patients in this study died however their prognostic risk may have been a contributory factor. We found that the small size of this group cannot allow for definite conclusions to be made with regards to AML patients with HIV and their survival after induction therapy. There were 23 HIV negative patients with AML, 11 of which survived induction therapy, 11 were deceased at the end of the study and 1 of the patient's survival was unknown. According to our results, HIV negative patients had an approximately 50% chance of favourable response to therapy.

Research conducted by Ravandi et al., and Bacher et al., are unconvinced by the efficacy of *NPM1* and *FLT3* as prognostic markers which this study, by virtue of its results, is largely in agreement.

In the course of our research, we found an association between high WBC counts at diagnosis and poor overall survival as well as an association between high WBC counts and mutated *NPM1* however we did not find a similar association between mutated *FLT3* and WBC counts despite literature to the contrary (Chauhan et al., 2011).

4.12 Recommendations for Future Investigations and Patient Management:

We recommend the following: an increase in the size of the sample size to enable trends with regard to the prognostic impact, if any, of mutated *NPM1* and *FLT3* on AML patients as well as the effect on survival of HIV positive patients with AML; WHO classification to be employed at IALCH to direct patient management as well as increased use of cytogenetics as a prognostic directive; the use of the HRM technique for the routine detection of mutated *NPM1* and direct sequencing or GeneScan for the detection of the *FLT3-ITD* mutation together with a positive control of which funding for this study did not allow.

APPENDIX A

UNIVERSITY OF
KWAZULU-NATAL

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

07 September 2010

Ms Horacia Naidoo
65 Idlewild, 92 Goble Road
Morningside
Durban
4001

Dear Ms Naidoo

PROTOCOL: The study of the impact of selected mutations- JAK2, NPMI and FLT3 and HIV status on patients with acute myeloid leukemia and their response to induction therapy. REF: BF261/09.

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application.

The study was provisionally approved by a quorate meeting of BREC on **08 December 2009** pending appropriate responses to queries raised. Your responses dated 16 August 2010 to queries raised on 13 May 2010 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 07 September 2010.

This approval is valid for one year from **07 September 2010.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC

Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/ResearchEthics11415.aspx.
BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The following Committee members were present at the meeting that took place on 08 December 2009:

Professor V Rambiritch	Pharmacology (Deputy Chair)
Ms J Hadingham	External
Dr T Hardcastle	Surgery - Trauma
Mr R Moore	IPO - Research Office
Prof Puckree	Physiotherapy
Prof Pudifin	Medicine
Dr J Singh	CAPRISA
Ms T Makhanya	External
Ms T Esterhuizen	Faculty of Medicine
Prof R Bhimma	Paediatrics and Child Health
Dr M A Sathar	Medicine
Professor S Collings	Psychology
Professor Madiba	General Medicine
Dr Z Khumalo	KZN Health (External)
Dr R Govender	Family Medicine

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

PROFESSOR D R WASSENAAR
Chair: Biomedical Research Ethics Committee

DEPARTMENT OF HEALTH

PROVINCE OF KWAZULU-NATAL

INKOSI ALBERT LUTHULI CENTRAL HOSPITAL
OFFICE OF THE MEDICAL MANAGER
800 Bellair Road, Mayville, 4058
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Tel: 031 240 1059 Fax. 031 240 1050
Email: Ursulanun@ialch.co.za

5 August 2010

Ms H Naidoo
Dept of Microbiology
IALCH

Dear Ms Naidoo

Re: Ref No: BF 261/09: The study of the impact of selected mutations – JAK2, NPMI and FLT3 and HIV status on patients with acute myeloid leukemia and their response to induction therapy.

As per the policy of the Provincial Health Research Committee (PHRC), you are hereby granted permission to conduct the above mentioned research once all relevant documentation has been submitted to PHRC inclusive of Full Ethical Approval.

Kindly note the following.

1. The research should adhere to all policies, procedures, protocols and guidelines of the KwaZulu-Natal Department of Health.
2. Research will only commence once the PHRC has granted approval to the researcher.
3. The researcher must ensure that the Medical Manager is informed before the commencement of the research by means of the approval letter by the chairperson of the PHRC.
4. The Medical Manager expects to be provided feedback on the findings of the research.
5. Kindly submit your research to:

   The Secretariat
   Health Research & Knowledge Management
   330 Langaliballe Street, Pietermaritzburg, 3200
   Private Bag X9501, Pietermaritzburg , 3201
   Tel: 033395-3123, Fax 033394-3782

Yours faithfully

.....

