Distribution of *PML-RARA* isoforms in Acute Promyelocytic Leukemia Patients from a tertiary hospital in KZN, South Africa using qPCR

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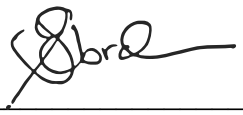
Declaration

Thesis submitted in fulfilment of the requirements for the degree of Masters in Medical Science in the Department of Haematology. This study represents original work by the author and has not been submitted in any form to another University. The work of other investigators cited in this thesis has been duly acknowledged.



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Mrs. S. Ebrahim Dr. M. Gordon

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Dedication

To my family and friends who have motivated and supported me through the years – Thank you. To my husband Farhad and my sons M.Suhail and Ahmad, this would not have been possible without your love, support and encouragement. Thank you for your patience and understanding. Most of all my biggest thank you is to God who makes everything possible.

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List of abbreviations

ABL	**:**	**Abelson murine leukemia viral oncogene homolog**
ALL	**:**	**Acute Lymphoblastic Leukemia**
AML	**:**	**Acute Myeloid Leukemia**
AMML	**:**	**Acute Monocytic Myeloid Leukemia**
ATO	**:**	**Arsenic trioxide**
APL	**:**	**Acute promyelocytic leukemia**
aPTT	**:**	**Activated partial thromboplastin time**
ATRA	**:**	**All trans- retenoic acid**
bcr	**:**	**Break point cluster region**
BREC	**:**	**Biomedical Research Ethics**
c DNA	**:**	**Complimentary DNA**
CML	**:**	**Chronic Myeloid Leukemia**
CNS	**:**	**Central nervous system**
CR	**:**	**complete remission**
CT	**:**	**chemotherapy**
del	**:**	**deletion**
DEPC	**:**	**Diethyl pyrocarbonate**
DFS	**:**	**Disease free survival**
DIC	**:**	**Disseminated intravascular coagulation**
DNA	**:**	**Deoxyribonucleic acid**
dNTP	**:**	**Deoxy nucleotide Triphosphate**
DTT	**:**	**Dithiotheitol**
EAC	**:**	**Europe against Cancer**
EDTA	**:**	**Ethylene Diamine Tetra Acetic acid**
EL	**:**	**Erythrocyte lysis buffer**
ELN	**:**	**European Leukemia Network**
FAB	**:**	**French-American-British system**
FDP	**:**	**Fibrinogen degradation product (D-dimers)**
Fib	**:**	**Fibrinogen**
FISH	**:**	**Florescent in situ hybridization**
FLT3	**:**	**fms-related tyrosine kinase 3**
FLAG-IDA	**:**	**fludarabine, cytarabine, idarubicin**
HAT	**:**	**Histone acetyltransferase**
Haem lab	**:**	**Hematology Laboratory**
Hb	**:**	**Hemoglobin**
HDAC	**:**	**Histone deacetylase**
HIV	**:**	**Human Immune Deficiency Virus**
Hz	**:**	**Hertz**
IALCH	**:**	**Inkosi Albert Luthuli Central Hospital**
INR	**:**	**International normalized ratio**
ISCN	**:**	**International System for Human Cytogenetic Nomenclature**
ITD	**:**	**Internal tandem duplication**
IVI	**:**	**Intravenous injection**
KZN	**:**	**Kwa-Zulu Natal**
LC 2.0	**:**	**Light cycler 2.0**
M3	**:**	**morphological classification according to FAB system**
M3v	**:**	**M3 variant**

mg/m^2	**:**	**Milligrams per meter square**
$MgCl_2$	**:**	**Magnesium Chloride**
min	**:**	**Minutes**
ml	**:**	**Millilitre**
ml	**:**	**Microlitre**
mm	**:**	**Mastermix**
MPO	**:**	**Myeloperoxidase**
MoAb	**:**	**monoclonal antibody**
MRD	**:**	**Minimal residual disease**
mRNA	**:**	**messenger ribonucleic acid**
N/A	**:**	**not applicable**
NB	**:**	**Nuclear bodies**
NHLS	**:**	**National Health Laboratory Services**
No	**:**	**Number**
NPM	**:**	**Nucleophosmin**
NuMA	**:**	**Nuclear mitotic Apparatus**
Onco-Haem:	**:**	**Oncology-Haematology**
OS	**:**	**Overall survival**
^0C	**:**	**Degree Celsius**
PCR	**:**	**Polymerase chain reaction**
Plts	**:**	**Platelets**
PML	**:**	**Progressive multi focal leukoencephalopathy**
PLZF	**:**	**Promyelocytic Leukemia Zinc Finger**
PO	**:**	**Per Oral**
PS	**:**	**Potamine sulphate**
RCC	**:**	**Red cell count**
RT-PCR	**:**	**Reverse Transcriptase Polymerase chain reaction**
rpm	**:**	**Revs per minute**
rx	**:**	**Reaction**
sec	**:**	**Seconds**
qPCR	**:**	**Quantitative Real Time PCR**
RARA	**:**	**Retinoic acid receptor alpha**
RARE	**:**	**Retinoic acid receptor elements**
RCPA QAP	**:**	**Royal College of Pathologists Quality Assurance Program**
RNA	**:**	**Ribonucleic acid**
RP3	**:**	***RARA-PML***
RXR	**:**	**Retinoid X Receptor**
t-APL	**:**	**therapy related APL**
UK	**:**	**United Kingdom**
UKNEQAS Program	**:**	**United Kingdom National External Quality Assessment**
UKZN	**:**	**University of Kwa-Zulu Natal**
US	**:**	**United States**
V	**:**	**Volts**
WCC	**:**	**White cell count**
WHO	**:**	**World Health Organization**
WBC	**:**	**White Blood Cells**
WCC	**:**	**White Cell Count**
%	**:**	**Percentage**

ABSTRACT

Background

The study of genetic epidemiology of cancers in Africa is unique as compared to first world countries, as it entails the combination of gene-environment interaction, poor socio-economic conditions and the high prevalence of infectious (e.g. tuberculosis and HIV) and non-infectious diseases. Acute Promyelocytic Leukemia (APL), a subtype of Acute Myeloid leukemia (AML) if not diagnosed within 24 hours because of its hemorrhagic tendencies, becomes a medical emergency. It has become one of the most treatment-responsive cancers due to its excellent response to all trans-retinoic acid (ATRA). Advances in molecular diagnostics have resulted in a reverse-transcriptase polymerase chain reaction (RT-PCR) test to detect the *PML-RARA* (retinoic acid receptor alpha) transcript found in APL's. Globally, many centers have investigated the different breakpoint cluster regions (bcr) to classify the patients into different prognostic groups for specific molecular targeted treatment. However, there are no reports from Africa on the frequency of the different isoforms in APL patients. In this study we aim to identify and determine the frequency of bcr isoforms in APL patients from a tertiary hospital in Kwa Zulu Natal (KZN) by quantitative RT-PCR (qPCR). The correlation of the hematological parameters with the different isoforms was analyzed by descriptive non-parametric statistical analysis. The qPCR confirmed bcr1 to be the predominant isoform (63,6%) followed by bcr3 (31,8%) and bcr2 (4,5%). There was a median age group of less than 45 in our patient cohort. Patients with the bcr3 isoform had a poor prognosis according to their clinical risk stratification but this did not necessarily result in poor overall survival when monitored for minimal residual disease (MRD). The HIV-infected APL patients with

different isoforms responded to “standard of care” treatment in the same way as non-infected HIV patients.

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CHAPTER ONE

INTRODUCTION & LITERATURE REVIEW

1.1 Introduction

Leukemia represents a group of hematological malignancies characterized by clonal expansion of hematopoietic cells with uncontrolled proliferation, decreased apoptosis and blocked differentiation. Among all malignancies, this group is ranked fifth for male and sixth for female mortality. In addition, it is the leading cause of death in young people with cancer, with 300 000 new cases and 222 000 deaths each year worldwide (Ahn Y O *et al*, 1991; Yang C & Zhang X, 1991; Jemal A *et al*, 2005; Parkin D M *et al*, 2005).

Due to the disease progression and hematopoietic lineages involved, (Bennet J M *et al*, 1985) leukemia can be divided into acute or chronic and lymphoid or myeloid types. Subtypes are further classified based on the lineage and stage of differentiation. Chromosomal abnormalities are the most important predictors of prognosis in AML. The most common abnormality in APL with (t15;17)(q22;q12), a subtype of AML, is the *PML-RARA* (retinoic acid receptor alpha) fusion gene resulting from translocations, leading to fusion of the *PML* gene on chromosome 15 in more than 90% of cases (Sazwal S *et al*, 2009). In the case of a variant APL, *RARA* gene is replaced by five different partners with the *PLZF* being the most common (Mistry A R *et al*, 2003; Campbell L J *et al*, 2013).

Acute Promyelocytic Leukemia has evolved from one of the most fatal cancers to one that maybe curable. Due to APL being a medical emergency because of its hemorrhagic tendencies, a rapid molecular diagnostic test such as PCR or fluorescent in situ hybridization (FISH) is essential, as other tests such as southern blotting or cytogenetics would take too long for the result to be obtained. Molecular diagnosis of the bcr isoforms in APL and early assessment of treatment

response that use methodologies capable of detecting submicroscopic disease can determine subgroups of patients with leukemia at differing relapse risk (Lo–Coco F *et al*, 2010).

Importantly, the responses of leukemia to therapies differ from one subtype to another, making individualized therapeutic strategies extremely useful. Translational research across bedside to bench may not only shed light on leukemogenesis and gain insights into therapeutic mechanisms, but also provide opportunities for designing sophisticated individualized therapeutic protocols as highlighted by the development of curative approaches for APL (Gupta K *et al*, 2011). Monitoring for MRD during induction and consolidation by the detection of molecular targets offers personalized medicine with the ultimate goal of disease-free survival of the patient.

Many studies have shown that detection of *PML-RARA* transcripts by qualitative nested RT-PCR predicts hematological relapse, whereas negative RT-PCR results are associated with long-term remission (Polampalli S *et a*l, 2011; Coombs C C *et al*, 2015; Park J H *et al*, 2011). The small number of reports that do not appear to follow this trend may reflect failure to collect specimens at critical time points, poor quality ribonucleic acid (RNA), and /or inherent differences in the sensitivities of the specific RT-PCR assays used (Lang L *et al*, 2007). Therefore, accurate diagnosis is important for effective treatment. Screening for known specific abnormalities by RT-PCR could lead to more definitive diagnosis (Nora-Athina Viniou *et al*, 1995; Sazwal S *et al*, 2009).

The interpretation of molecular assay results remains a contentious issue and highlights a need to define and standardize assays for detection of molecular relapse (van Dongen J J M *et al*, 2015). This is particularly important because clinical studies indicate that survival rates can be improved

by treating patients at molecular relapse (presence of *PML-RARA* transcripts) as opposed to frank hematological relapse (presence of abnormal promyelocytes and blasts on a differential count) (Slack J L *et al*, 2001; Sasaki K *et al*, 2015). Studies suggest that sensitive, quantitative methods will improve the predictive power of molecular monitoring of APL compared to qualitative methods (Hussain I *et al*, 2011; Mandelli F *et al*, 1997; Polampalli S *et al*, 2011).

The epidemiology and clinical outcome of AML in HIV-infected adults is poorly documented. In a large study (16 AML cases) from 12 hematology centers in France, complete remission was obtained in 11 out of 15 HIV-infected patients; no deaths were related to AML treatment (Sutton L *et al*, 2001). According to Statistics South Africa, the estimated overall HIV prevalence rate in South Africa is approximately 11.2%. The total number of people living with HIV is estimated at approximately 6.19 million in 2015. For adults aged 15-49 years, an estimated 16, 6% of the population is HIV positive (www.statssa.gov.za/publications/p0302).

1.2 Literature Review

Acute Promyelocytic Leukemia is an (AML) subtype and represents less than 10% of all AML, an aggressive cancer of the bone marrow. It was first characterized by Leif Hillestad in 1970 (Stavem P, 1978); however cases resembling APL were reported from as early as 1935 (Risak, 1935). It was called APL because the white blood cell population was dominated by promyelocytes. Hillestad (1990) and coworkers reported that patients with this form of AML had a 100% mortality rate, as there was a tendency for severe bleeding without effective treatment and concluded that APL was the most malignant form of acute leukemia.

In 1976, the French-American-British (FAB) Nomenclature Committee assigned APL as the M3 subtype of acute myeloid leukemia (AML: M3) as indicated in (Table 1.1), based on the unique morphology of promyelocytes in the bone marrow (Bennet J M *et al*, 1976). In 2008, the World Health Organization (WHO) revised the FAB classification to include recurrent genetic abnormalities of AML. Here, M3 was replaced with APL t (15; 17) (q22; q12) *PML-RARA* as depicted in (Table 1.2).

Table 1.1: FAB classification of AML

FAB subtype	*Name*	*Adult AML patients*
M0	Undifferentiated acute myeloblastic leukemia	**5%**
M1	Acute myeloblastic leukemia with minimal maturation	**15%**
M2	Acute myeloblastic leukemia with maturation	**25%**
M3	Acute promyelocytic leukemia	**10%**
M4	Acute myelomonocytic leukemia	**20%**
M4EOS	Acute myelomonocytic leukemia with eosinophilia	**5%**
M5	Acute monocytic leukemia	**10%**
M6	Acute erythroid leukemia	**5%**
M7	Acute megakaryocytic leukemia	**5%**

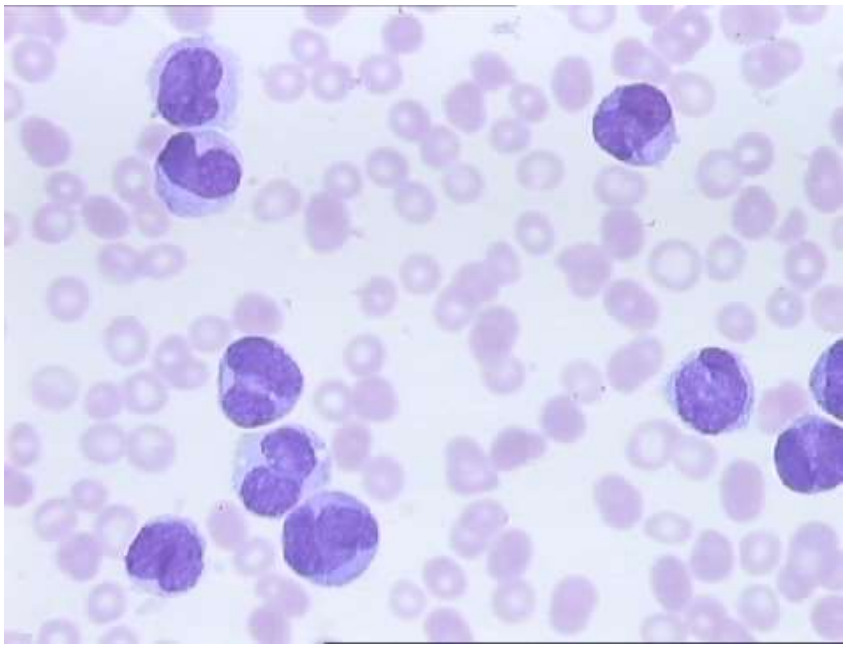
Table 1.2: WHO classification (2008) of AML and related neoplasm's

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBEB-MYH11* Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); *PML-RARA* AML with t(9;11)(p22;q23); *MLLT3-MLL* AML with t(6;9)(p23;q34); *DEK-NUP214* AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1* Provisional entity: AML with mutated NPM1 Provisional entity: AML with mutated CEBPA
AML with myelodysplasia-related change
Therapy-related myeloid neoplasms AML, not otherwise specified: Undifferentiated AML (M0) AML with minimal differentiation (M1) AML without maturation (M2) AML with maturation (M2) Acute myelomonocytic leukemia (M3) Acute monoblastic/monocytic leukemia (M4) Acute erythroid leukemia (M5) Pure erythroid leukemia (M6) Erythroleukemia, erythroid/myeloid (M6) Acute megakaryoblastic leukemia (M7) Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome: Transient abnormal myelopoiesis Myeloid leukemia associated with Down syndrome
Blasticplasmacytoid dendritic cell neoplasm

1.2.1 Clinical and morphological features

Acute Promyelocytic Leukemia is characterized by hyper granular morphology, severe coagulopathy, and an excellent clinical response to ATRA (Appelgate T L *et al*, 2002; Rego E M *et al*, 2015). Bleeding in APL is due to acute disseminated intravascular coagulopathy (DIC) which occurs in 85% of patients when there is sudden exposure of blood to procoagulants (e.g. tissue factor or tissue thromboplastin) which generates intravascular coagulation (Park J H *et al*, 2011). It is characterized by rapid increase in the number of immature white blood cells, resulting in rapid increase of malignant cells and crowding of the bone marrow. This results in very low red blood cell (anemia) and platelet counts, which exacerbate the bleeding diathesis in patients (Choudry A *et al*, 2012).

