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Detection of the Janus kinase 2 V617F mutation using molecular methods

by

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Declaration

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Haematology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed……………………………this…………………..day of…………………..2015

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Table of Contents

Page

Declaration ii

Acknowledgements iii

List of Figures viii

List of Tables x

List of Abbreviations xi

SUMMARY xiv

CHAPTER 1 1

INTRODUCTION 1

References 5

CHAPTER 2 10

LITERATURE REVIEW 10

2.1 Introduction 10

2.2 Normal haematopoiesis and JAK2 regulation 12

2.2 Myeloproliferative neoplasms 18

2.3 Philadelphia- chromosome negative classic MPNs 19

2.3.1 Polycythaemia Vera 19

2.3.2 Essential thrombocythaemia 23

2.3.3 Primary myelofibrosis 25

2.4 Mutations in myeloproliferative neoplasms 27

2.4.1 Janus kinase 2 V617F mutation 28

2.4.2 Janus kinase 2 exon 12 mutations 31

2.4.3 Calreticulin exon 9 mutations 31

2.4.4 Myeloproliferative leukaemia virus exon 10 mutations 32

2.4.5 Ten-eleven translocation oncogene family member 2 mutations 32

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2.4.6 Additional sex combs like 1 mutations 33

2.4.7 Casitas B lineage lymphoma proto oncogene mutations 33

2.5 Detection of JAK2 V617F mutation 33

2.5.1 Direct sequencing of amplified product 34

2.5.2 Restriction fragment length polymorphism 35

2.5.3 Allele specific polymerase chain reaction 35

2.5.4 Real time polymerase chain reaction 37

2.5.4.1 SYBR Green 1 chemistry 38

2.5.4.2 Hydrolysis probes 39

2.5.4.3 Hybridisation probes 40

2.5.4.4 Locked nucleic acid probes 41

2.5.4.5 Molecular beacons 42

2.6 Treatment of myeloproliferative neoplasms 42

2.7 Test method validation 43

References 47

CHAPTER 3 67

IDENTIFICATION OF THE JANUS KINASE V617F MUTATION USING ALLELE SPECIFIC POLYMERASE CHAIN REACTION AND SEQUENCING ASSAYS.......67

3.1 Introduction 67

3.2 Materials and Methods 68

3.2.1 Study setting 68

3.2.2 Specimens collection 68

3.2.3 Genomic DNA extraction 69

3.2.4 Genotyping by comparative method allele specific polymerase chain reaction 69

3.2.4.1 Genomic DNA amplification 70

3.2.4.2 Analysis of amplified products 71

3.2.5 Genotyping by sequencing of amplified products obtained from primers designed for in-house PCR assay 71

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3.2.5.1 Genomic DNA amplification and analysis of amplified products obtained from primers designed for in-house PCR assay 72

3.2.5.2 Sequencing of amplified products obtained from primers designed for in-house PCR assay 72

3.2.6 Data and statistical analysis 73

3.3 Results and Discussion 73

3.3.1 Nucleic acid concentration 73

3.3.2 Genotyping by comparative method allele specific PCR 74

3.3.2 Genotyping by sequencing of amplicon obtained from primers designed for in-house PCR assay 77

3.4 Conclusion 81

References 82

CHAPTER 4 85

IDENTIFICATION OF THE JANUS KINASE V617F MUTATION USING REAL TIME POLYMERASE CHAIN REACTION 85

4.1 Introduction 85

4.2 Materials and Methods 86

4.2.1 Study setting 86

4.2.2 Specimen collection 87

4.2.3 Genomic DNA extraction 87

4.2.4 Genotyping by comparative method allele specific polymerase chain reaction 87

4.2.5 Genotyping by sequencing of amplicon obtained from primers designed for in-house PCR assay 87

4.2.6 Locked nucleic acid probe real time PCR 88

4.2.6.1 Primer and probe design 88

4.2.6.2 Genomic DNA amplification using LNA probe real time PCR 89

4.2.6.3 Determining analytical sensitivity 90

4.2.7 SYBR Green real time polymerase chain reaction 90

4.2.7.1 Primer design 90

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4.2.7.2 Genomic DNA amplification 90

4.2.8 Data analysis 91

4.3 Results 91

4.3.1 Nucleic acid concentration 91

4.3.2 Locked nucleic acid probe real time PCR 92

4.2.3 Determining the analytical sensitivity of the LNA probe-based PCR 94

4.3.4 SYBR Green real time PCR assay results 94

4.4 Discussion 95

4.5 Conclusion 99

References 100

CHAPTER 5 105

CONCLUSION 105

5.1 Concluding remarks 105

5.2 Future research 106

References 109

APPENDIX A: Equipment and reagents 111

APPENDIX B: Nucleic acid concentration as measured by the Nanodrop 2000C spectrophotometer (Thermo Scientific) 114

APPENDIX C: Results of the three molecular assays 117

APPENDIX D: Threshold cycle values of the LNA probe real time PCR as generated by the Cepheid SmartCycler II system 120

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List of Figures

Page

Figure 2.1: The 2008 WHO Classification of MPN 10

Figure 2.2: The process of blood formation in the bone marrow. A pluripotent stem cell gives rise to common myeloid and lymphoid progenitor cells that further differentiate into erythroid, megakaryocytic, granulocytic, monocytic and lymphocytic lineages (Moratuwagama, 2014) 13

Figure 2.3: The JAK/STAT signalling pathway which is activated by binding of ligand to the receptor. Binding of ligand activates JAK2 molecules leading to gene transcription (Steensma, 2006). 17

Figure 2.4: The role of the JAK2 V617F mutation in the pathogenesis of MPN. In the presence of the V617F mutation, JAK proteins becomes active even when no ligand is bound to the receptor leading to uncontrolled proliferation of cells (Levine *et al.*, 2007) 29

Figure 2.5: The process of implementing a molecular genetic test for diagnostic use. The shaded arrows represent the two general routes to implementation, depending on the availability of a suitable performance specification: validation (lighter) and verification (Mattocks *et al.*, 2010) 44

Figure 3.1: Allele specific PCR gel with Roche master mix showing faint bands and non-specific amplification bands between 50 and 100 bp 75

Figure 3.2: Allele specific PCR gel, the mutation is represented by the presence of a band around 200 bp and another band just before 400 bp. Wild type samples showed only the 364 bp band 76

Figure 3.3: In-house PCR assay gel showing a clearly visible band around 300 bp 78

Figure 3.4: Sequencing chromatograms generated by the CLC main workbench software program (CLCBio) showing the presence of four mutant samples, one wild type sample and a homozygous control 79

Figure 4.1: An example of amplification curves generated by the Cepheid SmartCycler II system when LNA probe real time PCR was performed 92

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Figure 4.2: An example of wild type amplification represented by the presence of a single positive amplification curve 92

Figure 4.3: A heterozygous sample represented by the presence of two amplification curves. One curve indicates the presence of the mutant allele and the other that of the wild type allele 93

Figure 4.4: Analytical sensitivity curve showing positive amplification of the different dilutions as generated by the Cepheid SmartCycler II system 94

Figure 4.5: SYBR green melting curve results of mutant and wild type controls as generated by the Cepheid SmartCycler II system. The melting curve for mutant and wild type controls could not be distinguished from each other 95

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List of Tables

Page

Table 2.1: The WHO diagnostic criteria for PV, both 2001 and 2008 criteria are shown 21

Table 2.2: Complications that arise in patients with PV and causes of the complications 22

Table 2.3: The 2008 WHO diagnostic criteria for ET 24

Table 2.4: The 2008 WHO diagnostic criteria for PMF 26

Table 2.5: Calculation of diagnostic sensitivity and specificity of an assay 46

Table 3.1: Primers used in the AS PCR comparative assay duplicated in this study 70

Table 3.2: Primers designed for use with the in-house PCR assay for the detection of the JAK2 V617F mutation 71

Table 3.3: The overall number of samples that tested positive and negative with the AS-PCR and direct sequencing 77

Table 4.1: Primers and probes used for detection of the JAK2 V617F mutation using LNA probe real time PCR 88

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List of Abbreviations

ABL1:	Abelson leukaemia virus
AML:	Acute myeloid leukaemia
Apo-A1:	Apolipoprotein–Al
ARMS:	Amplification refractory mutation system
AS-PCR:	Allele specific PCR
ATP:	Adenosine triphosphate
ASXL1:	Additional sex combs like 1
BCR:	Breakpoint cluster region
BCSH:	British Committee for Standards in Haematology
CALR:	Calreticulin
CBL:	Casitas B lineage lymphoma proto oncogene
CEL:	Chronic eosinophilic leukaemia
CML:	Chronic myelogenous leukaemia
CNL:	Chronic neutrophilic leukaemia
C-terminus:	Carboxyl-terminus
Cy3:	Cyanine 3
ddNTPs:	Dideoxynucleotides
dHPLC	Denaturing high performance liquid chromatography
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
EPO:	Erythropoietin
ET:	Essential thrombocythaemia
EZH2:	Enhancer of zeste homolog 2
FAM:	6- Carboxy Fluorescein
FRET:	Fluorescence resonance energy transfer
G:	Guanine
gDNA:	Genomic deoxyribonucleic acid
G-CSF:	Granulocyte colony stimulating factor
GM-CSF:	Granulocyte monocyte colony stimulating factor
Hb:	Haemoglobin
HEX:	Hexacloro-Fluorescein

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HIF:	Hypoxia inducible factor
HSC:	Haemopoietic stem cell
IDH1/2:	Isocitrate dehydrogenase 1/2
IKZF1:	IKAROS family zinc finger 1
IL:	Interleukin
JAK2:	Janus kinase 2
laBkFQ:	Lower black fluorescence quencher
LDH	Lactate dehydrogenase
LNA	Locked nucleic acid
MAPK:	Mitogen activated protein kinase
MDS	Myelodysplastic syndrome
MPD:	Myeloproliferative disorder
MPL:	Myeloproliferative leukaemia virus oncogene
MPN:	Myeloproliferative neoplasm
NGS:	Next Generation Sequencing
NHLS:	National Health Laboratory Service
PCR:	Polymerase chain reaction
Ph	Philadelphia chromosome
PI3K:	Phosphoinositide 3-kinase
PMF:	Primary myelofibrosis
PV:	Polycythaemia vera
RFLP:	Restriction fragment length polymorphism
SNP:	Single nucleotide polymorphism
SRSF2:	Serine/arginine-rich splicing factor 2
STAT:	Signal transducer and activator of transcription
T:	Thymine
Taq:	*Thermus aquaticus*
TBE:	Tris borate ethylenediaminetetraacetic acid
T Cells:	Thymus cells
TET2:	Ten-eleven translocation oncogene family member
T_m	Melting temperature
TNF:	Tumour necrosis factor
TPO:	Thrombopoietin

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TP53:	Tumour protein p53
U2AF1:	U2 Small Nuclear RNA Auxiliary Factor 1
WBC:	White blood cell count
WHO:	World Health Organization

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Detection of the Janus kinase 2 V617F mutation using molecular methods

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SUMMARY

In 2005, a mutation located at exon 14 of the Janus Kinase gene on chromosome 9 was discovered in patients with Polycythaenia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF). The mutation (JAK2 V617F/G1849T) causes valine to be substituted by phenylalanine at codon 617. As a result the World Health Organisation (WHO) revised the diagnostic criteria of myeloproliferative neoplasms (MPN) in 2008 to include the detection of the JAK2 V617F mutation as a major diagnostic criterion for PV, ET and PMF.

Molecular assays with high sensitivity and specificity should be offered by diagnostic laboratories for this purpose. To comply with these requirements, commercial and in-house assays that offer different sensitivity and specificity levels have been developed. In addition to the performance characteristics of diagnostic assays used to detect the JAK2 V617F mutation, associated cost remains an important factor to consider when selecting the assay that is best suited to a particular laboratory. Commercial diagnostic kits are expensive which led to the development of more cost-effective in-house assays for use in routine diagnostic laboratories. The purpose of this study was to develop real time polymerase chain reaction

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(PCR) assays for the detection of the JAK2 V617F mutation that could be used in diagnostic laboratories.

Primers and locked nucleic acid (LNA) probes were designed. DNA was extracted from 60 fresh peripheral blood specimens. Allele specific PCR (AS-PCR) was performed on 59 DNA samples while direct sequencing and real time PCR assays were performed on all 60 samples. Performance of the different molecular assays was compared. In addition, the analytical sensitivity of the LNA real time PCR assay was determined by processing specimens comprising serial dilution of homozygous mutant in wild type DNA.

Allele specific PCR identified 26 of the 59 as positive for the JAK2 V617F mutation. The LNA real time PCR assay and direct sequencing both showed 24 of 60 samples to harbour the mutation. Diagnostic sensitivity and specificity of the developed real time PCR assay and sequencing assay was 88% and 100%, respectively. There was 100% agreement between the real time PCR and sequencing assays. Agreement between real time PCR and AS-PCR was calculated to be 94% with kappa value of 0.89.

The developed real time PCR assay showed acceptable performance when assessed against the comparative AS-PCR method. In addition, analytical sensitivity of 0.1% was demonstrated for this assay. The findings confirm the suitability of the developed LNA probe real time PCR assay to detect the JAK2 V617F mutation in a clinical laboratory.

Key words: Essential thrombocythaemia, Janus tyrosine kinase 2, Locked nucleic acid, Myeloproliferative neoplasms, Polymerase chain reaction, Polycythaemia vera, Primary myelofibrosis

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CHAPTER 1

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal disorders that result from transformation of a haematopoietic stem cell (HSC) (Quintás-Cardama *et al.*, 2010). This transformation of HSC causes uncontrolled proliferation of myeloid cell lines leading to overproduction of both mature and immature blood cells (Delhommeau *et al.*, 2010; Abdel-Wahab and Levine, 2011; Titmarsh *et al.*, 2014). Classic Philadelphia (Ph) chromosome negative MPNs include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Anderson and McMullin, 2014).

The annual incidence of MPNs ranges between 0.5 and 6.5 per 100 000 population (Cankovic *et al.*, 2009). Price *et al.* (2011) conducted a retrospective study to compare annual medical and pharmacy costs between patients with MPN and matched age-gender individuals without MPN. The study found that costs related to the treatment of patients with MPN are two to six times higher than those of patients without MPN (Price *et al.*, 2011). Another study found that annual medical and pharmacy costs were 1.5 to 3 times higher in MPN patients as compared to the matched control group of patients without MPN (Mehta *et al.*, 2014). Improved identification and reporting of MPNs remains important in order to facilitate allocation of resources for the management of patients with MPN (Titmarsh *et al.*, 2014).

