Genotypic and expression analysis of *CYP3A4* and *CYP3A5* in patients with chronic myeloid leukaemia

By

Gaynor Gillian Thompson

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Department of Haematology and Cell Biology

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Study Promoter: Prof. C.D. Viljoen

DECLARATION

I hereby certify that the dissertation submitted by me for the M.Med.Sc (Molecular Biology) degree at the University of the Free State is my independent effort and has not previously been submitted for a degree at another university or faculty. I furthermore, waive copyright of the dissertation in favour of the University of the Free State.

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Gaynor Thompson

I dedicate this dissertation to my father

M.D. Thompson

(1960-1999)

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I Can Do All Things Through Christ Who Strengthens Me.

Philippians 4:13

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

3’	3 prime
5’	5 prime
A	Alpha
B	Beta
℃	Degree Celsius
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar
%	Percentage
A	Adenine
ABL	Abelson gene
ADRs	Adverse drug reactions
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BCR	Breakpoint cluster region gene
C	Cytosine
c-abl	Normal *ABL* gene
cDNA	Complementary DNA
CML	Chronic myeloid leukaemia
c-kit	Cytokine receptor

CTAB	Cetyltrimethylammonium bromide
C_T	Threshold cycle
CYP450	Cytochrome P450
CYP3A4	Cytochrome 3A4 enzyme
CYP3A5	Cytochrome 3A5 enzyme
Da	Dalton
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
ELN	European LeukemiaNet
et al.	*Et alia* (and others)
ETOVS	Ethics committee of the Faculty of Health Sciences of the Free State
FAM	Fluorescein amidite
G	Guanine
g	Gram
GUS	β-Glucuronidase gene
HRM	High resolution melting
IRIS	International randomized study of Interferon versus STI-571
L	Litre
mg	Milligram
$MgCl_2$	Magnesium chloride
Ml	Millilitre
mM	Millimolar

mRNA	Messenger ribonucleic acid
NCCN	National Comprehensive Cancer Network
NH_4Cl	Ammonium chloride
NH_4HCO_3	Ammonium bicarbonate
Ng	Nanogram
Nm	Nanometre
P value	Probability value
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
pH	Percentage hydrogen
Ph	Philadelphia
Ph^+	Philadelphia chromosome positive
POP 7	Performance optimized polymer 7
qRT-PCR	Quantitative real-time polymerase chain reaction
rpm	Revolutions per minute
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
T	Thymine
TAE	Tris-acetate-EDTA
TAMRA	Tetramethylrhodamine
TE	Tris EDTA
TKIs	Tyrosine kinase inhibitors
Tm	Melting temperature
Tris	Tris hydroxymethyl aminomethane
UV	Ultraviolet

V	Volts
www	World wide web
WT	Wild type

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PREFACE

Chronic myeloid leukaemia (CML) is a malignant clonal disorder that leads to the uncontrolled proliferation of myeloid cells. The development of CML is due to a reciprocal translocation between chromosomes 9 and 22 resulting in the fusion of *BCR* and *ABL* genes that encodes an oncoprotein with constitutive kinase activity. The kinase disrupts normal cellular activity resulting in uncontrolled and poorly differentiated cellular proliferation.

The treatment of choice for CML is tyrosine kinase inhibitors such as imatinib, nilotinib and dasatinib. Imatinib is the first example of targeted therapy for the treatment of CML. The clinical use of imatinib has resulted in a favourable response rate in up to 85% of CML patients. However, there are reports that some patients experience adverse drug reactions (ADRs) to imatinib. Inter-individual differences in the metabolism of imatinib may be one of the reasons for varied response to imatinib.

Imatinib is primarily metabolised by the Cytochrome P450 (CYP450) enzymes, CYP3A4 and CYP3A5. SNPs in *CYP3A4* and *CYP3A5* have been described that result in altered catalytic activity. Reduced activity of these enzymes in CML patients being treated with imatinib can lead to an over exposure of the drug and result in an ADR. Conversely, an over active CYP3A4 and CYP3A5 can result in reduced efficacy of the drug. A limited number of studies have investigated the

effect that SNPs in *CYP3A4* and *CYP3A5* has on CML treatment. Thus the aim of this study was to screen *CYP3A4* and *CYP3A5* for SNPs and determine whether any of these are associated with changes in gene expression as well as with the presence of ADRs.

This dissertation contains a literature review, three research chapters and a conclusion. The literature review is a summary of the information regarding CML, its treatment with imatinib and the CYP3A4 and CYP3A5 enzymes responsible for its metabolism. The three research chapters are written in article format with each containing an introduction, materials and methods and results and discussion section. Although an effort has been made to avoid unnecessary duplication, some repetition between the introductions in the different chapters was unavoidable. Chapter two describes the detection of SNPs in *CYP3A4* and *CYP3A5* of CML patients. The CML patient cohort was the same for both chapter two and chapter four. Chapter four describes quantification of *CYP3A4* and *CYP3A5* mRNA and evaluates the association between gene expression and the SNPs described in chapter two. Chapter three is a research chapter assessing the stability of ultramer for use as copy number standard in real-time PCR. Chapter three forms part of a published article in Gene and has been adapted to include only data relevant to this dissertation. Although chapter three did not form part of the original study design, it became a necessary component of the dissertation since no commercial standards were available for the quantification of *CYP3A4* and *CYP3A5* described in chapter four. Therefore, we validated the use of

ultramer as copy number standards for the quantification of *CYP3A4* and *CYP3A5*. The final chapter (Chapter five), discusses and draws final conclusions regarding this study. A combined reference list for all the chapters was compiled. A summary in both English and Afrikaans is included at the end of the dissertation. An appendix section is included in the dissertation and is divided into sections A, B and C which corresponds to Chapter two, three and four, respectively. In this dissertation all figures and tables are contained within the text and numbered according to the chapter in which they occur. Throughout the dissertation, reference is made to specific genes and their protein products. Gene names are referred to in the *italic* form and the protein in normal text.

While reading this thesis it is important to note that this study was not intended to be a population study, but rather to determine the potential impact that SNPs in *CYP3A4* and *CYP3A5* have on gene expression and whether levels of *CYP3A4* and *CYP3A5* expression are associated with adverse drug reactions to imatinib. One of the limitations of this study was that only 36 CML patients were available to form part of the study cohort.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction to chronic myeloid leukaemia

1.1.1 Leukaemia

Leukaemia describes a group of neoplastic disorders that arise in haematopoietic cells and leads to the uncontrolled proliferation and accumulation of immature blood cells in the bone marrow and peripheral blood (Linet, 1985; Zeeb and Blettner, 1998). The most common symptoms of leukaemia include anaemia, neutropenia, thrombocytopenia, weakness and an increase in infections (Linet, 1985; Steward and Kheihues, 2003). If left untreated, leukaemia is fatal, often due to complications resulting from the leukemic infiltration of the bone marrow and the replacement of normal haematopoietic precursor cells (Pillar, 1997).

Leukaemia is categorised according to its clinical course as either chronic or acute depending on the degree of maturation of the malignant cell, and how fast the progression to a fatal clinical outcome is. The disease is further classified according to affected cell lineage, as either lymphoid (B-cells or T-cells) or myeloid (granulocytic, erythroid and megakaryocytic) leukaemia (Zeeb and Blettner, 1998). Factors such as morphology, degree of differentiation, immuno-phenotype and genetic characterisation of the malignant cell population may also be taken in to account when distinctions between the different types of leukaemia are made.

1.1.2 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a haematopoietic stem cell disorder, characterised by an increase in myeloid cells, predominantly granulocytes, in peripheral blood (Faderl *et al.,* 1999; Sawyers, 1999). The incidence of CML is approximately one to two in every 100,000 individuals per year and accounts for approximately 15% of newly diagnosed cases of adult leukaemia (Faderl *et al*., 1999; Sawyers, 1999; Johnson *et al.,* 2003). The median age at diagnosis is 53 years, with less than 10% of cases occurring in individuals younger than age 20 (Faderl *et al*., 1999; Sawyers, 1999; Cortes, 2004). The presenting characteristics of CML may vary between individuals and approximately 40% of patients are asymptomatic and diagnosed due to an atypical blood count with an increase in myeloid cells, erythroid cells as well as platelets in peripheral blood (Faderl *et al*., 1999; Sawyers, 1999; Cortes, 2004). Common symptoms of CML include fatigue, weight loss, splenomegaly, abdominal fullness, anaemia and thrombocytosis (Faderl *et al*., 1999; Sawyers, 1999).

1.1.3 Disease progression of CML

CML follows three phases of progression namely the chronic phase, accelerated phase and blast crisis (Table 1.1) (Sokal *et al*., 1988; Sawyers, 1999). In the majority of cases (80% to 90%), CML is diagnosed in the chronic phase of the disease (Cortes, 2004). In the chronic phase, patients have an increased number of myeloid cells in peripheral blood. During the chronic phase the myeloid cells retain their functionality and differentiate normally (Savage and Antman, 2002).

Although the chronic phase can last several years, if left untreated the disease usually progresses to the accelerated phase and blast crisis (Hochhaus *et al*., 2002; Calabretta and Perrotti, 2004). The accelerated phase marks the onset of advanced, rapidly progressive CML and is characterised by an increase in immature blast cells in the peripheral blood and bone marrow. If left untreated the progression from the accelerated to blast crisis typically occurs within two to eighteen months (Sawyers *et al*., 2002; Calabretta and Perrotti, 2004; Esfahani *et al.*, 2006; Radich, 2007). During the blast crisis additional genetic abnormalities may occur and the cancer can resemble acute myeloid leukaemia (Derderian *et al.*, 1993; Faderl *et al.,* 1999; Sawyers, 1999; Sawyers *et al.,* 2002). The prognosis for patients in blast crisis is poor, if left untreated the median survival is three to nine months (Savage *et al.*, 1997; Cortes, 2004).

1.2 Genetics of CML

1.2.1 The Philadelphia chromosome

CML was the first malignancy to be associated with a chromosomal abnormality, and its discovery was considered a breakthrough in cancer biology (Nowell and Hungerford, 1960; Sawyers, 1999). The cytogenetic basis of CML is the presence of the Philadelphia (Ph) chromosome first described as a “minute acrocentric chromosome” in patients with CML (Nowell and Hungerford, 1960). The Ph chromosome is a result of a reciprocal translocation between the long arms of chromosomes 9 and 22 (Figure 1.1) (Sawyers, 1999). The Ph chromosome is present in approximately 95% of CML patients. The remaining 5% of patients

have complex or variant translocations, usually including additional chromosomes (Sawyers, 1999). The translocation leads to the fusion of the Abelson (*ABL)* gene on chromosome 9 to the breakpoint cluster region (*BCR)* gene on chromosome 22, resulting in a *BCR-ABL* fusion oncogene (Faderl *et al*., 1999).

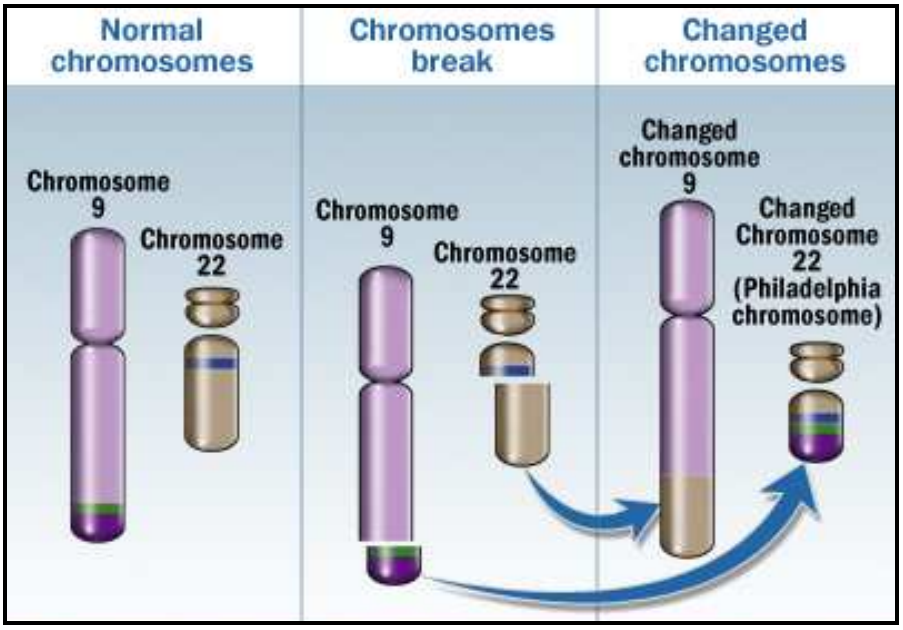


Figure 1.1: Schematic representation of the reciprocal translocation between the long arms of chromosomes 9 and 22. The translocation results in a longer chromosome 9 and a shortened chromosome 22, known as the Philadelphia (Ph) chromosome. The Ph chromosome contains the *BCR-ABL* oncogene (Copied from http://www.mayoclinic.com/health/medical/IM03579).

Table 1.1: Phases of chronic myeloid leukaemia according to the World Health Organisation and European LeukemiaNet criteria (Baccarani *et al*., 2006).

Phase	World Health Organisation Criteria	European LeukemiaNet Criteria
Chronic Phase	• None of the criteria for accelerated phase or blast crisis are met	
Accelerated phase	• Blasts 10 - 19% in peripheral blood and/or bone marrow cells • Peripheral blood basophils ≥ 20% • Persistent thrombocytopenia (<100×10^9/l) unrelated to therapy or persistent thrombocytosis (>1000×10^9/l) unresponsive to therapy • Increase in spleen size and increase in white blood cell count, unresponsive to therapy • Cytogenetic evidence of clonal evolution	• Blast cells in peripheral blood or bone marrow 15 - 29% • Blast cells and promyelocytes in peripheral blood or bone marrow >30%; with blast cells <30% • Basophils in peripheral blood ≥ 20% • Persistent thrombocytopenia (platelets <100×10^9/l) unrelated to therapy
Blast crisis	• Blasts ≥ 20% of peripheral blood white cells or nucleated bone marrow cells • Extramedullary blast proliferation • Large foci or clusters of blasts in the bone marrow biopsy specimen	• Blast cells in peripheral blood or bone marrow ≥ 30% • Extramedullary involvement (excluding liver and spleen)

1.2.2 The BCR-ABL tyrosine kinase

The *BCR-ABL* fusion gene encodes for a constitutively active tyrosine kinase (Sawyers, 1999; Faderl *et al.*, 1999). ABL is a non-receptor tyrosine kinase that plays an important role in signal transduction and the regulation of cell growth (Tang *et al.,* 2007). The function of BCR in normal cells remains unclear, although studies have suggested a role in signal transduction (Ma *et al*., 1997; Malmberg *et al.*, 2004; Oh *et al.,* 2010). The malignant nature of BCR-ABL lies in the fact that the normally well regulated tyrosine kinase activity of ABL is constitutively activated by the juxtaposition of the *BCR* sequence (Sawyers, 1999; Deininger *et al.*, 2000). The BCR-ABL tyrosine kinase catalyzes the transfer of phosphate from ATP to a tyrosine residue on a substrate protein. The uncontrolled kinase activity of BCR-ABL leads to increased proliferation of myeloid cells, decreased apoptosis and genetic instability of the leukemic cells.

1.3 Treatment of CML

In the past, treatment options for CML included allogeneic stem cell transplantation, cytoreductive chemotherapeutic drugs and interferon-α (Hehlmann *et al.*, 1994; Sawyers, 1999; Silver *et al.*, 1999; Baccarani *et al.*, 2002; Hehlmann *et al.*, 2003). Stem cell transplantation, although a curative treatment option for CML, is only accessible to a small number of patients due to the limitation in the availability of histo-compatible donors (Sawyers, 1999; Goldman and Melo, 2001). Chemotherapy treatments such as busulfan and hydroxyurea help to return blood cell counts to normal and reduce spleen size, but do not have a significant effect

on long term survival (Sawyers, 1999). Interferon-α is a glycoprotein which has antiviral and anti-proliferative properties (Savage and Antman, 2002). Although interferon-α prolongs survival in CML patients, side effects such as fatigue, myalgias, arthralgias, headaches, weight loss, depression, diarrhea, neurological symptoms, memory changes, hair thinning, autoimmune diseases, and cardiomyopathy makes it unsuitable as a long-term treatment option (Talpaz *et al*., 1991; Sacchi *et al*., 1995; Wetzler *et al.,* 1995; O'Brien *et al.,* 1996).

1.3.1 Treatment of CML with TKIs

Tyrosine kinase inhibitors (TKIs) were the first examples of targeted therapy for malignancies and have been approved for CML therapy since 2001 (Druker *et al.*, 2001). Imatinib mesylate, the first TKI developed, was designed to specifically inhibit the kinase activity of BCR-ABL. Imatinib inhibits tyrosine kinase activity by binding to amino acid residues in the ATP-binding site. The binding of imatinib alters the conformation of the BCR-ABL activation loop, locking the kinase in the inactive form. The kinase is considered inactive since ATP cannot bind and phosphorylation of the substrate is prevented and downstream signal transduction pathways are not activated (Schindler *et al*., 2000). Imatinib also inhibits other kinases including c-abl (normal ABL), PDGF-R (platelet derived growth factor) and c-kit (cytokine receptor) (Schindler *et al.,* 2000; Gambacorti-Passerini *et al.,* 2003).

The efficacy of imatinib was demonstrated by the International Randomized Study of Interferon and STI571 (IRIS) trial. The IRIS trial compared the efficacy and

safety of 400 mg per day of imatinib with that of interferon-α and low-dose cytarabine in 1106 patients with newly diagnosed chronic phase CML (O'Brien *et al.,* 2003a; Druker *et al.,* 2006). At the 6 year follow up of the IRIS trial, imatinib was associated with unprecedented response of an estimated event-free survival of 83%, while the estimated rate of freedom from progression to accelerated phase and blast crisis was 93%. The estimated overall survival was 88% (95% when only CML related deaths were considered) (Hochhaus *et al*., 2009). Although imatinib has proven to be highly effective, its use is complicated by the development of resistance or intolerance (O'Brien *et al.,* 2003a; Druker *et al.,* 2006; Hochhaus *et al.,* 2009).

Second generation TKIs were developed to overcome problems with intolerance and resistance in CML patients treated with imatinib (Shah *et al*., 2006; Talpaz *et al*., 2006). Second generation TKIs include nilotinib and dasatinib. Nilotinib has a similar chemical structure to imatinib and binds to the inactive conformation of BCR-ABL and is approximately 30 fold more potent than imatinib (O'Hare *et al*., 2005; Weisberg *et al.*, 2005). Similar to imatinib, nilotinib and dasatinib bind to the ATP binding site within the tyrosine kinase domain. Dasatinib has the advantage of binding to BCR-ABL regardless of the conformational state of the oncoprotein and has an *in vitro* potency of approximately 300 times that of imatinib (O'Hare *et al*., 2005; Talpaz *et al*., 2006). Originally approved for the treatment of CML patients who were resistant or intolerant to imatinib, both nilotinib and dasatinib gained approval in the front-line treatment setting in 2010 (NCCN, 2010; Tanaka *et*

al., 2012). Despite the approval of second generation TKIs in Europe and the United States as first line therapy, in a country such as South Africa, TKIs remain largely unaffordable in the public health setting which is accessed by about 80% of the population (Louw, 2012). The Glivec International Patient Assistance Program is a worldwide program designed to provide imatinib (Glivec) at no cost to patients who would not otherwise have access to treatment (Lassarat and Jootar, 2006; Mellstedt, 2006; Louw *et al*., 2011). Similar, but much smaller patient assistance programmes have recently been established in South Africa to facilitate access to nilotinib and dasatinib for patients with intolerance or resistance to imatinib. In South Africa imatinib is still recommended as the first-line therapy for patients with CML (Louw *et al*., 2011).

1.3.2 Monitoring response to CML treatment

A series of response criteria to treatment with a TKI were proposed by European LeukemiaNet (ELN) to determine if an optimal response, suboptimal response and/or failure to treatment are achieved (Baccarani *et al*., 2009). Imatinib is administered at a dosage of 400 mg/day in the chronic phase and 600 mg to 800 mg/day in the more advanced phases (Mauro and Druker, 2001; Sawyers *et al.,* 2002). For patients treated with imatinib, the initial goal is the achievement of a complete haematological response, defined as a normal peripheral blood count in association with less than 5% of blasts in the bone marrow (Baccarani *et al*., 2009) (Table 1.2). Response to treatment is then monitored by cytogenetic assessments with the next level of response being absence of the Ph chromosome which is

defined as a complete cytogenetic response (Baccarani *et al*., 2009). Most patients treated with imatinib from diagnosis have complete cytogenetic response within 12 months (O'Brien *et al*., 2003a). Further monitoring of *BCR-ABL* is then done by real-time PCR with a major molecular response being a three log reduction of *BCR-ABL* transcripts (Baccarani *et al*., 2009). Not reaching these response targets within a specific time frame is then considered as a suboptimal treatment response or in some cases treatment failure. It has been reported that up to 25% of patients discontinue imatinib due to treatment failure, suboptimal response and intolerance to imatinib (Marin *et al.*, 2008).

1.3.3 Intolerance to imatinib treatment

In a study by Hamdan *et al.* (2007), out of 216 CML patients treated with imatinib 29% required dose interruption, of which treatment was discontinued in 26% these patients. In the IRIS trial, approximately 4% of patients had to discontinue imatinib due to adverse effects (Druker *et al.,* 2006). In general, the severity of adverse drug reactions (ADRs) occurring with imatinib are mild to moderate in the majority of patients. Symptoms of ADRs to imatinib include oedema, muscle cramps, diarrhea, nausea, musculoskeletal pain, skin rash, abdominal pain, fatigue, joint pain, and headaches (Cohen *et al.,* 2002; Deininger *et al.,* 2003). Medication is used to provide symptomatic relief and manage mild to moderate adverse reactions to imatinib. For severe adverse effects such as neutropenia, thrombocytopenia and anaemia the management of treatment may require dose reduction, temporary interruption of treatment, discontinuation or replacement of

imatinib for another TKI (Johnson *et al.,* 2003; le Coutre *et al.,* 2004; Cohen and Tang, 2006)

Table 1.2: Definitions of haematological, cytogenetic, and molecular response criteria in CML according to European LeukemiaNet (Baccarani *et al.,* 2009*).*

Response	Criteria
Haematological Complete	Complete normalization of peripheral blood counts (leukocyte <10 x10^9/l) Platelet count <450 x10^9/l Basophils < 5% No myelocytes, promyelocytes, myeloblasts in the differential Spleen nonpalpable
Cytogenetic Complete Partial Minor None	 No Ph^+ metaphases 1%-35% Ph^+ metaphases 36% to 65% Ph^+ metaphases > 95% Ph^+ metaphases
Molecular Complete Major	 *BCR-ABL* mRNA undetectable by qRT-PCR Ratio of *BCR-ABL* to *ABL* (or *BCR* to *GUS*) ≤ 0.1% on the international scale

In general, more than 2 million cases of ADRs occur annually in the United States and have been reported to result in approximately 100,000 deaths (Lazarou *et al*., 1998). In the United Kingdom, ADRs were reported to occur at an incidence of 20,000 cases per year in 1999, compared to 250,000 cases in 2006. It is not known whether the increase in the incidence of ADRs is just a result of improved reporting (Veltmann 2005; Ushma *et al.* 2007). The cost of ADRs in 2006 in the United Kingdom was estimated to be approximately £466 million to the National Health Service (Wolf and Smith, 1999; Hitchen, 2006). Variability in drug response among patients is influenced by many factors including age, race, gender, and interactions with other drugs, concomitant disease, and renal and hepatic function (McKinnon and Evans, 2000; Leeder, 2001). However, genetic differences can play an important role and in some cases is considered to be the predominant factor influencing variability to drug response (Wolf *et al.,* 2000; Innocenti *et al.,* 2002).

1.4 Introduction to Cytochrome P450

Cytochrome P450 (CYP450) enzymes is a family of metabolising enzymes. Differences in the catalytic activity of the CYP450 enzymes have been hypothesized to be one of the main causes of variability in drug response (Evans and Relling, 1999; Wolf *et al.,* 2000; Innocenti *et al.,* 2002). The CYP450 enzymes are important catalysts for the oxidative and reductive metabolism of endogenous as well as exogenous compounds (Ingelman-Sundberg *et al.,* 1999; Rogers *et al.,* 2002). Single nucleotide polymorphisms (SNPs) occurring within the CYP450

genes have been reported to affect CYP450 enzyme activity (van Schaik *et al.,* 2001). Changes in CYP450 activity can alter the metabolic rate of the enzyme, which if decreased, results in the potential for adverse effects due to over exposure to the drug, and if increased, will lead to an increased clearance of a drug and possibly ineffective treatment (van Schaik *et al.,* 2001; Hirota *et al.,* 2004; Mathijssen and van Schaik, 2006; Schirmer *et al.,* 2007). However, the role that inter-individual variation plays in the metabolism of imatinib in CML patients is not well understood.

1.4.1 The nomenclature of Cytochrome P450

A total of 18 families, 43 sub families and 57 genes have been identified in humans (Nelson, 2009). Individual Cytochrome P450 (CYP450) enzymes are classified by their amino acid similarities and are designated by a family number, a subfamily letter, a number for an individual enzyme within the subfamily. Each allelic variant is indicated by an asterisk followed by a number (Nebert *et al.,* 1987; Nelson, 2009). For each CYP enzyme, the most common or “wild-type” allele is denoted as ‘*1’, for example *CYP3A4*1* (Nelson, 2009).

1.4.2 Introduction to *CYP3A4* and *CYP3A5*

Imatinib is metabolised by CYP3A4 and CYP3A5, with CYP1A2, CYP2C9, CYP2C19 and CYP2D6 playing a minor role (Cohen *et al*., 2002; Peng *et al*., 2005; de Kogel and Schellens, 2007). CYP3A4 and CYP3A5 form part of the CYP3A subfamily which is the most abundantly expressed CYP group in the liver

and small intestine. CYP3A enzymes have an important role in the metabolism of endogenous steroids, many procarcinogens and at least 50% of all pharmaceutical drugs (Westlind *et al.,* 1999; Boulton *et al.,* 2001; Lamba *et al.,* 2002). *CYP3A4* and *CYP3A5* are both located on chromosome 7q and consist of 13 exons, respectively (Finta and Zaphiropoulos, 2000). The CYP3A4 and CYP3A5 enzymes exhibit broad substrate specificity and therefore have the ability to metabolise a large number of structurally diverse compounds (Lamba *et al.,* 2002). CYP3A5 has an 84% amino acid sequence homology to CYP3A4 and the substrate specificity of these two enzymes appears to be similar though some differences in catalytic properties have been reported (Daly, 2006).