Either leukopenia (low white cell count) or leukocytosis (high white cell count) may be observed in the peripheral blood. Leukocytosis is only seen in about 25% of patients and organomegaly is rarely found on diagnosis (Chatterjee T *et al*, 2014). Hemorrhagic findings such as gingival bleeding; ecchymoses; epistaxis or menorrhagia has also been observed in a few patients (Yusof M *et al*, 2010).



<u>Figure 1.1</u>: APL (M3) scanned under 50 x oil objectives: showing typical morphology from Patient X diagnosed with APL and admitted to the Hematology Ward (A4W) at Inkosi Albert Luthuli Central Hospital on the 22/02/2011.

Abnormal promyelocytes are larger than their normal counterparts, with a nucleus that is bilobed, dumb-bell or kidney-shaped as depicted in (Figure 1.1). It is morphologically distinguished by the presence of hyper-granular (M3) blasts as shown in (Figure 1.1) and bundles of auer rods or faggot cells as shown in (Figure 1.2) (Ludwig W D *et al*, 2012). Anisocytosis, target cells and rouleaux formation of red blood cells may also be present.

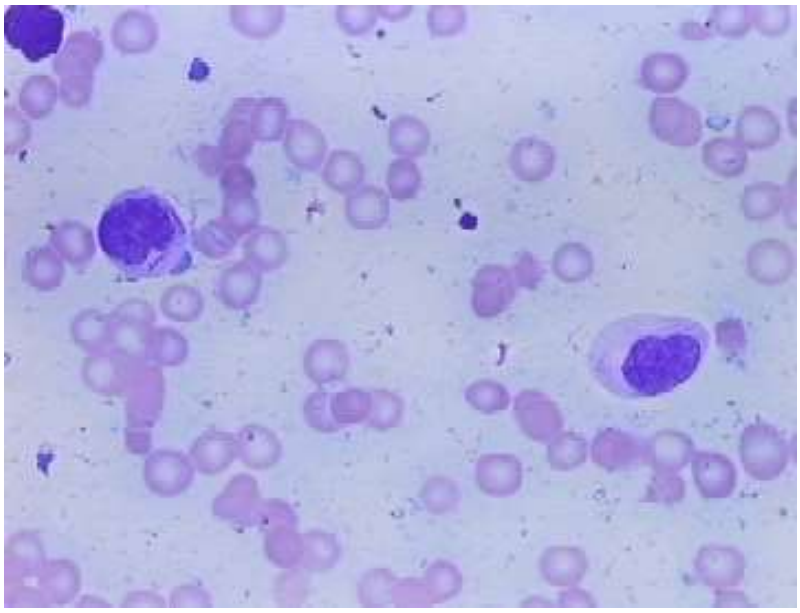


Figure 1.2: **APL M3 scanned under 50 x oil objectives: showing typical APL morphology with auer rods from Patient S diagnosed with APL and admitted to the Hematology Ward (A4W) at Inkosi Albert Luthuli Central Hospital on 31/07/2015.**

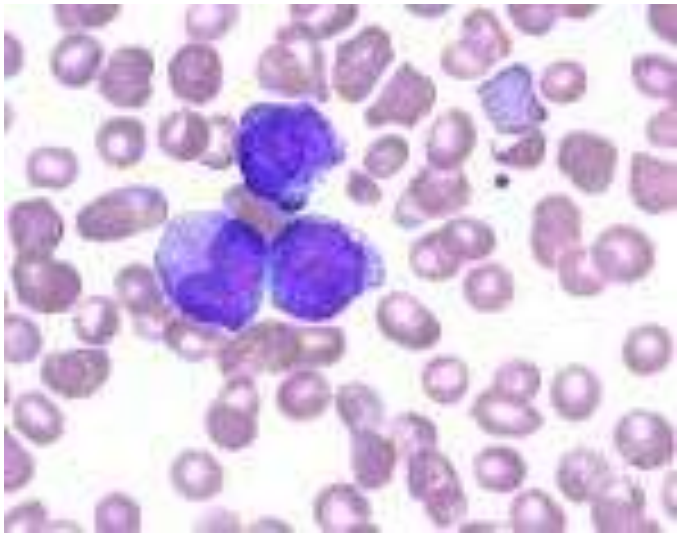


Figure 1.3: **AML M3v showing hypogranular promyelocytes; 50x (adapted from the American Society of Hematology Imagebank.org. The author John Lazarchick uploaded the slide on the 15/2/2004).**

About 25% of APL diagnosed is microgranular or hypogranular (M3v) as shown in (Figure 1.3). At higher magnification of the cells, the nuclear convolutions are more apparent. Azurophillic granules are present in the cell on the left (Figure 1.3). In APL, myeloperoxidase (MPO) is strongly positive in all leukemic promyelocytes; this is especially helpful in microgranular APL which is sometimes confused with Acute Monocytic Myeloid Leukemia (AMML) (Arber D A *et al*, 2008).

1.2.2 Role of *RARA* in granulopoeisis:

In APL, there is an abnormal accumulation of immature granulocytes called promyelocytes (http://www.ucsfhealth.org/adult/medical_services/cancer/leukemia/conditions/aml/signs.html). Despite reports implicating the fusion proteins in leukemia, little is known about how their function relates to the normal function of the *RARA* during granulopoeisis, the process by which immature myeloid cells, such as promyelocytes, differentiate to mature neutrophils (Kastner P *et al*, 2000).

The *RARA* gene is altered by chromosomal translocations in all cases of APL. These translocations lead to the synthesis of chimeric proteins, resulting from the fusion of novel N-terminal sequences to *RARA* amino acids (Melnick A *et al*, 1999). Retinoic acid (*RA*) has been shown to act as a differentiating agent for both normal and transformed immature myeloid cells (Breitman T R *et al*, 1980). At limiting *RA* concentrations, an increase in the proportion of unliganded *RAR* molecules may lead to a dominant-negative inhibition of *RA*-controlled differentiation. Retinoic acid appears to orientate the differentiation of pluripotent hematopoietic

progenitors toward the granulocyte lineage, indicating that *RA* may be involved in granulocyte fate specification (Tocci A *et al*, 1996).

1.2.3 The Normal and Mutated Function of *PML* and *RARA*

The normal function of the *RARA* gene provides instructions for making a transcription factor. A transcription factor is a protein that binds to specific regions of DNA and helps to control the activity of particular genes. The *RARA* protein controls the transcription of genes important for differentiation of immature white blood cells (promyelocytes). The *RARA* protein binds to specific regions of DNA and attracts other proteins that repress gene transcription, the first step to protein production. In response to a specific signal, the repressive proteins are removed and other proteins that induce transcription and cell differentiation binds to the *RARA* protein.

The *PML* gene provides instructions for a protein that acts as a tumor suppressor, which means it prevents cells from growing and dividing too rapidly in an uncontrolled way. The *PML* protein is found in distinct structures in the nucleus of a cell called *PML* nuclear bodies. Mutations can be acquired and present only in certain cells. These mutations are called somatic mutations, and are not inherited. A somatic mutation involving the *RARA* gene causes APL.

The *PML-RARA* protein product from this fused gene functions differently from protein products of the normal *PML* and *RARA* genes. The *PML* protein blocks cell growth, proliferation and induces apoptosis in combination with other proteins. The *PML-RARA* blocks differentiation of blood cells at the promyelocyte stage and allows abnormal cell proliferation. As a result excess promyelocytes accumulate in the bone marrow and normal white blood cells cannot form, leading to APL (https://ghr.nlm.nih.gov/).

1.2.4 *RARA* variants

World Health Organisation designated a subset of AML's morphologically similar to APL as shown in (Figure 1.5), but lacking both t (15; 17) by cytogenetics as shown in (Figure 1.4) and not detectable for *PML-RARA* by FISH (Arber D A *et al*, 2008). In addition to the *PML-RARA* rearrangement, four other APL associated translocation of the *RARA* gene have been characterized at the molecular level as shown in (Table 1.3). As with other hematological malignancies partner genes affect both neoplastic phenotype and response to treatment including ATRA, making their identification crucial in the evaluation of these patients.

At the molecular level, both *PML-RARA* and *PLZF-RARA* are strong repressors efficiently recruiting a complex containing the nuclear receptor corepressors N-COR/SMRT and histone deacetylase to target promoters. These repressive properties are linked with their leukemogenic potential, as histone deacetylase inhibitors can reverse the leukemic phenotype and lead to remission in transgenic mouse models of APL similar to what was found in humans (He L Z *et al*, 1998).

Since the *PLZF* gene was identified in an APL patient in 1993, a series of such cases have been reported. The findings indicated that these patients were resistant to ATRA and had a generally poor response to chemotherapy (Licht J D *et al*, 1995). In t(5;17) associated APL, *RARA* is translocated to a region on chromosome 5q35 containing the ubiquitous expressed and evolutionary conserved nucleophosmin (*NPM*) gene (Redner R L *et al*, 1996). Nuclear Mitotic Apparatus is an abundant, conserved and ubiquitously expressed protein involved in the

completion of mitosis and re-formation of nuclei in the post-mitotic daughter cells (He D Z *et al*, 1995). The *STAT5b-RARA* fusion is the newest fusion partner with *RARA*, identified in a patient with AML with minimal differentiation (M1) but with a proportion of blasts exhibiting microgranular APL morphology (Arnould C *et al*, 1999). Leukemic blasts from this patient failed to respond to ATRA *in vitro*.

Table 1.3: showing the *RARA* variants with their molecular and cytogenetic nomenclature

Name of RARA variant	**Cytogenetic/Molecular Description**	**Reference**
PML-RARA	t(15;17)(q22;21)	(Borrow J *et al*, 1990)
Nucleophosmin(*NPM-RARA)*	t(5;17)(q35;q21)	(Redner R L *et al*, 1996)
Promyelocytic Leukemia Zinc finger (*PLZF-RARA*)	t(11;17)(q23;21)	(Chen Z *et al*, 1993)
Nuclear Mitotic Apparatus(*NuMA -RARA*)	t(11;17)(q13q21)	(Wells R A *et al*, 1997)
STAT5b-RARA	t(17;17)(q11q21)	(Arnould C *et al*, 1999)

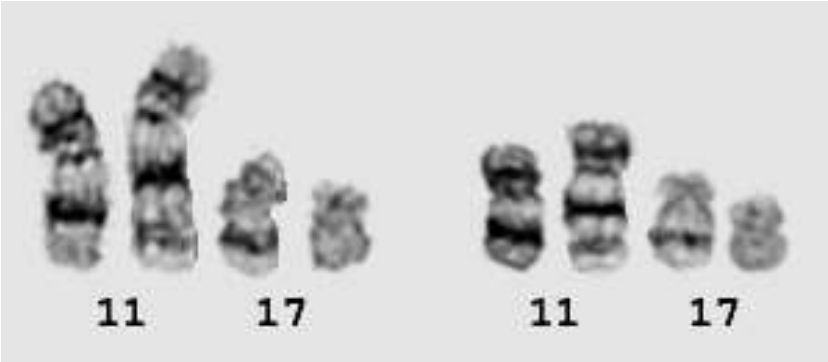


Figure 1.4: ***RARA-PLZF*** **t (11; 17) variant. Normal karyotypes for chromosomes 11 and 17 are shown on the left. The translocation of 11 to 17 found in patients with the *RARA-PLZF* t (11; 17) variant is seen on the right. Adapted from the Atlas Genetics and Cytogenetics Oncology, Haematology.1998; 2(3): 97-99, t (11; 17) (q23; q21) *ZBTB16/RARA* G- banding - Courtesy Diane H. Norback, Eric B. Johnson, Sara Morrison-Delap UW Cytogenetic Services**

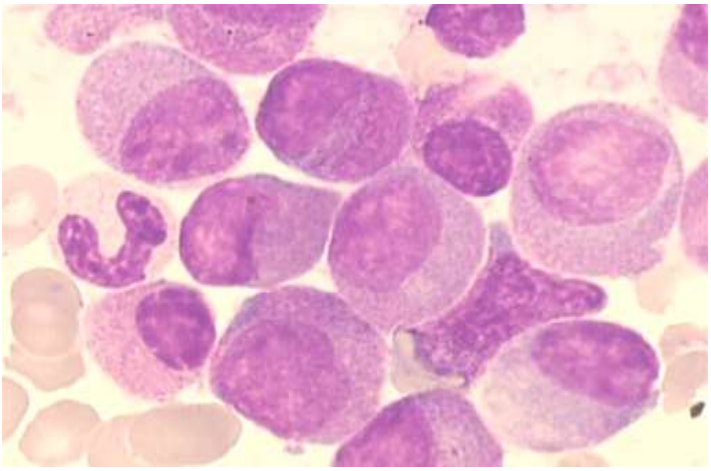


Figure 1.5: Slide with typical *RARA-PLZF* morphology with high rate of normal or dystrophic promyelocytes in peripheral blood and in bone marrow, no auer rods, myeloperoxidase reaction positive; immunocytochemical detection with an anti-*PLZF* shows a distinct punctuate nuclear distribution of the protein, suggesting its compartmentalization in the nucleus.

1.2.5 Molecular Pathogenesis of *PML-RARA* in APL

The high frequency (70 to 100%) of the *PML-RARA* fusion gene in APL patients and the tumor type specificity of the t (15; 17) suggests that chromosomal break-points maybe crucial in the pathogenesis of this malignancy (Bassi S C *et al*, 2012). Lo Coco (2006) and co-workers discussed that *RARA* fusion proteins may also interfere with cell survival, and this may contribute to their leukemogenic potential. Expression of *PML-RARA* in hematopoietic cells inhibits programmed cell death; whereas in non-hematopoietic cells it induces apoptosis. Progressive multi focal leukoencephalopathy possesses physiologic growth suppressor and pro-apoptotic properties that are disrupted by *PML-RARA* (Wang Z G *et al*, 1998). Treatment with ATRA restores normal localization of *PML*, allowing the resumption of its physiologic functions.

The molecular mechanisms by which *PML-RARA* deregulates *PML* intracellular pathways are not clear. Progressive multi focal leukoencephalopathy localizes within distinct nuclear bodies (Dyck J A *et al*, 1994). The *PML* nuclear bodies are destroyed in APL cells and are restored after ATRA treatment, as a direct consequence of *PML-RARA* degradation (Nervi C *et al*, 1998). Also, *PLZF* is a growth suppressor that localizes within nuclear bodies (Ruthardt M *et al*, 1994). Thus, nuclear bodies could be involved in negative growth control and their deregulation by *RARA*-fusion proteins might represent a critical step during promyelocytic leukemogenesis. *RARA* chromosomal rearrangements represent the genetic hallmark of APL. The tight association between *RARA* alterations and APL suggests that a normal *RARA*-dependent function must be altered to transform promyelocytes. Fusion of *RARA* with *PML* or *PLZF* has been shown to be required to induce APL (Kogan S C, 2000).

Acute Promyelocytic Leukemia patients expressing a bcr3 isoform of *PML-RARA* appear to undergo additional cytogenetic changes compared with patients expressing the bcr1 isoform (Slack J L *et al*, 1997). Using a murine APL model, Walter M J (2007) and coworkers also suggested that the bcr3 isoform of *RARA/PML* (RP3) may facilitate the acquisition of Del (2) in mice. The study of this mechanism may provide valuable insight into the ways by which cytogenetic changes involving chromosomal copy number may occur in some patients with AML.