The most common complications of MPNs, which also contribute significantly to morbidity and mortality, are increased thrombotic risk and bleeding tendencies (Quintás-Cardama *et al.*, 2010; Titmarsh *et al.*, 2014). In addition, between 10% and 30% of patients with MPN transform to secondary myelofibrosis and about 10% to acute myeloid leukaemia (Tefferi *et al.*, 2000).

Prior to 2005 no underlying genetic abnormality had been associated with classic Breakpoint cluster region-Abelson leukaemia virus (BCR-ABL1) negative MPNs. In 2005, a mutation at base 1849 in exon 14 of the Janus kinase 2 (JAK2) gene on chromosome 9 was discovered in patients with PV, ET and PMF (Baxter *et al.*, 2005; James *et al.*, 2005b; Kralovics *et al.*, 2005; Levine *et al.*, 2005). This somatic point mutation causes a substitution of guanine by thymine and the amino acid is changed from valine to phenylalanine in codon 617 of the

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JAK2 protein (Baxter *et al.*, 2005). The mutation is termed "JAK2 V617F" (Baxter *et al.*, 2005). The discovery has improved the understanding of the pathophysiology of MPN and has renewed interest and research into MPN biology and genetics (Vardiman *et al.*, 2009; Langabeer *et al.*, 2015). The identification of this mutation has also led to the development of therapies targeted at the inhibition of the JAK2 kinase (Levine and Gilliland, 2008; Quintás-Cardama *et al.*, 2010).

The JAK2 V617F mutation is detected in more than 95% of patients with PV and in 60% of patients with PMF and ET (Nangalia and Green, 2014). The discovery of this mutation has resulted in the revision of the diagnostic criteria of MPN by the World Health Organization (WHO) in 2008; a step that simplified the diagnosis but also ensured greater diagnostic accuracy.

As the demonstration of the JAK2 V617F mutation is now considered to be a major criterion for the diagnosis of MPNs, sensitive and specific assays are required for its detection (Greiner, 2006). Commercial kits are available and could arguably be used. The Ipsogen® JAK2 MutaScreen Kit represents an example of such an assay which uses the principle of allelic discrimination where two TaqMan® probes are used in a multiplex real-time PCR assay. One probe is a perfect match to the wild-type allele and the other to the mutant allele. This assay requires the use of 25 ng genomic DNA and an analytical sensitivity of 4% is claimed by the manufacturer (Ipsogen® JAK2 MutaScreen Kit Handbook, 2015). However, since the manufacturer has patency on the product, this assay has been found to be too expensive for routine use in a diagnostic laboratory. With this in mind, cost effective in-house assays are developed for diagnostic laboratories.

In-house assays that are currently being used for this purpose include allele specific polymerase chain reaction (AS-PCR) or amplification refractory mutation system (ARMS), direct sequencing, polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) and real time PCR (Poodt *et al.*, 2006; Greiner, 2006; Tan *et al.*, 2007).

Although the JAK2 V617F mutation was discovered by bidirectional sequencing of the coding exons and introns of JAK2 gene, this sequencing assay is not suitable for a routine diagnostic laboratory as it is time-consuming and laborious (Greiner, 2006). Studies have

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shown that sequencing has low detection sensitivity of between 5% and 20% (James *et al.*, 2005a; Lay *et al.*, 2006; Kannim *et al.*, 2009).

Allele specific PCR and PCR-RFLP assays are useful screening tools for mutations of this nature as the two assays are easy and inexpensive to perform (Steensma, 2006; Kannim *et al.*, 2009). The disadvantages of these assays include that post PCR processing is required which makes them time consuming and risks contamination (Soheili and Samiei, 2005). Allele specific PCR has been claimed to have analytical sensitivity of between 0.01% and 3% while PCR-RFLP has sensitivity of around 5% (Baxter et al., 2005; Steensma, 2006; Frantz *et al.*, 2007).

Real time PCR is a reliable assay to use in mutation detection as it is highly sensitive and can detect few gene copies in a reaction (Lim *et al.*, 2011). Cross contamination of PCR products is eliminated as no post-PCR steps are required (Lim *et al.*, 2011). Real time PCR assays based on hydrolysis probes for the quantification of JAK2 V617F have been developed with an analytical sensitivity of 0.01% (Hammond *et al.*, 2007; Kröger *et al.*, 2007).

Real time PCR with fluorescence resonance energy transfer (FRET) probes has also been developed by different groups (Murugesan *et al.*, 2006; Olsen *et al.*, 2006; Cankovic *et al.*, 2009). The reported analytical sensitivity of FRET assays using Lightcycler instruments (Roche Diagnostics GmbH, Mannheim, Germany) was about 5% (Lay *et al.*, 2006; Murugesan *et al.*, 2006; Olsen *et al.*, 2006). A study by Cankovic *et al.* (2009) reported a lower analytical sensitivity of only 10% for a similar assay performed on a Rotor-gene platform (Corbett Research, Sydney, Australia). The difference in analytical sensitivity may be influenced by the real time PCR instrument.

While laboratories may find it challenging to select the methodology and platform most suitable for their practice, detection of the JAK2 V617F mutation remains essential in the diagnostic algorithm of MPNs. In this regard a reliable, quick and sensitive assay that is able to detect 1% of mutant allele in wild type background is required (Gong *et al.*, 2013). The aim of this study was to develop real time PCR assays for the detection of the JAK2 V617F mutation for implementation in a routine clinical laboratory.

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The objectives were:

- To perform genomic DNA extraction on 60 samples of fresh whole blood collected by health care providers at the Steve Biko Academic Hospital
- To detect the mutation using conventional PCR assay
- To confirm wild type and mutant sequences by sequencing of the amplicons generated by the conventional PCR assay
- To detect the mutation using real time PCR assays (hydrolysis probe-based and SYBR green dye melt curve analysis)
- To ensure that the new assay has adequate sensitivity and specificity
- To incorporate the assay in the PCR diagnostic laboratory

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Myeloproliferative neoplasms (MPNs) are a rare group of clonal disorders of the bone marrow stem cells characterised by an overproduction of one or more of the myeloid lineages (Delhommeau *et al.*, 2010; Moulard *et al.*, 2014). These disorders are classified according to the 2008 WHO classification of myeloid neoplasms (Vardiman et al., 2009). Figure 2.1 illustrates the WHO classification of MPN (Vardiman *et al.*, 2009).

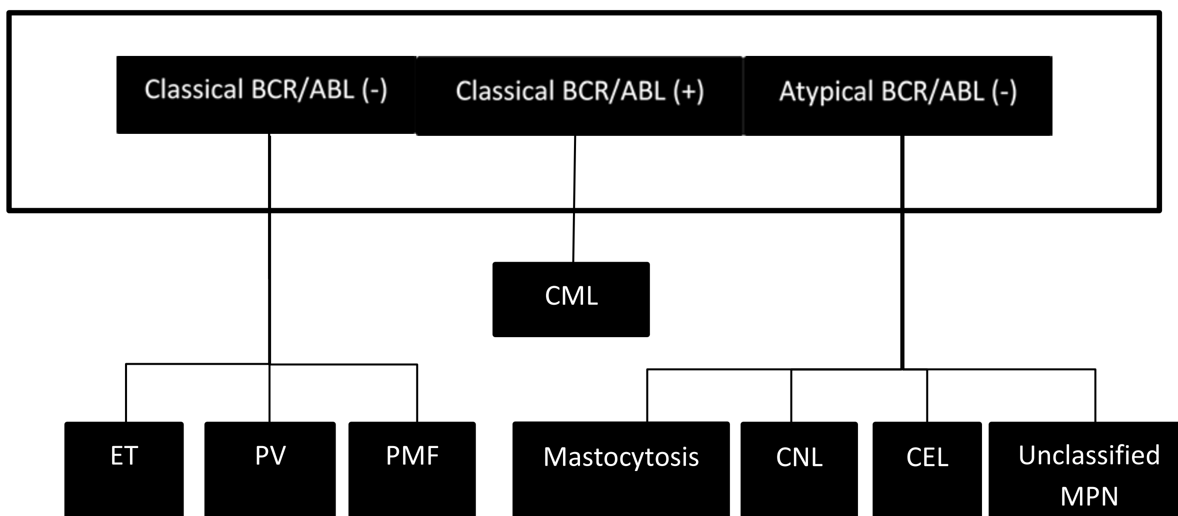

Figure 2.1: The 2008 WHO Classification of MPN

BCR/ABL: Breakpoint cluster region/Abelson leukaemia virus; CEL: chronic eosinophilic leukaemia; CML: chronic myeloid leukaemia; CNL: chronic neutrophilic leukaemia; ET: essential thrombocythaemia; MPN: myeloproliferative neoplasms; PMF: primary myelofibrosis; PV: polycythaemia vera (Vardiman et al., 2009).

Myeloproliferative neoplasms are classified as Philadelphia (Ph) chromosome positive and Ph chromosome negative MPNs on the basis of whether a reciprocal translocation between chromosome nine and 22 exists (Anderson and McMullin, 2014). Chronic myeloid leukaemia (CML) is a Ph positive MPN whereas polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) are Ph negative MPNs (Anderson

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and McMullin, 2014). In PMF, fewer blood cells are produced in the bone marrow as a result of fibrosis formation (Moulard *et al.*, 2014). Essential thrombocythaemia is characterised by an abnormal increase in platelets while in PV, there is an abnormal increase in red blood cells in the blood (Klco *et al.*, 2010; Moulard *et al.*, 2014).

Many patients with MPN suffer thrombotic and bleeding complications, which contribute significantly to morbidity and mortality in this group of patients (McMahon and Stein, 2013; Sun and Zhang, 2013). Risk factors for thrombosis in MPN include: (i) age over 60 years; (ii) a history of thrombosis; (iii) the presence of JAK2 V617F mutation; (iv) female gender and (v) leucocytosis (McMahon and Stein, 2013; Popov *et al.*, 2014). Myeloproliferative neoplasms may also progress to secondary myelofibrosis, myelodysplastic syndrome (MDS) or even acute myeloid leukaemia (AML) (Quintás-Cardama *et al.*, 2010; Titmarsh *et al.*, 2014).

In 2005 the JAK2 V617F mutation, located in exon 14 of the JAK2 gene on chromosome 9, was discovered in patients with PV, ET and PMF (Baxter *et al.*, 2005; James *et al.*, 2005b). The mutation was discovered by bidirectional sequencing of the coding exons and introns of the *JAK2* gene from peripheral-blood granulocytes and thymus (T) cells (Baxter *et al.*, 2005; James *et al.*, 2005b). This somatic point mutation causes a substitution of guanine by thymine and the amino acid is changed from valine to phenylalanine (Baxter *et al.*, 2005). This discovery improved the understanding of the molecular pathogenesis of PV, MPN and ET (Vardiman *et al.*, 2009). It led to the revision of the diagnostic criteria of MPN and provided new insights into MPN biology and genetics (Langabeer *et al.*, 2015). The discovery introduced the possibility of the development of therapies targeted at the inhibition of the JAK2 kinase activity (Levine and Gilliland, 2008; Quintás-Cardama *et al.*, 2010).

The JAK2 V617F mutation is detected in more than 95% of patients with PV and in 50 to 60% of patients with PMF and ET (Nangalia and Green, 2014). Currently there are hypotheses that seek to explain how a single mutation gives rise to three clinically different disorders (James, 2008). These hypotheses propose that the phenotype depends on different variables including the cell targeted by the mutation; the genetic background of the patient; the level of kinase activity generated by the mutant protein and lastly a pre-JAK2 event (James, 2008).

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The JAK2 V617F mutation comprises a single base change (James *et al.*, 2005b). Detection of single base changes requires extremely sensitive and specific assays (Greiner, 2006). Assays currently being used for this purpose include direct sequencing, allele specific polymerase chain reaction (AS-PCR), polymerase chain reaction restriction-fragment length polymorphism (PCR-RFLP) and real time PCR (Poodt *et al.*, 2006; Greiner, 2006; Tan *et al.*, 2007). Assays such as PCR-RFLP and direct sequencing are considered to be inappropriate for a routine diagnostic laboratory due to the low detection sensitivity (5% to 20%) and the time consuming nature (Lay *et al.*, 2006). Real time PCR and AS-PCR are the two most sensitive assays with sensitivities ranging between 0.01% and 5% (Steensma, 2006; Lay *et al.*, 2006).

This review will focus on the historical, clinical and diagnostic aspects of MPN. The genetic mutation described in patients with MPN and the assays used to detect the JAK2 V617F mutation will be reviewed in detail.

2.2 Normal haematopoiesis and JAK2 regulation

Haematopoiesis is a process in which the pluripotent stem cells in the bone marrow proliferate and differentiate to produce blood cells (Verfaillie, 1998). Mature blood cells originate from a self-renewing population of multi-potent haematopoietic stem cells that become committed to differentiate along the erythroid, megakaryocytic, granulocytic, monocytic and lymphoid lineages (Rane and Reddy, 2002; Ferrajoli *et al.*, 2006). In healthy persons haematopoiesis takes place within the bone marrow microenvironment where stem cells and their progeny interact with other cells and extracellular matrix ligands present in the medullary cavity (Verfaillie, 1998). The bone marrow stroma is composed of heterogeneous stromal cells and a microvascular network (Hoffbrand and Moss, 2011). The stromal cells include adipocytes, fibroblasts, endothelial cells and macrophages that secrete extracellular molecules such as collagen, glycoproteins and glycosaminoglycans to form an extracellular matrix (Hoffbrand and Moss, 2011). In addition, stromal cells also secrete several growth factors necessary for stem cell survival, proliferation and differentiation (Khwaja, 2006; Hoffbrand and Moss, 2011).

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Haemopoietic growth factors such as erythropoietin (EPO), thrombopoietin (TPO) and granulocyte colony stimulating factor (G-CSF) exert their effects by binding to specific receptors on developing cells (Lewis and Lee, 2007). Upon binding of the ligand to its receptor, a signal is sent to the cell to proliferate and differentiate (Lewis and Lee, 2007). Figure 2.2 illustrates the differentiation pathways of blood cells (Moratuwagama, 2014).