1.4.3 The impact of *CYP3A4* and *CYP3A5* SNPs on enzyme activity

Both *CYP3A4* and *CYP3A5* are highly polymorphic with *CYP3A4* having over 42 allelic variants while *CYP3A5* has approximately 26 (Dai *et al.,* 2001; van Schaik *et al.,* 2001). Various SNPs, resulting in altered catalytic activity have been identified in *CYP3A4* (Table 1.3) and *CYP3A5* (Table 1.4) (Dai *et al.,* 2001; Eiselt *et al.,* 2001; Murayama *et al.,* 2002). For example, a study by Dai *et al.* (2001), showed that *CYP3A4*17* has a decreased clearance rate of testosterone and chlorpyrifos *in vitro.* Similarly, *CYP3A5* allelic variants have been reported to encode enzymes that have altered catalytic activity (Table 1.4). For example, *CYP3A5*3* is a functionally significant variant, considered to account for the majority of *CYP3A5* variability (Kuehl *et al*., 2001). In *CYP3A5**3 a guanine replaces an adenine at

position 6986 in intron 3, resulting in a splice site which leads to a non functional CYP3A5 protein (Hustert *et al*., 2001). A limited number of studies have investigated the role of allelic variants of *CYP3A4* and *CYP3A5* in CML treatment response. Sailaja *et al.* (2010) found an association between the *CYP3A5*3* and increased risk of developing CML. Kim *et al*. (2009) reported that the presence of *CYP3A5*3* had an adverse impact on the achievement of a major or complete cytogenetic response in patients treated with imatinib. Although there is not a large amount of information regarding SNPs in *CYP3A4* and *CYP3A5* and the potential impact it may have on imatinib metabolism and treatment response, it is an important consideration especially when trying to obtain the most efficient treatment strategy possible.

1.4.4 The allelic frequency of *CYP3A4* and *CYP3A5* SNPs in different populations

Differences is allelic frequency in *CYP3A4* and *CYP3A5* SNPs are population specific (van Schaik *et al.,* 2001; Lamba *et al.,* 2002; Yamaori *et al.,* 2005). For example, a study by Lamba *et al*. (2002), determined that the most common variant of *CYP3A4, CYP3A4*1B*, thought to influence the expression of *CYP3A4*, is present in Caucasians at a frequency of 2.0 to 9.6%, 40% in African Americans but absent in the Japanese and Chinese. In South Africa, the same variant was detected in 81.3% of Indians, 42.9% of Caucasians and 16.4% of Africans (Chelule *et al.,* 2003). *CYP3A5*3* has also been reported to have varying frequencies in different populations including 27% in African Americans and 95%

in Caucasians (Hustert *et al*., 2001; Kuehl *et al*., 2001; van Schaik *et al*., 2001;). *CYP3A5*3* was detected in South African population at a frequency of 0.14 in Black patient, 0.94 in Caucasians and 0.59 in Coloured patients (Fukuen *et al*., 2002; Dandara *et al*., 2006). In a genetically diverse population such as the South African population there may be many undescribed SNPs with unknown impact on catalytic activity of CYP3A4 and CYP3A5 and therefore uncertainty about the impact it may have on treatment efficacy.

Table 1.3: *CYP3A4* allelic variants with altered catalytic activity. (Data was obtained and adapted from; [5]Dai *et al*., 2001; [1]Eiselt *et al*., 2001; [4]Fukushima-Uesaka *et al*., 2004; [6]Kang *et al.,* 2008; [3]Lamba *et al*., 2002; [2]Murayama *et al*., 2002 and [7]Wang *et al.,* 2011).

Allelic Variant	Position in AF280107 sequence	Base change	Amino Acid Change	Location	Effect of SNP on enzyme activity	
					In Vivo	***In Vitro***
*CYP3A4*8*	75944	G/A	R130Q	Exon 5	Unknown	Decreased[1]
*CYP3A4*11*	83903	C/T	T363M	Exon 11	Unknown	Decreased[1,2]
*CYP3A4*12*	83932	C/T	L373F	Exon 11	Unknown	Decreased[1]
*CYP3A4*13*	84062	C/T	P416L	Exon 11	Unknown	Decreased[1]
*CYP3A4*16*	77639	C/G	T185S	Exon 7	Unknown	Decreased[2,3,4]
*CYP3A4*17*	77651	T/C	F189S	Exon 7	Unknown	Decreased[5]
*CYP3A4*18*	82106	T/C	L293P	Exon 10	Decreased[5]	Increased[4,6]
*CYP3A4*22*	77425	C/T	NA	Intron 6	Decreased[7]	Unknown

Table 1.4: *CYP3A5* allelic variants with altered catalytic activity. (Data was obtained and adapted from; [2]Hustert *et al*. 2001; [1]Kuehl *et al* 2001 and [2]Lee *et al*. 2003).

Allele	Position in AC005020 sequence	Base change	Amino Acid Change	Location	Effect of SNP on enzyme activity	
					In Vivo	In Vitro
*CYP3A5*3*	6986	A/G	NA (Splice variant)	Intron 3	Decreased[1]	Decreased[1,2]
*CYP3A5*6*	14690	G/A	NA (Splice variant)	Exon 7	Unknown	Decreased[1]
*CYP3A5*8*	3699	C/T	R28C	Exon 2	Unknown	Decreased[3]
*CYP3A5*9*	19386	G/A	A337T	Exon 10	Unknown	Decreased[3]

1.4.5 The expression of *CYP3A4* and *CYP3A5*

CYP3A4 and CYP3A5 are expressed primarily in the liver but also in the gastrointestinal tract, lungs, leucocytes, kidneys, pituitary gland and prostate (Canaparo *et al*., 2007; Koch *et al.,* 2002; Yokose *et al*., 1999; Piipari *et al*., 2000; Nowakowski-Gashaw *et al*., 2002). There have been reports that there is a high inter-individual difference in expression of *CYP3A4* and *CYP3A5* (Kuehl *et al*., 2001; Yamaori *et al*., 2005). The difference in expression has been suggested to be due to sequence variation within the gene (Ozdemir *et al.,* 2000). The level of *CYP3A4* and *CYP3A5* expression may help determine which patients might experience side effects or even toxicity when the standard dosage of a drug is administered (Eichelbaum and Burk, 2001). Several studies have shown that CYP3A4 and CYP3A5 expression in the liver is correlated to hepatic *CYP3A4* and *CYP3A5* mRNA expression (Lin *et al*., 2002; Watanabe *et al*., 2004). Temesvári *et*

al. (2012) reported that mRNA expression of *CYP3A4* in leucocytes correlated to CYP3A4 hepatic activity. Since using liver samples for the detection of hepatic *CYP3A4* and *CYP3A5* mRNA expression comes with both practical and ethical problems, the use of peripheral blood, which can be obtained during routine medical examination, is a more convenient option.

1.5 Conclusion

CML can be effectively treated with imatinib. However, some individuals experience ADRs to imatinib. One of the reasons for varied treatment response among individuals may be as a result of inter-individual differences in the metabolism of imatinib. Imatinib is primarily metabolised by CYP3A4 and CYP3A5. Allelic variants as a result of SNPs in *CYP3A4* and *CYP3A5* have been described, some of which have been associated with altered catalytic activity. A decrease in catalytic activity of the enzymes may result in ADRs due to a prolonged exposure to the drug. Compared to this, an increase in catalytic activity could result in ineffective treatment. SNPs in *CYP3A4* and *CYP3A5* may impact the expression of these genes and result in a less favourable response to imatinib treatment. By understanding the impact of polymorphisms in *CYP3A4* and *CYP3A5*, a more accurate imatinib treatment regimen can be established for each patient, depending on which allelic variant is present.

CHAPTER TWO

DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN *CYP3A4* AND *CYP3A5* OF CML PATIENTS

2.1 Introduction

Imatinib mesylate is a potent and selective inhibitor of BCR-ABL tyrosine kinase activity, used in the treatment of chronic myeloid leukaemia (CML) (Druker *et al*., 2001; Peng *et al*., 2005). The treatment of CML with imatinib has shown to have remarkable efficacy with rates of complete haematological response of approximately 96% and event free survival reported in approximately 83% of patients (Druker *et al*., 2006; Hochhaus *et al*., 2009). Despite the outstanding results obtained with imatinib, cases of treatment failure and suboptimal response have been reported, with approximately 4% of patients discontinuing imatinib treatment due to adverse drug reactions (ADRs) (Hochhaus *et al.,* 2009).

Common side effects of imatinib include nausea, oedema, muscle cramps, diarrhea, skin rashes, neutropenia and anaemia (Cohen *et al.,* 2002; Deininger *et al.,* 2003; Johnson *et al.,* 2003; Henkes *et al.,* 2008). Less severe ADRs are treated with medication to provide symptomatic relief while more severe adverse effects such as neutropenia, thrombocytopenia and anaemia are managed by

dose reduction, temporary interruption of treatment, discontinuation of treatment, or substituting imatinib for another TKI (Johnson *et al.,* 2003; le Coutre *et al.,* 2004; Cohen and Tang, 2006). Although the ADRs related to treatment with imatinib have been well described, the pharmacokinetic link with ADRs has not been investigated extensively (Johnson *et al.,* 2003; Schmidli *et al.,* 2005; Henkes *et al.,* 2008). Pharmacokinetics focuses on the absorption, metabolism, distribution and excretion of a drug (Lin *et al.,* 2003; Ekins *et al.,* 2005). Imatinib is mainly metabolised by Cytochrome P450 enzymes CYP3A4 and CYP3A5, while CYP1A1, CYP1A2, CYP1B1, CYP2D6, CYP2C9 and CYP2C19 are thought to contribute to a minor extent (Cohen *et al.,* 2002; Gschwind *et al*., 2005; Peng *et al*., 2005). It has been suggested that the activity of CYP3A4 and CYP3A5 may play a role in the therapeutic efficacy, safety and inter-individual variability in patients treated with imatinib (Cohen *et al.,* 2002; Gambacorti-Passerini *et al.,* 2003). However, there are relatively few studies that have evaluated the drug metabolism of imatinib with therapeutic outcome (O'Brien *et al*., 2003b; Gréen *et al.,* 2010).

Several studies have reported that there is a large inter-individual variability in expression of CYP3A4 and CYP3A5 (Lamba *et al.,* 2002). For example, variation in CYP3A4 protein levels has been reported to differ up to 40 fold between individuals (Westlind *et al*., 1999). The genetic contribution to the inter-individual variation has been estimated to range from 60% to 90% (Ozdemir *et al*., 2000; Lamba *et al*., 2002). Several SNPs in *CYP3A4* and *CYP3A5* have been

associated with altered catalytic activity. For example, allelic variants *CYP3A4*8*, **11, *12, *13, *16, *17* and **22* are associated with decreased CYP3A4 catalytic activity (Dai *et al*., 2001; Eiselt *et al*., 2001; Murayama *et al.,* 2002; Wang *et al*., 2011). Similarly, allelic variants of *CYP3A5* (*CYP3A5*3, *6, *8* and **9*) have been reported to result in a significant decrease in CYP3A5 activity (Kuehl *et al*., 2001; Lee *et al.,* 2003; Haufroid *et al*., 2004; Wong *et al*., 2004; Josephson *et al*., 2007). Identifying potential influential SNPs may allow for the prediction of drug disposition in individual CML patients and therefore aid in treatment optimization. Thus the aim of the study was to screen the 13 exons of *CYP3A4* and 13 exons and one intron of *CYP3A5* for SNPs using high resolution melting curve (HRM) analysis in CML patients being treated with imatinib. Sequencing was used to characterize the SNPs detected by HRM analysis.

2.2 Materials and methods

The 13 exons of *CYP3A4* and the 13 exons and one intron of *CYP3A5* were screened for the presence of SNPs using HRM analysis followed by sequencing. The study population consisted of patients being treated for CML with imatinib at the Haematology Clinic at the University of the Free State, Bloemfontein. The study was conducted according to an approved ethics protocol (ETOVS 32/07) and informed consent was obtained from patients participating in the study. A unique patient number was assigned to each patient in order to identify their blood, DNA or RNA sample, while still allowing for patient anonymity. The study group consisted of patients of different ethnicities (Black n = 27; Caucasian n = 6 and

Coloured *n* = 3). The study group was divided into a control group which consisted of 27 CML patients who did not experience ADRs at the standard dose of 400 mg/day or higher of imatinib and an experimental group which consisted of nine CML patients reported to experience ADRs from imatinib (Table 2.1). The experimental group consisted of patients that were receiving a lower than standard dose of imatinib due to the development of ADRs as well as patients experiencing ADRs at 600 mg/day (Table 2.1).

2.2.1 TRI Reagent stabilization

Peripheral blood was collected in EDTA and approximately 20 ml was treated with 50 ml of lysis buffer (consisting of equal parts of 0.144 M NH_4Cl and 0.01 M NH_4HCO_3, pH 7.4). Samples were incubated for 10 minutes at room temperature followed by 10 minutes of centrifugation at 3,500 rpm. The supernatant was discarded and an additional 25 ml of lysis buffer added, followed by incubation for 5 minutes at room temperature. Thereafter, the sample was centrifuged at 3,500 rpm for 10 minutes and the supernatant discarded and the remaining white blood cell pellet dissolved in 1.6 ml of TRI Reagent (Sigma-Aldrich). The white blood cells were mixed by pipetting until the solution was homogenous. The sample was stored at -70ºC until used for nucleic acid extraction.

2.2.2 DNA extraction and DNA concentration determination

The 1.6 ml TRI Reagent homogenate was thawed at room temperature followed by the addition of 15 µl of proteinase K (20 mg/ml). The sample was then incubated at 65ºC for 20 minutes and mixed by inversion every 5 minutes followed by the addition of 350 µl of chloroform. The sample was placed on ice for 3 minutes followed by centrifugation at 12,000 rpm for 15 minutes. Approximately 1 ml of the aqueous phase was retained for RNA extraction which was performed as soon as possible to prevent RNA degradation (refer to chapter 4). The remaining inter-phase and organic phase was used for DNA extraction. The DNA sample was precipitated by the addition of 480 µl of absolute ethanol and incubated for 5 minutes at room temperature. This was followed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was discarded and the pellet washed by the addition of 1.6 ml of 0.1 M sodium citrate, followed by incubation for 10 minutes. The sample was centrifuged at 12,000 rpm for 5 minutes, the supernatant discarded and the sodium citrate wash repeated. The pellet was dissolved in 200 µl of 8 mM sodium hydroxide and incubated at 65ºC for 15 minutes, followed by the addition of 800 µl of CTAB buffer. The sample was incubated at 65ºC for 10 minutes, 333 µl of 6 M potassium acetate added followed by 30 minute incubation on ice. The sample was centrifuged at 13,000 rpm for 10 minutes. The DNA containing supernatant was retained and the protein precipitate was discarded. The supernatant was mixed with 1 ml of isopropanol and incubated on ice for 60 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes. The DNA precipitate was washed with 1 ml of 75% ethanol and incubated for 5 minutes

followed by centrifugation at 12,000 rpm for 5 minutes. The 75% ethanol wash step was repeated and the DNA pellet dissolved in 50 µl of nuclease free sterile water. The concentration of extracted DNA for each sample was determined fluorometrically using the Qubit dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's instructions. Two calibration standards, at 0 ng/µl and 10 ng/µl respectively, were prepared by the addition of 10 µl of each standard to 189 µl of Qubit dsDNA HS Buffer and 1 µl of Qubit dsDNA HS Reagent provided in the kit. The concentration of DNA was determined by the addition of 1 µl of extracted DNA to 198 µl of Qubit dsDNA HS Buffer and 1 µl Qubit dsDNA HS Reagent. The mixture was vortexed and centrifuged, followed by incubation at room temperature for 2 minutes before measuring the concentration of DNA using the Qubit fluorometer (Invitrogen). The extracted DNA was stored at -70ºC until used.

2.2.3 Primer design

The PCR reactions for each of the 13 exons of *CYP3A4* and the 13 exons and one intron of *CYP3A5* were performed using primers designed using the online program Primer3Plus (http://www.bioinformatics.nl/primer3plus) (Table 2.2 and Table 2.3). The primer binding sites were situated in the intronic regions to enable the PCR amplification of the entire exon. Exon 13 of *CYP3A4* consisted of 552 bases and since this fragment was larger than the recommended amplicon size for HRM analysis, 3 overlapping primer pairs were designed, denoted Exon 13.1, 13.2 and 13.3 respectively (Table 2.2). A primer pair was designed for intron 3 of *CYP3A5*, since a functionally significant SNP (A6986G) has been reported within

the intron region. AF280107 and AC005020.5 were used as reference sequences for *CYP3A4* and *CYP3A5*, respectively.

2.2.4 PCR

The PCR reaction contained 5 µl of 10 x PCR Gold buffer (Applied Biosystems), 3 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.16 µl of 5 U/µl AmpliTaq Gold (Applied Biosystems), 200 nM forward and reverse primer, respectively, 1 µl of approximately 20 ng/µl genomic DNA and nuclease free sterile water to a total volume of 50 µl. The reactions were performed on the GeneAmp 9700 (Applied Biosystems). The cycling conditions were as follows: 10 minutes at 95ºC followed by 40 cycles of 30 seconds at 95ºC, 1 minute at 58ºC, and 1 minute at 72ºC, followed by a final extension step of 7 minutes at 72ºC.

2.2.5 Gel electrophoresis

PCR amplicon was resolved on a 2% agarose gel, using 1 x TAE buffer (40 mM Tris, 40 mM acetic acid and 1 mM EDTA, pH 8) for approximately 25 minutes at 230 V. Thereafter, the gel was stained in an excess volume of ethidium bromide (0.5 µg/ml) for approximately 25 minutes and visualized under UV light and documented using the Kodak Gel Logic 200 imaging system with the Kodak1D v3.6.5 software.

Table 2.1: Summary of the CML patient characteristics, study groups and imatinib dosage.

Patient number	Gender	Race	Imatinib Dosage[1]	Study Group
1	Male	Black	400 mg	Control
3	Male	Black	600 mg	Control
7	Male	Caucasian	400 mg	Control
8	Male	Black	800 mg	Control
9	Male	Black	400 mg	Control
12	Female	Black	200 mg[2]	Experimental
13	Female	Black	300 mg[2]	Experimental
18	Female	Black	400 mg	Control
19	Male	Black	800 mg	Control
20	Female	Caucasian	400 mg	Control
22	Female	Black	200 mg[2]	Experimental
24	Male	Caucasian	400 mg	Control
25	Male	Black	300 mg[2]	Experimental
28	Male	Black	400 mg	Control
29	Female	Black	400 mg	Control
35	Male	Black	400 mg	Control
38	Female	Black	400 mg	Control
40	Male	Black	400 mg[3]	Experimental
42	Male	Coloured	400 mg	Control
44	Male	Black	800 mg	Control
48	Female	Black	300 mg[2]	Experimental
49	Male	Caucasian	800 mg	Control
50	Female	Black	300 mg	Control
55	Male	Black	400 mg	Control
56	Female	Black	400 mg	Control
57	Female	Coloured	400 mg	Control
59	Female	Black	300 mg[2]	Experimental
60	Male	Black	800 mg	Control
61	Male	Black	400 mg	Control
62	Female	Caucasian	300 mg[2]	Experimental
63	Male	Black	400 mg	Control
64	Female	Coloured	400 mg	Control
68	Female	Black	600 mg	Control
70	Male	Black	400 mg[3]	Experimental
72	Female	Caucasian	600 mg	Control
73	Female	Black	400 mg	Control

[1] Dosage at the time of sampling
[2] Imatinib dose reduced to lower than the standard 400 mg/day due to ADRs
[3] Imatinib dose reduced to 400 mg/day since ADRs occurred at 600 mg/day

Table 2.2: The sequence of the primers designed to PCR amplify the 13 exons of *CYP3A4.*

Primers	Primer sequence (5'-3')	Fragment	Annealing
Exon 1 F	AACAATCCAACAGCCTCACTG	245	58ºC
Exon 1 R	CCCAAGTCCAAGGAAACAGA		
Exon 2 F	TCGTTCTCTTGAGCATTCCA	247	58ºC
Exon 2 R	AAGCTGCTCTTGGCAATCAT		
Exon 3 F	GGCTTCGACTGTTTTCATCC	221	58ºC
Exon 3 R	TTGGGCTGAGACTGTCCTCT		
Exon 4 F	TGTAAAGTCAGGATCAAAGTCTGG	300	58ºC
Exon 4 R	TGGAACCTTCCTGGACATTT		
Exon 5 F	CCATGGAGACCTCCACAACT	227	58ºC
Exon 5 R	CTGTCCCCACCAGATTCATT		
Exon 6 F	GCCATGTCCTTCTGGGACTA	246	58ºC
Exon 6 R	GGAATAACCCAACAGCAGGA		
Exon 7 F	GGTAAAAAGGTGCTGATTTTAATTTT	342	58ºC
Exon 7 R	GATGATGGTCACACATATC		
Exon 8 F	TGCTCCAGGTAAATTTTGCAC	244	58ºC
Exon 8 R	AAATTATGAAAAACTAAACATCCTCCT		
Exon 9 F	AGATCAAGGACCACGCTTGT	249	58ºC
Exon 9 R	TGGCAGAAATTCTCATCATCC		
Exon 10 F	TGATGCCCTACATTGATCTGA	291	58ºC
Exon 10 R	TTCTCCTGGGAAGTGGTGAG		
Exon 11 F	CTGCATGGACTGAGTTAAAAGTT	354	58ºC
Exon 11 R	GGCAGAATATGCTTGAACCAG		
Exon 12 F	CATGTAACTCTTAGGGGTATTATGTCA	290	58ºC
Exon 12 R	AAAATACAGACCACTCAGTTAAAAGAA		
* Exon 13.1 F	ACTTTTGTTTATTTAATGCTTCTCAA	276	58ºC
* Exon 13.1 R	CCCGGTTATTTATGCAGTCC		
* Exon 13.2 F	TGTGCCTGAGAACACCAGAG	239	58ºC
* Exon13.2 R	GTGCTAACTGGGGGTGGTG		
* Exon13.3 F	GGAGGTAGATTTGGCTCCTCT	300	58ºC
* Exon13.3 R	TTGTTGGTTCTCATTCAGTTCTAT		

***** One primer pair was designed for each exon, with the exception of exon 13. Due to the large fragment size, exon 13 was amplified using three overlapping primer pairs.

Table 2.3: The sequence of the primers designed to PCR amplify the 13 exons and one intron of *CYP3A5.*

Primers	Primer sequence (5'-3')	Fragment	Annealing
Exon 1 F	CATAAATCTTTCAGCAGCT	247	58ºC
Exon 1 R	CCCAAGTCCAAGGAAACAAA		
Exon 2 F	TCACAATCCCTGTGACCTGA	208	58ºC
Exon 2 R	GGGGCATTTTTACTGATGGA		
Exon 3 F	CATTGGACGTGTTTTCA	261	58ºC
Exon 3 R	TTTGTATTTAGGTTGACAAGAGCTTCA		
* Intron 3 F	CGAATGCTCTACTGTCATTTCTAACC	114	58ºC
* Intron3 R	GCCACCCAAGGCTTCATATG		
Exon 4 F	GCAGAATCGGGCTAGTGAAG	203	58ºC
Exon 4 R	AAAAATTTAATCAGTGGATCAATCA		
Exon 5 F	CATGAAGATCACCACAACTAATGTGA	250	58ºC
Exon 5 R	TGGAACGGACTGTGATCTTACTTT		
Exon 6 F	TCTGGGACTTGAGTCTGCAC	190	58ºC
Exon 6 R	AAGGGCTCATGACAGCTCAG		
Exon 7 F	AGGACGGTAAGAGGTGCTGA	300	58ºC
Exon 7 R	GGAATTGTACCTTTTAAGTGGATGA		
Exon 8 F	GCTCCAGGTAAAGTTTGCATTT	230	58ºC
Exon 8 R	TCAAAACCTAAACATCGTCATTT		
Exon 9 F	AGGACTGCACTTTTGATTACTTCTG	253	58ºC
Exon 9 R	TGCTATGTGGCAAAAATTCTCATC		
Exon 10 F	TTCCTTCTTGGGATTAGAGAGCTTCA	300	58ºC
Exon 10 R	GGCTTCACCTCTTCCCTTCAT		
Exon 11 F	TGCATGGACTCAGTTGAGAGTT	354	58ºC
Exon 11 R	GGCAGAATATGCTTGAACCAG		
Exon 12 F	CTACTGGTTGGGAGGTGGAG	377	58ºC
Exon 12 R	TTTGGCCCATAGAATGAATTA		
Exon 13 F	ACTTCATTTATTAATTCTCCATATGCT	300	58ºC
Exon 13 R	GACTCTGGGAGAGCTCAATG		

***** Intron 3 primer pair was included in order to be able to detect A6986G (*CYP3A5*3*).

2.2.6 High resolution melting curve analysis

PCR amplicon was diluted 1:100, 000 or 1:1,000, 000 in nuclease free sterile water, in order to obtain a threshold cycle (C_T) value of between 20 and 25, for use as template for HRM analysis. HRM analysis was performed on the ABI 7500 Fast (Applied Biosystems). The reaction mixture consisted of 1 µl of diluted PCR product, 200 nM forward and reverse primer, respectively, 10 µl of MeltDoctor HRM master mix (Applied Biosystems) and nuclease free sterile water to a final volume of 20 µl. A no template control (NTC) containing no DNA as well as a wild type (WT) control was included in each run. The WT was a sample that was sequenced to confirm that it contained no SNPs. The cycling conditions were as follows: 95ºC for 10 minutes followed by 40 cycles of 95ºC for 10 seconds and 58ºC for 1 minute. This was followed by 95ºC for 30 seconds, 60ºC for 30 seconds, followed by an increase in temperature to 95ºC at a ramp rate of 0.03ºC/second and a 40ºC step for 30 seconds. Melting curve data were collected using the expert mode function of the 7500 Fast SDS version 1.4 Software. Melting curves were analysed using the HRM software version 2.0 (Applied Biosystems) with default settings.