Over the last few years, it has been established that the *RARA* variant genes(X) which results in the variant *RARA* fusion proteins (X-*RARA*), play a key role in the pathogenesis of APL through the recruitment of co-repressors and the histone deacetylase (HDAC)-complex, to repress genes implicated in myeloid differentiation (Mistry A R *et al*, 2003; Campbell L J, *et a*l, 2013).

1.2.6 *PML-RARA* Isoforms in APL

Based on the genomic breakpoint in the *PML* gene, three different *PML-RARA* isoforms are recognized: bcr3 within intron 3 and bcr1 and 2 within intron 6 as shown in (Table 1.4) (Sazwal S *et al*, 2009). The three respective mRNA types, short (S)-form, long (L) form and variable (V)-form, can exhibit different phenotypes but do not affect complete remission (CR) rate or disease-free survival (DFS).

The chromosomal breakpoints in *RARA* are localized within a 15kb DNA fragment of the *RARA* intron 2 on chromosome 17. In contrast, three regions of the *PML* locus on chromosome 15 are involved in the translocation breakpoints: intron 6 (bcr1; 55% of cases), exon 6 (bcr2; 5%) and intron 3 (bcr3; 40%) (van Dongen J J M *et al*, 1999). Chimeric *PML-RARA* and *RARA-PML*

transcripts are formed as a consequence of the reciprocal translocation between the *PML* and *RARA* loci. The existence of different breakpoint regions in the *PML* locus and the presence of alternative splicing of *PML* transcripts are responsible for the great heterogeneity of *PML-RARA* transcript sizes observed among APL patients (Biondi A *et al*, 1992). Moreover, the alternative usage of two polyadenylation sites generates additional *PML-RARA* transcripts of different sizes. The observation that *RARA-PML* transcripts are present in most but not all APL cases, has favored the use of *PML-RARA* transcripts as PCR targets for detection of APL cells at diagnosis and during monitoring (Grignani F *et al*, 1993; van Dongen J J M *et al*, 1999).

Several studies attempted to correlate the type of *PML-RARA* transcript either with clinico-biologic features at diagnosis or with treatment response and outcome (Sazwal S et al, 2009; Chatterjee T *et al*, 2014). The vast majority of analyzed series compared the two major *PML-RARA* isoforms, (*PML* bcr1 and bcr2) and (PML bcr3) as summarized in (Table 1.4). At diagnosis no correlations was found with respect to sex, platelet count, presence of coagulopathy or "differentiation syndrome", when comparing patients with L-type or S-type *PML-RARA* transcripts. However, patients with S-type transcripts had significantly higher white blood cell counts and more frequently display variant microgranular APL (M3v) morphology. Although S-type transcripts correlated with established adverse prognostic features at diagnosis (i.e. hyper-leukocytosis, M3v), compared to the L-type transcript, this association did not translate into poor treatment outcome such as relapse, in the context of combined ATRA and chemotherapy regimens (Hassan R *et al*, 2014).

Table 1.4: Differences in *PML-RARA* transcripts in APL Patients

L/V-type	S- type
PML bcr1 and 2	PML bcr3
Intron 6 and exon 6	Intron 3
Low and normal White Cell Count	High White Cell Count (hyperleucocytosis)
Good and intermediate prognosis	Intermediate and poor prognosis

1.2.7 Epidemiology of APL *PML-RARA* bcr isoforms

In a study by Kudo K (1998) and coworkers the development of therapy related (t-AML) AML was investigated in 17 APL patients of mixed race mostly Italians and Japanese. It was found that the development of t-APL is specific to genetic factors among different ethnic groups. A study in the United States (US) showed the percentage of African–Americans with APL with t (15;17) (q22;q12) and abnormal cytogenetics was almost twice that of whites. This could be due to more environmental exposures that predispose them to cancer and bone marrow stem cell disorders compared to whites http:file://E:\Differences in factors and outcomes in African Americans and whites with AML.mht. The study of Sazwal S and coworkers, (2009) in India showed the presence of bcr3 (S-isoform) in Indian APL patients to be higher than the bcr1 (L-isoform). Similarly, in the study of Hassan R (2014) and coworkers the S-form (35%) was mostly observed in Malay APL patients compared to Latinos (approximately 10%).

1.2.8 Laboratory Diagnosis

1.2.8.1 Routine Hematology tests

Blood or bone marrows are sent for routine investigation such as full blood count, differential count, DIC, and cytochemical stains (Sudan Black, myeloperoxidase (MPO) and combined esterase). The cytochemistry can be omitted if four–colour flow cytometry is available (Milligan D.W *et al*, 2006). A blood smear is scanned for auer rods or 'faggot cells" and blasts under the microscope.

1.2.8.2 Immunophenotyping

Immunophenotyping by flow cytometry was recommended for the management of AML in adults. It is available in most hematology diagnostic laboratories although different laboratories may use different acute panels of antibodies due to budget constraints. CD3, CD7, CD13, CD14, CD33, CD34, CD64, CD117 and cytoplasmic MPO are quantified at diagnosis using flow cytometry (Milligan D.W *et al*, 2006).

Acute Promyelocytic Leukemia cells are usually CD13 positive and CD33 positive, but are characterized by low or absent expression of HLA-DR, CD34, CD11a, CD11b, CD18 and CD117 (Paietta E *et al*, 2004). Hypogranular APL frequently co-expresses CD34 and CD2 (Exner M *et*

al, 2000). Expression of CD56 has been observed in about 20% of cases and confers a worse outcome (Ferrara F *et al*, 2000).

1.2.8.3 Specialized hematology tests

All molecular tests are considered specialized test. RT-PCR is used for the detection of the *PML-RARA* fusion gene and is an important guide for treatment management. The unique *PML-RARA* aberration serves as a marker for rapid diagnosis and prediction of response to ATRA and ATO therapies. Low cost *PML* nuclear staining is extremely useful in countries with limited resources as a rapid genetic diagnosis. Wild- type *PML* protein is localized in the nucleus of the cells, forming nuclear bodies (2-20 per nucleus that appear as speckled dots (Koken M H *et al*, 1994). With co-localization of *PML* and *RARA* in the nucleus, this changes the appearance to a microgranular pattern. The micro granular pattern reverts to normal after treatment with ATRA (Daniel M T *et al*, 1993). The *PML* pattern has proven useful for the diagnosis of *PML-RARA* positive APL.

1.2.8.4 Cytogenetic and Molecular Diagnostic assays

The combination of cytogenetic, FISH and molecular analysis provides a powerful approach for diagnosing and sub classifying malignant diseases into relevant clinical and biological subgroups. This is necessary in selecting appropriate therapy and in monitoring the efficacy of therapeutic regimens. In cryptic and complex APL, the classic t (15; 17) is absent on routine cytogenetic assays. However, the leukemia is morphologically and clinically similar to t (15; 17) positive APL and is treated as such (Grimwade D *et al*, 2000). In these unusual cases, other methods are

needed for identification, such as FISH, RT-PCR and direct sequencing. FISH uses a dual colour dual fusion probe to detect *PML-RARA* rearrangements. The typical normal FISH pattern has two red signals (2R) and two green signals (2G) for the *PML* and *RARA* loci respectively. When t (15; 17) is present, the characteristic FISH pattern has one red, one green and two fusion signals.

However, it is possible to detect false-negatives using standard FISH probes on t (15; 17) APL cases with insertions, as FISH is often not sensitive enough to detect small cryptic insertions (Wang Y *et al*, 2009). Therefore, RT-PCR should be used to confirm the detection of the *PML-RARA* fusion gene.

RT-PCR may also fail to identify poor quality samples (low copy number) and standardization has proven challenging (Grimwade D *et al*, 2014). Another disadvantage of this method is that it lacks the capacity to quantify the levels of leukemia-specific transcripts. Quantitative real-time PCR (qPCR) has definite advantages over RT-PCR as it enables absolute quantification of the leukemic target and endogenous control gene transcripts, which is useful in MRD monitoring and directed therapy. The standardized *PML-RARA* qPCR assay has been shown to increase MRD detection rates compared to conventional, nested RT-PCR. Although qPCR assays for the major leukemia-associated fusion transcripts have been standardized at the international level, there is still limited prospective data on the use of such assays to individualize therapy (Grimwade D *et al*, 2009).

1.2.9 Treatment

1.2.9.1 History of ATRA

All trans- retenoic acid (daily dose of 45mg/m^2) was first used to treat patients at diagnosis in Shanghai (Huang M E *et al*, 1988). After a collaborative meeting in Shanghai in 1987, the Chinese drug was kindly provided to treat patients in Paris, France (Castaigne S *et al*, 1990). Treatment of ATRA was proposed for newly diagnosed patients in Shanghai (Huang *et al*, 1988) and to treat relapsed patients in France. Instead of the normal initial worsening with chemotherapy, coagulopathy rapidly improved. The most remarkable features were the progressive change of malignant cells in the bone marrow (Castaigne S *et al*, 1990).

In relapsed APL, ATRA, idarubicin and ATO recommended by the National Comprehensive Cancer Network (NCCN) can induce complete morphological, cytogenetic and molecular remission (Coombs C C *et al*, 2015). Laboratory and clinical research studies have contributed to transform this once rapidly fatal disease into the most successful examples of translational research in medicine (Lo Coco F *et al*, 1999). Within the first 10 days of treatment, 5-10% of APL patients will develop fatal hemorrhage, especially in the CNS and lungs (Rodeghiero F *et al*, 1990). ATO and ATRA have both been shown to quickly correct this coagulation disorder, and the initiation of the latter has become a true emergency in any new APL patient. ATRA should be promptly started when APL is clinically and cytologically suspected even if cytogenetic and molecular confirmations of the diagnosis are pending (Sanz M A *et al*, 2009).

1.2.9.2 Mechanisms of ATRA

Phenotypically, pharmacological doses of ATRA lead to effective differentiation of immature APL cells to terminally differentiated granulocytes. The binding of ATRA to *RAR* receptors causes degradation of *PML-RARA* protein through the ubiquitin–proteasome and caspase system, leading to restoration of terminal differentiation of promyelocytes. Exposure of APL cells to ATRA in vitro or in vivo induces relocalization of *PML* and restores the normal structure. Under the action of ATRA, co repressor (CoR) is dissociated from the repressive complex, whereas the co activator (CoA) is recruited to the complex. As a result, the repression of transcriptional activation of target genes is relieved and the differentiation of promyelocytes is restored (Zhi-Xiang Shen, 2009). In vitro cell culture has shown that ATRA-induced differentiation also coincides with activation of apoptosis (Grignani *et al*, 1998). Although ATRA appears to be highly effective in clearing the bulk of proliferative tumor cells, a residual population of cells with detectable t (15; 17) almost invariably persists following treatment with this reagent alone; therefore additional chemotherapy, such as ATO is needed to achieve complete remission (Chen G *et al*, 1991).

1.2.9.3 Mechanism of ATO-based APL therapy

Compared to ATRA, ATO is less able to induce terminal differentiation of APL cells. *In vitro* studies using cultured cells have revealed a dose-dependent effect of this drug on differentiation and apoptosis (Chen G *et al*, 1997). At high concentrations (0.5-2.0 *uM*) ATO induced cell death by apoptosis, while at low concentrations (0.1-0.25 *uM*) this drug caused partial differentiation of APL cells along the granulocyte lineage (Cai X *et al*, 2000). Arsenic trioxide -mediated differentiation has been shown to become dramatically enhanced in the presence of cyclic adenosine monophosphate (cAMP). The mechanism responsible for this synergistic effect was

proposed to be the combined effect of ATO-induced *PML-RARA* degradation and *cAMP*-mediated inhibition of cell cycle progression (Zhu J *et al*, 2002).

At the molecular level, ATO exerts its therapeutic effect on APL in part by initiating a cascade of biochemical alterations that primarily affect the *PML* moiety of *PML-RARA*. In addition to affecting differentiation of leukemic cells, recent studies have also implicated ATO in clearance of leukemic-initiating cells (LICs), a small population of malignantly transformed cells with stem cell characteristics that are frequently refractory to cancer therapeutic drugs. *PML-RARA* expression has been reported to support properties of self-renewal of LICs (Wojiski S *et al*, 2009) ATO also increases radioactive oxygen species (ROS) production in malignant cells. As a consequence, this drug leads to disruption of the mitochondrial membrane potential, followed by cytochrome C release, capase activation and subsequent apoptotic cell death (Jing Y *et al*, 1999).

1.2.9.4 Current treatment

It is now apparent that the nature of the *RARA*- fusion partner is a critical determinant of response to ATRA and arsenic, underlining the importance of cytogenetic and molecular characterization of patients with suspected APL to determine the most appropriate treatment approach. Combined treatment with ATRA and anthracycline-based chemotherapy is highly successful in APL, providing longer remissions and probable cures in up to 70% newly diagnosed patients (Tallman M S *et al*, 2002). The persistence of resistant clones causing relapse and low survival still represents a problem in 15-25% of patients and can be identified by MRD monitoring (Mistry A R *et al*, 2003).

1.2.9.5 Future treatment

The challenge for future studies is to improve complete remission through reduction in induction deaths, identifying patients to stratify in different risk groups, thereby reducing the risks of treatment toxicity and development of secondary leukemia or myelodysplasia. With the advent of ATRA and arsenic, APL has already provided the first example of successful molecular targeted therapy and with further understanding of the pathogenesis of the disease, the next decade will find further improvements in the treatment of these patients (Mistry A R *et al*, 2003).

1.2.10 Minimal Residual Disease (MRD)

The study by Carlos S, (2007) and coworkers confirmed the need for MRD analysis by frequent sampling (at least every 4 months) in patients who have a molecular result with >10 normalized copy number (NCN) or reconfirmed positivity by RT-PCR. Studies of APL over the last decade have resolved some issues to predict clinical outcome but there are important issues to be clarified. Four treatment regimens have been instrumental in treatment of MRD in 2005. First, Fenaux P *et al*, (2009) found that concurrent treatment with ATRA and chemotherapy (CT) resulted in superior long-term outcome. Second, Lo-Coco F, (1999) and coworkers provided evidence that re-treatment at the time of molecular relapse during first remission provided improved clinical outcome. Third, Sanz M A, (2009) and coworkers defined pre-treatment criteria to classify patients for risk of disease recurrence following concurrent ATRA-CT and introduced risk-adapted consolidation therapy with dose intensification and ATRA supplementation for intermediate patients. Fourth, the availability of alternative agents, particularly ATO, has led to the formulation of novel induction regimens, which may add to the effectiveness of ATRA- AraC chemotherapy (ATRA-CT) or which may reduce or avoid the potential adverse effects of chemotherapy (Burnett A K *et al*, 1998). Papanikolaou NA, (2010)

and coworkers proposed that targeting APL with ATRA combined with small molecule inhibitors of cyclin-dependant kinases maybe more effective than current regimens affording reduced toxicity and relapse rates.

Detection of *PML-RARA* transcripts by molecular techniques constitutes an important tool for monitoring MRD and predicting evolution in APL patients especially at consolidation and maintenance (Sanz M A *et al,* 2005). Conventional qualitative RT-PCR has been widely used for genetic diagnosis and therapeutic monitoring of APL. Several reports have shown that RT-PCR positivity after consolidation therapy predicts hematology relapse, whereas persistent RT-PCR negativity is associated with long term survival and a low relapse rate (Jurcic J G *et al*, 2001; Mandelli F *et al*, 1997). However, RT-PCR have several disadvantages such as the occurrence of false positives due to cross-contamination, false negatives due to poor RNA quality or PCR inhibition, low sensitivity for measuring MRD, time and labor-consuming and inter-laboratory variability. Recently qPCR has become a new alternative for MRD. It has several advantages such as being highly sensitive and reproducible.

In a study by (Grimwade D *et al*, 2009) qPCR was used to monitor a large cohort of newly diagnosed patients with APL who were treated with standard ATRA and anthracyline-based chemotherapy to direct pre-emptive therapy with ATO and to guide transplantation in those who experienced failure with first-line therapy. It was found that a log reduction in leukemic transcripts after induction did not predict relapse risk.