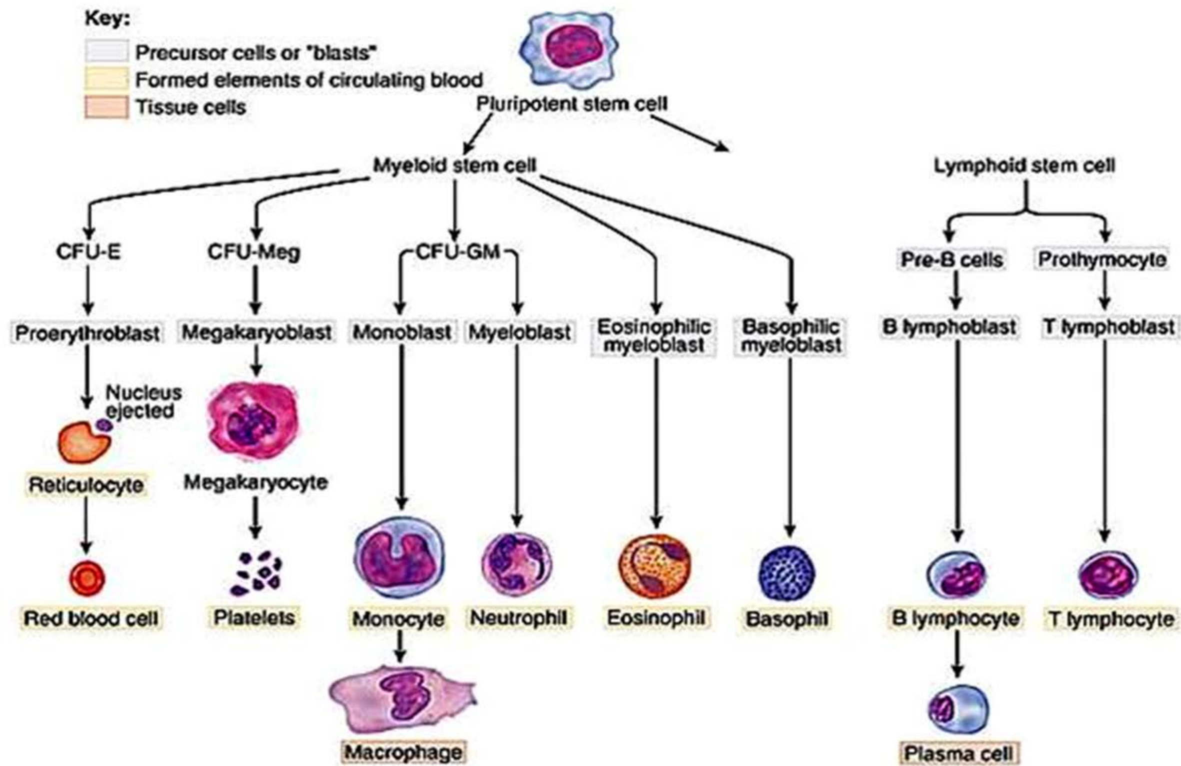

Figure 2.2: The process of blood formation in the bone marrow. A pluripotent stem cell gives rise to common myeloid and lymphoid progenitor cells that further differentiate into erythroid, megakaryocytic, granulocytic, monocytic and lymphocytic lineages (Moratuwagama, 2014)

CFU-E: colony forming unit-erythroid; CFU-Meg: colony forming unit-megakaryocyte; CFU-GM: colony forming unit-granulocyte/macrophage.

Erythropoietin is a glycoprotein hormone that is produced by interstitial peritubular cells of the kidney (Lewis and Lee, 2007). It promotes the survival, proliferation and differentiation

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of late erythroid progenitor cells (Lewis and Lee, 2007). Erythropoietin acts through an erythropoietin receptor that is expressed on the surface of precursor cells committed to the erythroid lineage (Lewis and Lee, 2007). Under normal conditions EPO is produced at a steady rate to maintain a normal level of erythropoiesis with hypoxic conditions resulting in increased synthesis (Lewis and Lee, 2007). In the hypoxic state, the transcription factor hypoxia inducible factor (HIF) is activated (Rankin *et al.*, 2007). Hypoxia inducible factor subsequently binds to hypoxia-response elements and activates genes such as those encoding EPO and influences adaptive responses to hypoxia by stimulating erythropoiesis and angiogenesis (Rankin *et al.*, 2007; Hoffbrand *et al.*, 2011). The level of EPO in the blood rises due to reduced oxygen tension and blood loss stimulating the production of red cells (Hoffbrand *et al.*, 2011). Prolonged EPO stimulation by HIF due to mutated proteins such as the von Hippel-Lindau tumour-suppressor protein can lead to polycythaemia (Hoffbrand *et al.*, 2011). Binding of EPO to EPO receptor results in the activation of the signal transduction pathway (Hoffbrand *et al.*, 2011). While EPO regulates erythropoiesis, megakaryocyte maturation and platelet production is regulated by TPO (Lewis and Lee, 2007).

Thrombopoietin produced constitutively by the liver and kidney is the primary regulator of megakaryocyte maturation and platelet production (Lewis and Lee, 2007). During normal homeostasis, the platelet count remains constant and circulating TPO is maintained at basal levels (Lewis and Lee, 2007). In thrombocytopenia the drop in platelet mass results in a reduced number of peripheral binding sites for TPO and in thrombocytosis, TPO binding by the elevated platelet mass reduces the level of circulating TPO, limiting thrombopoiesis (Lewis and Lee, 2007). Thrombopoietin promotes the proliferation and differentiation of megakaryocytes and their fragmentation into platelets (Lewis and Lee, 2007). Thrombopoietin receptors are found on megakaryocytes and platelets (Lewis and Lee, 2007). Binding of TPO to the Myeloproliferative leukaemia virus (MPL) receptor on megakaryocytes progenitors results in receptor dimerization and initiates the Janus kinase/ Signal transducer and activator of transcription (JAK/STAT) pathway that result in the proliferative response (Israels and Israels, 2002).

Granulopoiesis maintains the resting basal level of neutrophils and has the capacity to respond rapidly in the event of infection or inflammation (Israels and Israels, 2002). A series

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of cytokines such as G-CSF, granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-6 play a role in the regulation of granulopoiesis (Basu *et al.*, 2000). Inflammation-associated chemokines and cytokines stimulate increased granulopoiesis (Israels and Israels, 2002). The G-CSF cytokine is produced by neutrophils, endothelial cells, fibroblasts and other bone marrow stromal cells following stimulation by tumour necrosis factor, IL-1 and by endotoxin (Israels and Israels, 2002). These growth factors stimulate the proliferation of committed granulocyte progenitors, decrease apoptosis and reduce the maturation time from precursor to segmented neutrophil. Granulocyte colony stimulating factor receptors are present on promyelocytes, myelocytes, metamyelocytes and mature neutrophils (Israels and Israels, 2002). Binding of receptors induces phosphorylation of JAK-2 and JAK-1 leading to phosphorylation of the cytoplasmic region of the receptor (Israels and Israels, 2002). Phosphorylation then induces receptor dimerisation resulting in activation of intracellular signalling through the JAK/STAT, mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Israels and Israels, 2002). These intracellular signal transduction pathways are activated by dimerisation of TPO, EPO and G-CSF receptors (Hoffbrand and Moss, 2011).

Mitogen-activated protein kinase modules containing three sequentially activated protein kinases are key components of a series of vital signal transduction pathways that regulate cell proliferation, cell differentiation and cell death (Morrison, 2012). Mitogen-activated protein signalling pathways mediate intracellular signalling initiated by extracellular or intracellular stimuli, such as mitogens, cytokines, growth factors and environmental stressors (Kim and Choi, 2010). Activated MAPKs phosphorylate transcription factors resulting in regulation of a variety of cellular activities including cell proliferation, differentiation, migration, inflammatory responses and death (Kim and Choi, 2010). Activation by MAPK signalling cascades is achieved either through a series of binary interactions among the kinase components or through formation of a multiple kinase complex mediated by a scaffold protein (Kim and Choi, 2010). Deviation from the normal control of the MAPK signalling pathways have been implicated in the pathogenesis of many human diseases including cancer (Kim and Choi, 2010).

The PI3K is involved in cell survival pathways which regulate gene expression and cell metabolism (Cantley, 2002). Phosphoinositide 3-kinase transduce signals from various

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growth factors and cytokines into intracellular messages by generating phospholipids, which in turn activate the serine protein kinase B effector pathways (Liu *et al.*, 2009). Over-activation of the PI3K pathway is implicated in the development of diabetes mellitus and cancer (Cantley, 2002).

The JAK/STAT pathway plays a central role in the regulation of cell proliferation, differentiation, cell migration and apoptosis (Pfeifer *et al.*, 2008). The pathway is activated by cell-surface receptors that lack cytoplasmic kinase activity (Pfeifer *et al.*, 2008). Ligand binding to the extracellular domain of the receptor leads to conformational changes of the intracellular domain, bringing receptor-associated JAK proteins into close proximity and thereby enabling trans-phosphorylation of the JAKs (Pfeifer *et al.*, 2008). The activated JAKs then phosphorylate the receptor, generating binding sites for cytoplasmic proteins with STAT proteins (Pfeifer *et al.*, 2008). Monomeric STATs bind to specific phosphotyrosine residues of the activated receptor and are subsequently phosphorylated by JAK (Pfeifer *et al.*, 2008). Activated STATs dissociate from the receptor, dimerise rapidly and translocate to the nucleus (Pfeifer *et al.*, 2008). In the nucleus, STAT dimers together with cofactors bind to the DNA and activate transcription of target genes (Pfeifer *et al.*, 2008). Figure 2.3 shows the schematic representation of the JAK-STAT signalling pathway (Steensma, 2006).

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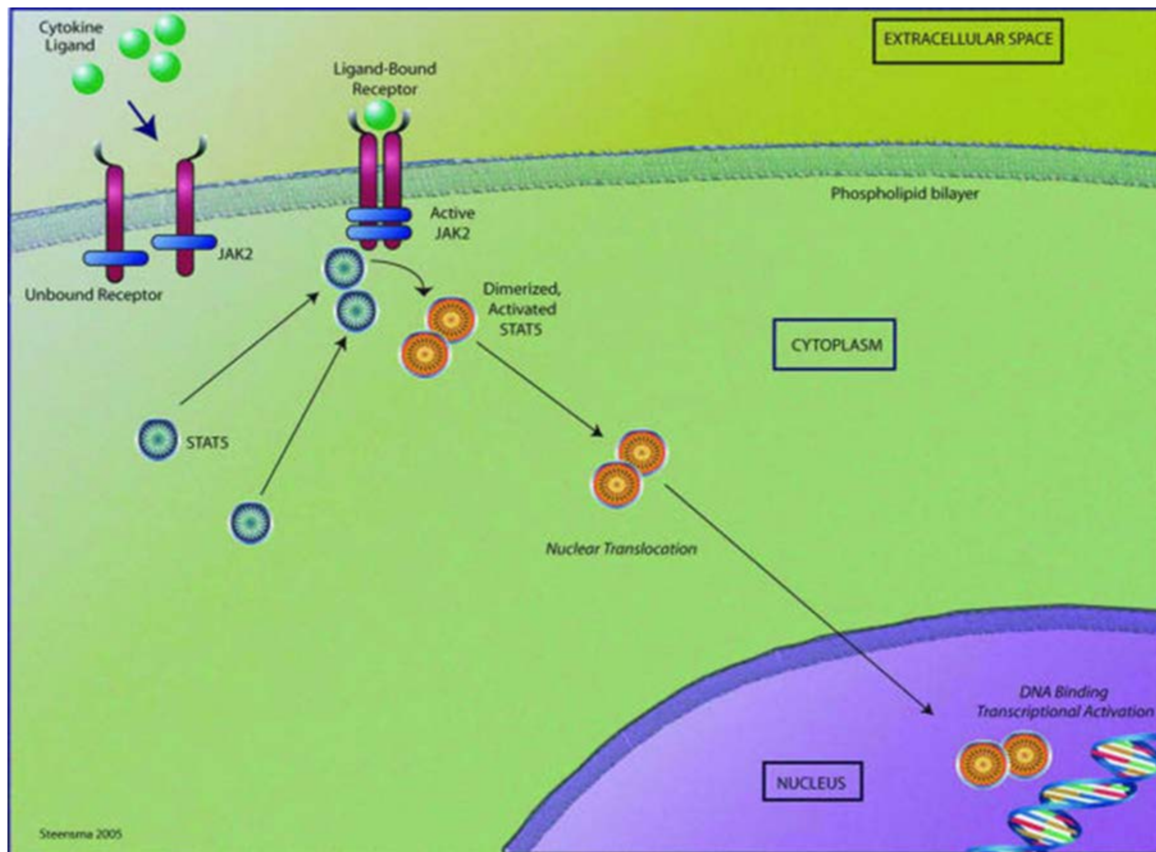

Figure 2.3: The JAK/STAT signalling pathway which is activated by binding of ligand to the receptor. Binding of ligand activates JAK2 molecules leading to gene transcription (Steensma, 2006).

Congenital defects in JAK-STAT signalling are associated with immunodeficiency states whereas acquired activating mutations and translocations contribute to the pathophysiology of haematological malignancies (Khwaja, 2006). Congenital defects in STAT pathways results in reduced responses to Gamma-interferon with a clinical picture of susceptibility to mycobacterial infections, viral infection and severe combined immune deficiency (O'Shea *et al.*, 2013). Janus kinase/STAT signalling is tightly regulated in normal cells but in malignant cells the control mechanisms fail due to over expression or mutations of one of the regulatory proteins involved (Pfeifer *et al.*, 2008). Gain of function mutations leading to constitutive activity of tyrosine kinases and therefore causing permanent STAT activation have been found in myeloproliferative neoplasms (Pfeifer *et al.*, 2008).

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2.2 Myeloproliferative neoplasms

The individual disorders that make up this group of diseases were first described in the nineteenth and twentieth century (Tefferi, 2008). In 1951 William Dameshek, described some similarities in bone marrow histology and clinical presentation among patients diagnosed with CML, ET, PV and PMF (Dameshek, 1951). The four diseases were grouped together and were termed "myeloproliferative disorders (MPD)" (Dameshek, 1951).