2.2.7 DNA sequencing

PCR amplicon was purified prior to DNA sequencing using ExoSAP-IT (AEC Amersham). ExoSAP-IT enzyme (4 µl) was added to 7 µl of PCR amplicon and the mixture incubated for 15 minutes at 37℃, followed by 15 minutes at 80℃ to inactivate the enzyme. The sequencing reaction was performed using the BigDye

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction consisted of 2 µl of Terminator mix, 1 µl of Sequencing buffer, 5 µl of purified PCR product and 0.8 pmol of primer. The cycling conditions were as follows: 1 minute at 96ºC, followed by 25 cycles of 10 seconds at 96ºC, 5 seconds at 58ºC, and 4 minutes at 60ºC. The sequencing products were purified using ethanol/sodium acetate precipitation according to the manufacturers' instructions by the addition of 3 µl of 3 M sodium acetate, 62.5 µl of absolute ethanol, 10 µl sequencing product and 14.5 µl of nuclease free sterile water. The sample was vortexed for 30 seconds, centrifuged for 10 seconds and incubated in the dark for 30 minutes at room temperature. This was followed by centrifugation for 30 minutes at 13,000 rpm. The supernatant was discarded and the pellet washed with 250 µl 70% ethanol followed by centrifugation for 10 minutes at 13,000 rpm. The supernatant was discarded and the DNA pellet dried at 90ºC for 1 minute. Thereafter, 25 µl Hi-Di Formamide (Applied Biosystems) was added to the sample. The sample was then incubated at 95ºC for 2 minutes, followed by incubation on ice for 5 minutes. The sample was loaded on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Separation of DNA fragments was performed using polymer POP-7 (Performance Optimized Polymer 7). The resulting data were analysed using the Sequence Analysis Software version 5.31 (Applied Biosystems) and visualized with Chromas software (http:www.technelysium.com.au). The online software LALIGN was used to align the sequence to a reference sequence (*CYP3A4*: AF280107 and *CYP3A5*: AC005020.5) (http://www.ch.embnet.org/software/LALIGN_form.html).

2.2.8 Statistical analysis

SNP data were analysed using SNPator, an internet based statistical analyses package (Morcillo-Suarez *et al.,* 2008). Allele and genotype frequencies were calculated and deviation from Hardy-Weinberg equilibrium assessed by Chi-square and Fisher's exact probability test. Confidence Intervals (95% CI) were calculated using the Odds Ratio with Haldane correction. Although this was not a population study, deviations from Hardy-Weinberg equilibrium were calculated for the CML study group as a whole as well as for Black patients, since the Caucasian and Coloured patient numbers were limiting. Associations were determined between SNPs and ADRs. The linkage between SNPs was determined using the software SNP & Variation Suite (SVS) version 7.7 (Golden Helix Inc., Bozeman, MA, USA). A P-value of less than 0.05 was considered statistically significant.

2.3 Results and discussion

2.3.1 Summary of SNPs detected in *CYP3A4* and *CYP3A5*

A total of ten SNPs were detected in *CYP3A4* of CML patients of which two have not previously been reported in literature (Table 2.4). One of the SNPs detected in *CYP3A4* (G20338A) has previously been associated with altered enzyme activity (Hu *et al*., 2007; Zhang *et al*., 2010; Yuan *et al.,* 2011) (Table 2.4). No mutations were detected in any of the patients in exons 1, 2, 3, 4, 5, 6, 8, 9, 12 and 13 of *CYP3A4*. For *CYP3A5,* a total of four SNPs were detected of which one has not been previously described in the literature (Table 2.5). Two of the allelic variants detected, *CYP3A5*3* and *CYP3A5*6,* have been associated with decreased

enzyme activity (Kuehl *et al*., 2001). No SNPs were detected in any of the patients in exons 1 to exon 3, exon 6 and exon 8 to exon 13 of *CYP3A5*.

2.3.2 Hardy-Weinberg equilibrium, allelic frequency and linkage analysis of *CYP3A4*

All the SNPs detected in *CYP3A4* in the combined study group were in Hardy-Weinberg equilibrium with the exception of G20338A and T15871G (Table 2.6). However, when considering only the Black population in the Hardy-Weinberg calculation both G20338A and T15871G were in Hardy-Weinberg equilibrium (Table 2.6). The T15871G SNP occurred at the highest allelic frequency (0.50) of all the SNPs detected in *CYP3A4* (Table 2.7). Previous studies have reported that this SNP has a frequency of 0.07 in Caucasians and 0.48 in African Americans (Table 2.8) (Lamba *et al.,* 2002). Although this research was not intended as a population study, the overall frequency of the T15871G SNP was 0.50 in the whole population tested and 0.56 in the Black CML patient group (Table 2.8). The linkage analysis showed that none of the SNPs showed statistically significant linkage to each other (all SNPs analyzed had a $r^2 < 0.05$). Interestingly the two SNPs that were not in Hardy-Weinberg equilibrium when analysing the CML patient group as whole, G20338A and T15871G, have previously been reported to be in partial linkage disequilibrium (Lamba *et al.,* 2002).

2.3.3 Novel *CYP3A4* SNPs

Two SNPs, A15649T and A15619G, that have not previously been described in literature, were detected. The A15649T SNP results in a change of the glutamine amino acid at position 200, to a histidine (Q200H). A15649T was detected in one patient from the Black CML group (Figure 2.1F). A15619G is a synonymous SNP detected in one individual in the Black CML patient group (Figure 2.1D). Although synonymous SNPs do not result in an altered amino acid residue they may have an impact on protein folding as a result of translational pause which may lead to an altered protein structure and/or altered protein activity (Kimchi-Sarfaty *et al*., 2007; Kudla *et al.,* 2009).

2.3.4 Association analysis of *CYP3A4* SNPs detected in CML patient with adverse drug reactions

The detected SNPs were analysed to determine whether they were associated with ADRs in CML patients being treated with imatinib. The G20338A (*CYP3A4*1G)* SNP had a significant association with the presence of ADRs (P = 0.0007) (Table 2.9). *CYP3A4*1G*, is located in intron 10 at base 82266 involving a G to an A nucleotide change. Data regarding the potential impact that the *CYP3A4*1G* variant has on CYP3A4 expression and/or activity is conflicting. Zhang *et al*. (2010) and Yuan *et al*. (2011) determined that patients homozygous for *CYP3A4*1G* had significantly lower CYP3A4 activity compared to wild type *CYP3A4*. Compared to this, Hu *et al*. (2007) found that patients with *CYP3A4*1G* had a faster mean drug clearance and reported an association between

*CYP3A4*1G* and increased CYP3A4 activity. The conflicting findings may be the result of the involvement of multiple CYP enzymes. The exact functional significance of the *CYP3A4*1G* polymorphism therefore warrants further investigation.

2.3.5 HRM analysis and sequencing of *CYP3A4*

In this study there was a 100% concordance between identifying sequence variants through HRM analysis and sequencing in *CYP3A4.* In exon 7 (Figure 2.1), exon 10 (Figure 2.2) and exon 12 (Figure 2.4) the HRM analysis plot distinguished between sequence variant samples based on zygosity. In exon 7, it appeared that zygosity of the T15871G SNP determined the variant calling, since the presence of additional SNPs (T15955A, T15901C, I193I, A15619G and A15649T) in certain patients did not affect the variant grouping (Figure 2.1). In exon 11, however, (Figure 2.3) the HRM analysis identified variant groups based on the presence of different SNPs and did not appear to distinguish variants based on zygosity.

Table 2.4: Summary of SNPs identified in the *CYP3A4* gene in CML patients treated with imatinib.

Allelic variant	*CYP3A4* nomenclature[1]	Nucleotide change	Position in AF280107 sequence	Distance from the start codon	Location	Amino Acid substitution	Described impact of SNP	Reference
	T15871G	T to G	77789	15753	Intron 7	NA	None	Dai *et al*. (2001)
	T15955A	T to A	77873	15837	Intron 7	NA	None	Lamba *et al*. (2002)
	T15901C	T to C	77819	15783	Intron 7	NA	None	Dai *et al*. (2001)
	I193I	C to T	77664	15628	Exon 7	None (I193I)	None	Sata *et al.* (2000)
	A15619G	A to G	77655	15619	Exon 7	None (G190G)	None	Not described
	A15649T	A to T	77685	15649	Exon 7	Q200H	None	Not described
CYP3A4*1G	G20338A	G to A	82266	20230	Intron 10	NA	•Decreased CYP3A4 activity[2], •Increased CYP3A4 activity[3] •Coronary heart disease [4]	Dai *et al.* (2001)[5]
	C23187T	C to T	85117	23081	Intron 11	NA	None	Dai *et al.* (2001)
	T363T	G to A	83904	21868	Exon 11	None (T363T)	None	Kesheva *et al.* (2004)
	I369V	A to G	83920	21884	Exon 11	I369V	None	Lamprecht (2009)

[1] The CYP nomenclature in this table is based on the manner that it has been reported in literature. Certain authors' base nomenclature on nucleotide position while others base nomenclature on amino acid position.

[2] Zang *et al.* (2010) and Yuen *et al*. (2011).

[3] Hu *et al*. (2007).

[4] He *et al*. (2011).

[5] Dai *et al* (2001) was the first study to describe G20338A.

Table 2.5: Summary of SNPs identified in the *CYP3A5* gene in CML patients treated with imatinib.

Allelic variant	*CYP3A5* Nomenclature[1]	Nucleotide change	Position in AC005020 sequence	Distance from start codon	Location	Amino Acid substitution	Described impact of SNP	Reference
*CYP3A5*3*	A6986G	A to G	22893	6986	Intron 3	Splice variant	Decreased CYP3A5 activity	Kuehl *et al*. (2001) Hustert *et al*.(2001)
	C7207T	C to T	23114	7207	Intron 3	NA	None	Solus *et al.* (2004)
	G7226A	G to A	23133	7226	Exon 4	None (T74T)	None	Not described
*CYP3A5*6*	G14690A	G to A	30597	14690	Exon 7	Splice variant	Decreased CYP3A5 activity	Kuehl *et al.* (2001)

[1]The CYP nomenclature in this table is based on the manner that it has been reported in literature. Certain authors' base nomenclature on nucleotide position while others base nomenclature on amino acid position.

Table 2.6: Hardy-Weinberg equilibrium of SNPs detected in *CYP3A4* of CML patients.

SNP	Observed	Expected	χ^2	P
C23187T	0.22	0.31	1.43	0.23
G20338A	0.11	0.27	5.01	0.03**[1]
A15619G	0.03	0.03	2×10^{-4}	0.99
I193I	0.03	0.03	2×10^{-4}	0.99
I369V	0.14	0.13	0.03	0.87
A15649T	0.03	0.03	2×10^{-4}	0.99
T15955A	0.08	0.08	5.6×10^{-3}	0.94
T15871G	0.14	0.50	19.70	0.00 **[2]
T15901C	0.03	0.03	2×10^{-4}	0.99
T363T	0.08	0.08	5.6×10^{-3}	0.94

***** Significant at P < 0.05

** Significant at P < 0.01

[1] The G20338A SNP was in Hardy-Weinberg equilibrium in the Black (n=27) CML population group (χ^2=0.44; P value=0.51)

[2] The T15871G SNP was in Hardy-Weinberg equilibrium in the Black (n=27) CML population group (χ^2=0.49; P value=0.48)

Table 2.7: Allele and genotype frequencies for *CYP3A4* SNPs detected in CML patients.

SNP	Allele Frequency		Genotype Frequency		
C23187T	C: 0.81	T: 0.19	TT: 0.08	CT: 0.22	CC: 0.70
G20338A	G: 0.84	A: 0.16	GG: 0.78	AG: 0.11	AA: 0.11
A15619G	A: 0.97	G: 0.01	AA: 0.97	AG: 0.03	GG: 0.00
I193I	C: 0.99	T: 0.01	CC: 0.97	CT: 0.03	TT: 0.00
I369V	A: 0.93	G: 0.07	AA: 0.86	AG: 0.14	GG: 0.00
A15649T	A: 0.99	T: 0.01	AA: 0.97	AT: 0.03	TT: 0.00
T15955A	T: 0.96	A: 0.04	TT: 0.92	AT: 0.08	AA: 0.00
T15871G	T: 0.50	G: 0.50	TT: 0.43	GT: 0.14	GG: 0.43
T15901C	T: 0.99	C: 0.01	TT: 0.97	CT: 0.03	CC: 0.00
T363T	G: 0.96	A: 0.04	GG: 0.92	AG: 0.08	AA: 0.00

Table 2.8: The allelic frequency of *CYP3A4* SNPs reported in literature compared to occurrence in the CML population as a whole as well as the Black CML patients.

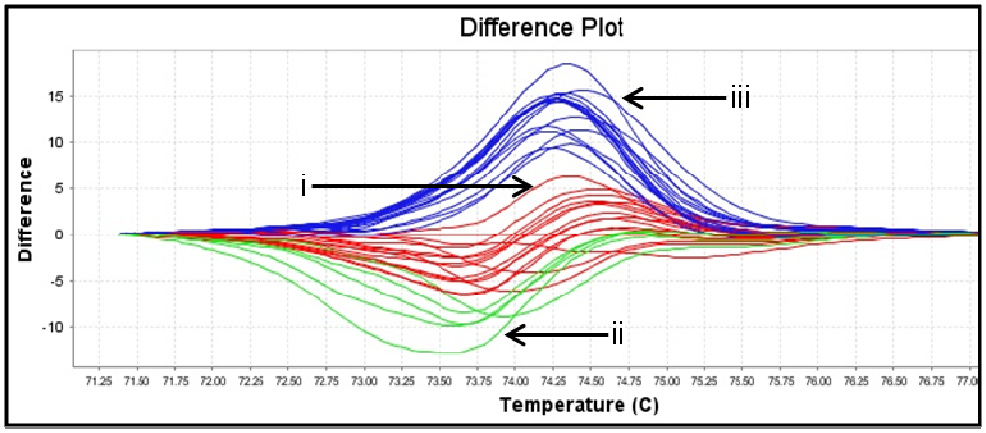
SNP	Frequency			Reference	CML patient SNP frequency	
	Caucasian	African American	Asian		Total	Black
T15871G	0.07	0.48	0.00	Lamba *et al*. (2002)	0.50	0.56
T15955A	0.00	0.05	0.00	Lamba *et al*. (2002)	0.04	0.06
T15901C	0.00	0.09	0.00	Dai *et al*. (2002)	0.01	0.02
I193I	0.00	0.05	0.00	Lamba *et al.* (2002)	0.01	0.02
A15619G	-	-	-	Not previously described	0.01	0.20
A15649T	-	-	-	Not previously described	0.01	0.02
G20338A	0.15	0.73	0.38	Dai *et al*. (2001)	0.16	0.20
	0.11	0.50	0.42 (Chinese) 0.18 (Japanese)	Lamba *et al.* (2002)		
C23187T	0.00	0.15	0.00	Lamba *et al.* (2002)	0.19	0.24
T363T	-	-	-	Not previously described	0.04	0.02
I369V	-	-	-	Not previously described	0.07	0.04

Table 2.9: The association between SNPs detected in *CYP3A4* SNPs and ADRs experienced by CML patients treated with imatinib.

SNP	P	Odds Ratio	CI 95	P-Fisher
C23187T	0.14	2.46	0.73 - 8.31	0.00
G20338A	7×10^{-4}**	8.33	2.15 - 32.32	0.00
A15619G	0.54	1.15	0.04 - 29.38	1.00
I193I	0.10	8.38	0.33 - 214.56	0.27
I369V	0.50	1.89	0.29 - 12.23	0.00
A15649T	0.54	1.15	0.04 - 29.38	1.00
T15955A	0.28	2.79	0.14 - 56.37	0.56
T15871G	0.60	1.32	0.47 - 3.69	0.00
T15901C	0.54	1.15	0.04 - 29.38	1.00
T363T	0.80	1.37	0.12 - 15.97	1.00

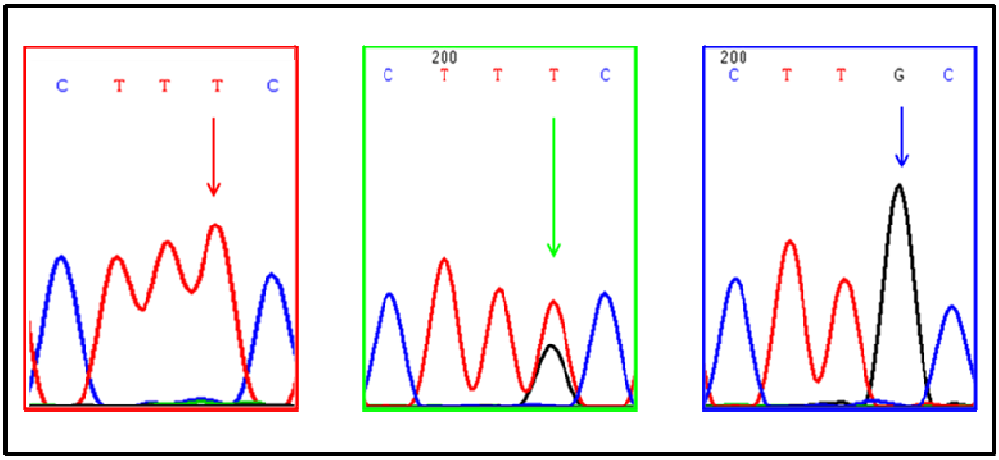
** Significant at P < 0.01

A



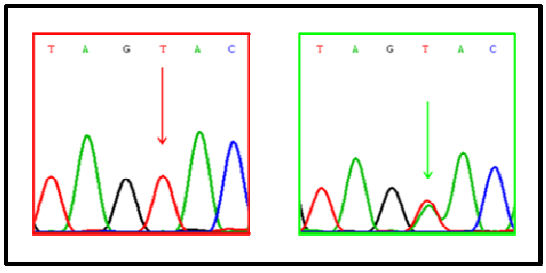
B

i Wild Type- T15871T ii T15871G-Heterozygous iii T15871G-Homozygous



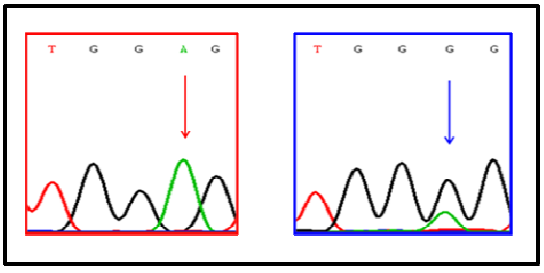
C

Wild Type- T15837T T15837A-Heterozygous



D

Wild Type- A15619A A15619G-Heterozygous



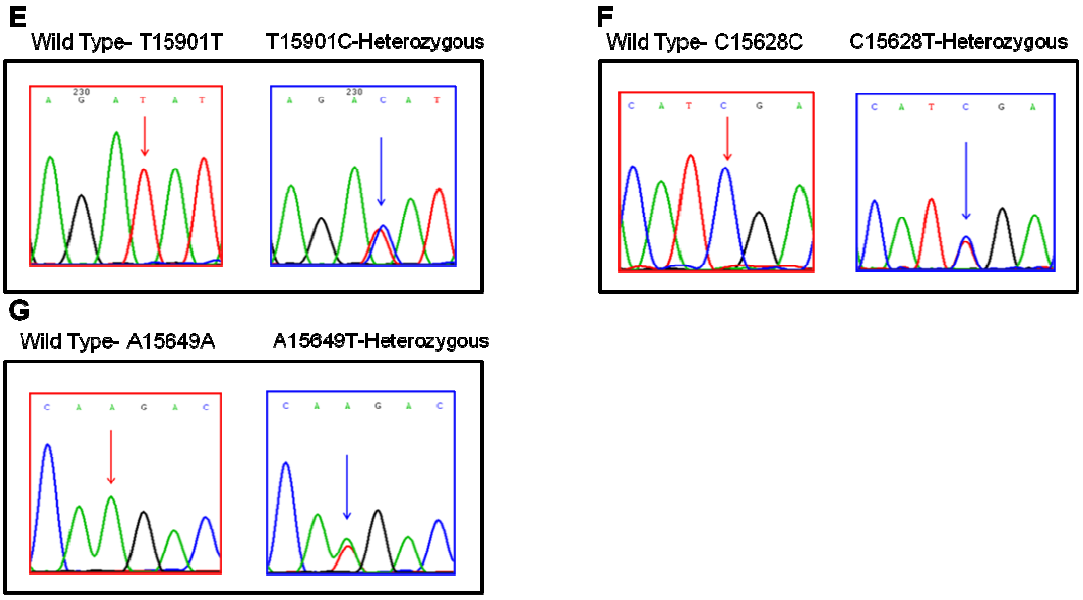


Figure 2.1: SNP result for exon 7 of *CYP3A4*. A: The difference plot of the exon 7, *CYP3A4*. Two variant groups were detected. The wild type (WT) group is depicted in red, and the two variant groups in green and blue. **B**: The sequencing results of the variant groups. B (i) The chromatograph of the WT sequence. B (ii) The chromatograph of the green variant group. The arrow indicates the T15871G SNP (heterozygous). B (iii) The chromatograph of the blue variant group. The arrow indicates the T15871G SNP (homozygous). **C:** The chromatograph for the T15837A SNP. The red box represents the WT and the green box the T15837A SNP (heterozygous). **D:** The chromatograph for A15619G .The red box represents the WT and the blue box the A15619G SNP (heterozygous). **E:** The chromatograph for T15901C .The red box represents the WT and the blue box the T15901C SNP (heterozygous). **F:** The chromatograph for C15628T. The red box represents the WT and the blue box the C15628T SNP (heterozygous). G The chromatograph for A15649T .The red box represents the WT and the blue box the A15649T SNP (heterozygous).

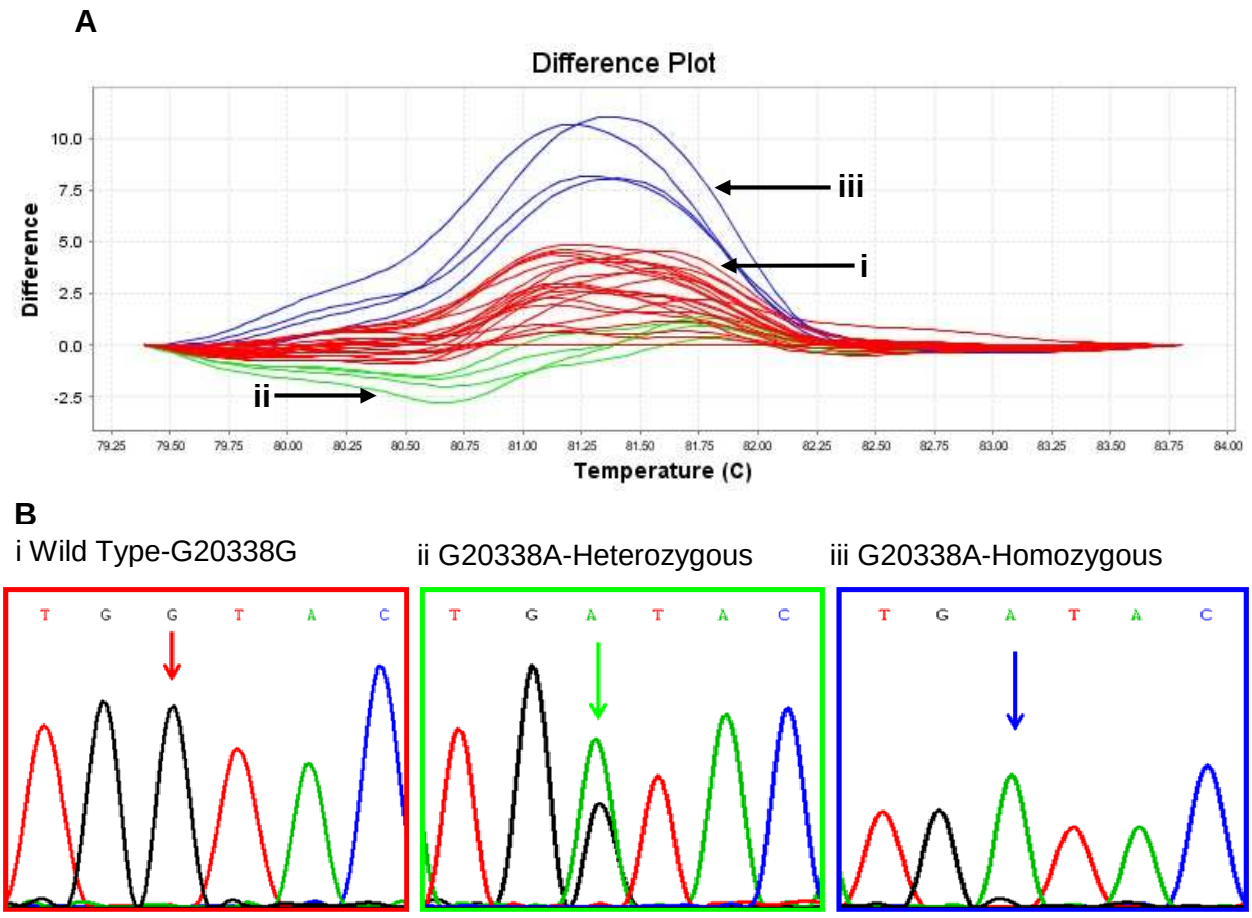


Figure 2.2: SNP results for exon 10 of *CYP3A4*. **A**: Difference plot of exon 10, *CYP3A4*. Two variants were grouped away from the wild type (WT). The WT group is depicted in red, and the two variant groups in green and blue. The blue variant contained the G20338A SNP in the homozygous form, while the green variant contained the same G20338A SNP in heterozygous form. **B:** The sequencing results of the variant groups. B (i) The chromatograph of the WT. B (ii) The chromatograph of the green variant group. The arrow indicates the G20338A SNP (heterozygous). B (iii) The chromatograph of the blue variant group. The arrow indicates the G20338A SNP (homozygous).