This study shows that sequential MRD monitoring provides an alternate strategy that allows additional therapy to be targeted to patients at risk for relapse which is also cost-effective. Due to

concerns that patients are currently over treated, there has been an increasing trend, with the availability of molecular targeted therapy towards de-intensification of APL therapy, with interest in chemotherapy-free schedules based on ATRA and ATO (Estey E H *et al*, 1999).

It was found by Carlos S (2007) and coworkers that no significant differences in *PML-RARA* NCN values post-induction were obtained between relapsed patients and those who remained in continuous complete remission. Also, the kinetics in tumor burden did not correlate with disease outcome. These results contrast with the findings in other leukemic disorders such as t (9; 22) and t (8; 21) in which successful induction therapy produces a 2 to 3 log reduction in the level of transcripts. The low number of positive cases detected at the end of consolidation limits the utility of qPCR to monitor MRD in APL patients.

1.2.11 Risk Stratification

Presently most centers use the Europe against Cancer (EAC) protocol (Gabert J *et al*, 2003) as explained in the methods section for risk stratification. Risk stratification is imperative in the treatment of APL patients, as those with low-risk disease (WBC <10000/ul) have a less intensive treatment regimen (Grimwade D *et al*, 2009). APL has evolved from the ATRA + chemotherapy backbone for all patients to ATRA and ATO and no chemotherapy in low-risk patients as a new standard of care. Given the favorable results from risk-adapted treatment strategies, first in the LPA99 trial followed by the LPA2005 trial, the National Comprehensive Cancer Network (NCCN) recommended an alternate approach in induction which includes ATRA plus idarubicin and ATO (Coombs C C *et al*, 2015).

It was found that ten percent of all APL cases lack the classic t (15; 17). This group includes cases with *PML-RARA* gene rearrangements like the t (5; 17) which are retinoid responsive and the t (11; 17) (q23; q21) that are retinoid-resistant. It is imperative to treatment to distinguish subtypes of APL. A European workshop was held in Monza, Italy in (1997) to develop a novel morphological classification system that takes into account the major nuclear and cytoplasmic features of APL (Danielle, S *et al*, 2000).

Several genetic and phenotypic characteristics of APL blasts provide relevant targets and the rationale for tailored treatment. Agents currently used are retinoids, ATO, anthracyclines and anti-CD33 monoclonal antibodies which have been taken off the market due to toxicity. Novel agents that maybe used in the future are histone deacetylase and *FLT3* inhibitors (Lo Coco F *et al*, 1999).

1.2.12 Cure rates of APL

Acute Promyelocytic Leukemia is the AML with the best prognosis. Currently around 90% of newly diagnosed patients with APL achieve complete remission and over 75% are curable (Degos L *et al*, 2003). Unfortunately the treatment outcome for patients in developing countries with APL is significantly inferior compared with that reported in Europe and United States (US). The International Consortium on APL (IC-APL) was established to create a network of institutions in developing countries to receive support from well-established US and European cooperative groups. This resulted in a decrease of almost 50% in early mortality and an improvement in OS of almost 30% compared with historical controls, resulting in OS and DFS similar to those reported in developed countries (Rego E M *et al*, 2013).

To further increase the CR and cure rates detection and diagnosis of this disease at its early stage is very important (Ohno R *et al,* 2003). A study by Park J H, (2011) and coworkers confirmed that the early death rate remains high despite the increased awareness of the disease and improved diagnostic tests and ATRA. APL needs to be recognized by all clinicians as a medical emergency and major improvements in the early death and cure rates of APL will not only depend on new drugs but on the education of health care providers across a wide range of medical fields.

1.2.13 HIV and AML

HIV-1 infected individuals have a markedly increased incidence of cancers, such as Karposi's sarcoma and non-Hodgkin lymphoma, however the risk of AML is at most slightly increased (Sutton L *et al,* 2001). The study by Sutton L (2001) and coworkers surveyed all Hematology centers from 1990 to 1996 for AML patients at diagnosis that were HIV sero-positive. This study showed a two-fold increase in the risk of AML in HIV-infected patients compared with the general population in France, although a larger cohort of patients need to be studied to confirm this. They found that HIV-1-associated opportunistic infections did not occur during chemotherapy of AML induced aplasia. CR and long- term leukemia- free survival were associated with a $CD4^+$ cell count above 200 x 10^6/l at AML diagnosis. On the other hand, known long term HIV infection or full blown AIDS at AML diagnosis were associated with poor survival.

It was reported by Allers K (2010) and co-workers that an HIV positive patient with AML was "cured" of HIV infection after a stem cell transplant combined with high dose chemo and radiation therapy. The stem cell donor had an extremely rare gene mutation (CCR5 gene spanning the Δ32-region from nucleotide 826 to 1138 on chromosome 3p21.31 (accession no: NM_000579) which protected him from HIV infection. Although ARV therapy to suppress HIV had been stopped, the patient had shown no signs of HIV for over 36 months since the transplant occurred. However the patient did relapse with AML but remains free from HIV.

1.2.13.1 APL in HIV-infected Patients

While the incidence of AML in HIV-infected patients has increased approximately twofold, the occurrence of APL is still exceedingly rare compared with the general population. Due to the very small number of reported HIV positive APL patients who have been treated with different therapies, as well as the variable outcome, the prognosis of APL in the setting of the HIV-infection is unclear. Due to considerable risk for relapse, standard treatment of APL together with HAART should be used in HIV infected patients (Boban A *et al*, 2009). Therapeutic strategies should target both pathologies, as ATRA has been found to induce apoptosis in HIV-infected leukemic cells and protease inhibitor therapy has been reported to be synergistic with ATRA in inducing differentiation of APL cell lines (Drilon AD *et al*, 2010).

The purpose of this descriptive study was to follow up patients for two years to detect MRD with qPCR. Due to APL's excellent response to molecular targeted therapy (ATRA) the occurrence of different isoforms and their effect on treatment response in APL, were also analyzed. We also

aim to correlate the hematological parameters, clinical features and prognostic groups to the bcr isoforms.

1.3 Rationale

- To our knowledge there are no reported findings among South African patients, with limited data on the frequency of APL patients, the distribution of bcr isoforms and their treatment outcome in Africa as a whole.
- In 2005, there were many reports showing that bcr3 patients had a poor prognosis than bcr1 or bcr2 patients; therefore it was important to use an assay that could identify the different bcr region to diagnose APL patients for treatment.

1.4 Aims and Objectives

Aim

To determine the distribution of *PML-RARA* isoforms in APL patients from a tertiary hospital in KZN, South Africa (S.A).

Objectives

1 To determine the occurrence of APL in a referral hospital in KZN, (S.A) from samples collected in 2007.

2 To identify *PML-RARA* bcr isoforms for the detection of the t (15; 17) translocation using a commercial kit.

3 To correlate the hematological parameters with the different *PML-RARA* isoforms.

4 To follow up the patients for the detection of (MRD) using qPCR to assess treatment outcomes for relapse/remission.

CHAPTER TWO

METHODOLOGY

2.1 Clinical Risk Stratification Protocol (IALCH) (Grimwade D *et al*, 2009)

On presentation patients were assigned to prognostic groups according to these hematological parameters:

1. Good prognosis: WBC (white blood cells) <10 and platelet count >40
2. Intermediate prognosis: WBC<10 and platelet count <40
3. Poor prognosis: WBC>10

2.2 Patient cohort

From 2007 to 2009, ninety one patients attending the Onco-Hematology outpatient's clinic at Inkosi Albert Luthuli Hospital (IALCH) with clinical signs and symptoms of Acute Leukemia were screened for this study. Twenty two sequential adult APL patients (over the age of 18 years) were chosen to be part of this study. There were nine pediatric APL patients excluded from the study. The study was conducted in accordance with the UKZN Biomedical Research Ethics guidelines (Ref no: BF070/07).

Residual peripheral blood and/or bone marrow samples that were sent for routine laboratory analysis at diagnosis were used for molecular analysis. The routine laboratory analysis included full blood count, platelet count, HIV testing, CD 4 count, international normalized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, protamine sulphate and fibrinogen degradation product (D-Dimers). Clinical diagnosis as well as preliminary laboratory tests such as the differential stain was used to diagnose the acute promyelocytic leukemia (APL-M3) according to WHO classification.

Immunophenotyping using single colour flow cytometry was performed to further subtype AML in another section of the laboratory. Florescent in situ hybridization and cytogenetic analysis were conducted in an external referral laboratory according to the International System for Human Cytogenetic Nomenclature (ISCN; 2005) on one patient to confirm the variant APL. A comprehensive dataset of the qPCR results in (Table 3.1) of each patient was recorded. Peripheral blood from five healthy donors as shown in (Table 3.3) was taken to serve as negative controls.

2.3 MRD Protocol for samples

Bone marrow aspirates and trephines were taken routinely at diagnosis. Bone marrow was also taken post induction of chemotherapy and after the three courses of consolidation. While on a maintenance program bone marrow trephines were performed six monthly. A bone marrow trephine was also done on completion of maintenance. In some cases, where the patient had adverse side-effects, blood was collected instead of bone marrow.

2.4 IALCH Standard Treatment Protocol for Clinical Management of APL-Front Line Therapy

For induction the following treatment was given:

ATRA 45mg/m^2 po (orally) plus Daunorubicin 70 mg/m^2 ivi x 3/7 (for 3 days) regardless of WBC. ATRA was given to the patient throughout induction. ATRA was continued until morphological remission (the patient cannot be diagnosed for APL by differential count). For consolidation three cycles of anthracycline was given based on the patient's response to therapy.

For patients with a good prognosis the treatment was as follows:

1. Idarubicin 5 mg/m^2 x ivi 4/7 (for 4 days) + ATRA 45mg/m^2 daily po x 2/52 (twice a year)

2. Mitoxantrone 10 mg/m^2 x ivi 5/7 (for 5 days) + ATRA 45mg/m^2 daily po x 2/52

3. Idarubicin 12 mg/m^2 x ivi 1/7 (for 1 day) + ATRA 45mg/m^2 daily po x 2/52

For patients with an intermediate or poor prognosis the treatment was as follows:

1. Idarubicin 7 mg/m^2 x ivi 4/7 + ATRA 45mg/m^2 daily po x 2/52

2. Mitoxantrone 10 mg/m^2 x ivi 5/7 + ATRA 45mg/m^2 daily po x 2/52

3. Idarubicin 12 mg/m^2 x ivi 2/7 + ATRA 45mg/m^2 daily po x 2/52

Maintenance therapy was carried out monthly for 24 months: 6-Mercaptopurine 100 mg/m^2 po daily; Methotrexate 10 mg/m^2 po weekly; ATRA 45 mg/m^2 po x 2/52 every three months (Dr S. Parsanath; Clin Haem; IALCH).

2.5 Erythrocyte Lysis and Trizol Treatment

A table for all reagents used is provided in the (Appendix 6.4). Samples were received at the laboratory within four hours of collection. The blood or bone marrow was immediately lysed and the RNA was extracted as described below.

Five volumes of EL Buffer (Qiagen; SA) was added to one volume of peripheral blood or bone marrow aspirate in the biosafety cabinet (LABAIRE, Microbiological Safety Cabinet, Class II) and placed on ice for 15 minutes. After vortexing (Heidolph, Reax Top, Germany, 230V, 50Hz), the tubes were centrifuged (Jouan BR4i, France, 3000rpm) for 10 minutes at 4^{o}C. The resulting supernatant was decanted and discarded. Two volume of cold EL Buffer was added to the white cell pellet. The contents were vortexed and centrifuged again for 10 minutes at 4^{o}C. The

supernatant was decanted and the pellet retained. Trizol reagent was added to the pellet and vortexed. The pellet was then disrupted and homogenized using an 18 gauge needle and syringe. Trizol reagent was added 10x the size of the pellet until a homogenous solution with reduced viscosity was achieved. The trizol pellets were then stored in 1ml aliquots in the -20 ^{o}C freezer.

2.6 RNA Isolation

Ribonucleic acid was isolated as per manufacturer's instructions, (Lifetech, S.A). Briefly**;** the centrifuge was cooled for 15 minutes at 2-8 ºC. The homogenates were thawed at room temperature and placed in a 37 ºC heating block (Biometra, TB1, Germany) for 5 minutes to permit the complete dissociation of nucleoprotein complexes. An aliquot of 400ul of chloroform was added to the Trizol homogenate and shaken vigorously for 15 seconds. The tubes were then placed on ice for 3 minutes.

Following centrifugation at (12 000 rpm for 15 minutes at 4° C) the sample was separated into three phases. The RNA was located in the upper phase and was clearly separated from the interphase and the lower phase, which contained DNA and protein. If the upper phase was not clear, additional chloroform may be added and the centrifugation repeated. Without disturbing the interphase, the upper phase was transferred to a labeled 1.5 ml microfuge tube. If there was disruption of the interphase, centrifugation was repeated. The protein and DNA were stored at -80 °C for DNA isolation if necessary. An aliquot of 500 ul cold isopropanol (Sigma; SA; Molecular Biology Grade) was added to the supernatant. The tubes were incubated on ice in the cold room (~4 ºC) for 2 hours or overnight.

After centrifugation (12 000 rpm for 10 minutes at 4º C) the supernatant was decanted without disrupting the pellet, and 1ml of cold 75% ethanol (Merck, BDH, Molecular Biology Grade) was added to the pellet. The tubes were centrifuged (8000 rpm for 5 minutes at 4 ºC). The wash step was repeated. The microfuge tubes were air dried in the biological safety cabinet for 15 minutes. An aliquot of 30 ul DEPC water was added to the pellet and placed on the heating block at 55 ºC for 10 minutes. The RNA was stored at -20 ºC until cDNA synthesis (within 3 days). The RNA was then stored at -80 ºC and the cDNA was stored for up to six months at -20 ºC.

2.7 Analysis of RNA

Ribonucleic acid quantity and quality was assessed using the Nanodrop ND-1000 spectrophotometer (Inqaba, SA). A volume of 1ul of sample was pipetted onto the testing pedal of the instrument and the concentration and purity was measured. The ratio of absorbance at 260 and 280 nm (i.e. the A_{260}/A_{280} ratio) was used to determine the purity of RNA. The A_{260}/A_{280} for the RNA should be ≥ 1.8 and the expected concentration of the RNA was greater than or equal to 200 ng/ul.

2.8 RT-PCR

RNA was converted to cDNA using the standardized EAC Reverse Transcription protocol (Gabert J *et al*; 2003). 2ul of RNA, 2ul of random hexamer (Promega, USA, 500ug/ml) and DEPC treated water was added to give a total volume of 9ul. This reaction was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Ver3.12; USA). It was denatured for 10 minutes at 70 ºC and then immediately cooled on ice for 5 minutes.

The EAC mastermix consisted of the following reaction components: 5 X Expand reverse transcriptase buffer (first strand) (Invitrogen, S.A), 5mM $MgCl_2$ (Roche), dNTP (10mM each), 100mM DTT (Anatech Instruments; SA), 40U/ul RNAse Inhibitor (Anatech Instruments; SA), 200U/ul Superscript III Reverse Transcriptase (Invitrogen; SA). A volume of 11ul of EAC mastermix was added to the denatured RNA isolates and made up to a total volume of 20ul with molecular grade PCR water (Roche; SA). These samples were quick spun at 12000 rpm and then run on the GeneAmp PCR System 9700 (Applied Biosystems, Ver3.12; USA) at 25 ^{o}C for 10 minutes, 42 ^{o}C for 45 minutes, 99 ^{o}C for 3 minutes and then cooled to 4 ^{o}C. The amplified cDNAs were spun at 12000 rpm and kept at 4 ^{o}C until required for the PCR reaction (immediate processing). For storage for more than a day the cDNA was kept at -20 ^{o}C.

2.9 Optimization of In-house Real-Time PCR assay

When this study began in 2007, a RT-PCR assay for the detection of *PML-RARA* isoforms was not available in our laboratory. At this time, commercial qPCR kits were also not available. The initial objective was therefore to develop a rapid in-house RT-PCR assay on the Light Cylcer 2.0 (Roche SA) (Appendix 3). Primers and probes as shown in Table 2.1, published in the journal, Leukemia (van Dongen J J M *et al*, 1999) were used.