In 1960, an abnormality on chromosome 22 was discovered in CML patients (Nowell and Hungerford, 1960). This abnormal chromosome 22 became known as the Ph chromosome, named after the city in which it was discovered (Nowell, 2007). In 1973, improved cytogenetic assays demonstrated that the Ph chromosome resulted from a translocation between the long arms of chromosome 9 and chromosome 22 [t (9; 22) (q34; q11.2)] (Rowley, 1973; Nowell, 2007). This novel chimeric chromosome comprised the fusion of Abelson leukaemia virus (ABL1) gene on chromosome 9 to the breakpoint cluster region (BCR) gene on chromosome 22 (Sawyers, 1999). About 95% of CML patients carry the Ph chromosome and 5% carry other variants such as t(7;22)(p22;q11), t(9;22;15)(q34;q11;q22), t(15;22)(p11;q11), t(1;9;22;3)(q24;q34;q11;q21) and t(12;22)(p13;q11) with the same end result as the Ph chromosome (Sawyers, 1999). The presence of this fusion gene in CML patients led to the division of MPD into BCR/ABL1 positive CML and BCR/ABL1 negative disorders (PV, ET, PMF) (Langabeer *et al.*, 2015).

Prior to 2005 the presence of the Ph chromosome and/or BCR-ABL1 fusion gene was considered diagnostic for CML (Jaffe, 2001). In contrast, the diagnosis of BCR-ABL1 negative MPDs was based only on clinical and laboratory features without the possibility of molecular testing (Jaffe, 2001). The discovery of the V617F mutation in the JAK2 gene in 2005 introduced the possibility of molecular diagnosis of the classic BCR-ABL1 negative MPD (Baxter *et al.*, 2005). As a result of the discovery, the WHO in 2008, revised the diagnostic criteria of MPDs to include testing of genetic markers, such as JAK2 V617F and exon 12 mutations (Vardiman *et al.*, 2009). This approach simplified the diagnostic process and enhanced diagnostic accuracy (Vardiman *et al.*, 2009). The term MPD was also changed to MPN to better reflect the neoplastic nature of this group of disorders (Vardiman *et al.*, 2009).

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2.3 Philadelphia- chromosome negative classic MPNs

Classic Philadelphia chromosome negative MPNs comprise three disease entities with different phenotypic expression namely PV, PMF and ET (Bench *et al.*, 2013). These three diseases are characterised by clonal expansion of bone marrow lineages (Klco *et al.*, 2010).

2.3.1 Polycythaemia Vera

Polycythaemia vera is characterised by clonal proliferation of the erythroid lineage (Klco *et al.*, 2010). This disease is associated with an increase in red blood cell production which results in increased red cell mass, haemoglobin level, haematocrit value and increased blood viscosity (Klco *et al.*, 2010). Polycythaemia vera was first reported by Vasquez, a French physician, in 1892 and defined more clearly by Osler in 1903, hence it was eponymously called Vasquez-Osler disease (Bilgrami and Greenberg, 1995). In keeping with prevailing trends to assign a medical name that describes the cause of the disease or its clinical signs, it was later renamed polycythaemia rubra vera. Polycythaemia rubra vera was also distinguished from secondary polycythaemia, which includes a heterogeneous group of disorders characterised by polycythaemia unrelated to a primary bone marrow disorder, and relative polycythaemia, which is caused by plasma volume contraction (Steensma, 2005). The reported annual incidence of PV increases with advanced age and varies from 0.7 to 2.6 per 100 000 inhabitants in Europe (Swerdllow *et al.*, 2008). The median age at diagnosis is 55 to 60 years and it is rarely found in patients younger than 20 years old of age (Swerdllow *et al.*, 2008). In 2003, the prevalence of PV was reported as 22 per 100,000 people in the state of Connecticut (Ma *et al.*, 2008).

The diagnosis of PV is based on the assessment of both clinical and laboratory features (Tefferi, 2011). Patients present with chest pains, hepatomegaly, night sweats, pruritus after bathing, splenomegaly, weight loss and neurologic symptoms, such as blurred vision dizziness and headache (Stuart and Viera, 2004). Haemoglobin and haematocrit levels are elevated in PV patients (Stuart and Viera, 2004). Haemopoietic cells of virtually all PV patients carry a mutation in the JAK2 protein (Kiladjian, 2012). When a patient is suspected of having PV, the presence of a JAK2 mutation is confirmatory to the diagnosis and its

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absence combined with normal or increased serum EPO level essentially excludes the diagnosis (Levine and Gilliland, 2007; Kiladjian, 2012).

The JAK2 V617F mutation is found in approximately 95% of patients with PV, the JAK2 exon 12 mutation in ~2% and only 1% of patients carry other JAK/STAT mutations (Tefferi, 2012). Homozygosity for the V617F mutation is found in 25% to 30% of PV patients (Vannucchi *et al.*, 2007). The homozygous state occurs as a result of mitotic recombination affecting the short arm of chromosome 9 (Scott *et al.*, 2007). Mitotic recombination is a mechanism that preserves genomic sequence integrity in the repair of double-strand DNA (Tischfield and Shao, 2003). When mitotic recombination occurs between homologs, one of which has a mutation in a tumour-suppressor gene, the normal allelic sequence may be lost, leading to loss of heterozygosity (Tischfield and Shao, 2003). Homozygous patients have a higher haemoglobin level, higher white cell count, lower platelet count and an increased incidence of pruritus (Vannucchi *et al.*, 2007). In 2008 the WHO revised the preceding 2001 classification of MPD to incorporate the JAK2 V617F and JAK2 exon 12 mutations as major diagnostic criteria in the diagnostic algorithm of PV as shown in Table 2.1 (Jaffe, 2001; Vardiman *et al.*, 2009).

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Table 2.1: The WHO diagnostic criteria for PV, both 2001 and 2008 criteria are shown

2001	2008
A1 Elevated red cell mass >25% above mean normal predicted value, or Hb >18.5 g/dL in men, 16.5 g/dL in women	**Major criteria** 1. Hb > 18.5 g/dL (♂); >16.5 g/dL (♀) 2. Presence of JAK2 V617F or JAK2 exon 12 mutation
A2 No cause of secondary erythrocytosis: Absence of familial erythrocytosis No elevation of EPO due to: Hypoxia (arterial paO2 ≤ 92%) High oxygen affinity haemoglobin Truncated EPO receptor Inappropriate EPO production by tumour	**Minor criteria** 1. Bone marrow trilineage myeloproliferation 2. Subnormal serum EPO 3. Endogenous erythroid colony formation in vitro
A3 Splenomegaly	
A4 Clonal genetic abnormality other than Philadelphia chromosome or BCR-ABL fusion gene in marrow cells	
A5 Endogenous erythroid colony formation in vitro	
B1 Thrombocytosis > 400 x10^9/L	
B2 WBC >12 x10^9/L	
B3 Bone marrow biopsy showing panmyelosis with prominent erythroid and megakaryocytic proliferation	
B4 Low serum EPO levels	

BCR-ABL: Abelson leukaemia virus-Breakpoint cluster region; EPO: Erythropoietin; Hb: Haemoglobin; WBC: White blood cells (Jaffe, 2001; Vardiman *et al.*, 2009).

The 2001 classification allowed for the diagnosis of PV when A1 and A2 together with any other category A criterion were present, or when A1 and A2 and any two category B criteria were present. In the 2008 classification, diagnosis requires the presence of both major and

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one minor criteria or the presence of the first major criterion together with two minor criteria (Jaffe, 2001; Thiele and Kvasnicka, 2009; Vardiman *et al.*, 2009).

Thrombosis occurs in 40% of patients with PV and is the cause of death in 20% to 40% of these (Spivak, 2002). In general, formation of a thrombus arises from abnormalities in blood flow, blood constituents and the presence of vessel wall damage (Makin *et al.*, 2002). The increase in haematocrit in PV results in increased blood viscosity resulting in impaired blood flow (Makin *et al.*, 2002). The impaired blood flow might lead to arterial or venous thrombosis (Klco *et al.*, 2010). Arterial thrombosis is more common than venous thrombosis in the context of MPN (Spivak, 2002). The predominant cause of mortality and morbidity is thrombosis occurring in the large arterial vessels that results in stroke, ischaemic attacks and myocardial infarction (Elliott and Tefferi, 2005). About 10% of patients develop intra-abdominal vein thrombosis (Elliott and Tefferi, 2005). Apart from thromboembolic phenomena, a range of complications result in clinical symptoms and physical signs (Spivak, 2002). Some of these complications of PV and their causes are listed in Table 2.2 (Spivak, 2002).

Table 2.2: Complications that arise in patients with PV and causes of the complications

Complication	*Cause*
Thrombosis	Increased red cell mass
Organomegaly	Increased red cell mass Extramedullary haematopoiesis
Pruritus, Peptic ulcer disease	Inflammatory mediators
Hyperuricaemia, gout, renal stones	Increased red cell turn over
Ocular migraine	Thrombocytosis
Haemorrhage	Increased red cell mass Acquired von Willebrand Disease
Myelofibrosis	Reaction to neoplastic clone by non-neoplastic stromal cells
Acute leukaemia	Clonal evolution

(Spivak, 2002)

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Patients may also present with haemorrhage which might result from thrombocytosis or acquired von Willebrand Disease (Spivak, 2002; Elliott and Tefferi, 2005). Between 30% and 50% of patients with PV present with splenomegaly that predisposes them to develop secondary myelofibrosis (Campbell and Green, 2011). Progression to myelofibrosis occurs in 10% to 20% of PV patients (Campbell and Green, 2011).

2.3.2 Essential thrombocythaemia

Essential thrombocythaemia is an acquired clonal stem cell disorder with expansion of the megakaryocytic line characterized by a sustained increase in the platelet count and a tendency to develop thrombosis or bleeding (Fabris and Randi, 2009). This disease was initially described in 1934 in an Austrian patient who presented with persistent elevation of platelet count with megakaryocyte hyperplasia, thrombosis and a bleeding tendency (Skoda, 2007; Tefferi, 2008). This disease was initially called haemorrhagic thrombocythaemia owing to the phenotype (Skoda, 2007; Tefferi, 2008).

The incidence of ET varies between 0.6 to 2.53 cases per 100 000 persons per year (Brière, 2007; Bittencourt *et al.*, 2012). The median age at onset is 50 to 55 years and it predominates in females with a ratio of 2:1 (Campbell and Green, 2011; Bittencourt *et al.*, 2012). The somatic mutation JAK2 V617F is found in 50% of patients with ET, with homozygosity found only in 2% to 4% (Vannucchi *et al.*, 2007; Fabris and Randi, 2009). Myeloproliferative leukemia virus oncogene (MPL) exon 10 mutations have been described in 2% to 5% of patients with ET (Delhommeau *et al.*, 2010). Mutations involving the Calreticulin (CALR) gene are found in about 15% to 25% of ET and PMF patients who do not carry either JAK2 or MPL mutations (Klampfl *et al.*, 2013). In addition to aiding in the diagnosis of patients with MPN, somatic mutations also allowed for prognostication. Patients with ET harbouring JAK2 mutations are generally older with higher haemoglobin levels, leukocytosis, lower platelet counts and a greater propensity for thrombosis (Barbui *et al.*, 2012). Calreticulin exon 9 mutations are found mostly in younger, especially male patients who have higher platelet counts and lower haemoglobin levels (Tefferi, 2014; Tefferi *et al.*, 2014b). Patients with CALR positive ET also have a better prognosis as they are at lower risk of developing thrombosis when compared to patients carrying JAK2 and MPL mutations (Rotunno *et al.*, 2014).

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At present, the diagnosis of ET is based on the 2008 version of the WHO classification of myeloid neoplasms, which is a revision of the 2001 classification scheme (Vardiman *et al.*, 2009). Table 2.3 shows the comparison between the 2001 and 2008 WHO diagnostic criteria for ET (Jaffe, 2001; Vardiman *et al.*, 2009). In the 2008 classification, diagnosis for ET requires meeting all four criteria (Vardiman *et al.*, 2009). The proposed revision of this diagnostic criteria seeks to also incorporate CALR and MPL mutations as clonal markers in the major diagnostic criteria (Barbui *et al.*, 2015).

Table 2.3: The 2008 WHO diagnostic criteria for ET

2001	2008
Positive criteria Sustained platelet count ≥ 600 x10^9/L Bone marrow biopsy specimen showing proliferation mainly of megakaryocytic lineage with increased numbers of enlarged mature megakaryocytes **Criteria of exclusion** No evidence of PV No evidence of CML No evidence of chronic idiopathic myelofibrosis No evidence of MDS	Sustained platelet count ≥ 450 x10^9/L Megakaryocyte proliferation with enlarged, mature megakaryocytes Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm Demonstration of JAK2 V617F or other clonal marker or no evidence of reactive thrombocytosis

CML: Chronic myeloid leukaemia; MDS: Myelodysplastic syndrome; PMF: primary myelofibrosis; PV: Polycythaemia Vera; WHO: World Health Organization (Jaffe, 2001; Vardiman et al., 2009).

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Patients with ET often present with thrombosis or bleeding. Between 2% and 10% of ET cases are expected to transform to PV, secondary myelofibrosis or acute leukaemia (Wolanskyj *et al.*, 2005; Beer, 2011; Campbell and Green, 2011). Thrombocytosis may cause acquired von Willebrand Syndrome that might result in bleeding episodes in patients with ET (Elliott and Tefferi, 2005). In pregnant women, ET may worsen the risk of thrombosis and late pregnancy loss is frequently observed (Brière, 2007).

2.3.3 Primary myelofibrosis

Primary myelofibrosis is a clonal stem cell disorder characterised by bone marrow fibrosis, cytopenias, splenomegaly and extramedullary haematopoiesis (Hoffman and Rondelli, 2007; Mitra *et al.*, 2013). The disease was first described in 1879 in Germany when Heuck noted two cases of leukaemia with peculiar bone marrow and blood findings (Weinstein, 1991; Tefferi, 2008). Heuck noted osteofibrosis of the bone marrow and extramedullary haematopoiesis in the liver and spleen (Weinstein, 1991). The disease has been called by different names and the most commonly used were idiopathic myelofibrosis and agnogenic myeloid metaplasia (Reilly, 1997).