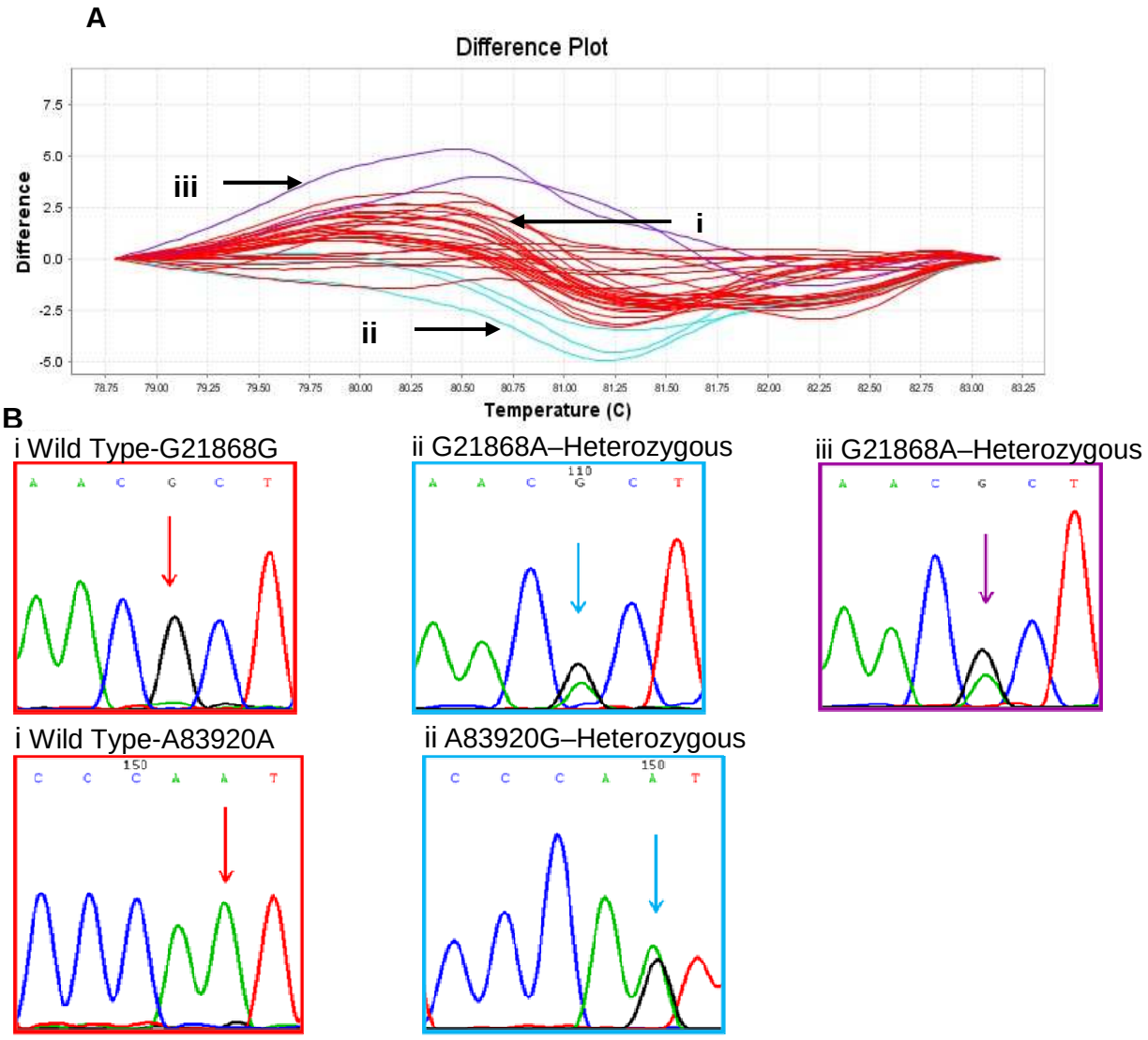


Figure 2.3: SNP results for exon 11 of *CYP3A4*. A: Difference plot of exon 11, *CYP3A4*. Two variants were grouped away from the WT. The WT group is depicted in red, and the two variant groups in blue and purple. The blue variant contained the A83920G (I369V) and the G21868A SNP. The purple variant group contains the G21868A SNP in heterozygous form. **B:** The sequencing results of the variant groups. B (i) The chromatograph of the WT. B (ii) The blue variant group contained two SNPs, G21868A and A83920G. Both were detected in the heterozygous form. B (iii) The chromatograph of the purple variant group. The arrow indicates the G21868A SNP (heterozygous).

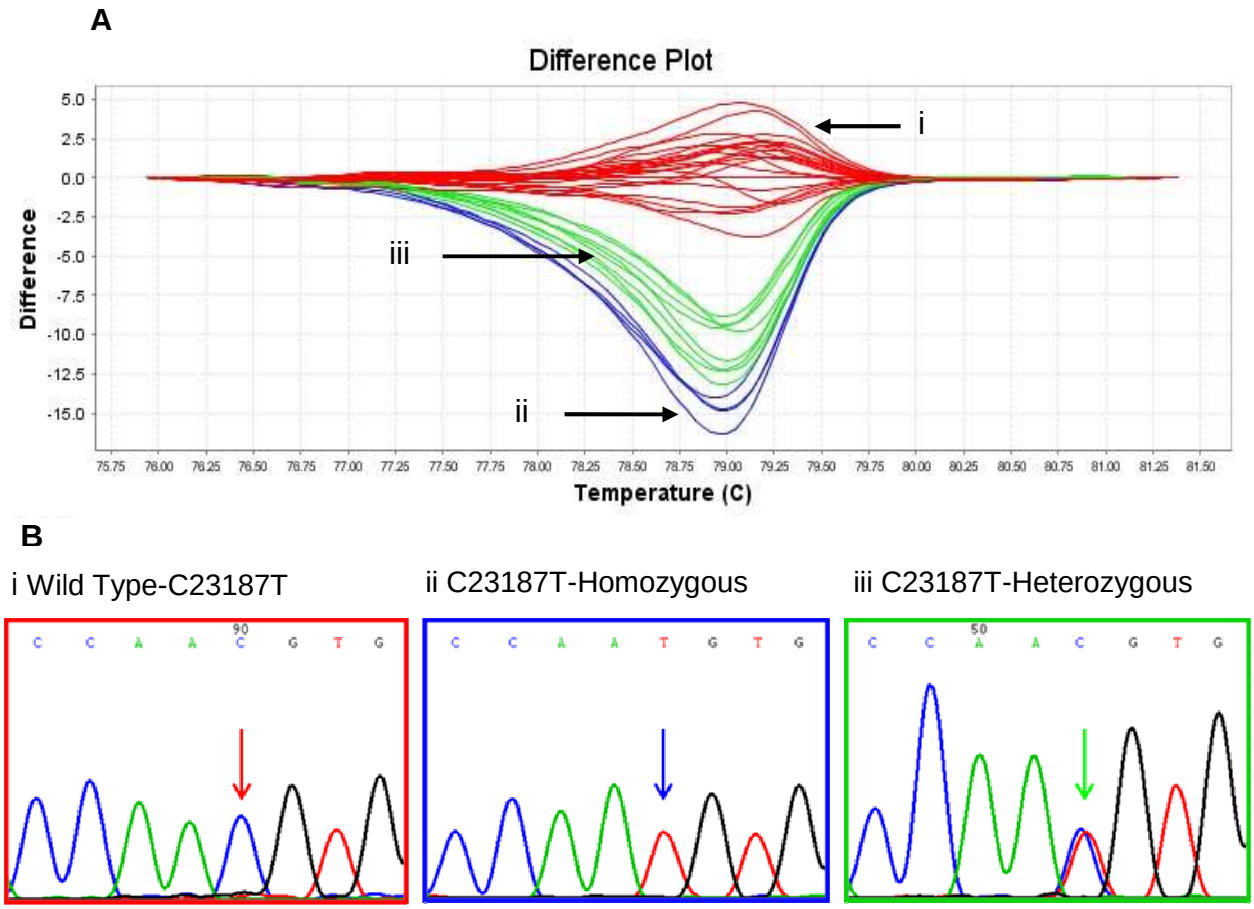


Figure 2.4: SNP results for exon 12 of *CYP3A4*. **A**: Difference plot of exon 12, *CYP3A4*. Two variants were grouped away from the WT. The WT group is depicted in red, and the two variant groups in green and blue. The blue variant contained the C23187T in homozygous form, while the green variant contained the same C23187T in heterozygous form. **B:** The sequencing results of the variant groups. B (i) The chromatograph of the wild type sequence. B (ii) The chromatograph of the blue variant group. The arrow indicates the C23187T SNP (homozygous). B (iii) The chromatograph of the green variant group. The arrow indicates the C23187T SNP (heterozygous).

2.3.6 Analysis of exon 5 of *CYP3A4*

When performing HRM analysis on exon 5 of *CYP3A4*, the difference plot identified 3 variant groups, with one patient sample in each of the variant groups (Figure 2.5). When the three patient samples were sequenced, they were found to contain 10 heterozygous SNPs that had not been previously described in literature. To verify this data, a BLAST (Basic Local Alignment Search Tool) analysis was performed. The BLAST results showed that the variant samples had 99% homology with *CYP3A7* versus 93% homology with *CYP3A4*. This suggested that *CYP3A7* was being amplified in these variant samples. When the real-time PCR conditions were made more stringent by increasing the annealing temperature from 58ºC to 61ºC, only *CYP3A4* was amplified. HRM analysis of *CYP3A4*, exon 5, at the more stringent annealing temperature did not identify the presence of variants compared to the wild type (Figure 2.6).

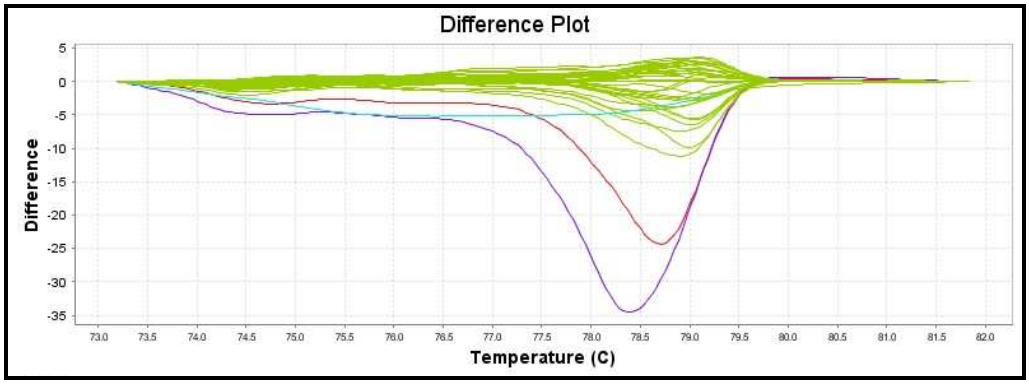


Figure 2.5: Difference plot of the exon 5 of *CYP3A4* before the optimisation of annealing temperature. The wild type variant is depicted in green. Three variant groups grouped away from the wild type, shown in blue, red and purple.

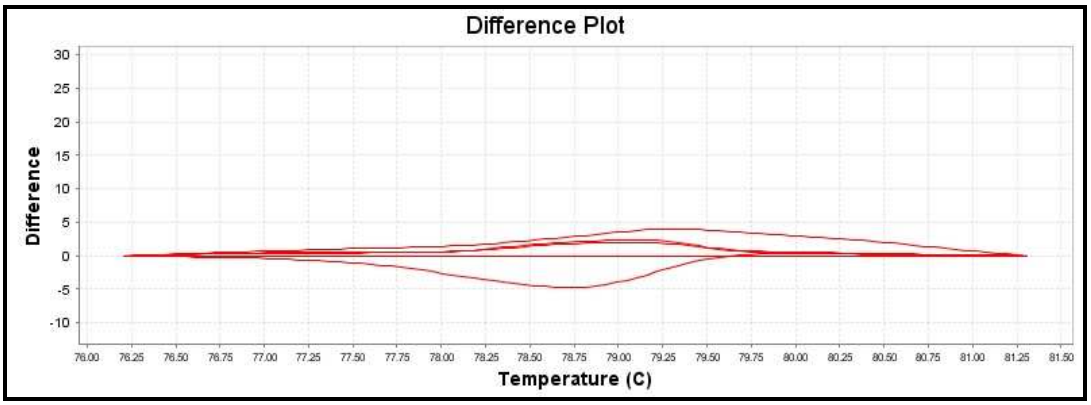


Figure 2.6: Difference plot of exon 5, *CYP3A4* after the optimization of annealing temperature increased to 61ºC.

2.3.7 Analysis of the derivative melting curve of exon 7 of *CYP3A4*

When performing HRM analysis on exon 7 of *CYP3A4,* a complex melting curve with multiple melting transition points was observed (Figure 2.7). Gel electrophoresis revealed that non specific amplification was not responsible for the multiple peaks since a single product of the expected size of 342 bp was produced (Figure 2.8). The exon 7 sequence was analysed using the internet based melting curve prediction software, uMELT (Dwight *et al*., 2011) (http://www.dna.utah.edu/umelt/umelt.html). The analysis of exon 7 with the prediction software indicated that there are multiple melting domains within the amplicon of exon 7 (Figure 2.9). Although the melting curve for exon 7 was complex, the various SNPs genotypes could be distinguished and were confirmed by sequencing.

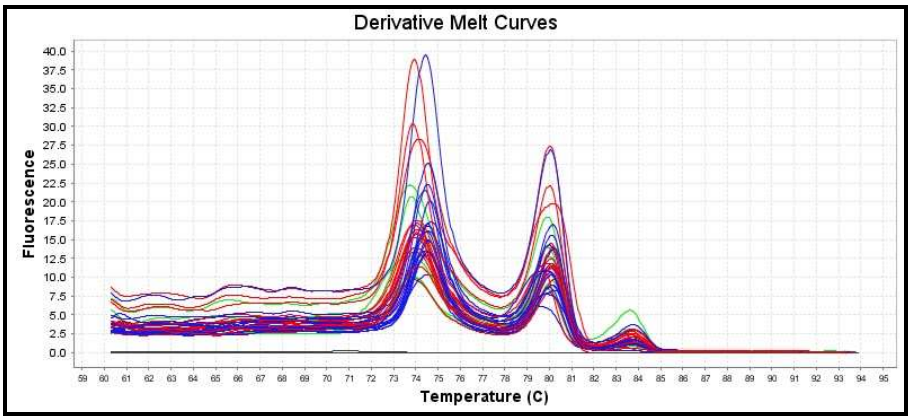


Figure 2.7: The derivative melting curve for exon 7 of *CYP3A4*. Multiple melting peaks were present.

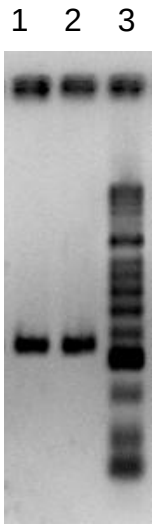


Figure 2.8: Gel electrophoresis image for exon 7 of *CYP3A4.* The HRM PCR product was resolved on a 2% agarose gel. The expected fragment size was observed. Lane 1, WT sample (342 bp); Lane 2, Patient 1 (342 bp); Lane 3, HyperLadder II.

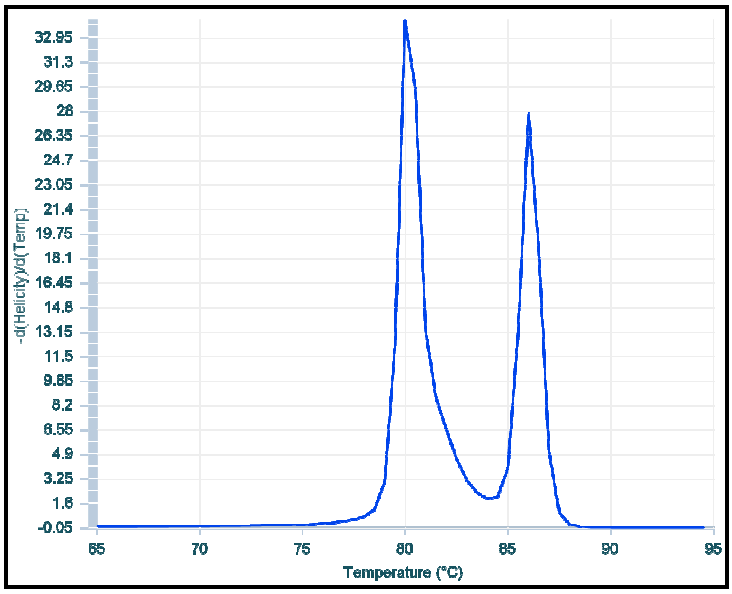


Figure 2.9: The predicted derivative melting curve for exon 7 of *CYP3A4* as determined by the uMelt software.

2.3.8 The Hardy-Weinberg equilibrium, allelic frequency and linkage analysis of *CYP3A5*

All the SNPs detected in *CYP3A5* in the combined study group were in Hardy-Weinberg equilibrium (Table 2.10). The G14690A (*CYP3A5*6)* SNP occurred at the highest allelic (0.07) frequency of all the SNPs detected in *CYP3A5* (Table 2.11). The *CYP3A5*6* variant is a functionally significant polymorphism that results in a splice variant causing decreased enzymatic activity of CYP3A5. It was detected in the CML population in all three ethnic groups. A previous study by Dandara *et al*. (2006) on a South African population reported a frequency of 0.21 in the Xhosa population and 0.12 in the Coloured patient group (Table 2.12). None of the SNPs detected in *CYP3A5* where in statistically significant linkage (r^2< 0.05).

Table 2.10: Hardy-Weinberg equilibrium of SNPs detected in *CYP3A5*.

SNP	Observed	Expected	χ^2	P
C7207T	0.05	0.05	0.00	0.97
A6986G	0.11	0.10	0.01	0.91
G14690A	0.08	0.13	0.68	0.41
G7226A	0.03	0.03	0.00	0.99

Table 2.11: Allele and genotype frequencies for *CYP3A5* SNPs.

SNP	Alleles		Genotype		
C7207T	C: 0.97	T: 0.03	CC: 0.96	CT: 0.04	TT: 0.00
A6986G	A: 0.95	G: 0.05	AA: 0.89	AG: 0.11	GG: 0.00
G14690A	G: 0.93	A: 0.07	GG: 0.89	AG: 0.08	AA: 0.03
G7226A	G: 0.99	A: 0.01	GG: 0.97	AG: 0.03	AA: 0.00

2.3.9 Novel *CYP3A5* SNPs

In this study the G7226A SNP detected has not previously been described in literature. It is a G to a A polymorphism that occurs within exon 4 of *CYP3A5*. It is a synonymous SNP and therefore does not result in the change of amino acid. This SNP was detected in one patient in the Black population in the control group.

2.3.10 Association analysis of *CYP3A5* SNPs detected in CML patients with adverse drug reactions

The detected SNPs were analysed to determine whether they were associated with ADRs. *CYP3A5*3* (A6986G) was the only allelic variant calculated to have a significant association with the experimental group (P = 0.03) (Table 2.13). The *CYP3A5*3* variant has been described as the most functionally important SNP in the *CYP3A5* gene. The SNP creates an alternative splice site resulting in a

truncated protein and thus decreased enzymatic activity. In the CML population tested in this study it was only detected in the Black population.

2.3.11 HRM analysis and sequencing results of *CYP3A5*

As was the case with *CYP3A4*, there was a 100% concordance between identifying sequence variants through HRM analysis and sequencing in *CYP3A5*. In intron 3 (Figure 2.10) and exon 7 (Figure 2.12) the HRM difference plot differentiated between variant samples based on zygosity. In exon 4 (Figure 2.11) however, the HRM variant grouping was dependent on the presence of different SNPs and did not differentiate between samples based on whether they were homozygous or heterozygous.

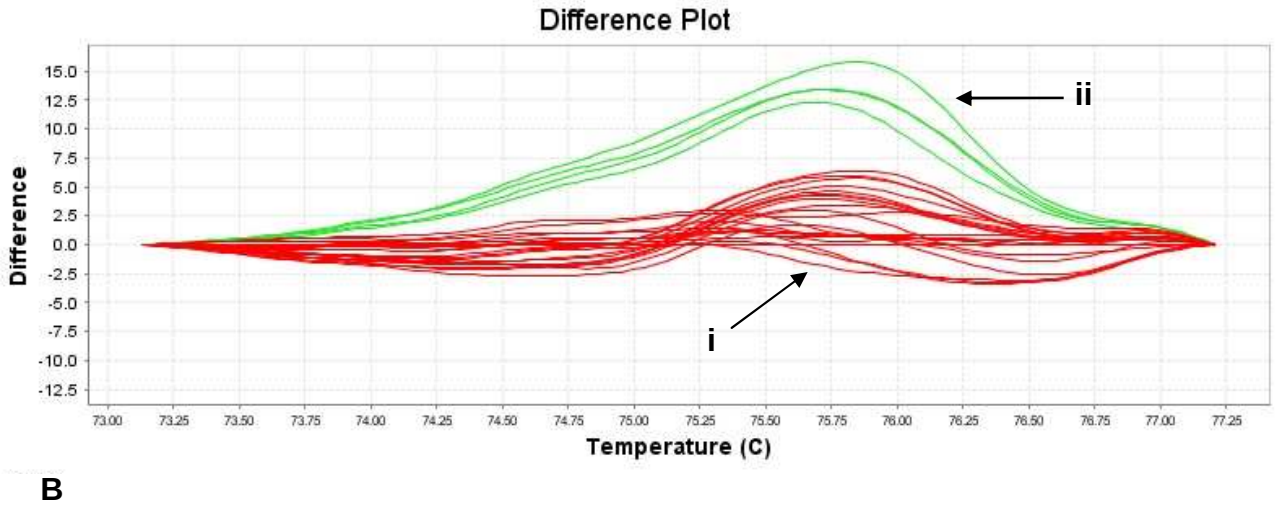
Table 2.12: The allelic frequency of *CYP3A5* SNPs reported in literature compared to in the CML population as a whole as well as the Black CML patients.

SNP	Frequency				Reference	CML patient SNP frequency	
	Caucasian	African American	Asian	South African population		Total	Black
C7207T	-	-	-	-	-	0.03	0.04
A6986G	0.85	0.48	0.77 (Japanese)	0.14 (Black) 0.94 (Caucasian) 0.59 (Coloured)	Kuehl *et al*. (2001) Fukuen *et al*. (2002) Dandara *et al*. (2006) Fernandez *et al*. (2010)	0.05	0.07
G14690A	0.00	0.08	0.00 (Japanese)	0.21 (Black) 0.12 (Coloured)	Kuehl *et al*. (2001) Fukuen *et al*. (2002) Dandara *et al*. (2006) Fernandez *et al*. (2010)	0.07	0.06
G7226A	-	-	-		Not previously described	0.01	0.02

Table 2.13: The association between SNPs detected in *CYP3A5* and ADRs experienced by CML patients on imatinib.

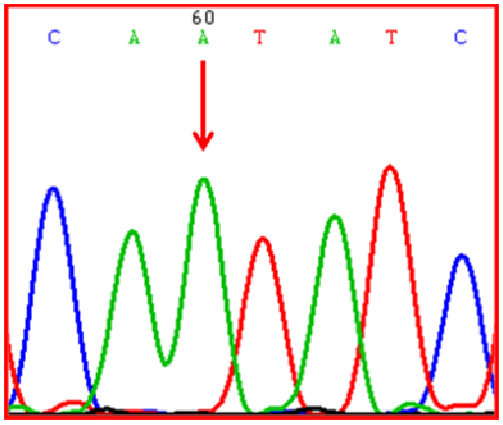
SNP	P	Odds Ratio	CI 95	P-Fisher
C7207T	0.46	2.79	0.17 - 46.84	0.47
A6986G	0.03*	9.35	0.91 - 95.96	0.00
G14690A	0.50	1.89	0.29 - 12.23	0.00
G7226A	0.54	1.15	0.04 - 29.38	1.00

* Significant at $P < 0.05$



B

i Wild Type-A6986A



ii A6986G- Heterozygous

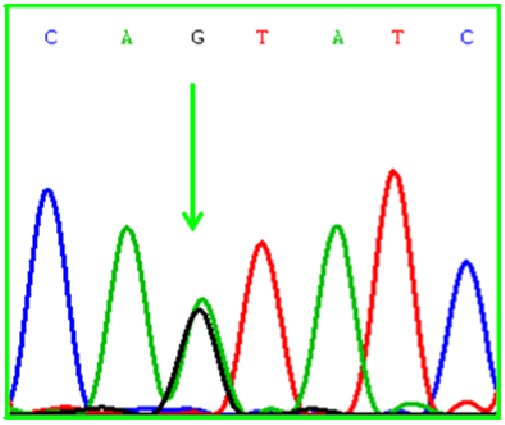


Figure 2.10: SNP results for intron 3 of *CYP3A5*. A: Difference plot of intron 3, *CYP3A5*. One variant was grouped away from the WT. The WT group is depicted in red, and the variant group in green. The green variant contained the A6986G SNP (*CYP3A5*3*) in the heterozygous form. **B:** The sequencing results of the variant group. B (i) is the chromatograph of the WT. B (ii) The chromatograph of the green variant group. The arrow indicates the A6986G SNP (heterozygous).