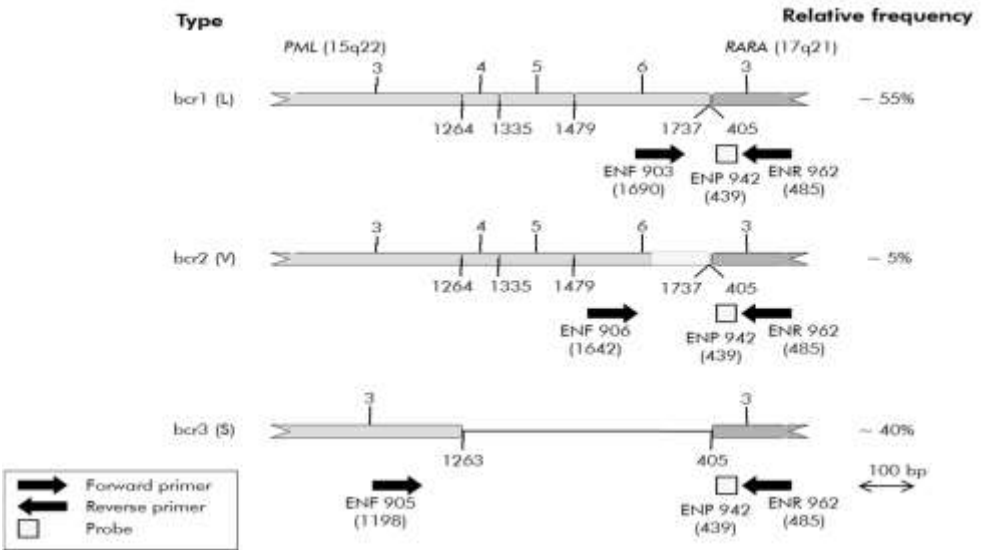
Table 2.1: Primers for RT-PCR analysis of t (15; 17) (q22;q21) with the *PML-RARA* fusion gene

Primer code	5_Position[a] (size)		Sequence (5_±3_)
PML-A1(F)	1438	(21)	CAGTGTACGCCTTCTCCATCA
PML-A2(F)	969	(18)	CTGCTGGAGGCTGTGGAC
RARA-B(R)	485	(20)	GCTTGTAGATGCGGGGTAGA
PML-C1(F)	1546	(21)	TCAAGATGGAGTCTGAGGAGG
PML-C2(F)	997	(19)	AGCGCGACTACGAGGAGAT
RARA-D(R)	426	(20)	CTGCTGCTCTGGGTCTCAAT
RARA-E3(R)_	682	(20)	GCCCACTTCAAAGCACTTCT

The HL60 and NB4 cell lines (Sigma; SA) were ordered to use as controls for the reaction. To attempt to make the PCR faster, a single round real time PCR was optimized with the assistance of Roche. The master mix for the in-house PCR consisted of the following in a final volume of 20ul: 2 ul cDNA; 200uM dNTP; 400nM primers; 2.5 mM. $MgCl_{2;}$ PCR buffer (20 nM Tris HCl, 50 mM KCl, pH 8.3) and 0.5U Amplitaq Gold. The run conditions were as follows for 35 cycles: 94 ^{0}C for 30s (melting), 65 ^{0}C for 60s (annealing) and 72 ^{0}C for 60s (extension). The same primers (Roche; SA) as listed in Table 2.1 were used in different combinations to amplify the different breakpoints (PML-A1+RARA-B: bcr1);(PML-A2+RARA-B: bcr2);(PML-A2+RARA-E3: bcr3). A primer stock concentration of 100 pmol was supplied. A working stock of primer concentrations from 8 pmol to 20 pmol were tested for the optimal concentration. Sensitivity assays were run on the LC 2.0 with dilutions of the NB4 cell line used as a positive control and HL60 as a negative control.

2.10 Quantitative Real-time PCR using the Ipsogen qPCR fusion kit

The IPSOGEN qPCR fusion kit assay (Barker Medical; France) was used to obtain the final gene transcript ratio in copy numbers and the bcr isoforms (presented in the thesis) used for data analysis in this study, due .to lack of sensitivity of the in-house real-time PCR assay.



<u>Figure 2.1:</u> Diagram of probe and primer set from Ipsogen bcr1, bcr2 and bcr3 handbook used in the qPCR assay

Schematic diagram of the *PML-RARA* FG transcript covered by the EAC qPCR primers and probe set. For type bcr1 (L): ENF903–ENP942–ENR962. For type bcr2 (V): ENF906–ENP942–ENR962. For type bcr3 (S): ENF905–ENP942–ENR962.

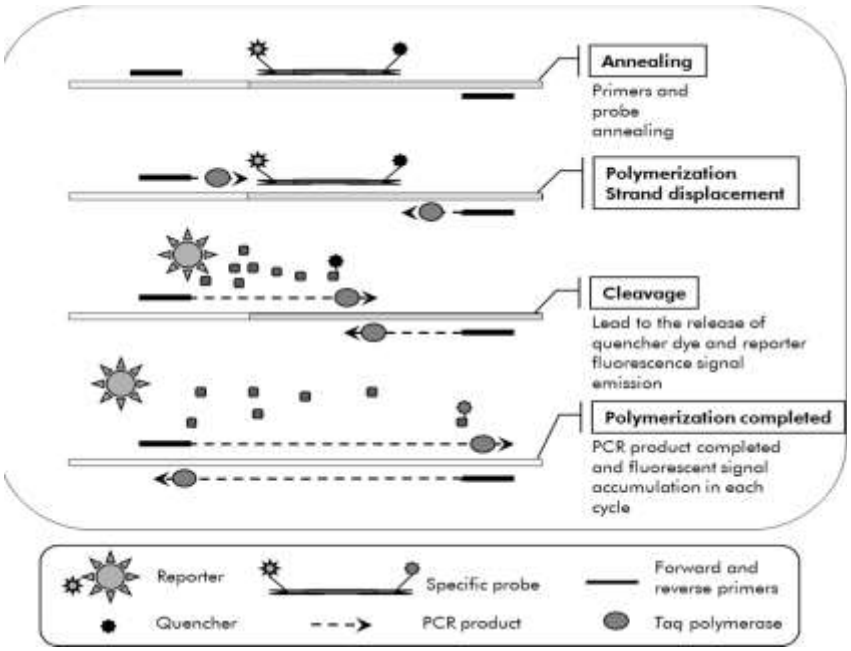
The number under the primers and probe refers to their nucleotide position in the normal gene transcript. Relative frequency refers to the proportion of each type of FG transcripts among *PML-RARA* variants.

The *PML-RARA* Ipsogen bcr1, bcr2 and bcr3 (Barker Medical; France) kits were used according to manufacturer's instructions. The kit contains different mastermix for both target and housekeeping genes. The probes used in the bcr1, bcr2 and bcr3 kits as shown in (Figure 2.1).

The principle of the reaction is described in (Figure 2.2). There are five plasmid DNA target (*PML-RARA*) standards supplied at a dilution of 1 million copies to 10 copies as per (Table 2.2). These standards are used to generate the standard curve for quantification of the *PML-RARA* gene. Due to the particular technological requirements, it was recommended that LC 2.0 experiments must be performed with the LC Taqman Master (Roche, South Africa). All reagents were thawed and placed on ice. The qPCR mastermix was prepared according to the number of samples being processed as shown in (Table 2.2).

Table 2.2: Preparation of qPCR mastermix for bcr isoforms

Component	1 rx (*ul*)	ABL: 14+1 rx (*ul*)	PML-RARA Bcr1:16+ 1rx(*ul*)	Final concentration
Freshly prepared LC Taqman MM (x5)	4.0	60	68.0	1x
Primers and Probe Mix (25x)	0.8	12	13.6	1x
Nuclease free PCR grade water	10.2	153	173.4	-
Sample to be added	5.0	5.0	5.0	-
Total volume	20.0	20.0	20.0	-



<u>Figure 2.2</u>: Reaction principle adapted from Ipsogen handbook:

cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM™–TAMRA™). The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5' 3' exonuclease activity of the *Taq* DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the FAM and a decrease in fluorescence from the TAMRA.

In each capillary 15ul of the qPCR mastermix and 5ul of the RT product (cDNA, ~100ng RNA equivalent) obtained from the RT-PCR (EAC; 2003) and the dilution standards and negative control was added for each experiment. The reaction was mixed gently by pipetting up and down. The capillaries were placed in the adapters provided with the apparatus and spun down at

3000 rpm for 30 seconds. The capillaries were then loaded into the thermal cycler and run at the program specified in (Table 2.3). It was recommended by the manufacturer to use the Automated (F'max) analysis on the LC 2.0 Software 4.0 to obtain reproducible results. Thermal cycling conditions were carried out according to manufacturer recommendations as shown in (Table 2.3).

Table 2.3: Thermal cycler conditions for LC 2.0

Mode of analysis	Quantification
Hold	Temperature: 95 ^{o}C
	Time: 10 minutes
	Ramp: 20
Cycling	50 times
	95 ^{o}C for 10 seconds; ramp:20
	60 ^{o}C for 1 minute; ramp: 20; with acquisition of
	FAM fluorescence: Single
Hold 2	45 ^{o}C for 1 minute;ramp: 20

2.11 Methods applied to the interpretation of qPCR data

2.11.1 Analysis of Data

Data was analyzed using the absolute quantification in the LC 2.0 software 4.1. Using standards with a known number of molecules, a standard curve was established to determine the precise amount of target present in the test sample. The Ipsogen standard curves are plasmid-based. A housekeeping gene was run simultaneously as a control for each unknown sample.

For each gene (*ABL* and *PML-RARA*), raw C_p values obtained from plasmid standard dilutions are plotted according to the log copy number. A negative control was used in all assays to control for amplicon contamination. A positive control was not run in every assay as the standards were

dilutions of known concentrations of the *PML-RARA* fusion gene. Also, an *ABL* housekeeping gene was run with every sample to control for the integrity of the RNA. If the housekeeping gene had a value below 1000 copies the RNA was considered to be degraded. When the positive control was run (usually twice a month or when a new kit was opened) it was a known positive patient's sample that was used or RNA extracted from the NB4 cell line lyophilized pellet (Sigma, Capital Labs). The LC 2.0 software has a feature to import previously run standard curves to analyze the data only if the standards run in the present assay passes after analysis in the absolute quantification of the LC 2.0 software 4.1. In addition, a PCR assay with efficiency less than 1.5 was considered unsuccessful and the assay was repeated. Also, a Cp value <10 or >35 indicated false positivity and amplification in the negative control indicated amplicon contamination. Initially samples were run on a gel to verify the products.

Table 2.4: Plasmid standards in the Ipsogen kit

Target Gene	Copy Numbers	Housekeeping Gene	Copy numbers
PML-RARA bcr1 10^1	F1- 10	ABL 10^3	C1- 1000
PML-RARA bcr1 10^2	F2- 100	ABL 10^4	C2- 10000
PML-RARA bcr1 10^3	F3- 1000	ABL 10^5	C3- 100000
PML-RARA bcr1 10^5	F4- 100000	-	-
PML-RARA bcr1 10^6	F5- 1 000 000	-	-

2.11.2 Normalized copy number (NCN)

The *ABL* standard curve equation was used to transform raw C_p values (obtained with PPC-*ABL*) for the unknown samples into *ABL* copy numbers (ABL_{CN}). The *PML-RARA* standard curve

equation was used to transform raw C_p values (obtained with PPF-*PML-RARA*) for the unknown samples, into *PML-RARA* copy numbers (*PML-RARA* $_{CN}$).

The ratio of these CN values gives the normalized copy number (NCN):

$$NCN = \frac{PML\text{-}RARA_{CN}}{ABL_{CN}} \times 100\%$$

The result was reported as a percentage of the target fusion gene and the housekeeping gene. The result was considered invalid and not reported if the housekeeping gene was below a 1000 copies. The sensitivity of the assay was 10^{-3} after serial dilutions was run in duplicate with positive patient's sample. Positive and negative samples were determined by the percentage of the target gene divided by the housekeeping gene. A result of 0% was considered negative and a result greater than 0, 1% was considered positive also, a crossing point ($C_{P)}$ of >35 was rejected as a false positive and a C_P <10 was considered negative.

2.11.3 MRD value

The minimal residual disease (MRD) value was the ratio between the CG normalized expression of the FG in the follow-up $(FG_{CN}/ CG_{CN})_{FUP}$ and diagnostic samples $(FG_{CN}/ CG_{CN})_{DX.}$

$$\textbf{MRD value (MRDv)} = \frac{(FGCN/CGCN)_{FUP}}{(FGCN/CGCN)_{DX}}$$

2.12 Verification of PML-RARA qPCR assay

In the Europe against Cancer Program (EAC) 25 laboratories from 10 countries collaborated to establish a standardized protocol for Taqman-based real-time quantitative PCR (qPCR) analysis of the main leukemia-associated fusion genes. Four phases were scheduled: training, optimization, sensitivity testing and patient sample testing. There were three quality control

rounds on a large series of coded RNA samples, which enabled the final validation of the primer and probe sets for ALL, AML and CML (Gabert J *et al*, 2003).

All three breakpoints for the *PML-RARA* were evaluated. The only cell line available for testing was the NB4 which has a bcr1 *PML* breakpoint. For the bcr2 and bcr3 primer/probe sets diagnostic patient's bone marrow RNA was used. EAC data revealed that no significant difference was observed in *PML-RARA* bcr3 expression when comparing peripheral blood and bone marrow on paired samples. Overall the frequency of false positivity was 6.9% and was limited to individual laboratories were the (crossing threshold) Ct value in the false-positive well was always more than 30 and most of the time higher than 35. From the 96 samples tested there were no false-negatives for bcr1, 2 or 3 and the assay had a sensitivity of 10^{-3} (Gabert J *et al*, 2003).

2.13 Statistical Analysis

Statistical analysis of data was performed in consultation with a statistician from the department of Bioethics and Research, UKZN, Dr Benn Sartorius (PHD) (UKZN). All tests were performed using Strata 13. Non parametric Fisher's exact test and Kruskal Wallis test were used to analyze the data due to the sample size being small.

CHAPTER THREE

RESULTS

3.1 Results

3.1.1 Patient cohort

Figure 3.1 shows the breakdown of Acute Leukemia patients attending the Onco-Haem clinic at IALCH, KZN, South Africa from January 2007 to December 2009. A total of 91 acute leukemia patients were referred to IALCH from peripheral hospitals in KZN during that period. The patients were classified by clinical hematologists into the different types of leukemia's using the WHO (2016) classification as shown in Chapter 1, Figure 1.2. The incidence of APL was 25% at our hospital. The frequency of AML in this cohort was 69% (63 out of 91) and out of these, 35% (22 out of 63) were identified as APL (by differential morphology screening and flow cytometry). The remainder of the AML patients (40 out of 63) were adults with translocations other than the t (15; 17) (q22; q12) translocation, or children. One patient out of the 63 AML patients had a variant t (15; 17).