Primary myelofibrosis usually affects elderly people (Cervantes and Pereira, 2012). The median age at diagnosis is about 65 years with less than 20% of patients younger than 50 years (Cervantes and Pereira, 2012). The average survival is less than five years although some patients survive for more than 20 years (Hoffman and Rondelli, 2007; Cervantes and Pereira, 2012). Mutations involving JAK2, MPL, Ten-eleven translocation oncogene family member (TET2), CALR and enhancer of zeste homolog 2 (EZH2) are found in some patients but the genetic trigger initiating PMF still remains unknown (Cervantes and Pereira, 2012). About 50% to 60% of patients with PMF carry the JAK2 V617F mutation, 5% to 10% of patients carry MPL mutations and 15% to 25% carry CALR mutations (Delhommeau *et al.*, 2010; Cervantes and Pereira, 2012). A study to assess the prognostic value of the V617F mutation in 152 PMF patients showed that patients who were positive for the mutation had more aggressive disease and reduced survival with a hazard ratio of 3.30 (Campbell *et al.*, 2006). Patients died of complications, such as leukaemic transformation and bone marrow failure (Campbell *et al.*, 2006). The authors recommended the need for a careful prospective study of the effects of the V617F mutation in PMF and suggest that the V617F mutation may

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be an important molecular prognostic marker for PMF (Campbell *et al.*, 2006). Myeloproliferative leukaemia virus mutated PMF is associated with the female gender, age above 56 years and a lower haemoglobin level that might predispose the patient to transfusion dependency (Tefferi, 2010).

Current diagnosis follows the 2008 WHO criteria and requires assessment of both clinical and laboratory features (Tefferi, 2011). Diagnosis is based on bone marrow morphology, the presence of fibrosis and mutations in the JAK2 or MPL oncogene. Table 2.4 shows the 2008 WHO diagnostic criteria for PMF (Vardiman *et al.*, 2009).

Table 2.4: The 2008 WHO diagnostic criteria for PMF

Major criteria
1. Megakaryocyte proliferation and atypical with either reticulin and/or collagen fibrosis or increased bone marrow cellularity (granulocytic hyperplasia with reduced erythropoiesis) 2. Not meeting WHO criteria for CML, PV, MDS or other myeloid neoplasm 3. Demonstration of JAK2 V617F or other clonal marker or no evidence of reactive marrow fibrosis
Minor criteria
Leukoerythroblastosis Increased serum LDH level Anaemia Palpable splenomegaly

CML: Chronic myeloid leukaemia; LDH: Lactate dehydrogenase MDS: Myelodysplastic syndrome; PV: polycythaemia vera; WHO: World Health Organization (Vardiman *et al.*, 2009)

Diagnosis requires meeting all three major and two minor criteria (Vardiman *et al.*, 2009). It is anticipated that the 2008 WHO-based diagnostic criteria for MPNs will be revised in 2016

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to include demonstration of the CALR mutation or other clonal markers as major diagnostic criteria (Barbui *et al.*, 2015). The 2012 British Committee for Standards in Haematology (BCSH) guidelines for investigation and management of myelofibrosis was recently modified to include testing for the CALR mutations into the major criteria alongside JAK2 V617F (Reilly *et al.*, 2014).

Assessment of bone marrow morphology is important since accumulation of fibrous tissue parallels disease progression (Swerdllow *et al.*, 2008). In the prefibrotic stage of the disease there is no significant increase in reticulin and collagen fibres whereas in the fibrotic stage, the bone marrow shows an increase in reticulin and collagen fibrosis (Swerdllow *et al.*, 2008). The presence of 10% to 19% blasts in the blood or bone marrow is defined as an accelerated phase of PMF, and 20% or more blasts in the blood or bone marrow indicate an acute leukemic transformation (Swerdllow *et al.*, 2008).

Patients present with progressive anaemia due to ineffective erythropoiesis and multi-organ extramedullary haemopoiesis that result in severe hepatosplenomegaly (Mitra *et al.*, 2013; Tefferi, 2013). Patients with advanced disease suffer from symptoms of massive splenomegaly (early satiety, abdominal pain, portal hypertension, splenic infarcts, etc.) and constitutional symptoms that include diffuse bone pain, unexplained fever, drenching night sweats and unintentional weight loss (Mitra *et al.*, 2013). The condition is frequently complicated by thrombosis and bleeding and transformation to leukaemia occurs in about 20% of cases (Hoffman and Rondelli, 2007; Tefferi, 2011). In most cases, death results from leukaemic transformation, progressive bone marrow failure, infection, bleeding, portal hypertension and vascular complications (Barbui *et al.*, 2010; Tefferi, 2013).

2.4 Mutations in myeloproliferative neoplasms

Since 2005 more than 20 novel somatic mutations have been described in MPN by exon sequencing (Tefferi, 2010). A series of mutations were described in MPN that directly or indirectly activate the JAK-STAT pathway, which may be the central theme in the development of this group of disorders (Rampal *et al.*, 2014b). These so-called "driver" mutations included the JAK2 and MPL mutations as well as mutations in the calreticulin gene (Tefferi, 2015). The JAK2 V617F mutation found in exon 14 of the JAK2 gene was the first

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mutation to be identified in Ph negative MPN patients (Baxter *et al.*, 2005; James *et al.*, 2005b; Kralovics *et al.*, 2005; Levine *et al.*, 2005). Mutations involving genes other than JAK2 are found in less than 20% of patients with MPN (Tefferi, 2012). The identification of the "driver" mutations in MPN provides a new diagnostic algorithm and offers the possibility of new therapeutic options to be used in the management of patients suffering from these disorders (Delhommeau *et al.*, 2010). Various other mutations were also described in patients with MPN and these involve genes responsible for RNA splicing (e.g. SRSF2, U2AF1), transcriptional regulation [e.g. TP53, IKAROS family zinc finger 1 (IKZF1)] and epigenetics [e.g. EZH2, TET2, additional sex combs like 1 (ASXL1) and Isocitrate dehydrogenase 1 and 2 (IDH1/2), casitas B lineage lymphoma proto oncogene (CBL), lymphocyte specific adaptor protein] (Brecqueville *et al.*, 2012; Tefferi, 2010; Tefferi, 2015). The pathogenesis of these mutations has not been completely elucidated and it is conceivable that they may cooperate with the driver mutations to cause disease progression (Chen *et al.*, 2015; Rampal *et al.*, 2014a). In addition, demonstration of mutations, such as EZH2, ASXL1, TET2, CBL and IDH1/2 cannot be used as diagnostic markers for MPN as they only exist in a small percentage of patients and are also found in other myeloid disorders (Ha and Kim, 2015).

2.4.1 Janus kinase 2 V617F mutation

Janus kinase 2, a cytoplasmic tyrosine kinase, is a key component in the JAK-signal transducer and activator of transcription (STAT) pathway (Baxter *et al.*, 2005; Tan *et al.*, 2007). Janus kinase- signal transducer and activator of transcription signalling is crucial for definitive erythropoiesis as well as the cytokine response by myeloid progenitors (Haan *et al.*, 2006). Binding of EPO to the EPO receptor activates JAK2 and it undergoes auto phosphorylation (Tan *et al.*, 2007). Janus Kinase 2 recruits STAT molecules which are subsequently phosphorylated and translocate to the nucleus to act as transcription factors (Tan *et al.*, 2007).

The JAK2 V617F protein located on the pseudo-kinase domain of JAK2 has increased kinase activity and it disrupts the auto-inhibitory JH2 domain of JAK2 (Khwaja, 2006). In the presence of JAK2 V617F, the JAK protein becomes active even when no growth factor is

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bound to the receptor resulting in uncontrolled cell proliferation (Steensma, 2006). Figure 2.4 illustrates the role of the JAK2 mutations in the generation of MPN (Levine *et al.*, 2007).

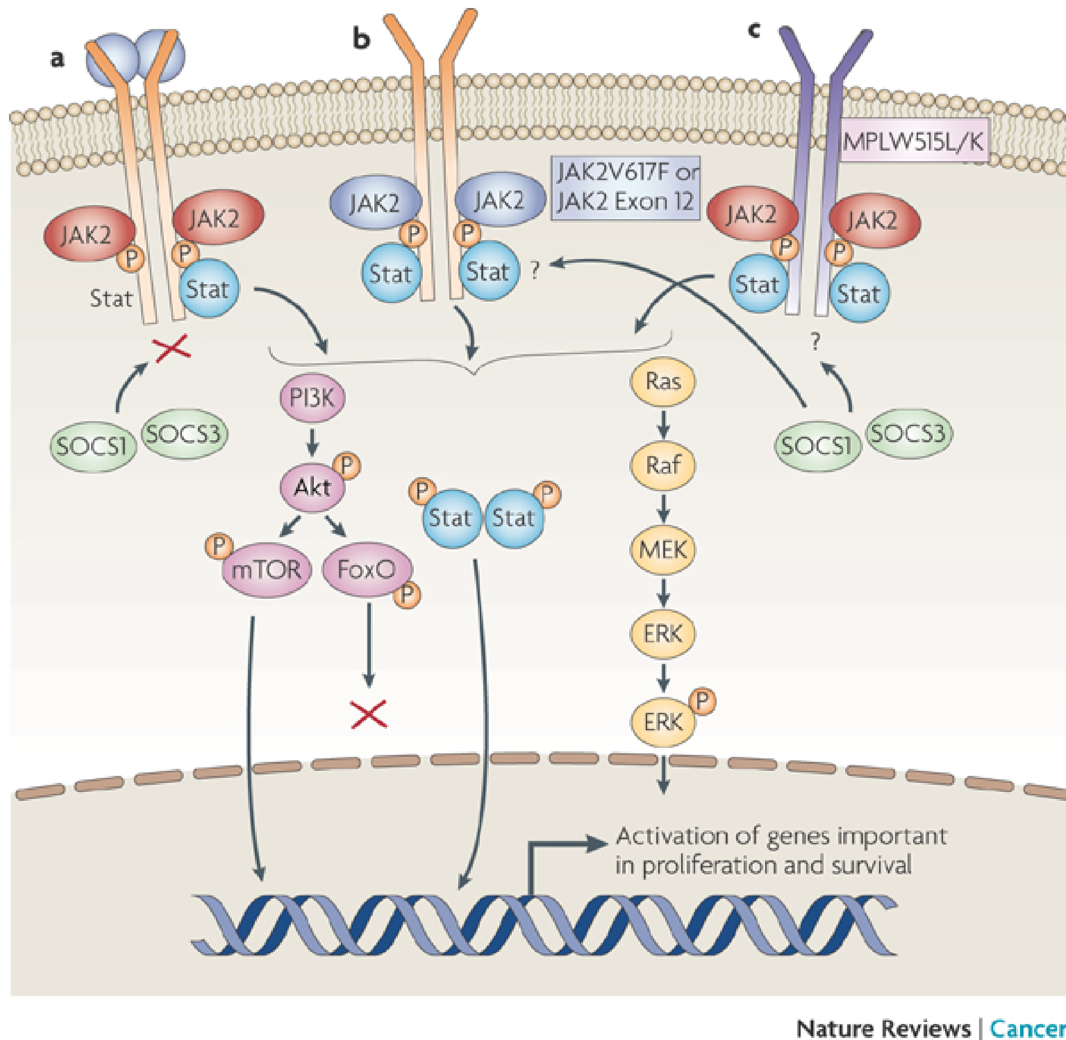

Figure 2.4: The role of the JAK2 V617F mutation in the pathogenesis of MPN. In the presence of the V617F mutation, JAK proteins becomes active even when no ligand is bound to the receptor leading to uncontrolled proliferation of cells (Levine *et al.*, 2007)

It is not known how a single mutation can cause three distinct disorders with different phenotypes (James, 2008; Chen *et al.*, 2010). Several hypotheses exist; the first is that the MPN phenotype depends on the cell targeted by the mutation. In this context it is proposed that should the mutation target a cell with self-renewal capability but which is committed to produce platelets with little capacity to produce other cell lines, then ET will develop (James, 2008). The second hypothesis is that the V617F mutation occurs in a HSC in all patients with MPN but the phenotype will depend on the genetic background of the patient (James, 2008).

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A comparative analyses of disease phenotypes of mice transplanted with JAK2 V617F transduced murine HSC was performed on C57Bl/6 and Balb/C mice (James, 2008). When C57Bl/6 mice were transplanted with JAK2V 617F transduced cells, mice developed a PV-like disease characterized by trilineage marrow hyperplasia, an enlarged spleen and polycythaemia (James, 2008). Polycythaemia was followed 3 months post-engraftment by a myelofibrotic stage (James, 2008). The outcome was different when Balb/C mice were used in the same experiments; the first step of the disease was not only characterised by polycythaemia but also by marked leukocytosis. The second stage of the disease was different, as the mice developed more pronounced myelofibrosis (James, 2008). The third assumption is that the disease phenotype depends on the intensity of the JAK2 V617F signal with high levels of activity favouring PV while low levels preferentially favour ET (James, 2008). Primary myelofibrosis is thought to arise due to the kinase activity level and duration of exposure (James, 2008). The final hypothesis relies on the assumption that another molecular event occurs before the JAK2 V617F mutation; it is suggested that a first event is responsible for the clonal expansion and JAK2 mutation occurs as a secondary event (James, 2008).

In addition to its effect on erythropoiesis, JAK2 V617F causes hypersensitivity signalling through the thrombopoietin / MPL pathway in megakaryocytes leading to an increased activation of STAT3 (Fleischman and Tyner, 2013). The activation of STAT3 results in increased ploidy and mobility of JAK2 V617F megakaryocytes, increased formation of pro-platelets and increased aggregation and thrombus formation of platelets (Fleischman and Tyner, 2013). The consequence of these changes is decreased bleeding volumes after injury with an increase in thrombotic events (Fleischman and Tyner, 2013).

The somatic point mutation, 1849 (G→T) in the JAK2 gene is present in most people with Ph negative MPNs but some studies suggest it can also be found in healthy individuals (Sidon *et al.*, 2006; Bellanne-Chantelot *et al.*, 2007). Sidon *et al.* (2006) detected the V617F mutation in a small number of haematopoietic cells in 10% of healthy individuals. The study was conducted in the Department of Medical Genetics, Free University of Brussels, Belgium where 57 healthy individuals age between 23 and 52 were investigated (Sidon *et al.*, 2006). It is suggested that the V617F mutation precedes the development of MPN, which needs several years to develop (Bellanne-Chantelot *et al.*, 2007). It should be noted that this

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mutation is indeed not specific to MPNs and has also been described in other myeloid malignancies, including acute myeloid leukaemia, myelodysplastic syndromes and chronic myelomonocytic leukaemia (Steensma *et al.*, 2005).