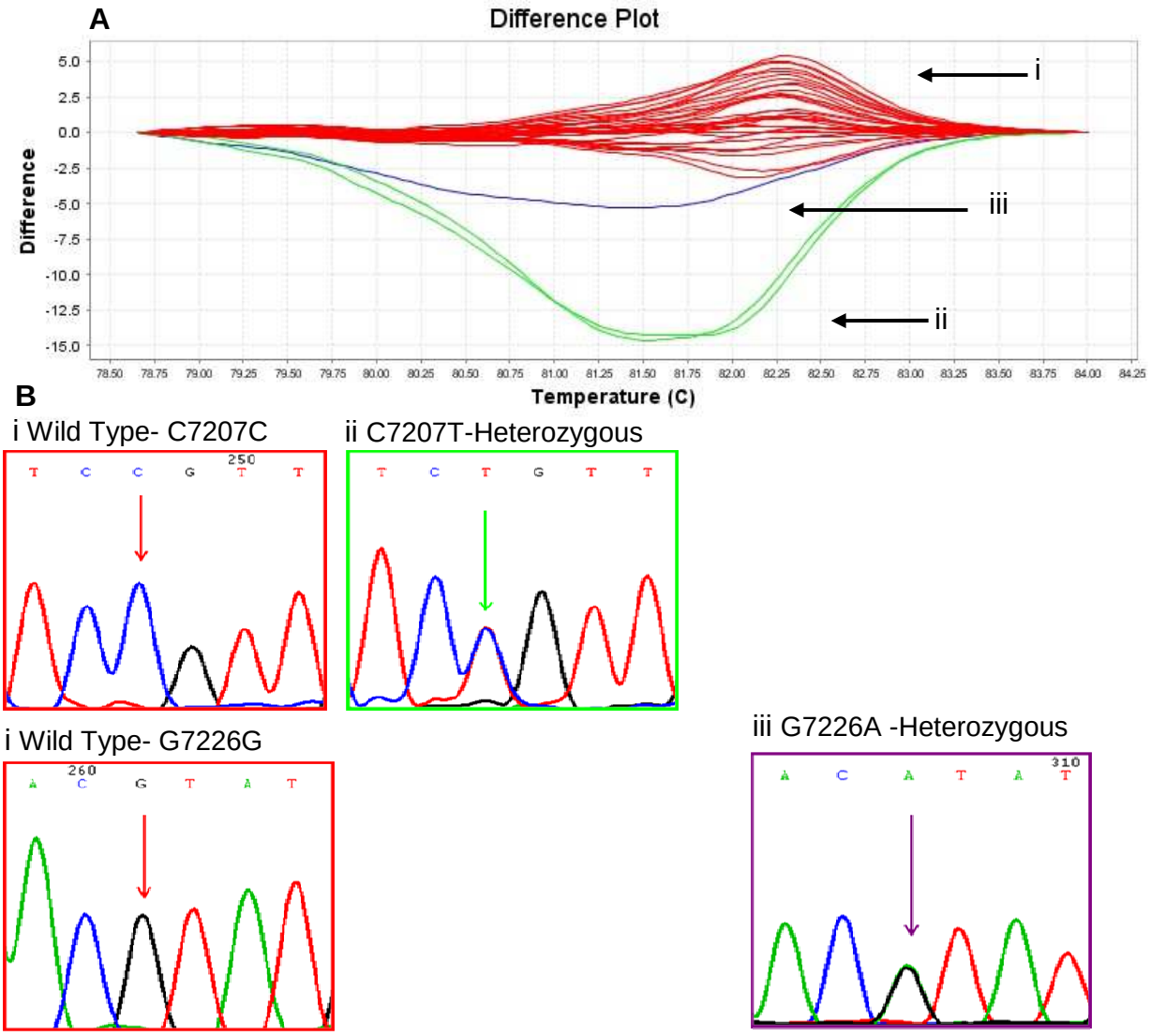


Figure 2.11: SNP results for exon 4 of *CYP3A5*. **A:** Difference plot of exon 4 region of *CYP3A5*. Two variants were grouped away from the WT. The WT group is depicted in red, and the two variant groups in green and blue. The blue variant contained the G7226A in heterozygous form, while the green variant contained the C7207T SNP in heterozygous form. **B:** The sequencing results of the variant groups. B (i) The chromatograph of the WT. B (ii) The chromatograph of the green variant group. The arrow indicates the C7207T SNP (heterozygous). B (iii) The chromatograph of the blue variant group. The arrow indicates the G7226A (heterozygous).

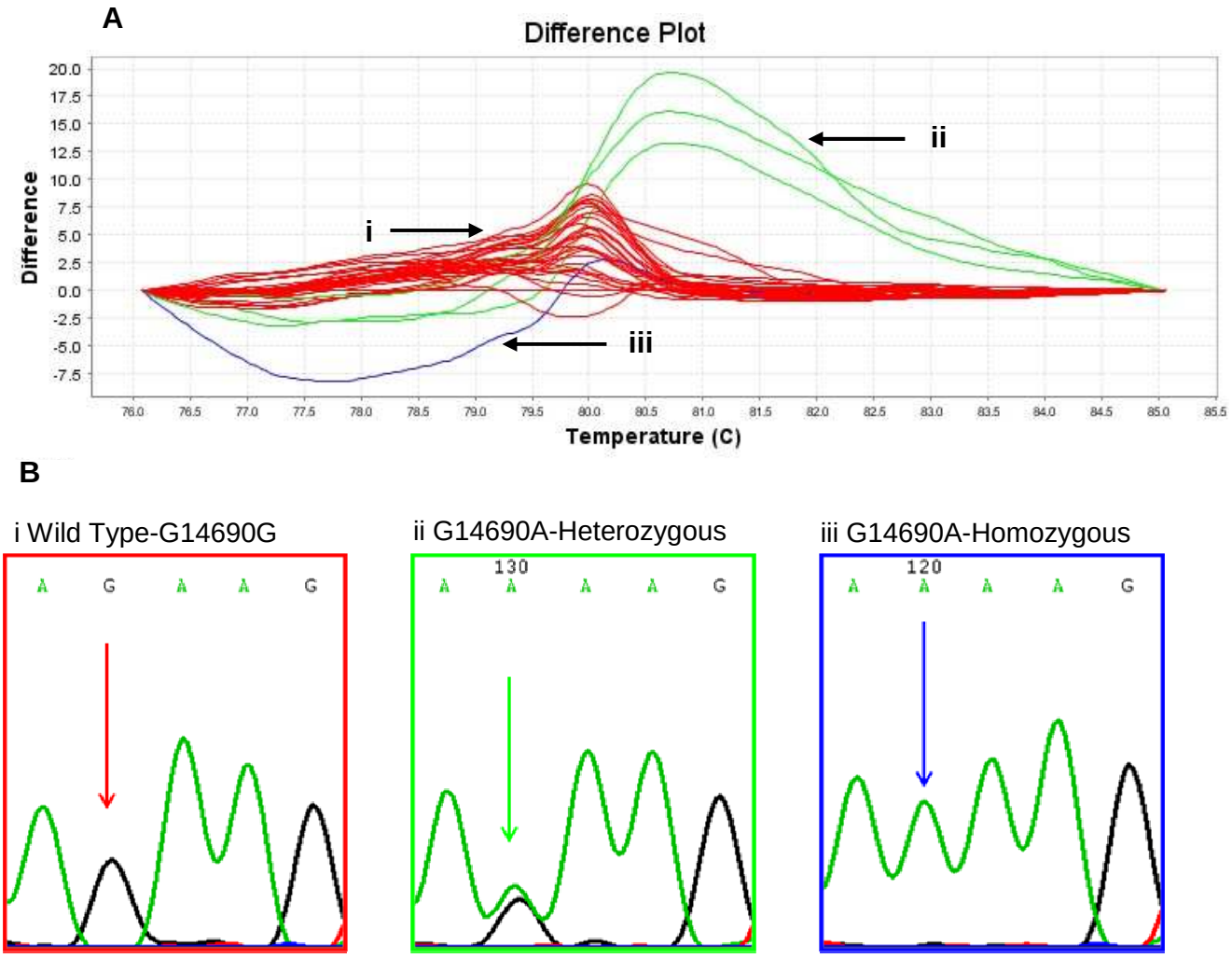


Figure 2.12: SNP results for exon 7 of *CYP3A5*. A: Difference plot of exon 7, *CYP3A5*. Two variants were grouped away from the WT. The WT group is depicted in red, and the two variant groups in green and blue. The blue variant contained the G14690A (*CYP3A5*6)* in homozygous form, while the green variant contained the same G14690A (*CYP3A5*6*) in heterozygous form. **B:** The sequencing results of the variant groups. B (i) The chromatograph of the WT. B (ii) The chromatograph of the green variant group. The arrow indicates the G14690A SNP (heterozygous). B (iii) The chromatograph of the blue variant group. The arrow indicates the G14690A SNP (homozygous).

2.4 Conclusion

In this study ten SNPs were detected in *CYP3A4* of which two, A15619G and A15649T, were not previously described in the literature. In *CYP3A5*, four SNPs were detected, one of which was not previously described in literature (G722A). Two known intron SNPs, G20338A in *CYP3A4* and A6986G in *CYP3A5* was significantly associated with the presence of ADRs in CML patients. With 36 CML patients and one Wild type sample, sequencing 29 fragments would have required a total of 2204 sequencing reactions since the sequencing was performed using forward and reverse primers separately. Through the use of HRM analysis as a screening tool this number could be reduced to approximately 380 sequencing reactions. This study was not intended to be a population study and future research should ideally include a larger population size.

CHAPTER THREE

STABILITY OF ULTRAMER AS COPY NUMBER STANDARDS IN REAL-TIME PCR[1,2]

3.1 Introduction

Real-time PCR has become a routine technique to quantify DNA or RNA copy number. However, one of the challenges in real-time PCR is the lack of commercially available copy number standards and/or reference material (Rutledge, 2004; Yan *et al.,* 2010). As a result, genomic DNA, cDNA, PCR amplicon, plasmid constructs or synthetic oligonucleotides are often used in serial dilution as standards (Yun *et al.,* 2006; Bustin *et al.,* 2009). Recent studies have investigated the stability of genomic DNA (Yun *et al.,* 2006; Röder *et al.,* 2010; Rossmanith *et al.,* 2011), PCR amplicon (Dhanasekaran *et al.,* 2010) and plasmid constructs (Dhanasekaran *et al.,* 2010; Martinez-Martinez *et al.,* 2011). It has been reported that although genomic DNA was more stable at -20^{o}C than 4^{o}C, degradation resulting in a change in copy number still occurred (Röder *et al.,* 2010; Rossmanith *et al.,* 2011). It has also been found that high concentrations of genomic DNA resulted in inhibition of PCR which can be overcome by additionally

[1] This chapter has been adapted from the article, Viljoen CD, Thompson GG, Sreenivasan S. Stability of ultramer as copy number standards in real-time PCR. *Gene* 2013; 516: 143-145, to include only data relevant to this dissertation.

[2] Although I am not the first author of this publication, I played an integral role in the formulation and execution of this research and its publication.

shearing the DNA (Yun *et al.,* 2006). In addition, freeze thawing also affects genomic DNA copy number standards adversely. Compared to this, while PCR products have been found to be more stable at -20^{o}C than 4^{o}C, storage still results in variation in copy number (Dhanasekaran *et al.,* 2010). Thus although different sources of nucleic acid are available for use as copy number standards, their stability in terms of storage remains a problem.

Due to advances in oligonucleotide synthesis, several studies have reported using ultramers in a native form as copy number standards for real-time PCR (Williams *et al.,* 2004; Zhang *et al.,* 2004; Vermeulen *et al.,* 2009; Flynn *et al.,* 2011; Ghiselli and Passamonti, 2011; Kavlick *et al.,* 2011; O'Callaghan and Fenech 2011). Ultramers have been used successfully as standards in either single stranded (Williams *et al.,* 2004; Zhang *et al.,* 2004; Flynn *et al.,* 2011; O'Callaghan and Fenech, 2011) or double stranded form (Kavlick *et al.,* 2011; Ghiselli and Passamonti, 2011). While some studies have reported on the storage conditions at -80^{o}C in TE (Kavlick *et al.,* 2011), or -20^{o}C in either water (O'Callaghan and Fenech, 2011) or buffer (Ghiselli and Passamonti, 2011), very few studies have investigated the effect of storage on ultramers. Kavlick *et al.* (2011) reported that ultramers were stable at -80^{o}C for over seven months. Compared to this, O'Callaghan and Fenech (2011) commented that ultramers were only stable for up to two weeks at 4^{o}C. From current studies it appears that the storage conditions for synthetic oligonucleotides used as standard in real-time PCR may have a considerable effect on copy number determination. Thus the aim of this study was

to determine the stability of ultramer as copy number standard when stored at 4^{o}C and -20^{o}C over a 30 day period using different mixing methods.

3.2 Materials and methods

3.2.1 Ultramer

A single stranded ultramer was synthesized for *CYP3A5* (152 bases) by Integrated DNA Technologies (Coralville, IA) (Table 3.1). Copy number was calculated according to Godornes *et al.* (2007) using the following formula:

$$\text{Number of copies} = \frac{\text{Mass(g) x Avogadro number}}{\text{Average molecular weight of a base x fragment length}}$$

$$= \frac{0.00018845 \times 6.022 \times 10^{23}}{330 \times 152}$$

$$= 2.262456756 \times 10^{15} \text{ copies}$$

$$= 2.262 \times 10^{15} \text{ copies}$$

The mass (g) of the ultramer was calculated based on the scale of synthesis provided by the manufacturer. The lyophilised ultramer was suspended in 0.1 x TE (10 mM Tris / 1 mM EDTA, pH 7.5). The concentration of the ultramer was confirmed using spectrophotometry. The ultramer was serially diluted in 0.1 x TE to obtain 10^2, 10^3, 10^4, 10^5 and 10^6 copies / 5 µl. The copy number standards

were mixed during preparation and prior to use by either vortexing, pipetting or inverting the tubes. The copy number standards were stored at 4ºC and -20ºC and used in real-time PCR assays at days 0, 7, 15 and 30, respectively.

3.2.2 Real-time PCR

Real-time PCR using the ultramer was performed on the ABI 7500 Fast. Sequences for primers and probes for *CYP3A5* were obtained from Fakhoury *et al.* (2005) and synthesized by Integrated DNA Technologies (Coralville, IA). The real-time PCR reactions were performed in duplicate and consisted of 2 x TaqMan Fast Advanced Mastermix (Applied Biosystems), 5 µl of copy number standard, 200 nM forward and reverse primer and 100 nM probe for *CYP3A5* made up to 20 µl with nuclease free sterile water. Thermal cycling conditions were 2 minutes at 50^{o}C, 10 minutes at 95^{o}C followed by 45 cycles of 15 seconds at 95^{o}C and 1 minute at 60^{o}C.

Table 3.1: Sequence, fragment length and scale of synthesis of the ultramer.

Gene	Ultramer sequence (5'-3')	Fragment length	Scale of synthesis
CYP3A5 (NM_000777)	TCTCTGGAAATTTGACACAGAGTGCTAT AAAAAGTATGGAAAAATGTGGGGAACGT ATGAAGGTCAACTCCCTGTGCTGGCCA TCACAGATCCCGACGTGATCAGAACAG TGCTAGTGAAAGAATGTTATTCTGTCTT CACAAATCGAAGGT	152 bases	4 nmol

3.3 Results and discussion

In contrast to O'Callaghan and Fenech (2011) who suggested that "working stocks of oligomers should be made fresh", ultramer copy number standards were found to be highly stable whether stored at 4ºC or -20ºC over a 30 day period in 0.1 x TE (Figure 3.1). Less than a 5% variance was observed in C_T values for the different copy number standards regardless of storage condition or mixing method over a period of 30 days. Furthermore, the method of mixing to prepare the ultramer and prior to use by either vortexing, pipetting or inverting appeared to make no difference in the reproducibility and linearity of results (Figure 3.2). Thus we conclude that 4°C is suitable for storage of ultramer copy number standard for up to one month and for longer term storage at -20°C.

The relative cost of using ultramers as copy number standards appears negligible compared to commercial standards (if available). We calculate that at a standard scale of synthesis (4 nmol), sufficient ultramer is synthesized for approximately 1 x 10^9 assays, using copy number standards in duplicate, for under $0.01 per assay (Table 3.2). Thus the use of ultramer is a highly cost efficient method of generating copy number standards that are up to 200 bases in size compared to commercially available standards.

The use of ultramer as copy number standards has several advantages over other sources of DNA for copy number standards: 1. Since the ultramer is synthetic, it is free of any biological contamination that could affect overall stability and

reproducibility; 2. Up to 200 bases of ultramer can be synthesized without additional preparation including cloning or extraction; 3. Ultramer is highly stable compared to genomic DNA or PCR amplicon; and 4. Preparations of different batches of ultramer appear to provide reproducible results. Based on these results, ultramer is stable, convenient and cost effective for use as copy number standards in real-time PCR.

Table 3.2: Cost efficiency of using ultramer as copy number standard.

Gene	Calculated copies of ultramer[a]	Theoretical number of assays[b]	Estimated cost per assay ($)
CYP3A5	2.26×10^{15}	1×10^{9}	<0.01

[a]At a standard scale of synthesis (4 nmol).

[b]Assuming that copy number standards are used in duplicate.

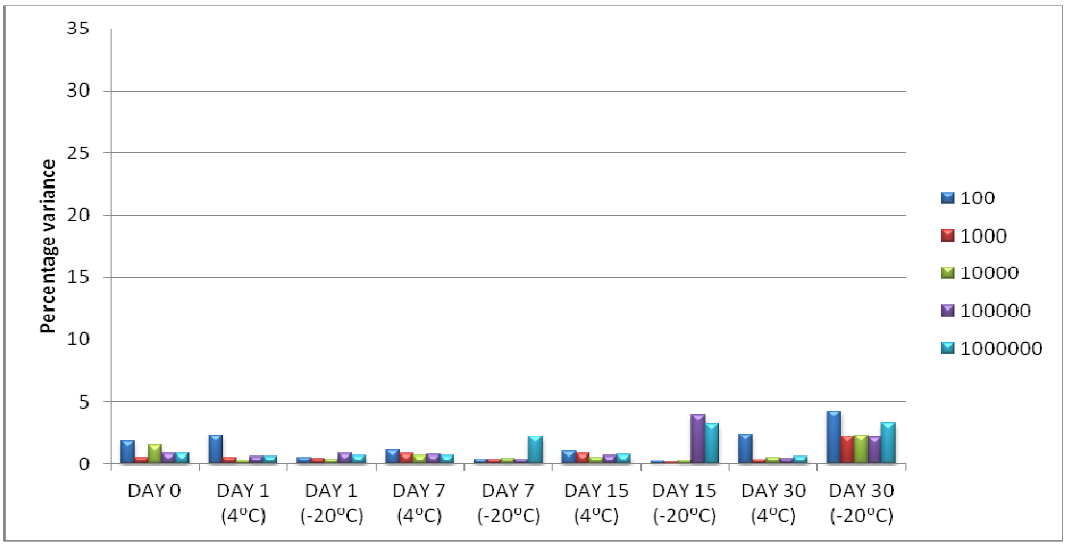


Figure 3.1: Mean percentage of variance in Ct value of *CYP3A5* for ultramer copy number standards (10^2, 10^3, 10^4, 10^5 and 10^6) at 0, 1, 7, 15 and 30 days at 4°C and -20°C was calculated using ANOVA. The data for each day represent a combination of mixing methods.

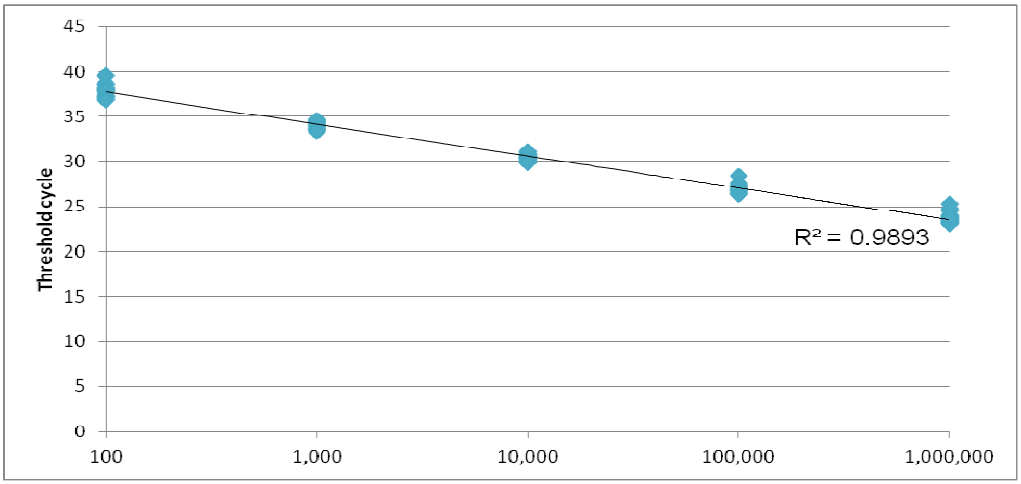


Figure 3.2: The plot represents a compilation of threshold cycle data from copy number standards (10^2, 10^3, 10^4, 10^5 and 10^6) for *CYP3A5* from 24 assays at 0, 1, 7, 15 and 30 days at 4°C and -20°C.

CHAPTER FOUR

GENE EXPRESSION OF *CYP3A4* AND *CYP3A5* IN CML PATIENTS

4.1 Introduction

Imatinib mesylate is a selective inhibitor of BCR-ABL kinase activity and has demonstrated excellent efficacy as first-line therapy for treatment of chronic myeloid leukaemia (CML) (Druker *et al*., 2001; Kantarjian *et al*., 2002; Talpaz *et al*., 2002; Druker *et al*., 2006). The treatment of CML with imatinib results in a durable response with prolonged patient survival (Talpaz *et al*., 2002; O'Brien *et al*., 2003; Hochhaus *et al.,* 2009). Imatinib has been shown to have a high inter-patient pharmacokinetic variability with blood plasma concentration varying between 40% and 60% (Peng *et al*., 2004). Pharmacokinetic variability is an important contributor to the difference of both the severity of side effects and therapeutic response among patients (Evans and Relling, 1988; Evans and Relling, 1999; Undevia *et al.,* 2005). Although several factors influence the pharmacokinetics of imatinib, metabolism is a crucial step of drug disposition and has been shown to be highly variable between individuals (Peng *et al*., 2004; de Kogel and Schellens, 2007; Undevia *et al.,* 2005).

Imatinib is metabolised primarily by the Cytochrome P450 enzymes, CYP3A4 and CYP3A5 (Cohen *et al*., 2002; Peng *et al*., 2005; de Kogel and Schellens, 2007). The expression of CYP3A4 and CYP3A5 has been reported to show high inter-individual variation (Wilkinson, 1996; Lamba *et al*., 2002). For example, the hepatic expression of CYP3A4 is reported to vary up to 40-fold between individuals in the same population (Shimada et *al.,* 1994; Westlind *et al*., 1999; Guengerich, 1999). Compared to this, CYP3A5 is only detectable in an estimated 10% to 30% of Caucasians and 50% to 60% of African Americans (Wojnowski, 2004; Xie *et al.,* 2004). Ozdemir *et al.* (2000) suggested that the inter-individual variation in expression is primarily due to genetic factors.

Several studies have focused on the identification of SNPs in *CYP3A4* and *CYP3A5* (Lamba *et al.,* 2002). SNPs in *CYP3A4* and *CYP3A5* have been found to be associated with altered protein is expression (Eiselt *et al.*, 2001; Kuehl *et al.,* 2001; Lamba *et al.*, 2002; Lee *et al*., 2003). For example, a SNP in intron 3 of *CYP3A5* (A6986G) denoted as variant *CYP3A5*3* results in a mRNA splice site, encoding a truncated CYP3A5 protein associated with decreased CYP3A5 expression (Kuehl *et al.,* 2001; Wong *et al*., 2004; Haufroid *et al*., 2004; Josephson *et al*., 2007). The presence of SNPs in either *CYP3A4* and/or *CYP3A5* may alter the rate of imatinib metabolism and result in a prolonged exposure to imatinib that may be the cause for the development of ADRs (Lin *et al.,* 2003; Peng *et al.,* 2005).

CYP3A4 and CYP3A5 are expressed primarily in the liver but also in the gastrointestinal tract, lungs, leucocytes, kidneys, and pituitary gland (Canaparo *et al*., 2007; Koch *et al.,* 2002; Yokose *et al*., 1999; Piipari *et al*., 2000; Nowakowski-Gashaw *et al*., 2002). Several studies have shown that CYP3A4 and CYP3A5 expression in the liver is correlated to levels of hepatic *CYP3A4* and *CYP3A5* mRNA, respectively (Lin *et al*., 2002; Watanabe *et al*., 2004). A recent study by Temesvári *et al*. (2012) reported that mRNA expression of *CYP3A4* in leukocytes correlate to CYP3A4 hepatic activity. The availability of hepatic samples is a challenge and therefore using peripheral blood would be a more feasible alternative. The aim of this study was to investigate the association between SNPs detected in *CYP3A4* and *CYP3A5* of CML patients treated with imatinib (refer to chapter 2) and *CYP3A4* and *CYP3A5* mRNA expression, respectively.

4.2 Materials and methods

The study group was the same as described in chapter 2, section 2.2.

4.2.1 TRI Reagent stabilisation

The white blood cells were stabilised in TRI Reagent (Sigma-Aldrich) as described in chapter 2, section 2.2.1.

4.2.2 RNA extraction and RNA concentration determination

RNA extraction was performed in a dedicated area where all equipment was treated with DEPC (Diethylpyrocarbonate) and RNase-Zap (Invitrogen) to prevent RNase contamination and RNA degradation. The previously prepared TRI Reagent homogenate (1.6 ml) was thawed at room temperature followed by the addition of 15 µl proteinase K (20 mg/ml). The sample was then incubated at 65ºC for 20 minutes followed by the addition of 350 µl of chloroform. The sample was placed on ice for 3 minutes followed by centrifugation at 12,000 rpm for 15 minutes. Approximately 1 ml of the upper aqueous phase was removed to which 1 ml of absolute isopropanol was added to precipitate the RNA. The sample was incubated on ice for 30 minutes followed by centrifugation at 12,000 rpm for 10 minutes. The RNA precipitate was washed twice by the addition of 1 ml of 75% ethanol. The sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant discarded. The pellet was air dried for 15 minutes after which it was dissolved in 50 µl of nuclease free sterile DEPC treated water. If necessary, the RNA was incubated at 55ºC for 15 minutes to dissolve the pellet. The sample was then placed on ice for 1 minute. The RNA concentration was determined fluorometrically using the Qubit RNA Assay Kit (Invitrogen) according to the manufacturer's instructions. Calibration standards (0 ng/µl and 10ng/µl RNA) included in the kit were prepared by the addition of 10 µl of standard to 189 µl Qubit RNA Buffer and 1 µl of Qubit RNA Reagent provided in the kit. To determine the concentration of RNA in the sample, 1 µl of extracted RNA was added to 198 µl of Qubit RNA Buffer and 1 µl Qubit RNA Reagent. The mixture

was vortexed and centrifuged, followed by incubation at room temperature for 2 minutes before measuring the concentration of RNA. Extracted RNA was stored at -70ºC until used for cDNA synthesis.