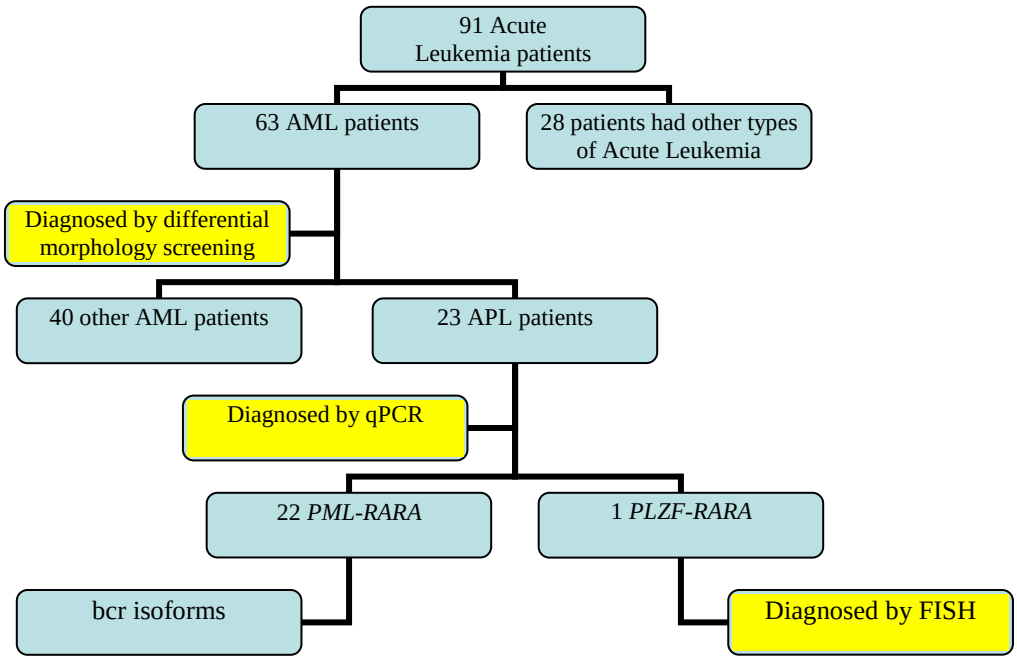
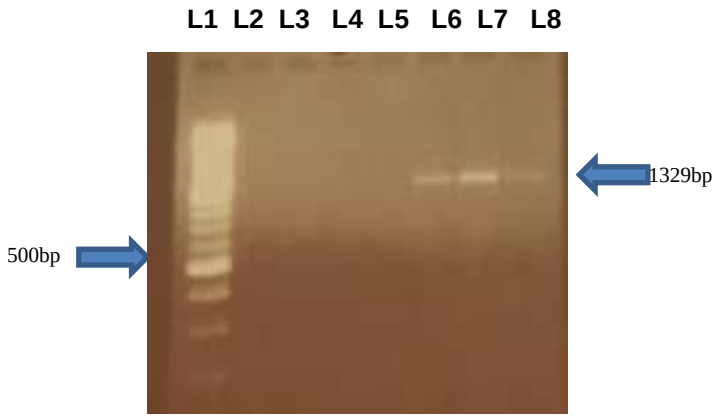


Figure 3.1: Diagram representing classification of Acute Leukemia patients visiting the Onco-Haem clinic from 2007 to 2009.

3.2 Optimization of In-house Nested RT-PCR

We attempted to develop an in-house RT-PCR assay, to determine the different bcr isoforms, based on the method described by van Dongen J J M *et al*,1999. Only three out of seven samples (Figure 3.2: lanes 6, 7 and 8) were amplifiable, producing a 1329bp product after the first round of PCR. Most of the samples that could not be detected had low concentrations of RNA (below 4ng/ul as shown in (Appendix 1)).



<u>Figure 3.2</u>: RT-PCR results from the first step of a nested PCR assay. Products were run on a 2% agarose gel. Lane 1 (L1) contained a 100bp ladder (Roche, SA). L2 to L8 had patient samples: 183, 189; 191, 197, 199, 202, and 203.respectively. L6 to L8 showed positive PCR products.

After a second round of amplification using internal primers to increase the sensitivity of the PCR, three out of seven samples were amplified producing a 381bp product. However, numerous non-specific bands were also seen as shown in Figure 3.3. This did not improve after the titration of the primer and Mg^{2+} concentration, or with the optimization of the annealing temperature.

Figure 3.3: RT-PCR results from the second round of nested PCR showing amplification of the positive products from the first round of PCR. L2, L8 and L15 were loaded with sample 199, 202 and 203 respectively.

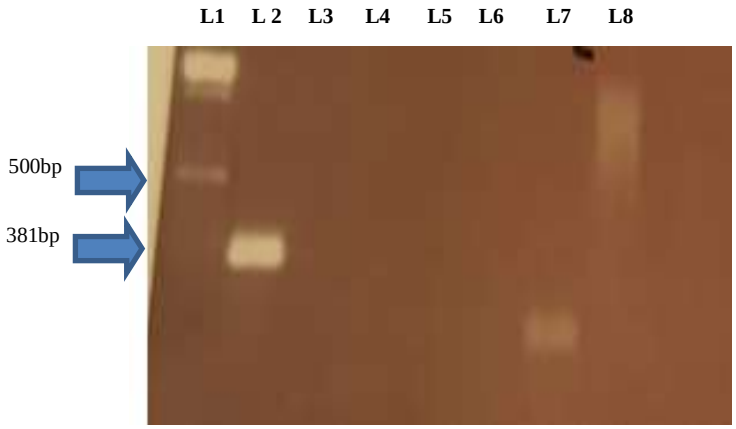


Figure 3.4: Sensitivity of the in-house RT-PCR assay. Lane 1 shows a 100bp ladder followed by serial dilutions of the NB4 cell lines in lanes 2 to 5 (from 10^7 copies to 10^4). The HL60 negative strain was run in Lane 6 and Lane 7.

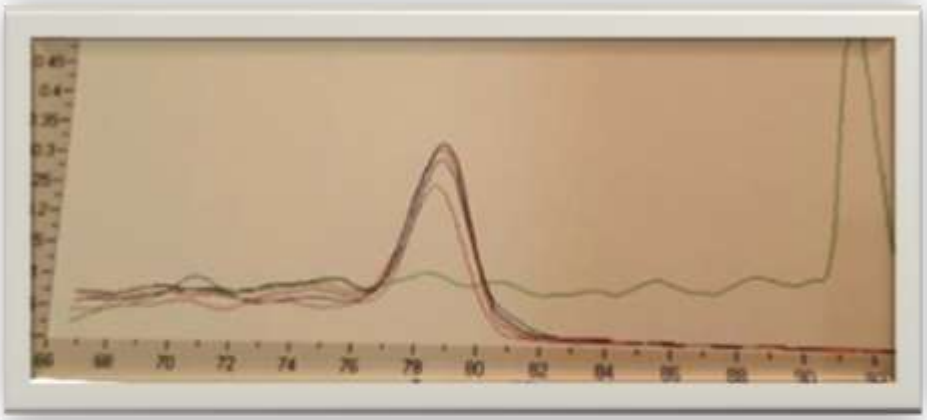
The sensitivity of the assay was also assessed using the NB4 cell line as a positive control (Figure 3.4). The NB4 cell line could only be detected at the highest concentration of 10^7 copies. No bands were seen for lower copy numbers. The HL60 cell line was used as a negative control.

PCR products were run on 2% agarose gel. The product was only visible at the undiluted concentration of the NB4 cell line showing that the PCR was not sensitive enough.

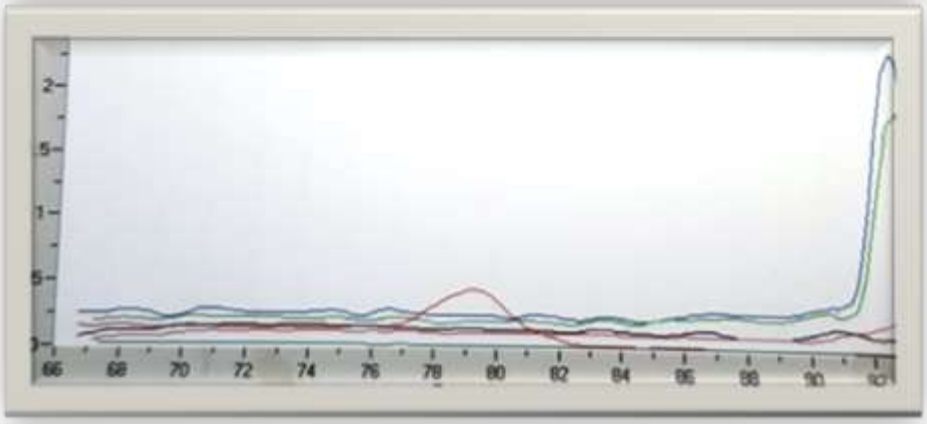
3.3 Optimization of In-house Real Time PCR on the LC 2.0

After numerous attempts at optimizing the in-house PCR assay, a new assay was developed on the Light Cycler 2.0 as a real-time PCR assay, as this technique was known for improved sensitivity and the generation of rapid results. The Fast Start Sybr Green kit (Roche; SA) was used to optimize the assay on the LC 2.0.

A



B



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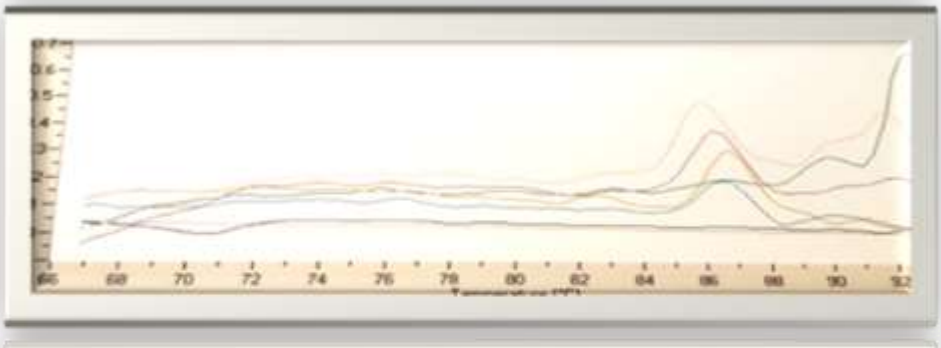
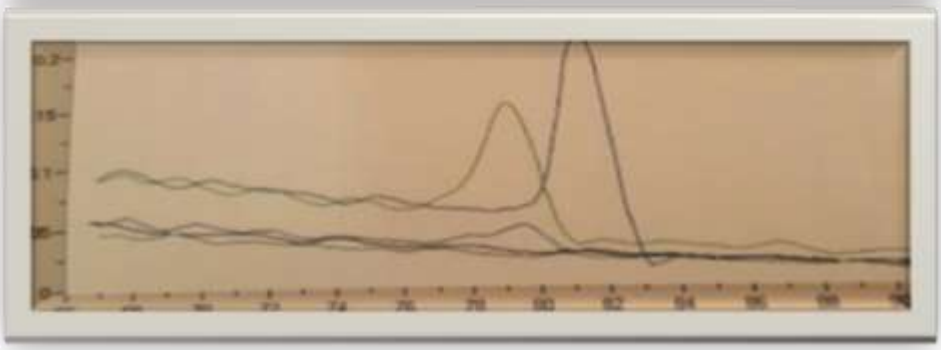


D

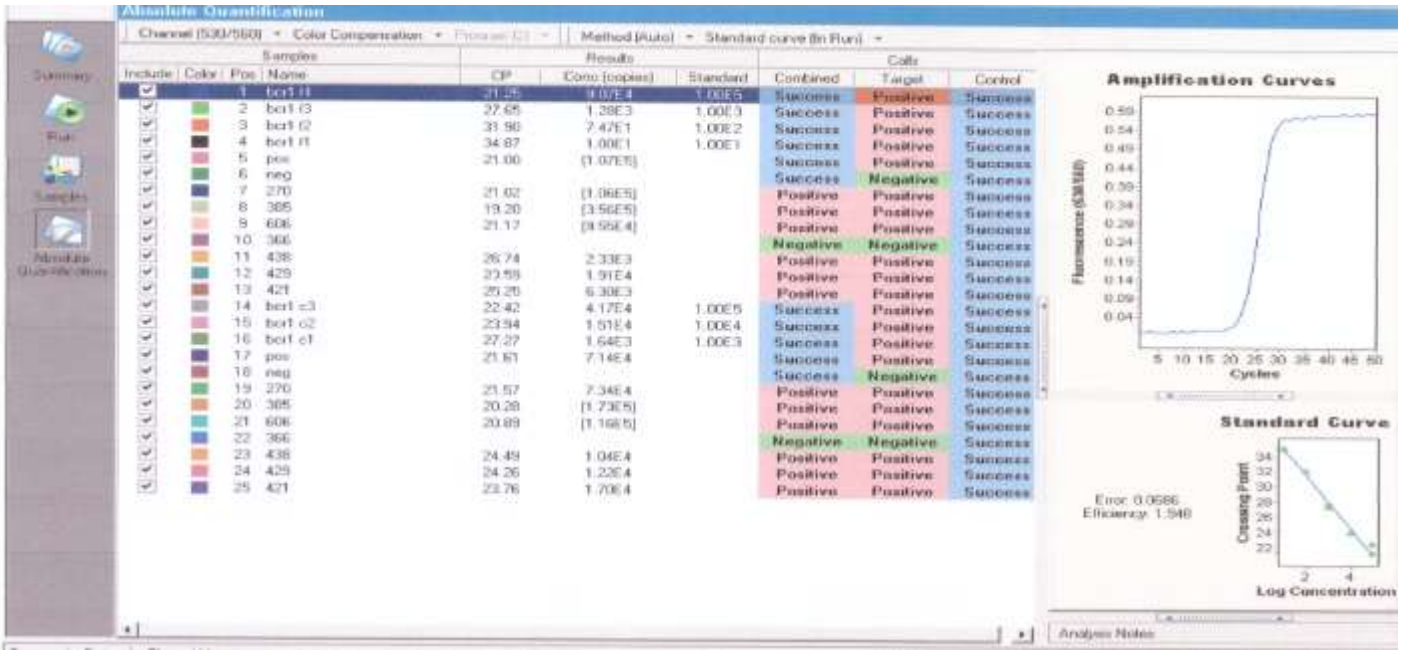


<u>Figure 3.5:</u> (A) LC 2.0 results showing optimization of annealing temperature. (B) LC 2.0 results showing $MgCl_2$ titration of a positive patient's sample. (C) LC 2.0 results showing primer titration of a positive patient's sample. (D) Serial dilutions of NB4 and HL60 cell lines run on LC 2.0 shows non-specific melt curve of 82^0C.

The annealing temperature was first titrated from 55 ^{o}C to 65 ^{o}C. Figure 3.5(A) shows the result of a positive sample that amplified at the correct melting temperature (Tm) of 92 ^{o}C when an annealing temperature of 65 ^{o}C (green) was used. The $MgCl_2$ was titrated from 3.0 mM to 1.5 mM. A concentration of 2.5 mM (shown in blue in Figure 3.5(B) was taken as optimum. Results for the primer titration are shown in Figure 3.5(C). The primers were titrated from 20 pmol/ul to 8 pmol/ul at intervals of 2 pmol/ul. The optimal primer concentration was found to be 8 pmol/ul (shown in black). Serial dilutions with the NB4 and HL60 cell line was unsuccessful as the PCR products failed to amplify at the correct melting temperature of 92 ^{o}C.

3.4 Ipsogen qPCR Results for PML-RARA bcr Isoforms of the Patients samples

Due to the in-house assays not being sensitive enough, a qPCR kit from Ipsogen (Barker Medical; France) was used to identify the PML-RARA bcr isoforms in APL patients with the t (15;17). In addition, the qPCR kit allowed for the quantitation of the transcripts which was helpful for the monitoring of MRD during patient follow-up after treatment. Serial dilutions (from 100 000 copies to 10 copies) of a plasmid standard of the PML-RARA bcr1 isoform was used to establish the standard curve for the target gene as shown in (Figure 3.6). Similarly, plasmid standards of the ABL gene (Barker Medical; France) with serial dilutions of a 100 000 copies to 10 copies were used to establish the standard curve for the housekeeping gene as shown in (Figure 3.6).



<u>Figure 3.6</u>: Results from the raw data of a bcr1 qPCR on the LC 2.0 which is tabulated in (Table 3.1). There are data sheets available for all patients in (Table 3.1). The efficiency of the PCR was 1.9. The positive, negative controls and the bcr1 standards all passed. The housekeeping gene for all the samples run was greater than 1000 copies.

One patient from the AML cohort presented with morphological and clinical characteristics of APL but did not have the *PML-RARA* transcript by qPCR. Subsequently, the variant *PML-RARA* transcript was detected by FISH in this patient, and was excluded from further analysis.

In the study 68% (15/22) peripheral bloods and 32% (7/22) bone marrow aspirates were received from patients started on ATRA and confirmed positive by qPCR t (15; 17) *PML-RARA (*Table 3.1).