2.4.2 Janus kinase 2 exon 12 mutations

Different mutations exist in exon 12 of the JAK2 gene, these include in frame deletions, point mutations and duplications (Tefferi, 2010). Defects in JAK2 exon 12 lead to a gain of protein functions that confer growth factor independency (Tefferi and Vainchenker, 2011). Janus kinase 2 exon 12 mutations activate the JAK/STAT signalling and lead to haemopoietic transformation (Tefferi and Vainchenker, 2011). Mutations in JAK2 exon 12 lead to erythrocytosis with possible transformation to secondary myelofibrosis (Pasquier *et al.*, 2014). The JAK2 exon 12 mutations are found in about 2% of patients having PV (Tefferi, 2012). Unlike the V617F mutation, exon 12 mutations are not found in people with ET and PMF (Vainchenker *et al.*, 2011). Mutations in JAK2 exon 12 have a very low prevalence, which makes it difficult to assess the prognostic impact of the mutation accurately (Campbell *et al.*, 2006).

2.4.3 Calreticulin exon 9 mutations

Calreticulin is a gene that encodes the endoplasmic reticulum associated calcium binding protein (Langabeer *et al.*, 2014). Mutations in the calreticulin gene comprise one of two variants represented by insertions or deletions (Vannucchi *et al.*, 2014). The first, type 1 CALR mutations, are characterised by a 52 base pair deletion (p.L367fs*46) and the second, type 2, is characterised by a 5 base pair TTGTC insertion (p.K385fs*47) (Tefferi, 2015). Based on the alpha-helix content of the mutant carboxyl-terminus (C-terminus), CALR variants that are neither type 1 or 2 are classified as type 1-like or type 2-like variants (Tefferi *et al.*, 2014a). The mechanism by which CALR mutations produce MPN is not completely understood and is the focus of ongoing research (Rotunno *et al.*, 2014).

Calreticulin exon 9 mutations are found mostly in younger patients who have higher platelet counts and lower haemoglobin levels (Tefferi, 2014). Patients with CALR positive ET have a better prognosis as they are at lower risk of developing thrombosis as compared to patients

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carrying JAK2 and MPL mutations (Rotunno *et al.*, 2014). Mutations involving the CALR gene are found in about 15% to 25% of ET and PMF patients who do not carry JAK2 and MPL mutations (Klampfl *et al.*, 2013).

2.4.4 Myeloproliferative leukaemia virus exon 10 mutations

Myeloproliferative leukaemia virus gene is located on the short arm of chromosome 1; it encodes the thrombopoietin receptor MPL (Tefferi, 2010). Myeloproliferative leukaemia virus is a key growth and survival factor for megakaryocytes (Tefferi, 2010). Binding of thrombopoietin to MPL activates JAK2, which in turn phosphorylates MPL and initiates signalling events that regulate proliferation and differentiation of cells (Gong *et al.*, 2013). Mutations of the MPL gene result in impaired function of the auto-inhibitory region and activation of the ligand independent TPO receptor (Gong *et al.*, 2013). Tyrosine kinases and transcription factors are activated that lead to the transformation of haematopoietic cells into cytokine independent clones resulting in megakaryocytic hyperplasia and marrow fibrosis (Gong *et al.*, 2013). Mutations in the MPL gene result in the substitution of a tryptophan 515 to a leucine, lysine, asparagine or alanine (Vainchenker *et al.*, 2011). These mutations are found in 5% to 10% of patients with PMF and 2% to 5% of patients with ET (Delhommeau *et al.*, 2010). Patients with MPL mutated ET are mostly of old age, having lower haemoglobin levels, higher platelet counts and a higher risk of developing arterial thrombosis (Tefferi, 2010).

2.4.5 Ten-eleven translocation oncogene family member 2 mutations

Ten-eleven translocation oncogene family member 2 (TET2) is a homologous gene located on the short arm of chromosome 4 (Tefferi, 2010). Ten-eleven translocation oncogene family member 2 mutations include frameshift, nonsense and missense mutations found across several of TET2's 12 exons (Tefferi, 2010). These mutations are found in 12% of MPN patients (Delhommeau *et al.*, 2010).

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2.4.6 Additional sex combs like 1 mutations

The Additional sex combs like 1 gene is found in the short arm of chromosome 20 (Tefferi, 2010). Additional sex combs like 1 has a role in the activation and silencing of development related genes through chromatin remodeling (Delhommeau *et al.*, 2010). Additional sex combs-like 1 mutations are seen in 8% of patients with MPN, 11% with myelodysplastic syndrome, 43% with chronic myelomonocytic leukaemia, 7% with primary and 47% with secondary AML (Tefferi and Vainchenker, 2011).

2.4.7 Casitas B lineage lymphoma proto oncogene mutations

Casitas B lineage lymphoma proto oncogene is located on the short arm of chromosome 11 and it encodes a cytosolic protein (Tefferi, 2010). This proto-oncogene negatively regulates signal transduction of activated tyrosine kinases (Delhommeau *et al.*, 2010). Casitas B lineage lymphoma proto oncogene mutations are found in less than 10% of patients with PMF and are also found in MPN patients who have transformed to AML (Delhommeau *et al.*, 2010).

2.5 Detection of JAK2 V617F mutation

The detection of the V617F mutation is of paramount importance in the diagnosis of MPNs. The ideal assay should be quick and easy to perform (Lay *et al.*, 2006). In most patients the JAK2 V617F mutation is present only in a small proportion of cells; therefore, detection methods used should be sensitive enough to pick up a mutant allele as low as 1% in the background of wild type allele (Wolstencroft *et al.*, 2007; Kouroupi *et al.*, 2012). Assays with different sensitivity and specificity values have been described for the detection of the V617F mutation (Greiner, 2006; Bench *et al.*, 2013). These methods were either designed to target the 1849G>T mutation or designed to scan for exon 14 that harbours the V617F mutation (Bench *et al.*, 2013). The use of sequence specific primers and probes improves the specificity and sensitivity of assays to differentiate between a mutant and a wild type allele (Bench *et al.*, 2013). Assays that are used to detect the mutation are PCR-RFLP, direct sequencing, ARMS or AS-PCR and real time PCR (Baxter *et al.*, 2005; Lay *et al.*, 2006; Murugesan *et al.*, 2006; Steensma, 2006).

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2.5.1 Direct sequencing of amplified product

The principle of this assay is primer extension reaction performed on PCR products using dideoxynucleotides (ddNTPs) to determine sequence information (Steensma, 2006). Sequencing allows direct visualisation of nucleotide order and it is of value in screening allelic polymorphisms (Gut, 2001; Steensma, 2006). The disadvantages of direct sequencing include limited sensitivity resulting from background noise in the generated chromatograms (Steensma, 2006). In addition, direct sequencing assay requires skilled personnel as well as expensive and specialized equipment (Steensma, 2006). It is also a labour intensive assay that requires a long time to complete and it is therefore not considered to be appropriate for use in a routine diagnostic laboratory setting (Steensma, 2006).

Several studies compared the sensitivity of direct sequencing to other assays used for the detection of the JAK2 V617F using a homozygous JAK2 V617F cell line diluted in JAK2 V617F wild type cells (James *et al.*, 2005a; Lay *et al.*, 2006; Kannim *et al.*, 2009). The sensitivity of the sequencing method was compared to that of hydrolysis and hybridization probes and it was less sensitive as it failed to detect mutant DNA below 5% in wild type background (James *et al.*, 2005a). This finding was confirmed by Kannim *et al.* (2009) who found that sequencing was unable to detect mutant DNA in less than 5% dilutions. Lay *et al.* (2006) demonstrated that sequencing was unable to produce a positive result when the mutation was present in less than 20% of the whole cell population.

Abdelhamid et *al.* (2013) quantified the JAK2 V617F mutation using next generation sequencing (NGS). This technology performs parallel sequencing during which millions of fragments of DNA from a single sample are sequenced simultaneously (Grada and Weinbrecht, 2013). The authors concluded that NGS allowed a better assessment of the percentage of V617F mutant alleles and therefore NGS can be used as a tool to monitor minimal residual disease and to assess therapeutic response (Abdelhamid *et al.*, 2013).

Mutation events that occur in exomes can give rise to indistinguishable clinical presentations, leaving the diagnosing physician with many possible causes for a given disease (Grada and Weinbrecht, 2013). Sequencing of the exome which is the protein-coding regions of the

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genome can facilitate the identification of disease-causing mutations in pathogenic presentations where the exact genetic cause is not known (Grada and Weinbrecht, 2013). The limitation of the NGS technology is that start-up costs and individual sequencing reactions can be very expensive (Grada and Weinbrecht, 2013). Data analysis is difficult and requires a skilled bioinformatics specialist with experience in the field (Grada and Weinbrecht, 2013). In addition. NGS assays generate a high volume of data which places substantial demands on data tracking and storage (Rizzo and Buck, 2012).

2.5.2 Restriction fragment length polymorphism

Polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) may be used for single nucleotide polymorphism (SNP) genotyping (Gut, 2001). The principle of the assay is based on the digestion of PCR products with restriction endonucleases (Gut, 2001). Restriction endonucleases should be specific for the base change at the position of the SNP so that a restriction cut for one allele can be generated (Gut, 2001). This assay has the potential to be used as a screening tool as it is easy and inexpensive to perform (Steensma, 2006). The disadvantage of PCR-RFLP is that there are limited numbers of restriction enzymes available (Gut, 2001; Steensma, 2006). This limitation may cause complex patterns to result, requiring abnormal results to be confirmed by another assay (Gut, 2001; Steensma, 2006). Another disadvantage of the PCR-RFLP assay is that it is time consuming as it requires additional time for restriction digestion and PCR products that are not completely digested could generate false positive results (Frantz *et al.*, 2007). A study to compare RFLP with a hydrolysis assay showed that it is less sensitive to detect the mutation in a population of cells as low as 5% (Lay *et al.*, 2006). The analytical sensitivity of PCR-RFLP was also found to be 5% in two other studies performed to compare different detection methods (Steensma, 2006; Frantz *et al.*, 2007).

2.5.3 Allele specific polymerase chain reaction

Allele specific PCR also known as the amplification refractory mutation system is one of the assays that is used to detect SNPs and other mutations (Wangkumhang *et al.*, 2007). Mutations are detected by analysing PCR products in agarose or polyacrylamide gels stained with ethidium bromide (Ugozzoli and Wallace, 1991). This assay requires primers that are

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perfectly annealed at their 3' ends for a DNA polymerase to extend those primers during PCR (Steensma, 2006). The success depends on good starting genomic DNA template, a high quality Taq polymerase and highly specific primers (Thornton and Basu, 2011). The advantage of this method is its high sensitivity to small amounts of mutant DNA in a wild type background (Steensma, 2006). It is also inexpensive to set up in a routine laboratory (Steensma, 2006; Kannim *et al.*, 2009). The disadvantages of AS-PCR is that it is time-consuming and labour intensive since it requires post-PCR processing (Soheili and Samiei, 2005).

Baxter *et al.* (2005) designed an allele specific PCR assay to detect the JAK2 V617F mutation in patients diagnosed with MPN. The assay uses two forward primers and one reverse primer (Baxter *et al.*, 2005). The first forward primer is specific for the mutant allele and contains an intentional mismatch (Baxter *et al.*, 2005). The second forward primer amplifies a 364 base pair (bp) product from both mutant and wild type alleles and serves as an internal control (Baxter *et al.*, 2005). The AS-PCR assay was able to detect the V617F mutation in 97% of PV patients as compared to conventional fluorescent dye chemistry sequencing that only detected the mutation in 73% (Baxter *et al.*, 2005). In patients with ET, AS-PCR was able to detect the mutation in 57% compared to sequencing, which allowed detection in only 12% of patients (Baxter *et al.*, 2005). Baxter *et al.* (2005) claimed to have designed AS-PCR primers that can detect V617F mutation present in as little as 3% of cells.

McClure *et al.* (2005) performed an ARMS assay using a forward primer that is specific to the mutant allele with a reverse primer specific to the wild type sequence to detect the V617F mutation. The designed ARMS assay only amplifies the mutant allele if present (McClure *et al.*, 2005). The amplified product was analysed using capillary electrophoresis (McClure *et al.*, 2005). Analytical sensitivity of the assay, determined by making cell dilutions of the JAK2 V617F positive and negative controls, was determined to be 0.01% (McClure *et al.*, 2005). The assay is suitable for use in a clinical laboratory setting as it yielded good intra-run and inter-run reproducibility results (McClure *et al.*, 2005).

Jones *et al*. (2005) designed an ARMS assay that differed from that designed by Baxter *et al.* (2005) and McClure *et al.* (2005) in that it uses two primer pairs (Jones *et al.*, 2005). This ARMS assay consisted of a forward outer primer, a reverse outer primer, a forward inner

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primer that is specific to the wild type sequence and a reverse inner primer specific to the mutant allele (Jones *et al.*, 2005). The forward primer from one set and the reverse from the other amplifies a positive control band (Jones *et al.*, 2005)

The binding of the reverse inner primer and the forward outer primer produce a fragment of 279 bp when the JAK2 mutation is present (Jones *et al.*, 2005). In the absence of the mutation the reverse outer primer and the forward primer produce a fragment of 229 bp (Jones *et al.*, 2005). Dilutions of mutant and wild type DNA were also used to determine the sensitivity of this assay and it was found to be between 1% and 2% (Jones *et al.*, 2005).

A study was performed in Algeria aimed to determine the prevalence of the JAK2 V617F mutation in patients suspected of having a diagnosis of MPN using ARMS assay (Benguella-Benmansour *et al.*, 2014). The assay used in this study was a modification from Jones *et al* (2005). The frequency of the V617F mutation was 81.6%, 58.6%, and 46.2% in PV, ET and PMF patients, respectively (Benguella-Benmansour *et al.*, 2014).