4.2.3 cDNA synthesis

The extracted RNA was used to synthesise cDNA. All cDNA synthesis reactions were performed in duplicate using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). The cDNA synthesis mixture contained 1 µg of RNA, 4 µl of High capacity RNA-to-cDNA Master Mix (Applied Biosystems) and nuclease free sterile water to a final volume of 20 µl. The mixture was incubated at 25ºC for 5 minutes followed by 30 minutes at 42ºC. The mixture was incubated for a further 5 minutes at 85ºC and cooled to 4ºC. The sample was stored at 4ºC until used.

4.2.4 Real-time quantitative PCR

Real-time PCR was performed with *CYP3A4* or *CYP3A5* as target genes and *GU*S as the reference gene. Real-time PCR reactions consisted of 2 x TaqMan Fast Advanced Mastermix (Applied Biosystems), 5 µl cDNA (equivalent to 0.25 µg of RNA), 200 nM forward and reverse primer, respectively, 100 nM probe and nuclease free sterile water to a final volume of 20 µl. The real-time PCR was performed on the ABI 7500 Fast. The thermal cycling conditions were 2 minutes at 50^{o}C, 10 minutes at 95^{o}C followed by 45 cycles of 15 seconds at 95^{o}C and 1 minute at 60^{o}C. The primer and probe sequences of the reference gene, beta-

glucuronidase (*GUS)* were obtained from Beillard *et al.* (2003) (Table 4.1). The sequence of the primer and probe for *CYP3A5* were obtained from Fakhoury *et al.* (2005) while the primer and probe for *CYP3A4* was designed using the online program Primer3Plus (www.bioinformatics.nl/primer3plus/). Primer and probe were synthesised by Integrated DNA Technologies. Commercial copy number standards were used to quantify *GUS* mRNA (Ipsogen) (10^3, 10^4 and 10^5 copies). Ultramer was synthesised by Integrated DNA Technologies and used as copy number standards for *CYP3A4* and *CYP3A5,* respectively, at 10^2, 10^3, 10^4, 10^5 and 10^6 copies (Table 4.2) (refer to chapter 3).

Table 4.1: The primer and probe sequences used for the real-time PCR amplification of *CYP3A4*, *CYP3A5* and *GUS*, respectively (F-forward primer; R-reverse primer; P-probe).

Gene	Primer/ Probe	Sequence (5'-3')	Reference
CYP3A4	F	TCCATTCCTCATCCCAATTC	Designed using Primer3Plus Software
	R	TGTGGGACTCAGTTTCTTTTGA	
	P	FAM ATGAAAGAAAGTCGCCTCGA MGB	
CYP3A5	F	CTGGAAATTTGACACAGAGTGC	Fakhoury *et al*. (2005)
	R	TTCGATTTGTGAAGACAGAATAACATT	
	P	FAM AACGTATGAAGGTCAACTCCCTGTGCTGG TAMRA	
GUS	F	GAAAATATGTGGTTGGAGAGCTCAT	Beillard *et al.* (2003)
	R	CCCGAGTGAAGATCCCCTTTTTA	
	P	FAM CCAGCACTCTCGTCGGTGACTGTTCA TAMRA	

Table 4.2: Characteristics of ultramer used as copy number standards for *CYP3A4* and *CYP3A5* real-time quantitative PCR, respectively.

Gene	Ultramer sequence (5'-3')	Fragment length	Scale of synthesis
CYP3A4 (AF182273)	CAGTCTTTCCATTCCTCATCCCAATTCTTGAA GTATTAAATATCTGTGTGTTTCCAAGAGAAG TTACAAATTTTTTAAGAAAATCTGTAAAAAGG ATGAAAGAAAGTCGCCTCGAAGATACACAAA AGCACCGAGTGGATTTCCTTCAGCTGATGAT TGACTCTCAGAATTCAAAAGAAACTGAGTCC CACAAAG	195 bases	4 nmole
CYP3A5 (NM_000777)	TCTCTGGAAATTTGACACAGAGTGCTATAAA AAGTATGGAAAAATGTGGGGAACGTATGAA GGTCAACTCCCTGTGCTGGCCATCACAGAT CCCGACGTGATCAGAACAGTGCTAGTGAAA GAATGTTATTCTGTCTTCACAAATCGAAGGT	152 bases	4 nmole

4.2.5 Statistical analysis

The *CYP3A4* and *CYP3A5* data were analysed to determine data distribution using the Shapiro-Wilk test. Non parametric statistical analysis was performed on data to assess the significance of the difference between groups by using the Mann-Whitney and the Kruskal-Wallis test. Spearman rank nonparametric correlation analysis was performed to evaluate the potential correlation between *CYP3A4* and *CYP3A5* mRNA. The Shapiro-Wilk, Mann-Whitney, Kruskal-Wallis and Spearman rank test were performed using the statistical software QI Macros version 2008.11 (KnowWare International Inc., CO, USA) used in combination with Microsoft Excel. The association between SNPs and gene expression was analysed using linear regression, with the additive model and Bonferroni correction using SNP & Variation Suite (SVS) version 7.7 software (Golden Helix Inc., Bozeman, MA, USA). The statistical significance was defined as $P < 0.05$.

4.3 Results and discussion

CYP3A4 mRNA was detected in 84% of individuals with 16% of patients having undetectable levels of *CYP3A4* mRNA. *CYP3A5* mRNA was undetectable in 5% of individuals tested. *CYP3A4* transcripts were the most abundant of the two genes quantified (Table 4.3) with *CYP3A4* and *CYP3A5* mean percentage differing by 189 fold. Temesvári *et al.* (2012) reported the *CYP3A4* mRNA expression in leukocytes to range between undetected and 0.218% while Haas *et al*. (2005) reported the *CYP3A4* gene expression ranged from undetected to 8.86%. The histogram (Figure 4.1 and Figure 4.2) depicting the distribution of *CYP3A4* and *CYP3A5* mRNA expression data, deviates from the expected bell shaped curve suggesting that the data is not normally distributed. The skewed distribution of *CYP3A4* and *CYP3A5* expression data was confirmed by the Shapiro-Wilk test ($P < 0.001$) (Figure 4.1 and Figure 4.2).

Table 4.3: Summary of *CYP3A4* and *CYP3A5* mRNA expression as a percentage ratio to *GUS*, in CML patients treated with imatinib.

	***CYP3A4*%**	***CYP3A5*%**
Mean	15.13	0.08
Standard deviation	46.18	0.10
Range	0.00 to 202.18	0.00 to 0.36
Median	0.24	0.04
Variance	2132.10	0.01

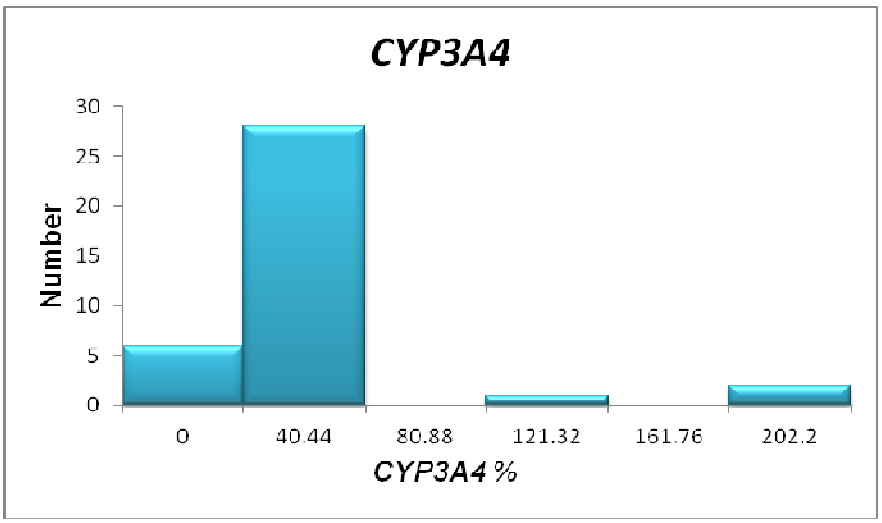


Figure 4.1: Histogram plot of *CYP3A4* mRNA expression data.

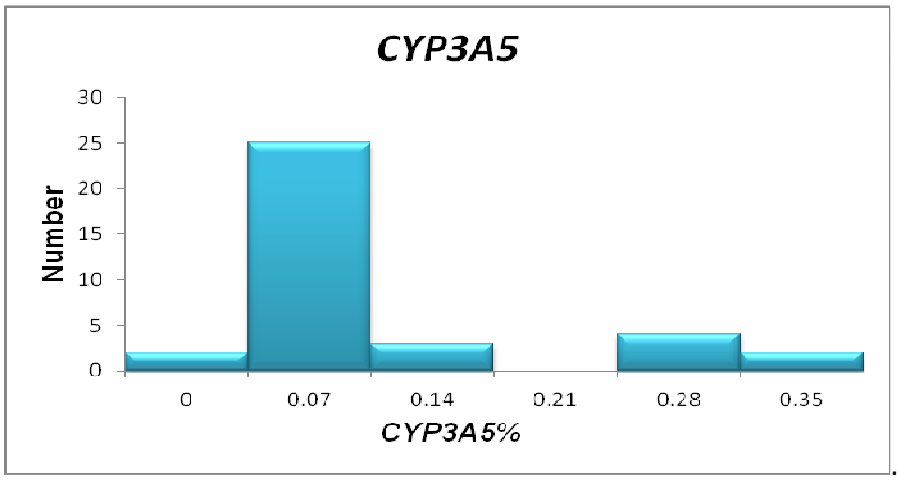


Figure 4.2: Histogram plot of *CYP3A5* mRNA expression data.

4.3.1 Association of SNPs and *CYP3A4* and *CYP3A5* mRNA expression.

The association between SNPs detected in *CYP3A4* and *CYP3A5* (refer to chapter 2) and gene expression was analysed using linear regression, with the additive model. Taking into account that multiple SNPs were tested in parallel, associations between the SNPs and gene expression were assessed after Bonferroni correction for multiple testing. The analysis showed that I193I was associated with increased *CYP3A4* mRNA expression (Table 4.4). The I193I SNP was detected in one Black CML patient who was on a decreased imatinib dosage (200mg per day) due to the occurrence of ADRs. However, I193I was not statistically associated with the presence of ADRs (refer to chapter 2), which may be due to a low frequency (0.01) and small population group. It is interesting to note that the I193I was associated with increased *CYP3A4* expression in a patient experiencing ADRs, since it would rather be expected that a decrease in expression would explain the presence of ADRs. I193I is a synonymous SNP and the amino acid sequence is thus unchanged. However, it has been suggested that synonymous SNPs could affect the stability and/or expression of a protein through altered miRNA binding, protein folding and mRNA splicing (Hunt *et al.,* 2009: Ho *et al.*, 2011). The impact of I193I on catalytic activity of CYP3A4 is unknown and no association studies have been reported in literature.

None of the SNPs detected in *CYP3A5* was statistically associated with altered *CYP3A5* expression (Table 4.5). However, this does not exclude the possibility of SNPs affecting protein activity which was not investigated in this study.

An association between *CYP3A5*3* and decreased gene expression was expected, since it has been reported in literature that *CYP3A5*3* is associated with decreased protein expression. A possible explanation may be that all the patients that had the A6986G SNP (*CYP3A5*3*) had it in the heterozygous form. It has been reported that the presence of at least one wild type *CYP3A5* allele will result in approximately two to three fold higher total CYP3A5 activity compared to having a homozygous *CYP3A5*3* variant (Kuehl *et al*., 2001; Lin *et al*., 2002).

Table 4.4: Association of *CYP3A4* mRNA and SNPs detected in *CYP3A4* of CML patients treated with imatinib.

SNP	P value	Bonferroni Corrected P Value
T15871G	0.26	1.00
T15955A	0.56	1.00
I193I	$4x10^{-5}$*	$4x10^{-4}$*
T15901C	0.96	1.00
A15619G	0.75	1.00
A15649T	0.96	1.00
G20338A	0.02*	0.196
I369V	0.61	1.00
T363T	0.80	1.00
C23187T	0.26	1.00

* Significant at P<0.05

Table 4.5: Association of *CYP3A5* mRNA and SNPs detected in *CYP3A5* of CML patients treated with imatinib.

SNP	P value	Bonferroni corrected P value
A6986G	0.17	0.68
C7207T	0.76	1.00
G7226A	0.61	1.00
G14690A	0.52	1.00

4.3.2 The correlation analysis of *CYP3A4* and *CYP3A5* mRNA expression

It has been reported in literature that mRNA expression of *CYP3A4* and *CYP3A5* are inversely correlated (Lin *et al*., 2002). In the current study since the expression data were not normally distributed, the Spearman rank correlation coefficient was used to determine if there was any correlation between *CYP3A4* and *CYP3A5* expression. In contrast to Lin *et al*. (2002) the Spearman correlation coefficient was 0.108 (P=0.393), indicating that there was not a statistically significant correlation between *CYP3A4* and *CYP3A5* expression in this study (Figure 4.3).

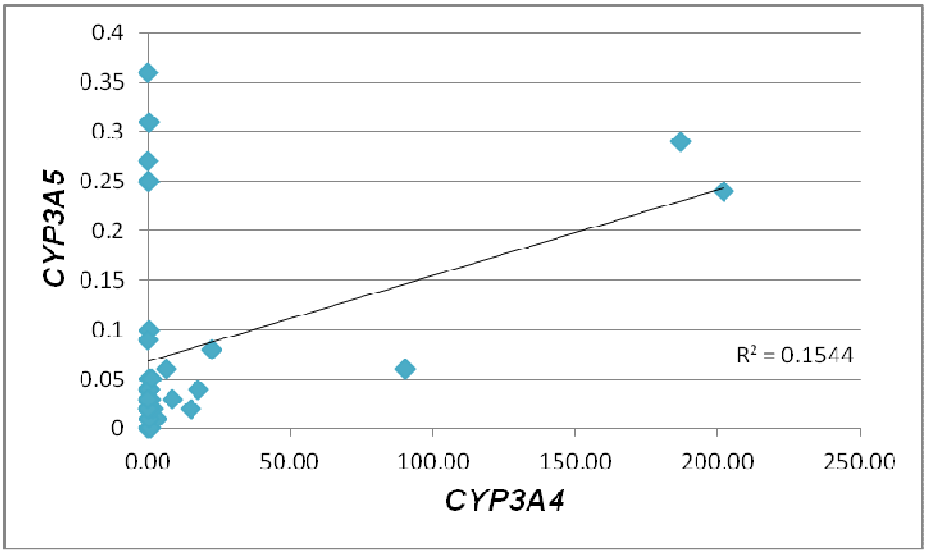


Figure 4.3: Correlation of mRNA levels of *CYP3A4* to *CYP3A5* in CML patients.

4.3.3 Expression levels of *CYP3A4* and *CYP3A5* mRNA in different gender and ethnicity groups

It has been reported that gender and ethnicity of an individual is significantly associated with *CYP3A4* and *CYP3A5* expression in the liver (Yamaori *et al*., 2005; Lin *et al.,* 2002). In this study *CYP3A4* expression was not significantly different between different races or gender (Table 4.4). There was a statistically significant difference between males and females for *CYP3A5* expression however no significant difference in *CYP3A5* level between different ethnicities (Table 4.4). However, it should be noted that this was not a population study and that the number of Caucasian (n=6) and Coloured (n=3) patients were limited.

Table 4.6: The results of the Mann-Whitney and Kruskal-Wallis test to determine association between *CYP3A4* and *CYP3A5* expression and gender and ethnicity respectively.

Gene	Gender (P-Value)	Ethnicity (P-Value)
CYP3A4	0.98	0.18
CYP3A5	0.04*	0.90

* Significant at P<0.05

4.4 Conclusion

Several studies have reported that altered expression of *CYP3A4* and *CYP3A5* is associated with SNPs. This study demonstrated an association between I193I and *CYP3A4* expression. However, none of the other SNPs detected had a significant association with mRNA expression. Contrary to what has been reported in literature, there was also no correlation between *CYP3A4* and *CYP3A5* expression. In this study gender and ethnicity also did not have an association with *CYP3A4* mRNA expression. However, *CYP3A5* did show a significant difference between males and females. Because of the importance of CYP3A4 and CYP3A5 in metabolism of not only imatinib but approximately 50% of all therapeutic drugs the impact of variation in gene expression should be carefully evaluated.

CHAPTER FIVE

CONCLUSION

The use of imatinib in the treatment of chronic myeloid leukaemia (CML) has achieved excellent response rates resulting in prolonged survival in a majority of CML patients. Despite the exceptional results achieved with imatinib, some patients experience adverse effects which in certain cases are severe enough to consider discontinuation of imatinib treatment. It has been suggested that one of the reasons for the varied response to imatinib may be due to inter-individual differences in drug disposition especially metabolism. Patients with decreased metabolic activity may experience adverse drug reactions (ADRs) due to prolonged exposure to imatinib and conversely an increase in metabolic activity may lead to ineffective treatment as a result of increased clearance of the drug.

Imatinib is metabolised by CYP3A4 and CYP3A5 and some SNPs in these genes have been associated with altered catalytic activity of the enzymes. Thus, SNPs in *CYP3A4* and *CYP3A5* may impact the expression of the genes and result in a less favourable response to imatinib treatment. However there is limited research regarding SNPs in *CYP3A4* and *CYP3A5* with the association with CML treatment and response to imatinib. This study investigated the presence of SNPs in *CYP3A4* and *CYP3A5* and a total of ten SNPs were detected in *CYP3A4* and four in *CYP3A5,* respectively. Of the SNPs detected in *CYP3A4* and *CYP3A5*, three were not previously described in literature namely, A15619G, A15648T and G7226A. The newly described SNPs were

all detected in a relatively low frequency. However since this was not intended to be a population study, the allelic frequencies described may not be a true indication of what the frequency of these SNPs would be in a larger population. Two of the SNPs detected were associated with ADRs namely, G20338A (*CYP3A4*1G*) in *CYP3A4* and A6986G (*CYP3A5*3*) in *CYP3A5*.

A statistically significant association was observed between the I193I SNP in *CYP3A4* and increased expression of *CYP3A4*. This SNP was detected in 1 CML patient in the experimental group, being treated with a lowered dosage of 200 mg/day of imatinib due to ADRs. However there was no statistical association between ADRs and I193I. It would have been expected that patients with ADRs would have decreased *CYP3A4* expression and not increased as was the case with this patient. In addition, this patient also had high levels of *CYP3A5* expression within the upper quartile of the expression data. In CML patients with *CYP3A5*3* there was no statistical association with low mRNA expression as was expected. A possible explanation for this may be that all the patients that had the *CYP3A5*3* variant had it in the heterozygous form. It has been reported that having at least one wild type allele will result in approximately two to three fold higher total *CYP3A5* expression compared to having the *CYP3A5*3* variant in homozygous form. One of the limitations of this study was that the effect of SNPs on protein activity was not investigated and could be considered for future studies.

In conclusion, this study identified ten SNPs in *CYP3A4* of which two were newly described. In *CYP3A5* four SNPs were detected of which one has not previously been

reported. The previously undescribed SNPs detected in this study may be unique to the South African population since they have not been reported in other population groups. While the results from this study do not exclude the role of *CYP3A4* and *CYP3A4* in individual patients, it does not appear that either *CYP3A4* or *CYP3A5* expression is directly linked in ADRs to imatinib.

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SUMMARY

Chronic myeloid leukaemia (CML) is a haematological malignancy characterised by the *BCR-ABL* fusion oncogene which encodes for a constitutively active tyrosine kinase. Imatinib mesylate is a tyrosine kinase inhibitor that has effectively been used in the treatment of CML. However, some individuals experience adverse drugs reactions (ADRs) to imatinib. One of the reasons for varied treatment response among individuals may be as a result of inter-individual differences in the metabolism of imatinib.

Imatinib is metabolised by the drug metabolizing enzymes CYP3A4 and CYP3A5. Single nucleotide polymorphisms (SNPs) in *CYP3A4* and *CYP3A5* have been described, some of which have been associated with altered catalytic activity of these enzymes. SNPs in *CYP3A4* and *CYP3A5* may also impact the expression of these genes and result in a less favourable response to imatinib treatment. Patients with a decrease in catalytic activity of CYP3A4 and CYP3A5 may experience ADRs due to prolonged exposure to imatinib. On the other hand an increase in activity may lead to ineffective treatment as a result of increased clearance of the drug. Thus the aim of this study was to screen *CYP3A4* and *CYP3A5* for SNPs using high resolution melting curve analysis (HRM) and determine the impact of these SNPs on gene expression in CML patients treated with imatinib.

A total of ten SNPs were detected in *CYP3A4*, of which two SNPs, namely A15619G and A15649T have not been previously described in literature. A15619G was a synonymous SNP while A15649T resulted in a change in amino acid from glutamine to a histidine. A total of four SNPs were detected in *CYP3A5*, of which one SNP namely, G7226A, had not previously been reported in literature and did not result in an amino acid change. Out of all the detected SNPs in *CYP3A4,* the G20338A SNP was statistically associated with the occurrence of ADRs but not with mRNA expression. The I369V SNP was statistically associated with increased *CYP3A4* mRNA expression. None of the SNPs detected in *CYP3A5* significantly affected mRNA expression. Expression of *CYP3A4* and *CYP3A5* was not dependent on ethnicity or gender, with the exception of *CYP3A5* which showed a statistically significant difference between males and females.

Currently a limited amount of literature exists regarding SNPs in *CYP3A4* and *CYP3A5* and CML treatment. Given the potential impact that SNPs can have on the CYP3A4 and CYP3A5 enzymes and therefore imatinib treatment, it is an important issue that needs to be investigated. Determining the potential impact of SNPs and differential gene expression of *CYP3A4* and *CYP3A5* is important as it may allow for more effective imatinib treatment.

Keywords: Chronic myeloid leukaemia (CML), Cytochrome P450, *CYP3A4, CYP3A5*, single nucleotide polymorphism (SNP), mRNA expression, imatinib mesylate, BCR-ABL.

OPSOMMING

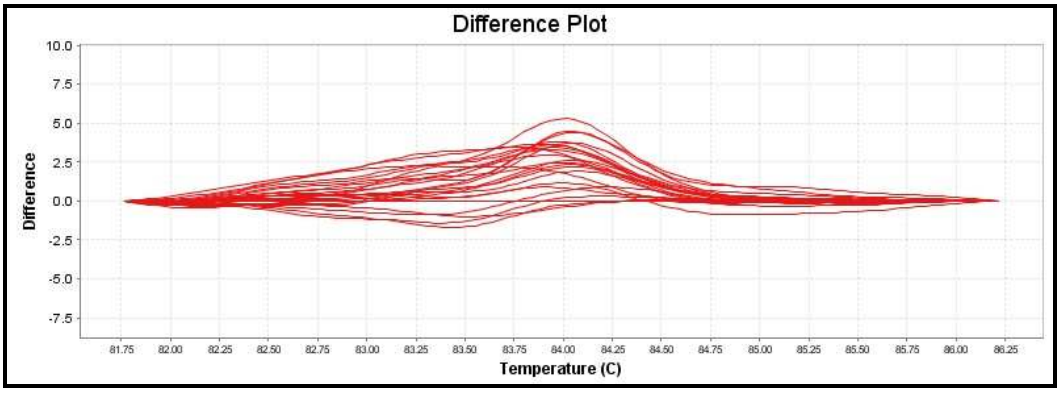
Chroniese miëloïede leukemie (CML) is ’n hematologiese maligniteit wat deur die *BCR-ABL*-fusie-onkogeen wat vir ’n wesentlik aktiewe tirosienkinase kodeer, veroorsaak word. Imatinib is ’n tirosienkinase-inhibeerder wat effektief aangewed word in die behandeling van CML, maar ongelukkig ervaar sommige pasiënte newe-effekte van imatinib. Individuele verskille in die afbraak van imatinib is ’n moontlike rede vir die variasie van hoe pasiënte op die vorm van behandeling respondeer.

Imatinib word deur metabolise ensieme CYP3A4 en CYP3A5 afgebreek. Daar is gevind dat enkelnukleotiedpolimorfismes (SNPs) in *CYP3A4* en *CYP3A5* geassosieer is met veranderde katalitiese aktiwiteit van hierdie ensieme. SNPs in *CYP3A4* en *CYP3A5* kan die uitdrukking van hierdie gene beïnvloed en sodoende veroorsaak dat die behandeling met imatinib minder doeltreffend is. Pasiënte met verlaagde ensiemaktiwiteit van CYP3A4 en CYP3A5 kan newe-effekte teenoor imatinib ervaar as gevolg van verlengde blootstelling daaraan. In teenstelling hiermee kan verhoogde ensiemaktiwiteit weer lei tot n verhoogde afbraak van imatinib en verlaagte blootstelling daaraan. Die doel van hierdie studie was om *CYP3A4* en *CYP3A5* te sif vir ENPs deur middel van die tegniek hoë-resolusie-smeltkromme-analise (HSA) en om die effek van enige SNPs op geenuitdrukking te bepaal in CML pasiënte wat met imatinib behandel word.

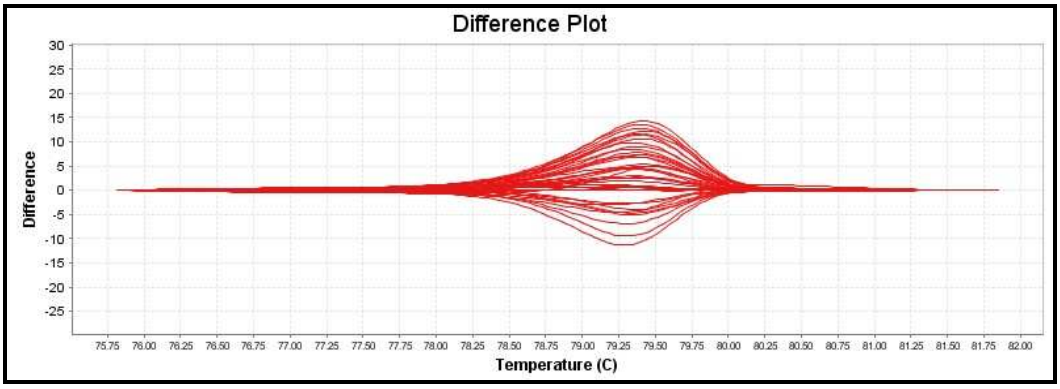
‘n Totaal van tien SNPs is by *CYP3A4* gevind, waarvan twee, naamlik A15619G en A15649T nie van te vore beskryf is nie. A15619G is ’n sinonieme SNP terwyl A15649T ’n aminosuurverandering van glutamien na histidien tot gevolg het. ’n Totaal van vier SNPs is by *CYP3A5* gevind, waarvan een, naamlik G7226A, ’n sinonieme SNP is wat nie voorheen beskryf is nie. Hierdie SNP het nie n aminosuurverandering tot gevolg nie. Van al die SNPs wat by *CYP3A4* gevind is, is net G20338A statisties betekenisvol met newe-effekte geassisieer, maar nie geenuitdrukking nie. Die I369V SNP was wel statisties betekenisvol met verhoogte *CYP3A4* geenuitdrukking geassosieer. Geeneen van die SNPs wat by *CYP3A5* gevind is, is statities betekenisvol met verhoogde geenuitdrukking geassosieer nie. Geenuitdrukking van *CYP3A4* en *CYP3A5* was ook nie aan ras en geslag gekoppel nie, met die uitsondering van *CYP3A5* wat n statisties betekenisvolle verskil tussen mans en vroue getoon het.