Table 3.1: Demographic positive qPCR results of APL patients at diagnosis with bcr1 isoform (pink) and bcr3 isoform (blue) and bcr2 isoform (white)

PATIENT ID	LAB NO	SAMPLE	TARGET GENE PML- RARA/ABL	HOUSE KEEPING GENE - ABL	RATIO	%
1	385	BONE MARROW	356000	173000	2.06	205.8
2	443	BLOOD	3030	7450	0.41	40.67
3	429	BLOOD	19100	12200	1.57	156.6
4	490	BLOOD	250000	94500	2.65	264.6
5	438	BLOOD	2330	10400	0.22	22.4
6	1064	BONE MARROW	101000	83900	1.2	120.38
7	270	BLOOD	106000	73400	1.44	144.4
8	674	BLOOD	29400	4960	5.93	592.7
9	1069	BLOOD	62300	83900	0.74	74.25
10	606	BLOOD	95500	116000	0.82	82.33
11	366	BONE MARROW	3390	4480	0.76	75.67
2	1859	BLOOD	24800	390000	0.06	6.36
13	421	BONE MARROW	6300	17000	0.37	37.06
14	1996	BLOOD	11500	5970	1.93	192.63
15	1664	BLOOD	73300	60300	1.2	121.56
16	1772	BLOOD	7030	27400	0.3	25.66
17	319	BONE MARROW	1470	73100	0.02	2.01
18	306	BONE MARROW	21200	39500	0.54	53.67
19	581	BLOOD	24300	3340	7.28	727.5
20	1529	BLOOD	106000	132000	0.8	80.3
21	374	BONE MARROW	10700	63100	0.17	16.96
22	2065	BLOOD	739	4070	0.18	18.16

All the housekeeping genes were over 1000 copies which showed good quality of the sample (a value below 1000 copies indicated degradation of the RNA).

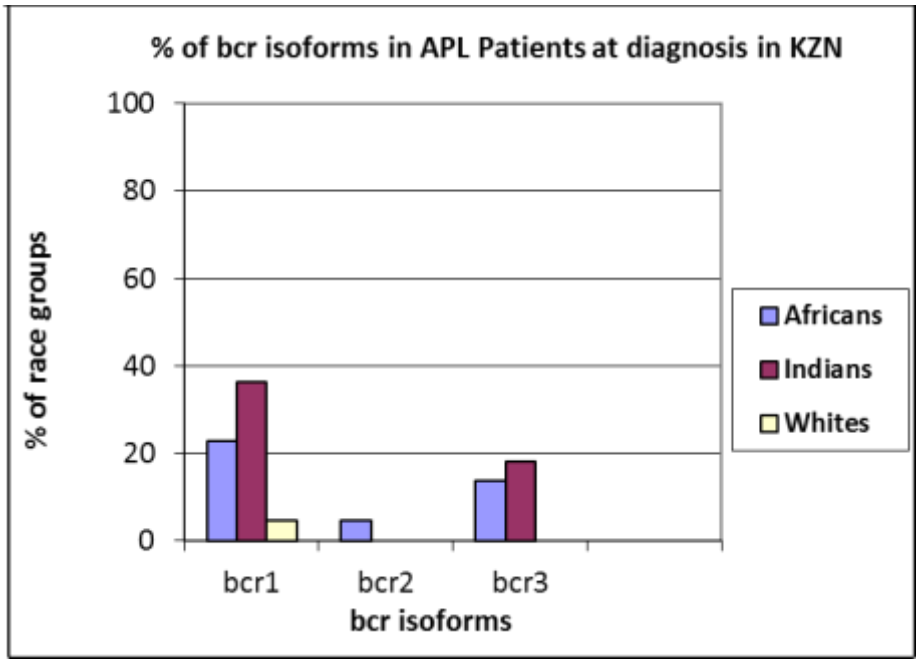


Figure 3.7: Bar graph showing qPCR results of APL patients at diagnosis from data in (Table 3.1)

The frequency of PML-*RARA* (L) bcr1 isoform in 22 positive patients was (1/22) 4,5% in Whites; (8/22) 36,4% in Indians and (5/22) 7% in Africans. PML-*RARA* (V) bcr2 isoform was (1/22) 4,5% and PML-*RARA* (S) bcr3 isoform was (4/22) 18,2% in Indians and (3/22) 13;6% in Africans (as shown in Figure 3.7). The Indian population showed predominance in the bcr1 and bcr3 isoforms while the bcr2 had only one African positive patient.

3.5 Clinical and Hematological Parameters

The 22 newly diagnosed adult APL patients were followed up for two years. A summary of their patient characteristics are given in Table 3.2. Clinical features and hematological parameters such as Hb, WCC, and platelet count (Table 3.5) were used to stratify patients into different prognostic groups (as described in section 2.1).

Table 3.2: Summary of patient's hematological parameters

No. of patients	22
M:F ratio	1:1
Median Age	30 years (Range 21-68 years)
Median Hb	7.5g/dl (Range 4.5-11.4 g/dl)
Median Aptt	23.4 seconds (Range 25-40 seconds)
Median D-dimer	4.04 ug/ml (Range 2.5-16.0 ug/ml) (Normal value <0.25 ug/ml)
Median Fibrinogen	3.0 g/l (Range 1.0-6.9 ug/ml) (|Normal value 1.5-4.5 g/l)
Median INR	1.15 (Normal value <1.2)
Median Platelet Count	45 $x10^9$/l (Range 4.6-430 $x10^9$/l) Normal value (171-454 $x10^9$/l)
Median WCC	5.0 $x10^9$/l (Range 0.83-29 $x10^9$/l) Normal value (3.90-12.6 $x10^9$/l)
Low risk group	1 patient
Intermediate risk group	15 patients
High risk group	6 patients

As shown in Table 3.2, there was an equal male to female ratio in the 22 patients. The median age group for our cohort was 30 years of age. Majority of the patients had a low platelet count with a median of 45 $x10^9$/l and fell into the intermediate risk group. Six patients were high risk according to the risk stratification explained in section 2.1. The median Hb was 8.0g/dl, the median white cell count was 5.0 $x10^9$/l and the median D-dimer 4.04 ug/ml.

Table 3.3: Patients hematological parameters at diagnosis compared to results after treatment

PATIENT ID	DATE	DIAGNOSIS		DATE	RESULT AFTER TREATMENT	
		WCC	PLTS		WCC	PLTS
1	2/27/2007	29	4.55	08/02/2007	7.32	8.6
2	5/2/2007	13.7	11	05/03/2007	demised	demised
3	4/17/2007	2.51	9.2	10/09/2007	3.03	176
4	7/5/2007	3.89	54	07/08/2007	4.85	109
5	4/24/2007	4.93	216	08/14/2007	6.23	237
6	6/19/2008	1.44	37	07/23/2008	2.11	213
7	9/19/2006	8.31	36	10/02/2006	4.04	46
8	11/22/2007	7.7	177	01/30/2008	7.36	190
9	6/23/2008	0.83	92	07/14/2008	6.30	134
10	9/19/2007	0.84	35	12/18/2007	6.30	134
11	1/23/2007	2.72	266	03/14/2007	8.96	273
12	6/19/2009	20.7	34	07/02/2009	10.39	15
13	4/10/2007	6.7	285	05/28/2007	3.99	178
14	8/26/2009	3.22	109	09/01/2010	3.49	220
15	3/26/2009	1.31	53	04/27/2009	3.49	192
16	05/13/2009	1.54	17	06/19/2009	6.75	242
17	11/16/2006	13.84	33	01/02/2007	7.34	283
18	02/11/2006	7.77	21	03/11/2006	3.13	46
19	8/28/2007	9.33	296	09/25/2007	9.10	332
20	2/5/2009	0.9	31	03/02/2009	10.05	315
21	2/7/2007	3.21	315	03/08/2007	5.77	241
22	9/28/2009	6.04	430	10/27/2009	4.69	375

Most of the patients in all isoforms presented with pancytopenia and thrombocytopenia as shown in Table 3.3. After treatment with ATRA all patients (Table 3.3) showed corrected WCC and increased platelet counts which is a significant prognostic indicator.

Table 3.4: Summary of statistical analysis

Statistic Analysed	p-value
bcr isoform compared to the patients that died	1.000
Patients died by HIV status	1.000
Bcr isoform compared by race	0.870
bcr isoform compared by gender	0.814
bcr isoform compared by age	Significant difference by age 0.03
bcr isoform compared by WCC/Plts	no significance 0.9274

There was no significant association of the bcr isoforms with statistical data analyzed such as, HIV-1 infection, race, gender and WCC and platelets (Table 3.4) by the Fisher's exact test. However, there was a significant association between the patient's age and bcr isoforms with a probability of 0.03 (Table 3.4) based on the Kruskal Wallis test. Bcr1 and bcr2 isoforms were significantly associated with younger patients (20-30 years), while the bcr3 isoform (was associated with older patients (> 40 years), with a significance of $p<0.05$.

3.6 Bcr Isoforms and treatment outcome:

Knowledge of bcr isoforms by qPCR provides independent prognostic information for treatment stratification (Table 3.5 and Table 3.6). In this study, qPCR was used to monitor patients for MRD (i.e. relapse or remission). The patients were listed in Table 3.5 and 3.6 in order of their follow-up dates to the Onco-Haem Clinic at IALCH. The majority of the patients completed their induction and maintenance as per treatment guidelines stipulated in section 2.4.1. A few (n=5) of the patients had to have alternate individualized therapy depending on the adverse effects reported. As part of patient management, APL patients are closely monitored by clinical hematologists to prevent relapse and minimize adverse side effects of the chemotherapy by individualizing treatment for each patient as stipulated in Tables 3.5 and 3.6.

Table 3.5: APL Patients with bcr1 isoform – presenting clinical features and treatment regimen

Pt ID	HIV	Hb (13-17)	Plt (150-400)	PROGNOSIS	WCC (4-10)	INDUCTION	CONSOLIDATION	MAINTENANCE	STATUS	COMMENT
7	Neg	8.2	26	Intermediate	6.26	completed	completed	completed	lost to f/up	HIV later
13	Neg	11.4	15	Intermediate	3.77	completed	completed	completed	remission	
1	Neg	7.8	<10	Intermediate	<4	completed	completed	not completed-hypoplastic-ATRAx3	remission-no neurological deficits	intracranial bleed
10	Pos	8.3	29	Intermediate	0.69	Daunorubicin	Idarubicin x3 -Mitoxantrone not available	completed	remission-well	relocated to JHB 2011
4	Neg	4.5	7	Intermediate	4.97	Daunorubicin +ATRA	Idarubicin x3	completed	remission-well	continues to follow-up
3	Neg	8.5	38	Intermediate	3.08	Daunorubicin +ATRA	Idaubicin, Mitoxantrone and Idarubicin	completed	remission-well	continues to follow-up
12	Neg	8	34	poor	22.48	Daunorubicin +ATRA	Idaubicin, Mitoxantrone and HIDAC	completed	remission-well	continues to follow-up
5	Pos	8.8	36	Intermediate	9.56	Daunorubicin +ATRA	Idarubicin x3 and ATRA	completed	remission-well	continues to follow-up
9	Neg	9.4	92	good	0.83	Daunorubicin +ATRA	Idaubicin, Mitoxantrone and Idarubicin	completed	remission-well	continues to follow-up
2	Neg	6.3	11	Intermediate	13.7	died within 24 hours of	N/A	N/A	demised	N/A
6	Neg	7.4	14	Intermediate	1.4	Daunorubicin +ATRA	defaulted follow up	N/A	demised	N/A
8	Neg	7.7	16	Intermediate	1.38	Daunorubicin +ATRA	idarubicin x3, Mitoxantrone and HD cytarabine	completed	remission-well	continues to follow-up
11	Neg	8.5	20	Intermediate	3.4	Daunorubicin +ATRA	idarubicin x3, Mitoxantrone and HD cytarabine	completed	remission-well	continues to follow-up
14	Neg	9.1	21	poor	0.76	Daunorubicin +ATRA	Etoposide and Cytarabine	not in remission	lost to f/up	

Table 3.6. APL Patients with bcr 3 isoform- presenting clinical features and treatment regimen

Pt ID	HIV	CD4	Hb	Plt	WCC	PROGNOSIS	INDUCTION	CONSOLIDATION	MAINTENANCE	STATUS	COMMENT
18	NEG	0	6.2	53	10.32	poor	Daunorubicin + ATRA	N/A	N/A	demised-post 2 days of	N/A
20	POS	290	8.1	13	2.24	intermediate	Daunorubicin + ATRA	Idarubucin-Mitixantrone + Idarubicin	completed-2yrs	Alive-well in remission	N/A
15	NEG	0	7.6	53	1.3	poor	Daunorubicin + ATRA Mitoxanthrone/ATR	Idarubicin high risk dose 4 days/ATRA-HIDAC/ATRA	completed	Alive-well in remission	N/A
19	NEG	0		296	9.33	intermediate	Daunorubicin + ATRA	Mitoxanthrone/ATRA-Daunorubicin and cytosar daunoruicin	completed	Alive-well in remission	N/A
16	NEG	0	9	21	2.1	intermediate	Daunorubicin + ATRA	Daunorubicin, cytarabine and ATRA- Idarubicin-HIDAC-Idarubicin	completed	Alive-well in remission	Intracranial bleed
17	NEG	0	5.2	33	73	poor	Daunorubicin + ATRA	Idarubicin/ATRA-x3 cytarabine	none	Alive-well in remission	N/A
21	NEG	0	8.8	20	4.6	intermediate	Daunorubicin + ATRA	molecular relapse mioxanthrone and cytarabine	completed-2yrs	Alive-well in remission	N/A

CHAPTER FOUR

DISCUSSION

4.1 DISCUSSION

Lack of population–based registries in developing countries has made it difficult to determine the real incidence of APL in countries like Africa, Latin America and India. This is due to the lack of well-structured networks and the accrual of large number of patients. The number of samples is limited as APL is a subordinate, sub-class of AML which is further classified into the different bcr isoforms. In 2004, the International American Society of Hematology (ASH) proposed the creation of a group, the International Consortium of APL (IC-APL) for collaboration of data in developing countries (Niemeyer C *et al*, 2006).

Inkosi Albert Luthuli Central Hospital is the only referral center for all acute leukemia patients in KZN, as only chronic leukemia patients are seen at other peripheral hospitals. It has been reported in a study by (Herbst M C, 2015) that approximately 25% of acute leukemia patients are AML and from this 10% are diagnosed with APL in South Africa The prevalence of APL was found to be higher, 36,5% of AML's compared to other South African and South American studies. Rego and coworkers, (2013) have reported that APL accounts for 28,2% of all AML cases in centers in Brazil. This information is ratified by other studies, Mexico (20%), Peru (22%) and Venezuela (27,8%) (Rego E M *et al*, 2013). However, this is much higher than reported cases found in Eastern countries. In a study in India it was found that APL accounts for 10% of AML cases in adults (Bajpai J *et al*, 2011; Sazwal S *et al*, 2009 and Dutta *et al*, 2000).

Molecular techniques such as qPCR have made it possible to diagnose and quantify the leukemia burden of APL patients according to their *PML-RARA* bcr isoforms. This is important for MRD monitoring which is pivotal for individualized treatment regimens and molecular targeted

therapy such as ATRA which tends to induce terminal differentiation of the *PML-RARA* transcript.

In this study bcr1 was found to be the predominant *PML-RARA* isoform by qPCR which is in keeping with studies published by western countries (Douer D *et al*, 2003; Lo Coco F *et al*, 2003 and Grimwade D *et al*, 1998). This was also the main isoform found in our South African cohort, who was mainly of Indian and African ethnic group due to the population demographics found in KZN.

It has been reported that patients with the bcr3 isoform generally have a poor prognosis (Slack J L *et al*, 2000; Chatterjee T *et al*, 2014). The bcr3 is associated with a shorter remission duration and overall survival compared to the other isoforms (Gonzalez M *et al*, 2001; Sanz M A *et al*, 2000). In the study of Chatterjee T *et al*, (2014) five patients died during the induction phase, three of which had the bcr3 isoform. Due to the large Indian population in our cohort (12 out of 22 patients) we compared our findings to the studies in India. We did not find an over-expression of the bcr3 isoform in our Indian patients as compared to studies from India (Sazwal S *et al*, 2009 and Dutta *et al*, 2000).