Dr M E L Joshua
Medical Manager

Informed Consent

Consent Document

Consent to Participate in Research:

Good Day Madam/Sir

Dr V.L Naicker (Haematology, IALCH), Prof E. Kormuth (Genetics, UKZN) and Miss H.Naidoo will be the researchers on this study. Miss H.Naidoo will be using this research project to obtain her Master's Degree.

This study will compare the molecular changes in patients with AML who are HIV positive and in patients with AML who are HIV negative. Their response to treatment will also be compared.

You have been asked to participate in this research study by allowing us access to your residual blood and bone marrow samples to run molecular tests on and allow us access to your medical history so that we may monitor your response to treatment. Your samples will be stored securely in the Haematology Laboratory with access only to the investigators for a period of 1 year. The samples will be refrigerated at -20C in a secure laboratory free from contamination. At the end of the study, all samples will be destroyed. Patient samples will not be used for other studies unless new ethical approval is applied for and obtained.

Your HIV status will be made available only to the researchers and will be kept confidential.

Your personal and medical details will be kept confidential and you will remain anonymous in the study. You will not be at any risk by being part of this study and will not need to pay any money for the additional laboratory tests.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop at any time. If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

You have been informed about the study by.....

You may contact Horacia Naidoo at 031 240 2742 or Dr V.L Naicker at 031 240 2739 at any time if you have questions about the research. You may contact the Biomedical Research Ethics Office on 031 260 4769 or 260 1074 if you have any questions about your rights as a research participant.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate. I have been given an opportunity to ask any questions that I might have about participation in the study.

Please tick if you agree to allow us to run more tests on your residual blood samples _____

Please tick if you agree to allow us access to your medical files _____

.....	.....
Signature of Participant	Date
.....	.....
Signature of Translator (if applicable)	Date
.....	.....
Signature of Witness	Date

Imvume Yokuzimbandakanya ku Cwaningo

Dokotela V.L Naicker (Haematology, IALCH), Professor E Kormuth (Genetics, UKZN) kanye no Nkosazana H Naidoo bazabekubenza ucwaningo ukulungiselela iziqu zakhe ze Masters.

Lolucwaningo luzobe luqhathanisa molecular ushintsho kwiziguli ezine AML abane sandulela gciwane le ngculazi kanye nabane AML abangenaso usandulela ngculazi. Kuzo bhekisiswa ukuthi ikhambi libasiza kangakanani.

Ucelwe ukuthi uzimbandakanye kulolueweiningo ukusigungaza ukuthola igazi kanye nomukantsha wokuhlolwa (Molecular), kanye nomlando wezokwe lapha, khona isizobona ukuthi likuphatha kanjani ikhambi. Amasampula azogcinwa endaweni evikelekile eHaematology Laboratory. Lapho kuzobe kuvunyelwe abacwaningi kuphela uicungena khona, lizogeinwa isikhathi esingango nyaka cwodwa. Amasampula azogeinwa kwisiqandisi esingu-20°C endaweni evikelekile engenawu amageiwane. Ekugeineni kwalolucweiningo wonke amasampula azolahlwa. Amasampula eziguli awazuku phinda asetshenziswe futhi ngaphandle uma kutholakale elinge igunya.

Imininingwane yakho ngeke idalulwe kucwaningo ngeke ube sengcupheni ngokuba yingxenye yalolu kwaningo, kanti futhi awuzolindeleka ukuthi ukhokhe imali yezindleko zase laboratory.

Ukuzimbandakanya kulolucwaningo akuphoqelekile, kanti awuzojeziswa kumbe uluze uma ungathandi okanye ushiye phakathi nocwaningo. Uma uvumelana kumbe uhambisana nokuzi mbandakanya nalolucwaningo uzonikezwa ikhaphi esayiniwe mayelana nalolucwaningo, kanye ne phepha elikuchazela kabanzi njengoba uqoke ukuzi mbandakanya kulolucwaningo.

Usucaciselekile ngalolucwaningo…….

Ungaxhumana no Horacia Naidoo ku 031-240 2742 kumbe u Dokotela V.L Naicker ku 031-240 2739 noma yingasiphi isikhathi uma unemibuzo ngalolucwaningo. Ungaphinda futhi uthintane ne Biomedical Research Ethics Office kulenombolo 031-260 4769/1074. Uma unemibuzo mayelana namalungelo akho njengokuqoka kwakho ukuzimbandakanya kulolucwaningo ungasebenzisa zona lezizinombolo.