2.5.4 Real time polymerase chain reaction

This assay is a valuable method for mutation detection and genotyping studies (Wilhelm and Pingoud, 2003). It requires the introduction of fluorescence dyes or probes in the initial reaction mixture in order to monitor the amount of product formed during the course of the reaction (Bustin, 2005; Kubista *et al.*, 2006). The advantage of real time PCR is the fact that it is highly sensitive as it can detect a few gene copies in a reaction (Lim *et al.*, 2011). Cross contamination of PCR products is eliminated as no post-PCR steps are required (Lim *et al.*, 2011). The disadvantages of real time PCR are that specialized equipment/instruments are needed and reagents are relatively expensive in comparison to conventional PCR (Wong and Medrano, 2005).

Chemistries that underlie real time PCR include hydrolysis probes, locked nucleic acid probes, intercalating dyes, molecular beacons, hybridization probes, sunrise primers, scorpion primers, and light-up primers (Gudnason *et al.*, 2007). Among this, SYBR green and hydrolysis probes are the most commonly used chemistries (Lim *et al.*, 2011).

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2.5.4.1 SYBR Green 1 chemistry

SYBR Green I is a fluorogenic minor groove DNA binding dye that intercalates double stranded DNA molecules (Arya *et al.*, 2005). This dye does not bind in a sequence specific manner (Arya *et al.*, 2005). The principle of the SYBR Green chemistry is that SYBR Green I produces little fluorescence when unbound in solution but emits a strong fluorescent signal of a defined wavelength upon binding (Soheili and Samiei, 2005; Gudnason *et al.*, 2007). The excitation of SYBR Green I is at 494 nm and the emission at 521 nm (Soheili and Samiei, 2005). Detection takes place at the extension step of real time PCR (Soheili and Samiei, 2005). As the cycle number increases, the signal intensity also increases due to the accumulation of PCR products (Soheili and Samiei, 2005). Fluorescent dyes enable analysis of targets without probes (Soheili and Samiei, 2005). Non-specific PCR products and primer-dimers also contribute to the fluorescent signal (Soheili and Samiei, 2005). Melting curve generated by monitoring the fluorescence properties of the PCR amplification products during a melting phase are used to differentiate between non-specific and specific products (Ririe *et al.*, 1997; Gašparič *et al.*, 2008). The advantage of SYBR Green I assay over other assays is that it is cheaper (Ponchel *et al.*, 2003).

A successful real time PCR reaction requires efficient and specific amplification of the product (Seifi *et al.*, 2012). Amplicon length and the quality of primer can affect the efficiency of real time PCR (Thornton and Basu, 2011). Primers should be designed properly, since the dye cannot distinguish between specific and non-specific PCR products as it intercalates into the DNA double strands (Thornton and Basu, 2011). Primers should be designed to be between 18 and 24 bp in length, to have a guanine-cytosine (GC) content of 30% to 70% and a melting temperature ($T_{m)}$ between 50ºC and 65ºC (Raymaekers *et al.*, 2009). The amplicon should be designed to be between 75 and 200 bp, since short amplicons can be amplified with higher efficiency (Seifi *et al.*, 2012). An amplicon of 75 bp is easily distinguished from primer dimers that might form (Seifi *et al.*, 2012).

Components of a SYBR Green I real time PCR reaction is PCR master mix, template and primers (Seifi *et al.*, 2012). The performance of SYBR Green I real time PCR should be determined by identifying the optimal annealing temperature and by constructing a standard curve (Seifi *et al.*, 2012). To determine the optimal annealing temperature, a range of

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annealing temperatures above and below the calculated T_m of the primers should be tested (Raymaekers *et al.*, 2009). The efficiency and reproducibility can be determined by using serial dilutions of a known template (Seifi *et al.*, 2012). A literature search for the use of the SYBR Green I assay to detect the JAK2 V617F mutation yielded no results.

2.5.4.2 Hydrolysis probes

Fluorescently labelled probes are nucleotide sequences that provides real time PCR specificity and sensitivity as only the desired amplicon is detected and reported (Soheili and Samiei, 2005). Hydrolysis probes (e.g TaqMan probes) are sequence-specific nucleotide sequences that carry a reporter and a quencher dye, the reporter is attached at the 5` end and the quencher dye is located at the 3` end of the probe (Didenko, 2001). During the combined annealing/ extension phase of real time PCR, the probe is cleaved by the 5′→3′ exonuclease activity of the *Taq* DNA polymerase separating the reporter and the quencher (Soheili and Samiei, 2005). Cleaving results in fluorescence that is proportional to the amount of amplicons generated (Soheili and Samiei, 2005). Melt curve analysis is impossible because the separation of the reporter dye from the quencher is irreversible (Kaltenboeck and Wang, 2005).

Primers should be designed to have a length of 18 to 24 nucleotides and an annealing temperature between 55ºC and 60ºC (Lim *et al.*, 2011). The probe should have a T_m 5ºC to 10ºC higher than that of the primers. Probes should be verified for possibilities to form secondary structures and that no self and cross hybridization with the primers will occur (Lim *et al.*, 2011). Probes should contain more cytosines than guanines to produce a greater normalized change in fluorescence (Raymaekers *et al.*, 2009).

Hammond *et al.*, (2007) developed a hydrolysis probe assay for the quantification of JAK2 V617F and reported an analytical sensitivity of 0.01% when mutated DNA was diluted with wild type DNA. Another study reported an analytical sensitivity of 5% when DNA from homozygous cell lines (positive for mutation) were diluted (Bousquet *et al.*, 2006). Kröger *et al.*, (2007) designed a Hydrolysis assay with a sensitivity of 0.01%. The specificity of the assay was assessed by investigating 60 DNA samples from healthy donors in three independent PCR reactions, also a no template control, a 0.01% and a 10% positive control

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were processed together with the 60 samples. In all three experiments none of the 60 healthy donor DNA samples was found to be positive and both the 0.1% and 10% positive controls yielded positive results (Kröger *et al.*, 2007). Reproducibility of the results was assessed by running PCR using low template concentrations in triplicate of the JAK2 mutant DNA in four different experiments and excellent reproducibility was attained (Kröger *et al.*, 2007).

2.5.4.3 Hybridisation probes

Hybridization probes also referred to as fluorescence resonance energy transfer (FRET) probes consist of two fluorescently labelled oligonucleotides (Arya *et al.*, 2005). The probes are designed to anneal next to each other in a head to tail configuration after the denaturation step of the real time PCR assay (Caplin *et al.*, 1999; Arya *et al.*, 2005). One probe is labelled with a donor fluorophore at its 3` end and the other is labelled with an acceptor fluorophore at its 5`end (Arya *et al.*, 2005). When probes hybridise to target sequences during PCR, the donor fluorophore emits energy, which excites the acceptor fluorophore of the second probe, which then emits fluorescence light at a longer wavelength (McChlery and Clarke, 2003). Hybridization probes are highly specific because two probes are required to simultaneously hybridise to adjacent target sequences (Kaltenboeck and Wang, 2005). Melt curve analysis can be performed, since the probes are not degraded and fluorescence is reversible (Kaltenboeck and Wang, 2005). For mutation detection, T_m difference between the two probes should be 5ºC to 10ºC (Caplin *et al.*, 1999).

A study by Lay *et al.* (2006) compared four different assays for the detection of the JAK2 V617F mutation and it was clear from their results that the FRET assay has several advantages; in addition to its high sensitivity, results were also easy to interpret (Lay *et al.*, 2006). The FRET assay was able to reproduce the same results down to the 5% level of mutant DNA (Lay *et al.*, 2006). McClure *et al.* (2005) designed a FRET assay with a sensitivity of 1% to 10% mutant DNA in a wild type background. A study by Cankovic *et al.*, (2009) showed an analytical sensitivity of 10% when mutant DNA was serially diluted into wild type DNA. Olsen *et al.* (2006) evaluated the ability of the JAK2 hybridisation assay to identify low concentrations of the JAK2 V617F by titrating homozygous mutant cell line DNA with homozygous wild type cell line. The authors recorded an assay sensitivity of 5% (Olsen *et al.*, 2006). Murugesan *et al.,* (2006) reported an analytical sensitivity of 5%

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when wild type DNA was diluted with varying amounts of homozygous mutant DNA and subjected to PCR and melting curve analysis. Murugesan *et al.,* (2006) claimed that the assay showed high reproducibility in three different experiments with 100% concordance (Murugesan *et al.*, 2006).

2.5.4.4 Locked nucleic acid probes

Locked nucleic acid (LNA) probes contain synthetic nucleic acid analogues that anneal to complementary target sequences with very high affinity (Simeonov and Nikiforov, 2002; You *et al.*, 2006). These nucleic acid analogs increase duplex stability, specificity and mismatch discrimination resulting in improved genotyping (Ballantyne *et al.*, 2008; Owczarzy *et al.*, 2008). Locked nucleic acid monomers contain a modified ribose moiety, which bridges the 2' and 4' carbons of the ribose ring (You *et al.*, 2006). The bridge locks the ribose in the N-type conformation resulting in enhanced base stacking and phosphate back bone pre-organisation leading to an improved affinity for complementary DNA sequences (You *et al.*, 2006). Due to the very high affinity of these molecules very short probes can be used (Simeonov and Nikiforov, 2002). Josefsen *et al.,* (2009) conducted a study in which they compared the sensitivity of LNA to that of hydrolysis probes; LNA probes produced lower threshold cycle values and higher proportion of positive PCR results than the hydrolysis probes when analysing less than 150 DNA copies.

Sidon *et al* (2006) used an assay that combines both molecular beacon and LNA probes in a single tube. The LNA probe was used to block the amplification of the wild type sequence and the assay was able to detect up to 0.01% of mutated alleles (Sidon *et al.*, 2006). A study to evaluate analytical and clinical performance of real time PCR assay using hydrolysis probes containing LNA bases was conducted (Denys *et al.*, 2010). The assay used in this study was a modification of the molecular beacon technology as previously described (Sidon *et al.*, 2006; Denys *et al.*, 2010). The assay has a reproducible quantitative sensitivity of 0.4% (Denys *et al.*, 2010).

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2.5.4.5 Molecular beacons

Molecular beacons are single stranded nucleic acid molecules with a stem and hairpin loop structure, with the loop complementary to a target nucleic acid (Mhlanga and Malmberg, 2001; Bustin, 2005). The stem is formed by the annealing of complementary sequences that are on either side of the probe sequence (Mhlanga and Malmberg, 2001). A reporter fluorophore is attached to the 5` end of the stem and the quencher on the 3` end (McChlery and Clarke, 2003). In solution, the stem keeps the arms in close proximity resulting in the proximal quenching of the reporter fluorophore (Bustin, 2005). During the annealing step of the PCR, the loop will hybridise to the specific target sequence, which results in a conformational transition separating the stem, which causes the reporter and quencher dyes to separate resulting in fluorescence (Kaltenboeck and Wang, 2005). The hairpin structure makes molecular beacons highly specific in recognizing nucleotide sequence mismatches (Mhlanga and Malmberg, 2001). As previously mentioned Sidon *et al.* (2006) used molecular beacon probes to test for the JAK2 mutation and reported their assay to have an analytical sensitivity 0.01% (Sidon *et al.*, 2006).

2.6 Treatment of myeloproliferative neoplasms

Allogeneic stem cell transplant is the only curative therapy for this group of disorders (Geyer and Mesa, 2014). However, transplant-associated mortality or severe morbidity occurs in more than 50% of transplant patients, which necessitates judicious risk stratification (Geyer and Mesa, 2014).

Current drug therapy for MPNs is not curative or able to prevent disease progression (Tefferi and Pardanani, 2015). Since MPNs are characterised by complications, such as bleeding, thrombosis and splenomegaly, therapy is mainly targeted at preventing thrombosis and alleviating symptoms (Langabeer *et al.*, 2014). Drugs available for the treatment of MPN patients include cytoreductive agents, single-agent JAK inhibitors, combination approaches with a JAK inhibitor base and non-JAK-targeted agents (Geyer and Mesa, 2014). Phlebotomy to a haematocrit target of 45% in patients with PV has been well-established (Tefferi, 2015). In addition, aspirin therapy has been recommended for all patients with PV

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and JAK2-mutated ET (Tefferi, 2015). High risk patients with PV or ET require cytoreductive therapy with hydroxyurea as drug of choice (Tefferi, 2015).

The discovery of the JAK2 V617F mutation in patients with MPNs resulted in the development of JAK2 inhibitors that are effective in controlling hyper-proliferation of haemopoietic cells (Verstovsek, 2008). Janus kinase 2 inhibitors compete with Adenosine triphosphate (ATP) for the ATP-binding catalytic site in the kinase domain (Passamonti *et al.*, 2011). The benefit of using JAK inhibitor therapies is that they control disease in patients who have splenomegaly and constitutional symptoms (Verstovsek, 2008). A number of JAK2 inhibitors including Ruxolitinib, Momelotinib, Pacritinib and Fedratinib have been developed and phase II and III clinical trials are currently underway (Geyer and Mesa, 2014). Single-agent JAK inhibitor Ruxolitinib has been approved for PMF management in the United States and the European Union, since it reduces splenomegaly, improves symptoms and survival (Geyer and Mesa, 2014).

The British Committee for Standards in Haematology guidelines for investigation and management of myelofibrosis 2012 was recently revised to formally recommend the use of ruxolitinib as first line therapy for symptomatic splenomegaly and constitutional symptoms regardless of the JAK2 V617F mutation status (Reilly *et al.*, 2014). Despite this, current experience with JAK inhibitors has been disappointing in that none of these drugs affected the JAK2 allele burden, induced complete remissions or reversed marrow fibrosis (Cervantes *et al.*, 2013; Pardanani *et al.*, 2014).

2.7 Test method validation

Test validation is a process that establishes the performance characteristics of a given test (Conraths and Schares, 2006). The validation process involves assessing the performance of the test in comparison with a 'gold standard' or reference test that is capable of determining the sample status without error (Mattocks *et al.*, 2010). If a suitable performance specification is available, it is necessary to establish that the new test meets this specification within the laboratory; this process is called verification (Mattocks *et al.*, 2010). Verification can be seen as a process to determine that 'the test is being performed correctly' (Mattocks *et*

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al., 2010). Other applications of verification may include a new test being implemented using a technology that is already well established in a laboratory or a test for which a suitable performance specification is available from another laboratory in which the test has already been validated (Mattocks *et al.*, 2010). Figure 2.5 highlights the process of implementing a diagnostic molecular test (Mattocks *et al.*, 2010).