Daar is tans ’n beperkte wetenskaplike publikasies oor die invloed van SNPs by *CYP3A4* en *CYP3A5* op CML behandeling. Gegewe die potensiële invloed wat SNPs op CYP3A4 en CYP3A5 en daardeur op die behandeling met imatinib kan hê, is dit ’n belangrike onderwerp wat ondersoek regverdig. Die bepaling van die effek van SNPs op geenuitdrukking van *CYP3A4* en *CYP3A5* is belangrik omdat dit tot meer effektiewe behandeling van CML met imatinib kan lei.

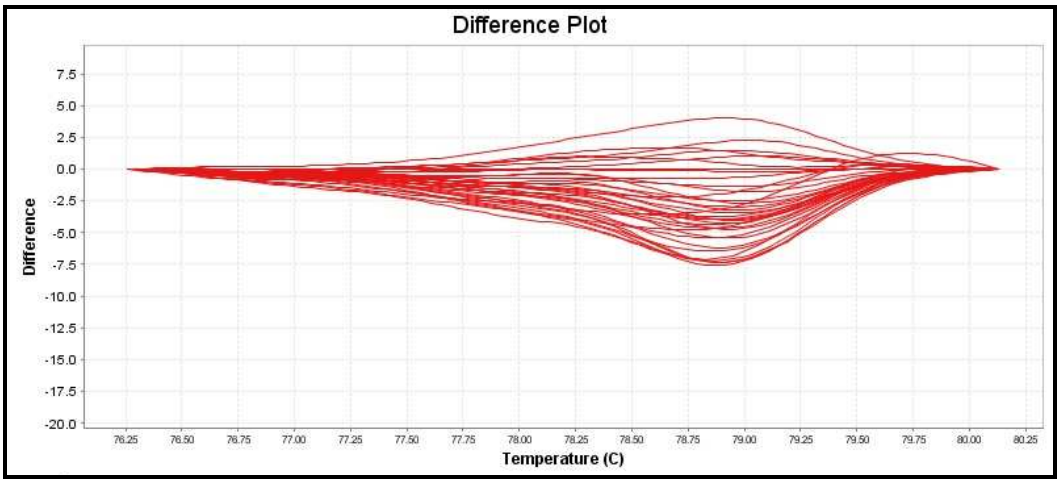
APPENDIX A (CHAPTER TWO)



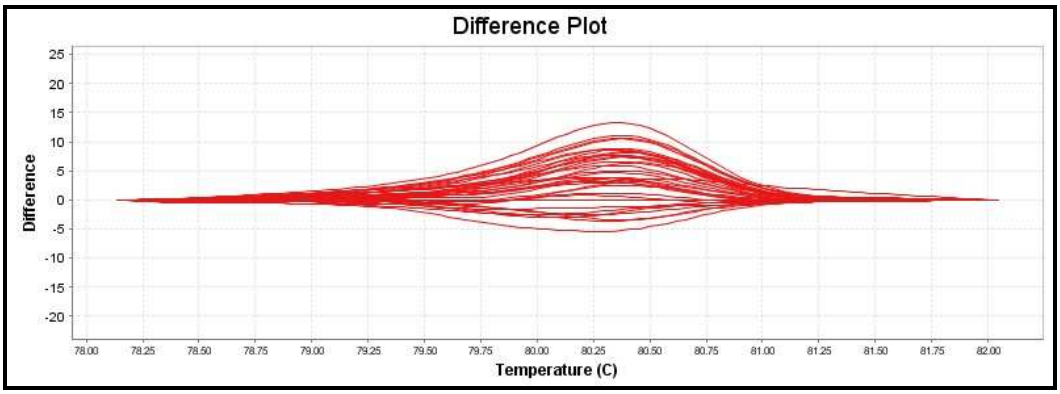
I. The HRM difference plot of exon 1 of *CYP3A4.*



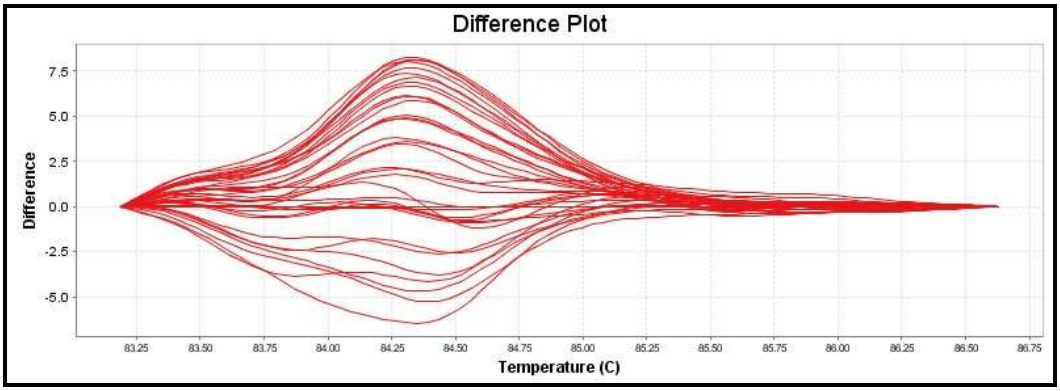
II. The HRM difference plot of exon 2 of *CYP3A4.*



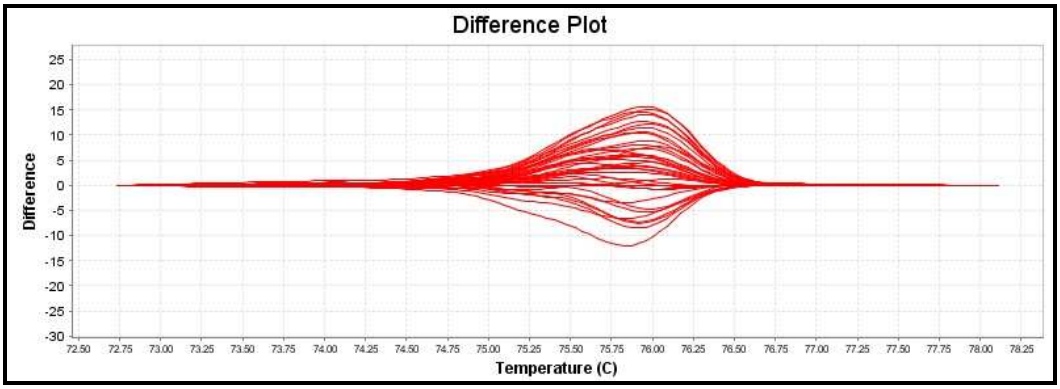
III. The HRM difference plot of exon 3 of *CYP3A4.*



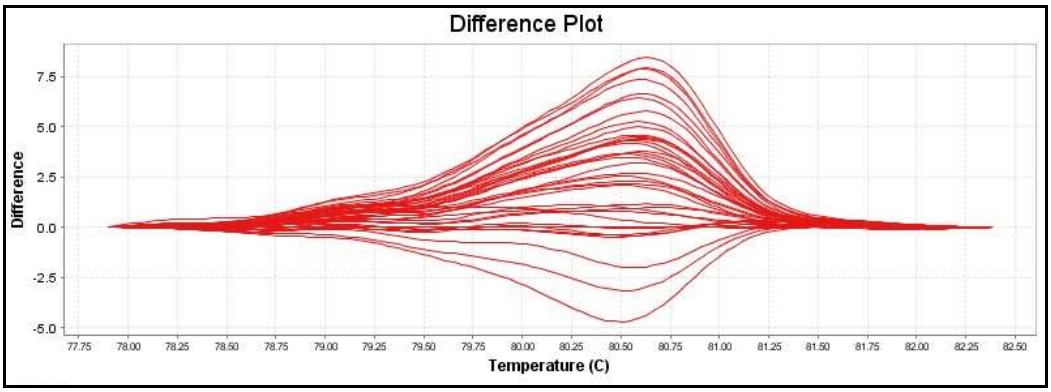
IV. The HRM difference plot of exon 4 of *CYP3A4.*



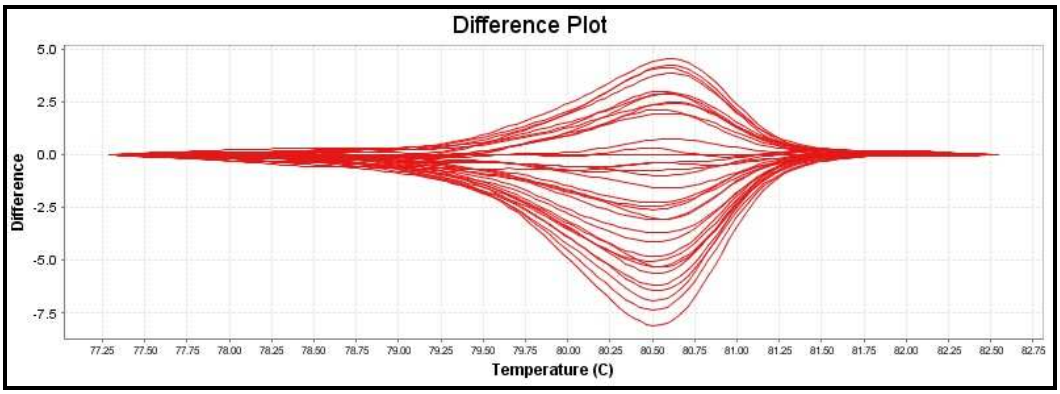
V. The HRM difference plot of exon 6 of *CYP3A4.*



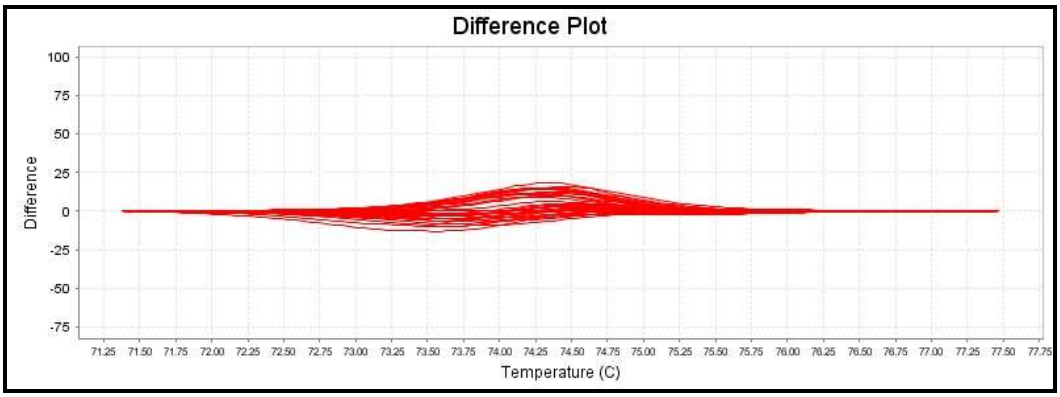
VI. The HRM difference plot of exon 8 of *CYP3A4.*



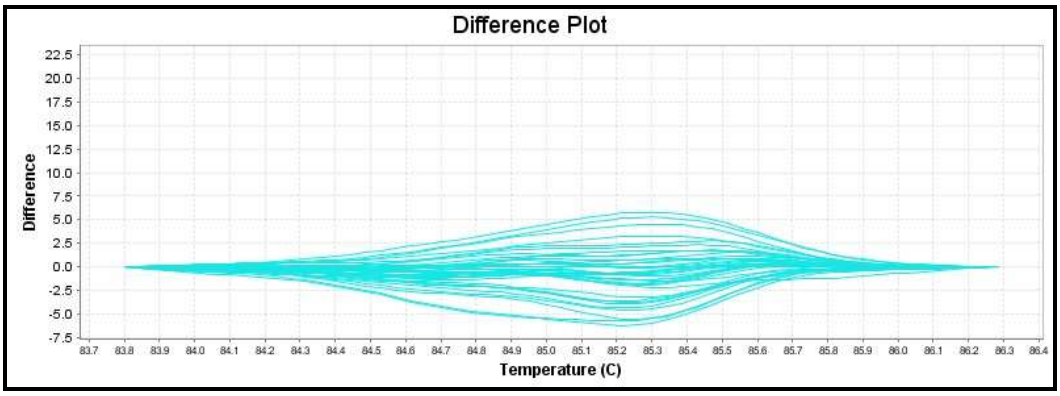
VII. The HRM difference plot of exon 13.1 of *CYP3A4.*



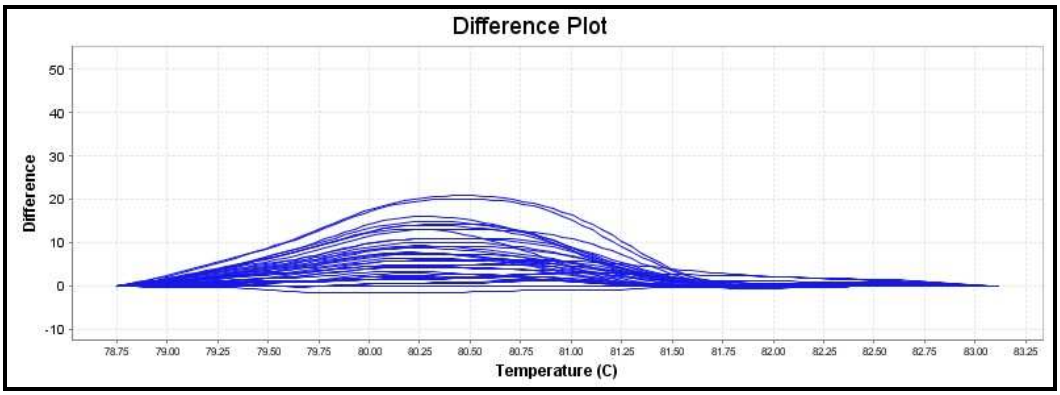
VIII. The HRM difference plot of exon 13.2 of *CYP3A4.*



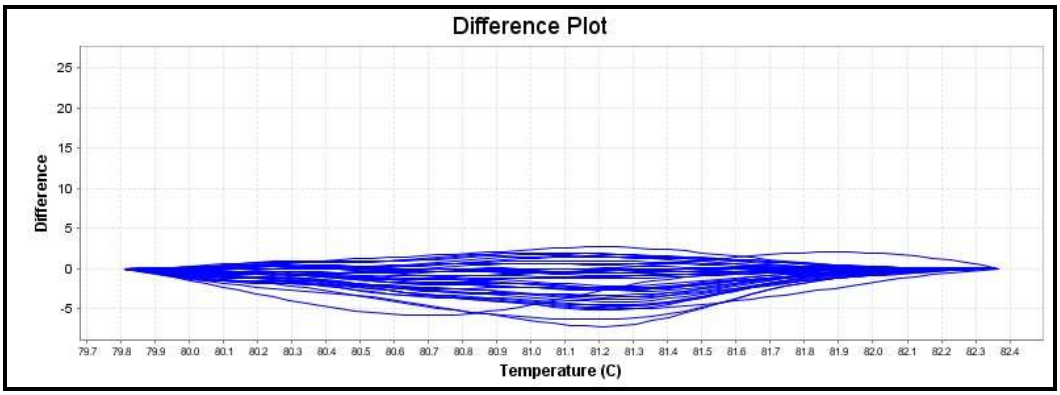
IX. The HRM difference plot of exon 13.3 of *CYP3A4.*



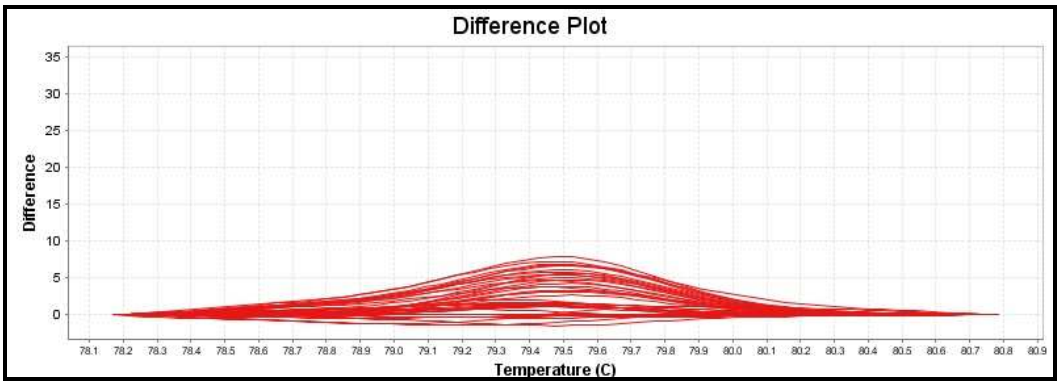
X. The HRM difference plot of exon 1 of *CYP3A5.*



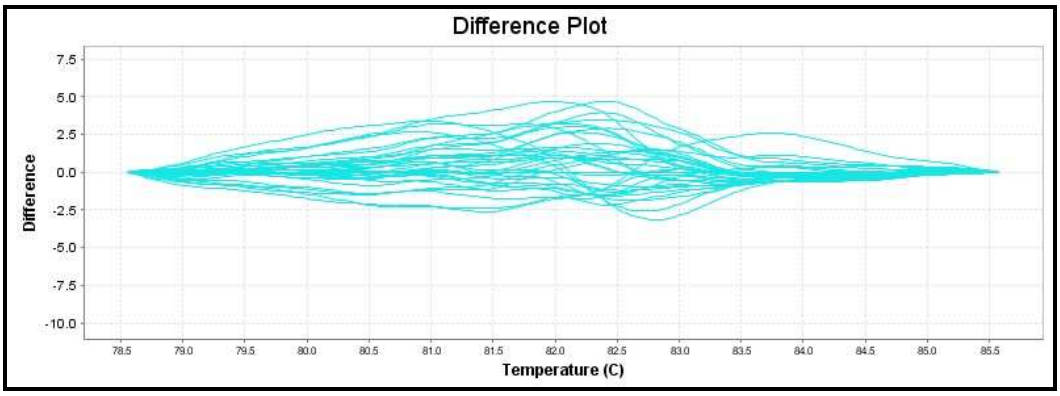
XI. The HRM difference plot of exon 2 of *CYP3A5.*



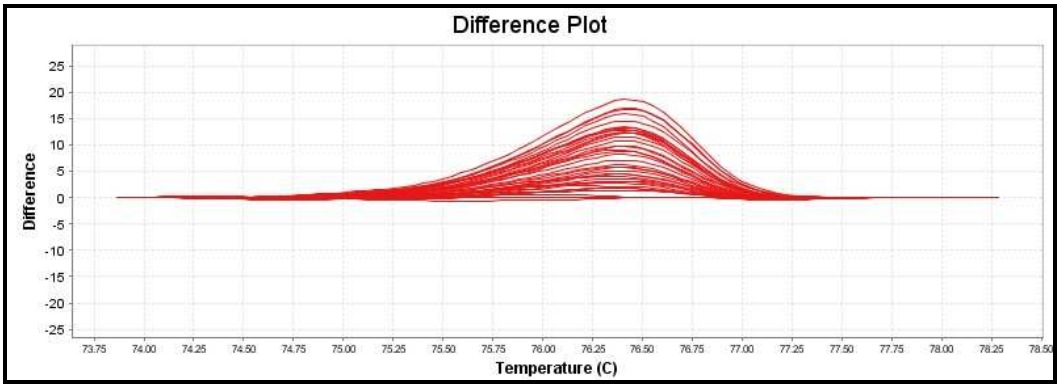
XII. The HRM difference plot of exon 3 of *CYP3A5.*



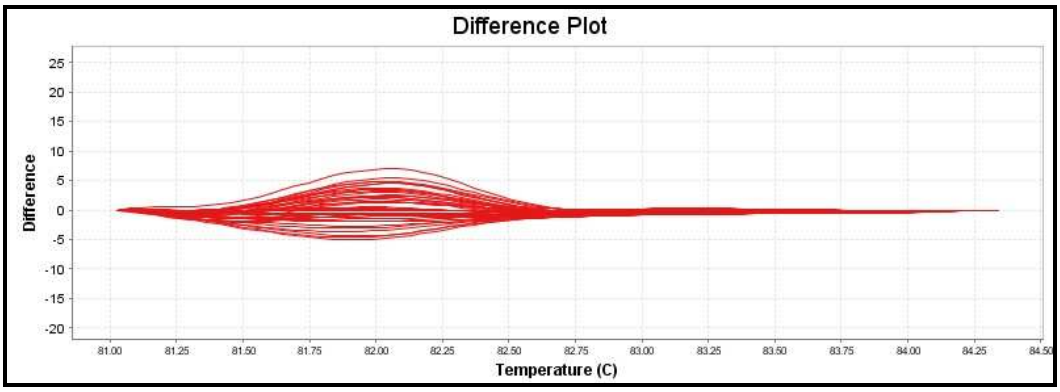
XIII. The HRM difference plot of exon 5 of *CYP3A5.*



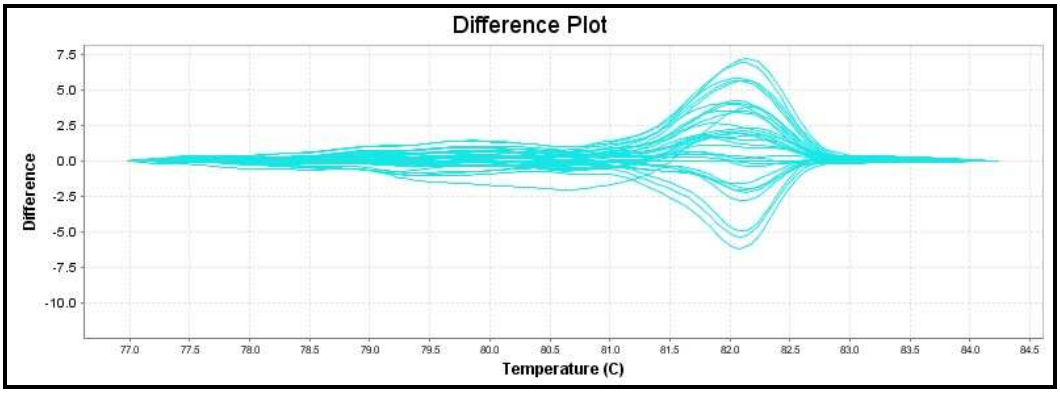
XIV. The HRM difference plot of exon 6 of *CYP3A5.*



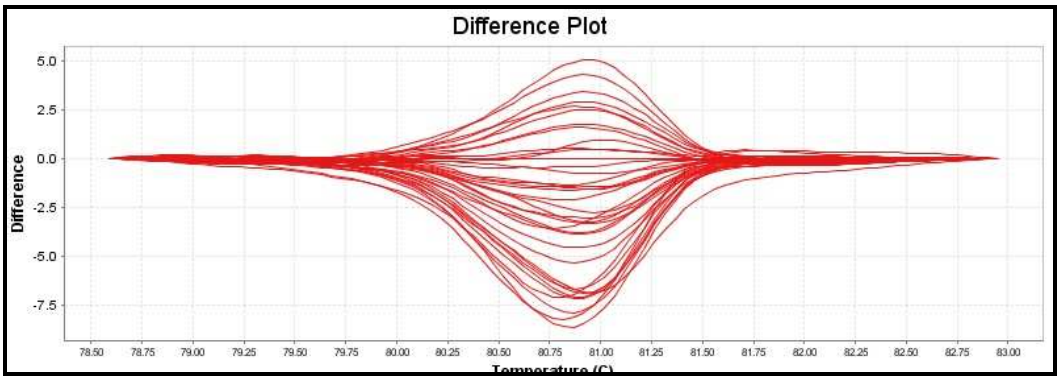
XV. The HRM difference plot of exon 8 of *CYP3A5.*



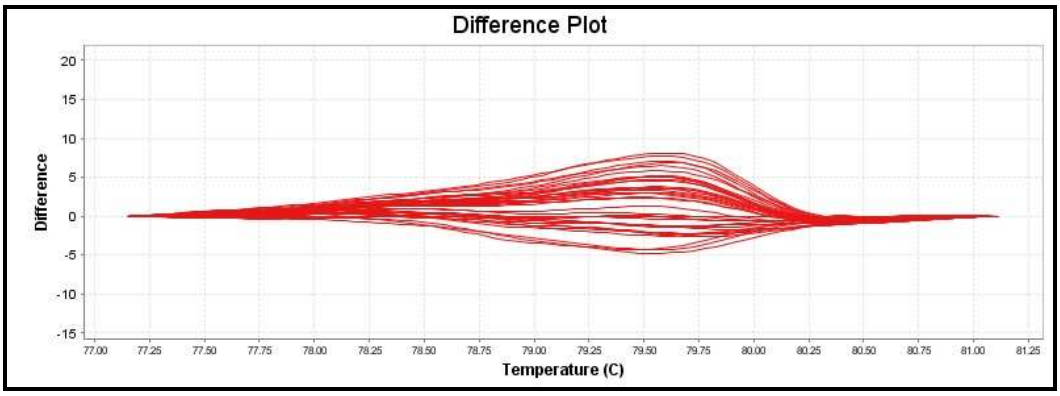
XVI. The HRM difference plot of exon 9 of *CYP3A5.*



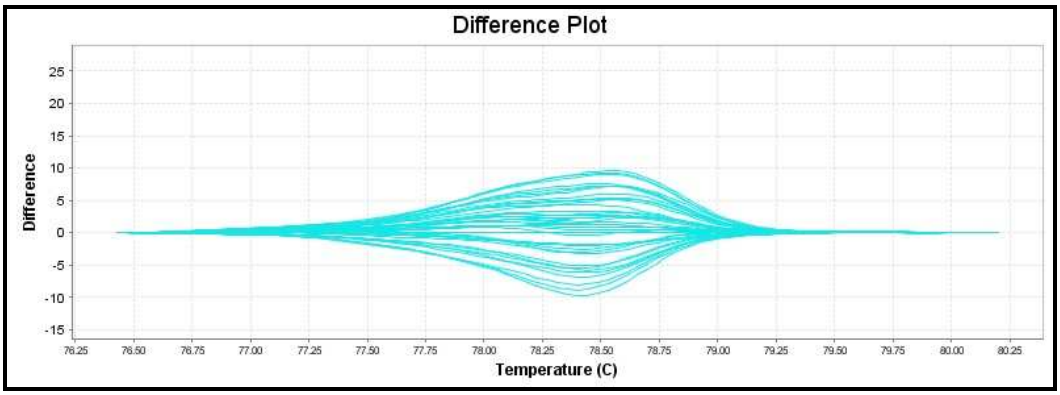
XVII. The HRM difference plot of exon 10 of *CYP3A5.*



XVIII. The HRM difference plot of exon 11 of *CYP3A5.*



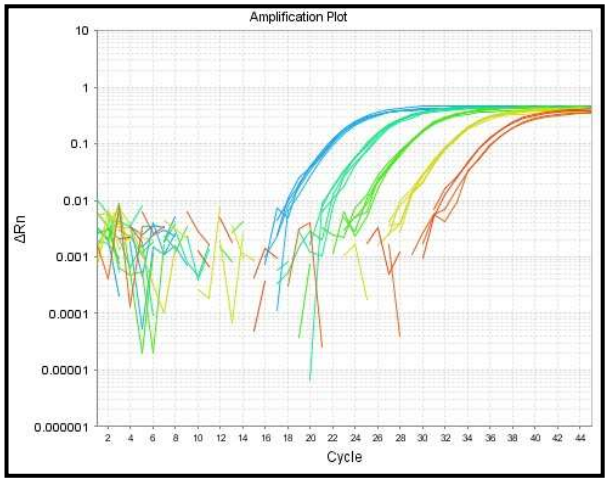
XIX. The HRM difference plot of exon 12 of *CYP3A5.*

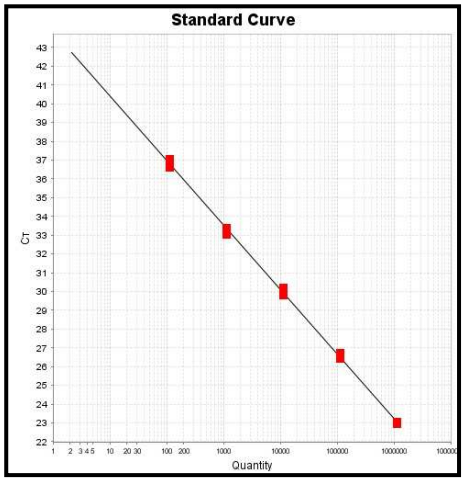


XX. The HRM difference plot of exon 13 of *CYP3A5*

Figure I to XX: The high resolution melting curve analysis difference plot allows for the visual differentiation of variant sample from the wild type group. The examples of difference plots within text (refer to chapter 2) had multiple variant grouping, each containing a specific SNP. This appendix is a compilation of difference plots that did not have variant grouping and therefore did not contain any SNPs. The figure legend indicates which exon and which gene the HRM plot refers to.