Ulwazi ngalolucwaningo ngizhaziswele lona ngomlomo. Ngiyaqanda ukuzimbandakanya kwami kulona bekungeyona impoqo. Nginikeziwe ithuba lokubuza imibuzo noma iyiphi ebenginayo.

Thikha uma uvumelana nokuthi sithathe isampula legazi lakho….....

Thikha uma uvumelana nokuthi sithole amafayela amayelana nempilo yakho……

……	……
Sayina – Patient	Usuku
……	…….
Sayina – Translator/Witness	Usuku

APPENDIX B

PRIMER SEQUENCES

Name of Gene	Strand	Sequence
ABL	Forward	5’- CCTTCAGCGGCCAGTAGC-3’
	Reverse	5’-GGACACAGGCCCATGGTAC -3’
FLT3	Forward	5’-TGCAGAACTGCCTATTCCTAACTGA -3’
	Reverse	5’- TTCCATAAGCTGTTGCGTTCATCAC-3’
NPM1	Forward	5’-TGATGTCTATGAAGTGTTGTGGTTCC -3’
	Reverse	5’-CTCTGCATTATAAAAAGGACAGCCAG -3’

APPENDIX C

REAGENT PREPARATION

1×TBE Buffer

To make up one liter of 1×TBE Buffer, add 900ml of autoclaved water to 100ml of 5×TBE Buffer.

Gel Red 5% Agarose Gel

To make up a large agarose gel, add 3g of Metaphor Agarose powder to 150ml of cold 1× TBE Buffer and mix completely. Refrigerate for 15 minutes. Microwave until solution is completely clear (agarose is dissolved). After cooling to 40 ˚C, add 15ul of GEL RED and swirl. Cast gel immediately in casting tray and refrigerate until use.

2% Agarose Gel

To make up a large 2% agarose gel, 6 Agarose Tablets are added to 150ml of 1×TBE Buffer and left to stand for 4-6 minutes. Microwave until solution is completely clear (agarose is dissolved). After cooling to 40 ˚C, cast gel immediately in casting tray and refrigerate until use.

75% Ethanol

To make up 50ml of 75% of Ethanol, add 12.5ml of autoclaved water to 37.5ml of absolute ethanol. Refrigerate at 4˚C.

0.1M Sodium Citrate in 10% Ethanol

To make up 0.1M Sodium Citrate Dihydrate, add 2.941g to 90ml of autoclaved water and 10ml of 100% ethanol.

RNase A (10mg/ml)

1. Sodium Acetate 0.01M
2. dH_20 15ml

0.027g of sodium acetate was dissolved in 15ml of distilled water, the pH adjusted to 5.2 and the volume made up to 20ml with distilled water. 20mg of RNase A (Boehringer Mannheim) was dissolved in 1ml of sodium acetate. The enzyme preparation was heated to 100˚C for 15 minutes and allowed to cool slowly to room temperature. It was then stored at -20˚C until further use.

Gel Loading Buffer

3. Bromophenol Blue (Merck) – 0.25%
4. Sucrose (NT Laboratory Supplies) in water – 40% (w/v)

4g of Sucrose and 0.025g of bromophenol blue was added to 10ml of distilled water. The loading buffer was aliquoted and stored at -20˚C.

DEPC Treated Water

0.1ml of DEPC was added to 100ml of solution to be treated and shaken vigorously. The solution was incubated for 12 hours at 37˚C. The solution was the autoclaved for 15 minutes to remove any trace of DEPC.

APPENDIX D

PATIENT HISTORY

Patient Name	
Sample Name	
Hospital No	
Age	
Sex	
Race	
HIV Status	
Clinical Diagnosis	
White Cell Count @ Presentation	
Cytogenetics	
Informed Consent	
mRNA expression levels (presentation/relapse)	
FLT3	
NPM1	
HRM Results	
FLT3	
NPM1	
mRNA expression levels (post induction)	
FLT3	
NPM1	
HRM Results	
FLT3	
NPM1	

LAB BOOK CHECKLIST FOR DNA AND RNA ISOLATION

Date		
KZ No		
Sample No		
DNA Isolation Numbers:	**Concentration (ng/uL)**	**Purity (260/280)**
-		
-		
-		
RNA Lysis		
Total amount of PBC		
1 step EL Buffer added (5x)		
Trizol (factor of 1.2ml)		
RNA Isolation Numbers:	**Concentration (ng/uL)**	**Purity (260/280)**
-		
-		
-		
Storage Samples (-80C):		
-		
-		
-		
-		
Additional Notes:		