Although KZN, South Africa has a high African and Indian population, our study is comparable to findings from other studies in the West where the predominant population is white. Therefore, we can conclude that the ethnic group of the patient does not influence the bcr isoform or the prognosis; rather this may be due to geographic or environmental factors and socio-economic conditions.

There were no reports of the unusual features of gum hypertrophy in our patients which was seen in the other studies in India (Chatterjee T *et al*, 2014). This study did not show an association between the bcr3 isoform and high WCC and the *PML-RARA* variant bcr2 isoform and high WCC at presentation like the other studies (Sazwal S *et al*, 2009). The patients are significantly younger in our cohort as previous studies have reported a median age of 45 (Chatterjee T *et al*, 2014). This could have attributed to the overall wellness of our patient cohort and the majority of patients responding positively to treatment.

Only one patient was diagnosed with the variant bcr isoform, also called bcr2. This is usually associated with a poor prognosis; however, after standard treatment of induction with ATRA and Daunorubicin and then consolidation twice with Idarubicin and ATRA, He was reported to be well after 23 months of treatment.

One patient diagnosed with the variant *PLZF-RARA* rearrangement using FISH was excluded from the qPCR isoform analysis. The *PLZF-RARA* rearrangement has been reported to have atypical APL morphology, such as regular nuclei and hypo or microgranular cells, abundant cytoplasm, more condensed chromatin pattern in blasts, absence of auer rods and increased number of Pelgeroid neutrophils (Sainty D *et al*, 2000). However, our patient did not show this atypical morphology, as the slides presented with normal granules and auer rods. The APL variant form although rare, offers a challenge to its early recognition which can be achieved through careful morphological analysis, clinical presentation, cytogenetic tests and FISH (Sandra S R, 2011). From a therapy point of view, usually t (11; 17) strongly blocks differentiation, so

the *PLZF-RARA* variant is characterized by poor response to retinoids as a single agent and also to arsenic trioxide (Mistry A R, 2003). Conversely, a number of studies have suggested that this subset of APL is not completely resistant to differentiation and therefore would respond to ATRA. In this study, the single patient with the PLZF variant was ATRA responsive achieved a short remission, after which the patient relapsed and thereafter demised (Lo Coco F *et al*, 1998; Bajpai J *et al*, 2011).

One patient was positive for all three isoforms bcr1, 2 and 3. However, this was not confirmed by sequencing. There are no other reported cases of patients with all three bcr isoforms worldwide. A larger cohort of samples needs to be investigated to determine if this is common in KZN. Despite an initial intracranial bleed this patient responded to individualized therapy.

Abnormal results were observed for all hematological parameters for the majority of patients. The most significant characteristic observed in our cohort of patients was a median platelet count of 45. This low platelet count can be attributed to the DIC observed in majority of these patients which is consistent with clinical features of APL. In previous studies of cytotoxic chemotherapy, early deaths during induction in patients with APL occurred primarily as a consequence of intracranial hemorrhage (Breccia M *et al*, 2010; Park J H *et al*, 2011; Bai H E *et al*, 2013.

There was no significant correlation between the *PML-RARA* isoforms and hematological parameters with response to treatment (p=1.000). While this finding is similar to previous studies (Grimwade D *et al*, 1996; van Dongen JJM *et al*, 1999; Gabert J *et al*, 2003), a limitation of this study is that the sample size may not have the power to detect such a relationship.

Minimal residual disease monitoring with qPCR for the detection of the *PML-RARA* transcript is known to be crucial for APL patients as it identifies the risk of relapse and predicts long–term remission (Mandelli F *et al*, 1997). The overall survival of our patients was 90.9% and it was found that 72.7% (16 out of 22 patients) had a disease free survival. At present three patients have demised and three have been lost to follow up but were well and in remission up until 2011. Presently sixteen patients remain well and in remission (Unpublished communication, S.Parsanath).

While long-term prognosis of APL in the setting of HIV-1 is currently unknown, the two APL patients with HIV in our study (one bcr1 and one bcr3) responded well to ATRA and chemotherapy while on anti-retroviral therapy and remain well and in remission, suggesting that APL patients with HIV do not necessarily have a poor survival rate. This compares well with complete remission and long term survival rates of 70-95% in uncomplicated APL. Other studies have also found that remission can be successfully induced and maintained with standard therapy and little interference of HAART (Sutton L *et al*, 2001; Boban A *et al*, 2009). Although $CD4^+$ cell counts were reduced during chemotherapy, this increased once treatment was completed, and the patients did not suffer any AIDS-associated complications (Kudva G C et al, 2004).

4.2 Conclusion

A higher prevalence (36,5%) of APL was found in our cohort of AML patients during the study period as compared to other studies. The bcr1 (63,6%) was found to be the predominant isoform in our population which is in accordance with reports from western countries (Grimwade D *et al*, 1996; van Dongen JJM *et al*, 1999). Our study confirmed other published data, (Vahadat L *et al*, 1994; Sazwal S *et al*, 2009; Chatterjee T *et al*, 2014) which have reported the bcr3 isoform to have poor prognosis and overall survival in APL patients.

Although it is a small group of patients, we can conclude from the statistical analysis of our patients that the good response to treatment across all isoforms maybe attributed to the majority of young adults (below 35 years) in our cohort who may tolerate the aggressive treatment better than their older counterparts. There are no previous reports on the significance of age in APL patients and the treatment outcomes; therefore further studies on this finding are needed.

There was no significant correlation between different hematological parameters and response to treatment. We have found no difference in prognosis and treatment outcomes in our HIV-infected and non-infected APL patients. The *PML-RARA* bcr subtype also had no effect on achieving remission earlier in HIV infected APL patients in our study. The HIV positive patients responded well to treatment with no adverse effects. Due to the limited number of HIV positive APL patients which was also found in other studies (Sutton L *et al*, 2001) we could not establish a conclusive result on the effect of the bcr isoform on treatment response in HIV patients.

Patients that remain without a detectable level of *PML-RARA* transcripts remain well and in remission for many years while patients that had detectable levels of *PML-RARA* transcripts had poor treatment outcomes. Quantitative Real Time PCR monitoring of MRD patients is a useful diagnostic tool to monitor APL patients throughout their treatment for hematological and molecular relapse.

4.3 Future Work

Prospective studies, on a larger cohort of patients are needed to confirm unique and novel findings in the molecular pathogenesis of APL patients. A correlation between other diagnostic methods such as immunophenotyping, FISH and qPCR at diagnosis and during MRD will be interesting to investigate. For qPCR to be used as an effective diagnostic tool for the detection of *PML- RARA* transcripts, standardization of the housekeeping gene with regards to the expression level will be required so that it is possible to monitor the low *PML-RARA* copy number patients as in the Chronic Myeloid Leukemia patients. Further studies with a larger cohort are required to determine if the FLT3 is predominant in the bcr3 isoform and if it contributes to a poor prognosis in APL patients (Sazwal *et al*, 2009).

CHAPTER FIVE

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CHAPTER SIX

APPENDICES

Appendix 6.1: RNA concentrations for Figure 3.2

Plots | Report

Test type: Nucleic Acid | 01/02/2017 9:20 AM | Exit

Report Name | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
MRNA 108	Default	01/02/2017	9:14 AM	3.09	0.077	0.033	2.32	0.48	40.00	230	0.161	0.016
MRNA 136	Default	01/02/2017	9:15 AM	3.56	0.089	0.055	1.61	0.29	40.00	230	0.307	0.063
MRNA 199	Default	01/02/2017	9:16 AM	4.96	0.124	0.083	1.49	0.07	40.00	230	1.789	0.014
MRNA 197	Default	01/02/2017	9:17 AM	0.19	0.005	0.006	0.81	0.01	40.00	230	0.929	0.017
MRNA 191	Default	01/02/2017	9:18 AM	2.06	0.051	0.055	0.93	0.04	40.00	230	1.458	0.017
MRNA 203	Default	01/02/2017	9:19 AM	4.76	0.119	0.083	1.44	0.23	40.00	230	0.514	0.106
MRNA 202	Default	01/02/2017	9:20 AM	12.08	0.302	0.192	1.57	0.18	40.00	230	1.689	0.297

Appendix 6.2: Raw Data for Statistical Analysis Results

No significant difference in died outcome by PCR result using the non-parametric Fishers exact test:

```
. tab pcrresultcleaned died, row exact

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

PCR RESULT |          died
   CLEANED |         0          1 |     Total
-----------+----------------------+----------
      BCR1 |        12          2 |        14
           |     85.71      14.29 |    100.00
-----------+----------------------+----------
      BCR2 |         1          0 |         1
           |    100.00       0.00 |    100.00
-----------+----------------------+----------
      BCR3 |         6          1 |         7
           |     85.71      14.29 |    100.00
-----------+----------------------+----------
     Total |        19          3 |        22
           |     86.36      13.64 |    100.00

           Fisher's exact =                 1.000
```

No significant difference in died outcome by HIV status using the non-parametric Fishers exact test:

```
. tab HIV died, exact row

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |          died
       HIV |         0          1 |     Total
-----------+----------------------+----------
         0 |        17          3 |        20
           |     85.00      15.00 |    100.00
-----------+----------------------+----------
         1 |         2          0 |         2
           |    100.00       0.00 |    100.00
-----------+----------------------+----------
     Total |        19          3 |        22
           |     86.36      13.64 |    100.00

           Fisher's exact =                 1.000
   1-sided Fisher's exact =                 0.740
```

No significant difference in BCR by race using the non-parametric Fishers exact test:

```
. tab RACE pcrresultcleaned , row exact

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |        PCR RESULT CLEANED
      RACE |      BCR1       BCR2       BCR3 |     Total
-----------+---------------------------------+----------
     ASIAN |         8          0          4 |        12
           |     66.67       0.00      33.33 |    100.00
-----------+---------------------------------+----------
     BLACK |         5          1          3 |         9
           |     55.56      11.11      33.33 |    100.00
-----------+---------------------------------+----------
     WHITE |         1          0          0 |         1
           |    100.00       0.00       0.00 |    100.00
-----------+---------------------------------+----------
     Total |        14          1          7 |        22
           |     63.64       4.55      31.82 |    100.00

           Fisher's exact =                 0.870
```

No significant difference in BCR by gender using the non-parametric Fishers exact test:

```
. tab GENDER pcrresultcleaned , row exact

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

           |        PCR RESULT CLEANED
    GENDER |      BCR1       BCR2       BCR3 |     Total
-----------+---------------------------------+----------
         F |         6          0          4 |        10
           |     60.00       0.00      40.00 |    100.00
-----------+---------------------------------+----------
         M |         8          1          3 |        12
           |     66.67       8.33      25.00 |    100.00
-----------+---------------------------------+----------
     Total |        14          1          7 |        22
           |     63.64       4.55      31.82 |    100.00

           Fisher's exact =                 0.814
```

Additional summary of continuous variables to include median and interquartile range (p25-p75):

```
. tabstat AGE WBC PLTS, stats(n mean sd median p25 p75 min max) col(stats)

    variable  |          N       mean         sd        p50        p25        p75
min       max
-------------+--------------------------------------------------------------------------------
---------
         AGE  |         22   33.77273   12.50498         31         24         43
21        68
```

```
          WBC  |         22       6.93    7.07173      4.965       1.54       8.31
.83        29
         PLTS  |         22   117.3977   127.6404         45         31        237
4.55       430
-------------------------------------------------------------------------------
--------

. tabstat AGE WBC PLTS, stats(n mean sd median p25 p75 min max) col(stats) by(
pcrresultcleaned) long

pcrresultcleaned      variable   |          N       mean          sd        p50
p25       p75       min       max
------------------------------+------------------------------------------------
-------------------------
BCR1                        AGE  |         14   28.78571   7.297824       27.5
22        34        21        43
                            WBC  |        14  7.751429  8.203698      5.06      2.72
8.31       .83        29
                           PLTS  |         14   99.05357   100.2496       45.5
34       177      4.55       285
------------------------------+------------------------------------------------
-------------------------
BCR2                        AGE  |          1         25          .         25
25        25        25        25
                            WBC  |         1      6.04          .      6.04      6.04
6.04      6.04      6.04
                           PLTS  |          1        430          .        430
430       430       430       430
------------------------------+------------------------------------------------
-------------------------
BCR3                        AGE  |          7         45   14.62874         44
30        55        25        68
                            WBC  |         7  5.414286  4.982766      3.21      1.31
9.33        .9     13.84
                           PLTS  |          7   109.4286    134.541         33
21       296        17       315
------------------------------+------------------------------------------------
-------------------------
Total                       AGE  |         22   33.77273   12.50498         31
24        43        21        68
                            WBC  |        22      6.93   7.07173     4.965      1.54
8.31       .83        29
                           PLTS  |         22   117.3977   127.6404         45
31       237      4.55       430
-------------------------------------------------------------------------------
-------------------------
```

Significant difference in age by BCR based on Kruskal Wallis test (non-parametric):

```
. kwallis AGE, by( pcrresultcleaned)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | pcrres~d | Obs | Rank Sum |
  |----------+-----+----------|
  |     BCR1 |  14 |   127.00 |
  |     BCR2 |   1 |     8.00 |
  |     BCR3 |   7 |   118.00 |
  +---------------------------+
```

```
chi-squared =     7.013 with 2 d.f.
probability =     0.0300

chi-squared with ties =     7.057 with 2 d.f.
probability =     0.0294
```

No significant difference in WCC/PLT by BCR based on Kruskal Wallis test (non-parametric):

```
. kwallis WBC , by( pcrresultcleaned)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | pcrres~d | Obs | Rank Sum |
  |----------+-----+----------|
  |     BCR1 |  14 |   166.00 |
  |     BCR2 |   1 |    12.00 |
  |     BCR3 |   7 |    75.00 |
  +---------------------------+

chi-squared =     0.151 with 2 d.f.
probability =     0.9274

chi-squared with ties =     0.151 with 2 d.f.
probability =     0.9274

. kwallis PLTS , by ( pcrresultcleaned)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | pcrres~d | Obs | Rank Sum |
  |----------+-----+----------|
  |     BCR1 |  14 |   156.00 |
  |     BCR2 |   1 |    22.00 |
  |     BCR3 |   7 |    75.00 |
  +---------------------------+

chi-squared =     2.759 with 2 d.f.
probability =     0.2516

chi-squared with ties =     2.759 with 2 d.f.
probability =     0.2516
```

Appendix 6.3 Raw data for graph in Figure 3.7

Type of Isoform	% Africans	% Indians	% Whites
bcr1 (14)	22.7	36.4	4.5
bcr2 (1)	4.5	0	0
bcr3 (7)	13.6	18.2	0

Appendix 6.4 List of reagents and consumables

Reagent/Consumable	Catalogue no.	Supplier
Promex 18G needles	NHN-18-1.5	Scientific Group
Trizma Base	93352	Capital Labs
Promex syringes	LPFMSYR-010-LYS	Scientific Group
PCR nucleotide mix	C1141	Anatech
Trizol reagent	ISS96018	Lifetech
RNAse ZAP	R2020	Capital Labs
Propanol MB grade	19516	Monitoring and Control
Ethanol MB grade	437433T	Monitoring and Control
Chloroform-UNIVAC	1.0244	Merck
EL Buffer	1014614	Whitehead Scientific
Superscript III	1880-044	Whitehead Scientific
Random Hexamers	C1181	Anatech
RNAsin+RNAse inhibitor	N2611	Anatech
Trisure Bio	38032	Celtic Diagnostics
LC Capillaries	119093390001	Roche
LC Taqman Master	04735536001	Roche
PPF BCR1	1071785	Whitehead Scientific
PPC BCR1	1071652	Whitehead Scientific
PPF BCR2	1071801	Whitehead Scientific
PPC BCR2	1071652	Whitehead Scientific
PPF BCR3	1071802	Whitehead Scientific
PPC BCR3	1071652	Whitehead Scientific
Gel Red	41003	Anatech
Metaphor Agarose	50180	Whitehead Scientific
Trizma base	93352	Capital ab
EDTA	E4884	Capital lab
Lightcycler 2.0 (t15;17) kit	40-0135-16	Roche
Boric acid	B6768	Capital lab