APPENDIX B (CHAPTER THREE)





Appendix B is a compilation of the raw data obtained from the analysis done in chapter three. This includes an example of an amplification plot, standard curve and the C_T values. The amplification plot and standard curve are that of the ultramer copy number standards (10^2, 10^3, 10^4, 10^5 and 10^6 copies).

Standard	Day 0		
	Vortex	Pipette	Invert
1	38.56	37.2	38.05
2	33.89	33.67	33.53
3	30.76	30.38	29.85
4	26.8	26.79	26.4
5	23.48	23.18	23.11

Standard	Day 1						Day 7					
	4ºC			-20ºC			4ºC			-20ºC		
	Vortex	Pipette	Invert	Vortex	Pipette	Invert	Vortex	Pipette	Invert	Vortex	Pipette	Invert
1	38.82	37.42	37.24	38.54	38.14	38.22	38.17	38.63	37.76	38.07	37.77	37.83
2	34.4	34.18	34.01	34.22	34.12	34.41	34.57	34.23	33.97	34.4	34.65	34.59
3	30.54	30.54	30.39	30.51	30.41	30.64	30.58	30.65	30.26	31.1	30.84	30.93
4	27.02	26.81	26.7	27.03	27.27	26.8	27.57	27.16	27.33	27.32	27.42	27.21
5	23.68	23.84	23.55	23.3	23.59	23.3	23.94	23.96	23.66	23.46	23.77	24.49

Standard	Day 15						Day 30					
	4ºC			20ºC			4ºC			20ºC		
	Vortex	Pipette	Invert	Vortex	Pipette	Invert	Vortex	Pipette	Invert	Vortex	Pipette	Invert
1	37.81	37.23	37.05	37.08	36.98	37.13	37.77	38.23	39.57	36.78	39.58	36.82
2	34.09	33.82	33.5	33.69	33.64	33.75	34.32	34.41	34.56	33.54	33.27	34.69
3	30.4	30.29	30.08	30.16	30.3	30.14	30.71	30.52	30.85	31.16	29.94	29.94

Table I: C_T values for the ultramer copy number standards. The stability of the standard was tested under different storage temperatures (4ºC and -20ºC), duration of storage (Day 0, 1, 7, 15 and 30) and mixing methods (vortex, pipette and invert).

APPENDIX C (CHAPTER FOUR)

Table: Copy number and C_T values of CYP3A4, CYP3A5 and GUS of patients.

Sample	Replica	C_T	Copies	CYP3A4 %	Result	Standard deviation
Patient 1	GUS a)	22.27	169499.46	0.51%		
Patient 1	GUS b)	22.24	174009.52	0.42%	0.47%	0.01%
Patient 1	CYP3A4 a)	33.85	858.72			
Patient 1	CYP3A4 b)	34.07	739.1			
Patient 3	GUS a)	23.59	67022.05	0.00%		
Patient 3	GUS b)	23.48	72647.62	0.00%	0.00%	0.00%
Patient 3	CYP3A4 a)	43.33	1.61			
Patient 3	CYP3A4 b)	44.02	1.02			
Patient 7	GUS a)	22.9	108851.19	0.26%		
Patient 7	GUS b)	22.83	114613.04	0.19%	0.23%	0.05%
Patient 7	CYP3A4 a)	35.5	288.01			
Patient 7	CYP3A4 b)	35.93	215.95			
Patient 9	GUS a)	23.09	95414.39	0.00%		
Patient 9	GUS b)	22.96	104610.45	0.00%	0.00%	0.00%
Patient 9	CYP3A4 a)	Undetermined	0			
Patient 9	CYP3A4 b)	Undetermined	0			
Patient 12	GUS a)	26.01	12143.39	205.12%		
Patient 12	GUS b)	25.93	12835.6	199.23%	202.18%	4.16%
Patient 12	CYP3A4 a)	28.76	24908.92			
Patient 12	CYP3A4 b)	28.72	25572.86			
Patient 13	GUS a)	22.16	183112.94	1.08%		
Patient 13	GUS b)	21.97	210449.09	0.76%	0.92%	0.23%
Patient 13	CYP3A4 a)	32.59	1979.24			
Patient 13	CYP3A4 b)	32.92	1589.98			
Patient 19	GUS a)	21.84	230788.41	0.00%		
Patient 19	GUS b)	21.88	223634.42	0.00%	0.00%	0.00%
Patient 19	CYP3A4 a)	Undetermined	0			
Patient 19	CYP3A4 b)	Undetermined	0			

Sample	Replica	C_T	Copies	CYP3A4 %	Result	Standard deviation
Patient 20	GUS a)	23.52	70347.58	0.10%		
Patient 20	GUS b)	23.42	75398.28	0.10%	0.10%	0.00%
Patient 20	CYP3A4 a)	37.65	68.97			
Patient 20	CYP3A4 b)	37.47	78.14			
Patient 22	GUS a)	25.8	14056.23	186.57%		
Patient 22	GUS b)	25.95	12684.91	187.69%	187.13%	0.79%
Patient 22	CYP3A4 a)	28.69	26224.98			
Patient 22	CYP3A4 b)	28.83	23808.64			
Patient 24	GUS a)	22.5	145008.53	0.31%		
Patient 24	GUS b)	22.28	168515.07	0.38%	0.35%	0.05%
Patient 24	CYP3A4 a)	34.81	453.42			
Patient 24	CYP3A4 b)	34.28	645.94			
Patient 25	GUS a)	23.76	59424.93	3.33%		
Patient 25	GUS b)	23.55	69028.53	2.46%	2.90%	0.62%
Patient 25	CYP3A4 a)	32.59	1981.62			
Patient 25	CYP3A4 b)	32.82	1699.65			
Patient 28	GUS a)	22.91	108044.54	0.50%		
Patient 28	GUS b)	23.01	101172.02	0.46%	0.48%	0.03%
Patient 28	CYP3A4 a)	34.53	544.97			
Patient 28	CYP3A4 b)	34.78	463.89			
Patient 29	GUS a)	22.48	146218.21	0.10%		
Patient 29	GUS b)	22.39	156426.43	0.08%	0.09%	0.01%
Patient 29	CYP3A4 a)	36.56	142.18			
Patient 29	CYP3A4 b)	36.82	119.63			
Patient 33	GUS a)	22.66	129552.98	1.67%		
Patient 33	GUS b)	22.29	167321.89	1.24%	1.46%	0.31%
Patient 33	CYP3A4 a)	32.45	2168.81			
Patient 33	CYP3A4 b)	32.52	2073.39			
Patient 35	GUS a)	22.03	202094.55	0.36%		
Patient 35	GUS b)	22.04	199770.66	0.33%	0.34%	0.02%
Patient 35	CYP3A4 a)	34.12	717.66			
Patient 35	CYP3A4 b)	34.26	651.31			

Sample	Replica	C_T	Copies	CYP3A4 %	Result	Standard deviation
Patient 38	GUS a)	23.74	60219.37	0.00%		
Patient 38	GUS b)	23.75	59954.95	0.00%	0.00%	0.00%
Patient 38	CYP3A4 a)	Undetermined	0			
Patient 38	CYP3A4 b)	Undetermined	0			
Patient 40	GUS a)	23.77	59124.99	15.32%		
Patient 40	GUS b)	23.72	61289.45	14.61%	14.97%	0.50%
Patient 40	CYP3A4 a)	30.29	9057.91			
Patient 40	CYP3A4 b)	30.31	8955.67			
Patient 44	GUS a)	24.51	35004.72	1.06%		
Patient 44	GUS b)	24.62	32344.49	1.18%	1.12%	0.08%
Patient 44	CYP3A4 a)	35.12	370.18			
Patient 44	CYP3A4 b)	35.08	380.62			
Patient 48	GUS a)	22.2	178226.56	0.02%		
Patient 48	GUS b)	22.13	188048.75	0.08%	0.05%	0.04%
Patient 48	CYP3A4 a)	38.6	36.88			
Patient 48	CYP3A4 b)	36.4	158.06			
Patient 55	GUS a)	23.8	56522	0.09%	0.13%	0.02%
Patient 55	GUS b)	23.7	61667	0.16%		
Patient 55	CYP3A4 a)	38	52			
Patient 55	CYP3A4 b)	37	101			
Patient 56	GUS a)	24.4	38193	0.08%		
Patient 56	GUS b)	24.3	40844	0.05%	0.06%	0.02%
Patient 56	CYP3A4 a)	38.8	30			
Patient 56	CYP3A4 b)	39.5	19			
Patient 59	GUS a)	22.9	103187	0.20%		
Patient 59	GUS b)	22.9	103484	0.27%	0.24%	0.05%
Patient 59	CYP3A4 a)	36	208			
Patient 59	CYP3A4 b)	35.5	281			
Patient 62	GUS a)	26.5	8938	9.92%		
Patient 62	GUS b)	26.2	11290	7.13%	8.52%	1.97%
Patient 62	CYP3A4 a)	33.8	886			
Patient 62	CYP3A4 b)	34	805			

Sample	Replica	C_T	Copies	CYP3A4 %	Result	Standard deviation
Patient 64	GUS a)	23.3	82949	0.04%		
Patient 64	GUS b)	23.1	93072	0.02%	0.03%	0.01%
Patient 64	CYP3A4 a)	38.7	32			28
Patient 64	CYP3A4 b)	39.2	23			35.5
Patient 70	GUS a)	23.9	52636	0.00%		
Patient 70	GUS b)	23.8	56014	0.00%	0.00%	0.00%
Patient 70	CYP3A4 a)	44.8	1			
Patient 70	CYP3A4 b)	Undetermined	0			
Patient 73	GUS a)	24.2	43841	0.05%		
Patient 73	GUS b)	24	51610	0.04%	0.04%	0.01%
Patient 73	CYP3A4 a)	39.4	20			0
Patient 73	CYP3A4 b)	39.5	20			0
Patient 18	GUS a)	24.4	39418	0.23%		
Patient 18	GUS b)	24.4	38710	0.11%	0.17%	0.09%
Patient 18	CYP3A4 a)	37.2	92			
Patient 18	CYP3A4 b)	38.3	42			
Patient 42	GUS a)	24.9	24893	0.09%		
Patient 42	GUS b)	25.0	23509	0.11%	0.10%	0.01%
Patient 42	CYP3A4 a)	39.2	23			
Patient 42	CYP3A4 b)	39.0	25			
Patient 57	GUS a)	25.3	18963	92.48		
Patient 57	GUS b)	25.3	19337	88.41	90.45%	2.88%
Patient 57	CYP3A4 a)	29.3	17538			
Patient 57	CYP3A4 b)	29.3	17096			
Patient 49	GUS a)	23.9	49714	0.66%		
Patient 49	GUS b)	23.5	64310	0.65%	0.65%	0.00%
Patient 49	CYP3A4 a)	35.2	326			
Patient 49	CYP3A4 b)	34.8	419			
Patient 50	GUS a)	24.9	25169	0.04%		
Patient 50	GUS b)	24.7	18923			
Patient 50	CYP3A4 a)	40.3	11			
Patient 50	CYP3A4 b)	Undetermined	0			
Patient 60	GUS a)	24.1	42533	1.95%		
Patient 60	GUS b)	23.6	60744	1.11%	1.53%	0.59%
Patient 60	CYP3A4 a)	33.8	828			
Patient 60	CYP3A4 b)	34.1	673			

Sample	Replica	C_T	Copies	CYP3A4 %	Result	Standard deviation
Patient 61	GUS a)	24.8	26435	0.81%		
Patient 61	GUS b)	24.8	26707	0.17%	0.18%	0.0%
Patient 61	CYP3A4 a)	38.1	47			
Patient 61	CYP3A4 b)	38.1	46			
Patient 63	GUS a)	25.2	20933	22.70%		
Patient 63	GUS b)	24.4	34549	12.50%	17.60%	7.21%
Patient 63	CYP3A4 a)	31.2	4752			
Patient 63	CYP3A4 b)	31.4	4318			
Patient 68	GUS a)	24.6	30378	5.89%		
Patient 68	GUS b)	24.6	31167	6.92%	6.41%	0.73%
Patient 68	CYP3A4 a)	32.7	1790			
Patient 68	CYP3A4 b)	32.4	2157			
Patient 72	GUS a)	23.5	63047	0.26%		
Patient 72	GUS b)	23.7	58393	0.24%	0.25%	0.02%
Patient 72	CYP3A4 a)	36.2	166			
Patient 72	CYP3A4 b)	36.5	140			
WT	GUS a)	24.2	41318	22.44%		
WT	GUS b)	24.2	40958	21.75%	22.09%	0.02%
WT	CYP3A4 a)	30.2	9272			
WT	CYP3A4 b)	30.3	8908			

Sample	Replica	C_T	Copies	CYP3A5 %	Result	Standard deviation
WT	GUS a)	26.1	23530	0.00%		
WT	GUS b)	26.0	25157	0.17%	0.08%	0.04%
WT	CYP3A5 a)	Undetermined	0			
WT	CYP3A5 b)	39.8	42			
Patient 1	GUS a)	24.9	52253	0.01%		
Patient 1	GUS b)	24.6	63147	0.02%	0.01%	0.01%
Patient 1	CYP3A5 a)	43.2	5			
Patient 1	CYP3A5 b)	41.6	13			
Patient 3	GUS a)	25.1	44640	0.03%		
Patient 3	GUS b)	24.7	59850	0.04%	0.04%	0.01%
Patient 3	CYP3A5 a)	41.7	12			
Patient 3	CYP3A5 b)	40.5	26			
Patient 7	GUS a)	23.8	107164	0.03%		
Patient 7	GUS b)	23.7	112256	0.03%	0.03%	0.00%
Patient 7	CYP3A5 a)	40.1	33			
Patient 7	CYP3A5 b)	40.3	30			
Patient 8	GUS a)	25.7	15765	0.00%		
Patient 8	GUS b)	25.8	15142	0.00%	0.00%	0.06%
Patient 8	CYP3A5 a)	Undetermined	0			
Patient 8	CYP3A5 b)	Undetermined	0			
Patient 9	GUS a)	24.6	63660	0.21%		
Patient 9	GUS b)	24.3	77757	0.30%	0.25%	0.06%
Patient 9	CYP3A5 a)	38.0	133			
Patient 9	CYP3A5 b)	37.1	233			
Patient 12	GUS a)	26.9	13632	0.33%		
Patient 12	GUS b)	26.7	15452	0.14%	0.24%	0.14%
Patient 12	CYP3A5 a)	39.6	45			
Patient 12	CYP3A5 b)	40.8	21			
Patient 13	GUS a)	24.2	81552	0.05%		
Patient 13	GUS b)	23.7	111114	0.02%	0.03%	0.02%
Patient 13	CYP3A5 a)	39.9	38			
Patient 13	CYP3A5 b)	40.6	25			

Sample	Replica	C_T	Copies	CYP3A5 %	Result	Standard deviation
Patient 18	GUS a)	24.4	39418	0.02%		
Patient 18	GUS b)	24.4	38710	0.06%	0.04%	0.03%
Patient 18	CYP3A5 a)	40.1	8			
Patient 18	CYP3A5 b)	38.4	24			
Patient 19	GUS a)	24.2	83557	0.01%		
Patient 19	GUS b)	24.2	84094	0.03%	0.02%	0.02%
Patient 19	CYP3A5 a)	41.9	11			
Patient 19	CYP3A5 b)	40.4	29			
Patient 20	GUS a)	25.4	37640	0.05%		
Patient 20	GUS b)	24.8	53220	0.06%	0.05%	0.01%
Patient 20	CYP3A5 a)	41.1	18			
Patient 20	CYP3A5 b)	40.2	32			
Patient 22	GUS a)	27.2	10632	0.43%		
Patient 22	GUS b)	27.2	11315	0.16%	0.29%	0.19%
Patient 22	CYP3A5 a)	39.6	46			
Patient 22	CYP3A5 b)	41.1	18			
Patient 24	GUS a)	24.1	89938	0.04%		
Patient 24	GUS b)	24.0	92193	0.04%	0.04%	0.00%
Patient 24	CYP3A5 a)	40.2	32			
Patient 24	CYP3A5 b)	39.9	38			
Patient 25	GUS a)	25.2	40465	0.00%		
Patient 25	GUS b)	24.7	58883	0.03%	0.01%	0.02%
Patient 25	CYP3A5 a)	Undetermined	0			
Patient 25	CYP3A5 b)	41.1	17			
Patient 28	GUS a)	27.6	8160	0.11%		
Patient 28	GUS b)	27.5	8727	0.38%	0.25%	0.19%
Patient 28	CYP3A5 a)	42.2	9			
Patient 28	CYP3A5 b)	40.1	34			
Patient 29	GUS a)	24.5	67914	0.04%		
Patient 29	GUS b)	24.8	53237	0.04%	0.04%	0.00%
Patient 29	CYP3A5 a)	40.6	24			
Patient 29	CYP3A5 b)	40.8	22			
Patient 33	GUS a)	23.4	141232	0.01%		
Patient 33	GUS b)	23.2	164660	0.01%	0.01%	0.00%
Patient 33	CYP3A5 a)	41.0	19			
Patient 33	CYP3A5 b)	41.4	15			

Sample	Replica	C_T	Copies	CYP3A5 %	Result	Standard deviation
Patient 38	GUS a)	25.5	34399	0.00%		
Patient 38	GUS b)	25.6	31416	0.06%	0.03%	0.04%
Patient 38	CYP3A5 a)	Undetermined	0			
Patient 38	CYP3A5 b)	41.1	18			
Patient 40	GUS a)	23.3	89574	0.01%	0.02%	0.01%
Patient 40	GUS b)	23.2	91494	0.02%		
Patient 40	CYP3A5 a)	38.3	13			
Patient 40	CYP3A5 b)	37.6	20			
Patient 42	GUS a)	23.9	58876	0.03%		
Patient 42	GUS b)	23.7	66840	0.03%	0.03%	0.00%
Patient 42	CYP3A5 a)	37.6	20			
Patient 42	CYP3A5 b)	37.7	19			
Patient 44	GUS a)	25.5	20071	0.07%		
Patient 44	GUS b)	25.4	21299	0.03%	0.05%	0.03%
Patient 44	CYP3A5 a)	38.1	15			
Patient 44	CYP3A5 b)	39.6	6			
Patient 48	GUS a)	27.5	5482	0.43%		
Patient 48	GUS b)	27.5	5293	0.30%	0.36%	0.09%
Patient 48	CYP3A5 a)	37.4	23			
Patient 48	CYP3A5 b)	38.0	16			
Patient 49	GUS a)	23.9	53726	0.00%		
Patient 49	GUS b)	23.9	54955	0.00%	0.00%	0.00%
Patient 49	CYP3A5 a)	Undetermined	0			
Patient 49	CYP3A5 b)	Undetermined	0			
Patient 50	GUS a)	25.6	16635	0.00%		
Patient 50	GUS b)	25.1	23338	0.03%	0.02%	0.02%
Patient 50	CYP3A5 a)	Undetermined	0			
Patient 50	CYP3A5 b)	40.2	8			
Patient 55	GUS a)	25.5	20511	0.15%		
Patient 55	GUS b)	25.4	21100	0.05%	0.10%	0.07%
Patient 55	CYP3A5 a)	37.0	30			0.9
Patient 55	CYP3A5 b)	38.7	10			1.4

Sample	Replica	C_T	Copies	CYP3A5 %	Result	Standard deviation
Patient 56	GUS a)	26.0	14900	0.15%		
Patient 56	GUS b)	25.8	16984	0.03%	0.09%	0.08%
Patient 56	CYP3A5 a)	37.5	22			
Patient 56	CYP3A5 b)	39.5	6			
Patient 57	GUS a)	25.1	26732	0.06%		
Patient 57	GUS b)	25.1	26825	0.06%	0.06%	0.00%
Patient 57	CYP3A5 a)	38.0	16			1.4
Patient 57	CYP3A5 b)	38.1	15			0.4
Patient 59	GUS a)	21.7	252495	0.01%		
Patient 59	GUS b)	21.5	297623	0.01%	0.01%	0.00%
Patient 59	CYP3A5 a)	37.0	30			
Patient 59	CYP3A5 b)	36.7	38			
Patient 60	GUS a)	24.0	56039	0.01%		
Patient 60	GUS b)	23.5	77650	0.02%	0.02%	0.01%
Patient 60	CYP3A5 a)	39.2	7			4.2
Patient 60	CYP3A5 b)	37.7	19			2.0
Patient 61	GUS a)	22.0	205728	0.02%		
Patient 61	GUS b)	22.1	192443	0.02%	0.02%	0.00%
Patient 61	CYP3A5 a)	36.4	46			
Patient 61	CYP3A5 b)	36.6	40			
Patient 62	GUS a)	23.8	61480	0.03%		
Patient 62	GUS b)	24.0	56140	0.03%	0.03%	0.00%
Patient 62	CYP3A5 a)	37.8	18			
Patient 62	CYP3A5 b)	38.1	15			
Patient 63	GUS a)	25.0	28767	0.06%		
Patient 63	GUS b)	24.5	39259	0.01%	0.04%	0.03%
Patient 63	CYP3A5 a)	37.9	17			
Patient 63	CYP3A5 b)	39.9	5			
Patient 64	GUS a)	23.0	107399	0.02%		
Patient 64	GUS b)	23.0	109779	0.03%	0.02%	0.01%
Patient 64	CYP3A5 a)	37.4	23			
Patient 64	CYP3A5 b)	37.0	31			
Patient 68	GUS a)	25.0	28896	0.07%		
Patient 68	GUS b)	25.1	26987	0.05%	0.06%	0.01%
Patient 68	CYP3A5 a)	37.6	20			
Patient 68	CYP3A5 b)	38.2	14			

Sample	Replica	C_T	Copies	CYP3A5 %	Result	Standard deviation
Patient 70	GUS a)	26.9	8015	0.05%		
Patient 70	GUS b)	26.7	9300	0.00%	0.03%	0.03%
Patient 70	CYP3A5 a)	40.0	4			
Patient 70	CYP3A5 b)	43.7	0			
Patient 72	GUS a)	27.4	5693	0.24%		
Patient 72	GUS b)	27.4	5710	0.38%	0.31%	0.09%
Patient 72	CYP3A5 a)	38.2	14			
Patient 72	CYP3A5 b)	37.5	21			
Patient 73	GUS a)	26.5	10126	0.09%		
Patient 73	GUS b)	26.4	11120	0.45%	0.27%	0.26%
Patient 73	CYP3A5 a)	38.9	9			
Patient 73	CYP3A5 b)	36.2	50			

The appendix C is a compilation of raw data from the mRNA quantification of *CYP3A4* and *CYP3A5* described in chapter 4. This includes information regarding C_T values and copy number *CYP3A4*, *CYP3A5* and *GUS* respectively, in CML patients.