PCR Protocol Sheet for RNA conversion to cDNA

Sample/Purity : Concentration (ng/ul) ÷ 1000 (ug/ul) × _____ (uL) ≥ 2ug/ul

Sample	**Purity**	**Concentration**	**Calculation**	**Amount of RNA added (ul)**

Serial dilutions for highly concentrated samples (≥2000ng/ul) - 1:10; 1:100

Sample & Dilution	**Purity of Dilution (260/280)**	**Concentration of Dilution (ng/ul)**	**Calculation**	**Amount of diluted RNA added (ul)**
cDNA Sample: RNA Isolate + DEPC water + 2ul Random Hexamer = 11ul				

cDNA Sample Name	**Amount of RNA Isolate (ul)**	**Amount of DEPC water (ul)**	**Random Hexamer (2ul)**	**Total (ul)**

PCR Renature Conditions	**PCR Conditions Post Addition of Mastermix (9ul per sample)**

EAC Mastermix	**× 1**	**× ____**
5X FS Buffer	4	
$MgCl_2$ (5mM)	1	
dNTP	1	
DTT	2	
Rnase Inhibitor	0.5	
Superscript III	0.5	
	9	

Expression Analysis Protocol Sheet

Mastermix for FLT3 or ABL (10pmol)	× 1	× ___	
Water	3		
Forward Strand Primer - 10pmol	0.5		
Reverse Strand Primer - 10pmol	0.5		
SyBr Green Dye	5		
	9		1ul of 10ng/ul DNA in each well
			Negative Controls

PCR Conditions

95 °C, 5 min, 4.4 °C/s
95 °C, 15s, 4.4 °C/s; 60 °C, 15s, 2.2 °C/s; 72 °C, 15s, 4.4 °C/s
95 °C, 5s, 4.4 °C/s; 65 °C, 1 min, 2.2 °C/s; 97 °C, -, 0.11 °C/s
40 °C, 30s, 2.2 °C/s

Mastermix for NPM1 (6pmol)	× 1	× ___	
Water	3.4		
Forward Strand Primer - 10pmol	0.3		
Reverse Strand Primer - 10pmol	0.3		
SyBr Green Dye	5		
	9		1ul of 10ng/ul DNA to each well
			Negative Controls

PCR Conditions

95 °C, 5 min, 4.4 °C/s
95 °C, 15s, 4.4 °C/s; 60 °C, 10s, 2.2 °C/s; 72 °C, 10s, 4.4 °C/s
95 °C, 5s, 4.4 °C/s; 65 °C, 1 min, 2.2 °C/s; 97 °C, -, 0.11 °C/s
40 °C, 30s, 2.2 °C/s

	Serial Number	Manufacturer
Lightcycler 480		

High Resolution Melting Analysis Protocol Sheet

Mastermix for FLT3 (3mM $MgCl_2$)	**× 1**	× ____	
Water	1.8		
$MgCl_2$ (25mM)	1.2		
Forward Strand Primer - 10pmol	0.5		
Reverse Strand Primer - 10pmol	0.5		
SyBr Green Dye	5		
	9		1ul of 10ng/ul DNA to each well
			Negative Controls

Mastermix for NPM1 (4mM $MgCl_2$)	**× 1**	× _____	
Water	1.4		
$MgCl_2$ (25mM)	1.6		
Forward Strand Primer - 10pmol	0.5		
Reverse Strand Primer - 10pmol	0.5		
SyBr Green Dye	5		
	9		1ul of 10ng/ul DNA to each well
			Negative Controls

PCR Conditions

95 °C, 5 min, 4.4 °C/s
95 °C, 10 s, 4.4 °C/s; 65 °C, 10s, 2.2 °C/s; 72 °C, 10s, 4.4 °C/s
95 °C, 1 min, 4.4 °C/s; 40 °C, 1 min, 2.2 °C/s; 65 °C, 1s, 4.4 °C/s; 95 °C, - , 0.02 °C/s
40 °C, 30 s, 2.2 °C/s

	Serial Number	Manufacturer
Lightcycler 480		

Sequencing of PCR Products

	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												
	RUN 1		RUN 2		RUN 3		RUN 4		RUN 5		RUN 6	

Date of Run	
Name of Plate	

Seq MM	×1	
Terminator Rxn Mix	0.4	
5x Sequencing Buffer	2	
Primer (1.6pmol)	2	
Deionised Water	0.6-4.6	
TOTAL VOL	9ul	

Trough Mix	×1	
3M NaOAc pH 5.2	1	
100% Ethanol	25	
TOTAL VOL	26	

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