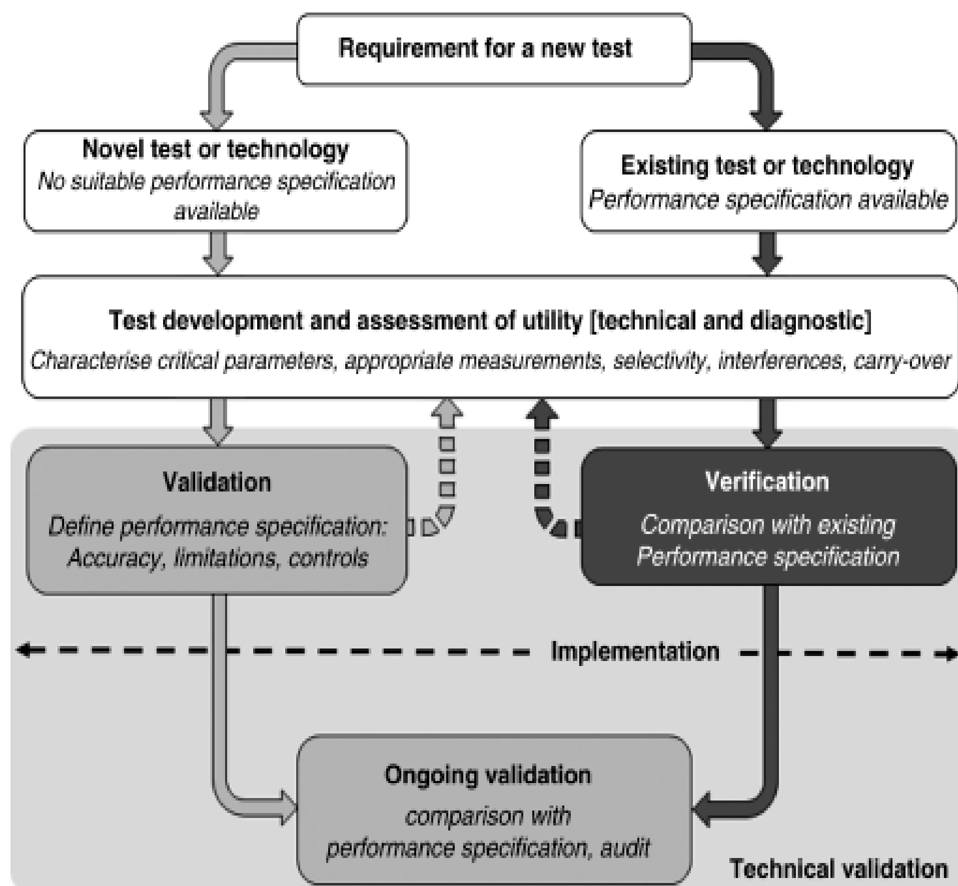

Figure 2.5: The process of implementing a molecular genetic test for diagnostic use. The shaded arrows represent the two general routes to implementation, depending on the availability of a suitable performance specification: validation (lighter) and verification (Mattocks *et al.*, 2010)

After making a decision to set up a diagnostic test, the technology to be used must be chosen and built into a suitable laboratory process (Mattocks *et al.*, 2010). Assessment of both the diagnostic and technical process is performed to ensure that the measurements obtained are relevant to the diagnostic question and that the analyte can be unambiguously identified (Mattocks *et al.*, 2010). The final stage of the laboratory process is to determine whether the

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performance of the test, in terms of accuracy, meets the required diagnostic standards (Mattocks *et al.*, 2010). The results of the analytical validation or verification determine whether, and how, the test will be implemented and set the requirements for performance monitoring of the test (Mattocks *et al.*, 2010). Performance can be established by performing method comparison to identify accuracy, replication experiments to check for precision and limit of detection experiments to check for the lowest concentration that can be detected (Burd, 2010). Test characteristics that need to be established include amplification efficiency, robustness, linearity, analytical sensitivity and specificity, diagnostic sensitivity and specificity, accuracy, precision, repeatability and reproducibility (Burd, 2010).

To calculate the PCR efficiency of an assay a dilution series is prepared from a specific positive material with all the targets considered in the assay to check if competition occurs between the targets and to evaluate if all perform equally well (Broeders *et al.*, 2014). Each dilution point is analysed in six-fold and four runs are performed (Broeders *et al.*, 2014). The difference between the PCR efficiencies obtained for each target of the multiplex assay should not exceed 15% to limit the impact of the possible competition between the different targets (Broeders *et al.*, 2014). Linearity for each assay target in a multiplex PCR assay is performed by serial diluting a positive material with all the targets (Broeders *et al.*, 2014). Analysis on the obtained data using Microsoft Excel® provides the correlation coefficient linearity of the curve which is a measure of the linearity of the PCR reaction. The linearity for each target should be ≥0.98 (Broeders *et al.*, 2014). Analytical sensitivity also referred to as limit of detection is used to describe the lowest concentration of an analyte in a specimen that can be measured by an analytical assay (Burd, 2010). Analytical sensitivity is achieved by making serial dilutions of the target using target negative material as the diluent (Forbes, 2003). The lower the detection limit of a test, the higher the analytical sensitivity (Burd, 2010). Analytical specificity refers to the assay's ability to measure a particular substance, rather than others, in a sample (Saah and Hoover, 1997).

Diagnostic sensitivity of an assay is the number of people with a given disease who are correctly identified by the test as having a disease (Mattocks *et al.*, 2010). Diagnostic

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specificity is the ability to identify a patient without a disease (Forbes, 2003). Table 2.5 gives the method of calculating diagnostic sensitivity and specificity (Jennings *et al.*, 2009).

Table 2.5: Calculation of diagnostic sensitivity and specificity of an assay

	Test positive	**Test negative**
Disease present	True positive	False negative
Disease absent	False positive	True negative

The diagnostic sensitivity is calculated as true positive/(true positive + false negative) and the specificity as true negative/(false positive + true negative) (Mattocks et al., 2010).

Another important parameter to consider when performing test validation is the Cohen's Kappa which is used to check for agreement between methods (McHugh, 2012). A kappa value of one indicates perfect agreement and a value above 0.75 is well acceptable (McHugh, 2012).

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CHAPTER 3

IDENTIFICATION OF THE JANUS KINASE V617F MUTATION USING ALLELE SPECIFIC POLYMERASE CHAIN REACTION AND SEQUENCING ASSAYS

3.1 Introduction

Myeloproliferative neoplasms (MPNs) are a group of disorders resulting from the abnormal transformation of a multipotent haematopoietic progenitor stem cell leading to increased production of blood cells (McLornan *et al.*, 2006; Chen *et al.*, 2010). Classical Philadelphia (Ph) chromosome negative MPN comprise of polycythaema (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Anderson and McMullin, 2014). These three disorders are characterised by an increase in red blood cells, platelets and bone marrow fibrosis, respectively (Dos Santos *et al.*, 2014). Patients present with a range of symptoms and signs, which may include features of hyperviscosity, hypervolaemia and hypermetabolism as well as that of anaemia (Titmarsh *et al.*, 2014). In addition, splenomegaly is frequently present (Titmarsh *et al.*, 2014). As a group, these disorders are complicated by thrombosis and bleeding, which contribute to higher mortality and morbidity (Passamonti *et al.*, 2012; Titmarsh *et al.*, 2014).

The description of the Janus kinase 2 (JAK2) V617F mutation in Ph chromosome negative MPNs patients renewed interest in the genetics and biology of MPNs. It also paved the way for the development of JAK2 inhibitors as possible treatment agents for these disorders (Langabeer *et al.*, 2015). This mutation was shown to constitutively activate the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway leading to uncontrolled proliferation of myeloid lineages (Sun and Zhang, 2013). The JAK2V617F mutation is present in 95% of patients with PV and in more than 50% of those with ET and PMF (Kouroupi *et al.*, 2012). Detection of this mutation has become an essential diagnostic tool (Kouroupi *et al.*, 2012). Assays that have been described include allele specific PCR (AS-PCR) and direct sequencing of PCR products (Baxter *et al.*, 2005; Lay *et al.*, 2006; Murugesan *et al.*, 2006).

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Allele specific PCR or sequence specific PCR utilises primers that are designed to match only a specific DNA point mutation making it suitable to distinguish between polymorphic alleles (Steensma, 2006). The 3'-end of the primer is often designed to be a perfect complement to the allele present in the input sample (Wangkumhang *et al.*, 2007). A control is included in the reaction to ensure that absence of PCR product from a given sample is not due to failure of the PCR reaction (Steensma, 2006). Allele specific PCR requires the use of hot start *Taq*DNA polymerases to prevent nonspecific primer annealing (Steensma, 2006). The advantage of this assay is its ability to detect small amounts of mutant DNA in a wild-type background with high sensitivity (Steensma, 2006). The analytical sensitivity of AS-PCR has been shown to be 1% (Jones *et al.*, 2005).

Direct DNA sequencing using the Sanger assay provides detailed nucleotide sequence information but limited sensitivity, due to the background noise in the chromatograms, restricts its usefulness in the diagnostic laboratory (Steensma, 2006). In addition, the assay is also considered to be relatively labour intensive and time consuming (Steensma, 2006). The aim of this study was to detect the JAK2 V617F mutation using AS-PCR and newly developed in-house method with sequencing.

3.2 Materials and Methods

3.2.1 Study setting

The study was conducted in the Department of Haematology at the Tshwane Academic Division of the National Health Laboratory Service (Pretoria South Africa). The Faculty of Health Sciences Research Ethics Committee of the University of Pretoria approved the study (protocol number 32/2012).

3.2.2 Specimens collection

Specimens of whole blood collected in EDTA, that were submitted by clinicians in the Steve Biko Academic Hospital to investigate the presence of the JAK2 V617F mutation in patients suspected of suffering from MPNs, were used in this study. Aliquots of a total of 60 such

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specimens were obtained from the reference PCR laboratory at the Charlotte Maxeke Johannesburg Academic Hospital where diagnostic testing has been performed. The specimens that were included in this study comprised of 30 that were shown to be positive for the JAK2 V617F mutation and 30 that were negative for this mutation as assessed by the reference laboratory. Historic results for all 60 specimens were also provided by the reference laboratory.

3.2.3 Genomic DNA extraction

Genomic deoxyribonucleic acid (gDNA) was extracted from 0.2 mL of peripheral blood using the GenElute™ blood genomic DNA kit (Sigma Aldrich, St. Louis, MI) according to the manufacturer's instructions. Briefly, whole blood was lysed in a chaotropic salt containing solution in order to denature the nucleic acids. The addition of ethanol to the lysate caused binding of DNA to the silica membrane when the lysate was spun at a rate of 6500 revolutions per minute (rpm) in a Mikro 200R micro-centrifuge (Hettich, Tuttlingen Germany). A washing step was performed to remove contaminants and the DNA was eluted in 200 µL of 10 mM Tris-HCl, 0.5 mM EDTA solution (Sigma Aldrich). Refer to Appendix A for reagents and a detailed extraction method. The concentration of DNA was calculated by measuring the absorbance at 260 nm and 280 nm using the NanoDrop 2000c (Thermo Scientific, Waltham, MA). Extracted DNA was stored at -20ºC until analysis.

3.2.4 Genotyping by comparative method allele specific polymerase chain reaction

Primers described by Baxter *et al.* (2005) were used to investigate the gDNA extracted from all of the samples (n=60) for the presence of the JAK2 V617F mutation. This assay was introduced by the reference laboratory in Johannesburg, without any amendments, to detect this mutation. Because this method was being used as diagnostic tool in the work-up of patients with MPN at the time of planning the study, it was included in this study as the comparative method. Part of the intent was to duplicate this assay in this study. The method utilises two sets of forward primers. The first forward primer is specific for the mutant allele and the second primer amplifies a 364 base pair (bp) product, from both the mutant and wild type alleles, that serves as an internal control (Baxter *et al.*, 2005). The primer sequences used in this assay are shown in Table 3.1.

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Table 3.1: Primers used in the AS PCR comparative assay duplicated in this study

Primer	Sequence	Expected PCR product sizes (bp)
Forward primer	ATCTATAGTCATGCTGAAAGTAGGAGAAAG	364
Reverse intronic primer	CTGAATAGTCCTACAGTGTTTTCAGTTTCA	
Forward primer specific for the mutant	AGCAATTTGGTTTTAAATTATGGAGTATATT	203

(Baxter *et al.*, 2005).

To confirm the reference laboratory's results, the method was duplicated in this study. In an attempt to duplicate the method in its entirety the PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), as employed by the reference laboratory, was used.

3.2.4.1 Genomic DNA amplification

Five microliters of genomic DNA (<100 ng) was added to PCR Master Mix (Roche) containing 0.4 µM of each of the forward primer and 0.8 µM of the reverse primer. Polymerase chain reaction assay was performed using the DNA Engine Peltier thermo cycler (Bio Rad Laboratories GmbH, Munich, Germany) under the following conditions: initial denaturation at 94°C for 7 min, followed by 35 cycles of amplification with denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 45 s and final extension of 5 min at 72°C. Amplification conditions used were those employed by the reference laboratory. Visualisation of amplified products using gel electrophoresis revealed the presence of faint bands and non-specific binding. The PCR Master Mix (Roche) was substituted with MyTaq™ Mix (Bioline, Taunton, MA).

Five microliters of DNA (<100 ng) was added to the MyTaq™ Mix (Bioline) containing 0.4 µM of each of the forward primer and 0.8 µM of the reverse primer. The PCR assay was performed using the DNA Engine Peltier thermocycler (Bio Rad) with the following cycling

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conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension of 5 min at 72°C.

3.2.4.2 Analysis of amplified products

Amplified products were separated on a 2% (m/v) molecular grade agarose gel (Bio Rad) in 1X Tris-borate EDTA (TBE) buffer [45mM Tris-borate (pH 8.0), 1mM EDTA (Sigma Aldrich). Ethidium bromide solution 10 mg/ml (Bio Rad) was used for visualisation. The voltage for electrophoresis was set at 80 V for 1 h and 30 min. The O` GeneRuler™ 50 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA) was loaded in a specified lane as a reference to verify that products of the desired size were indeed being amplified. Following electrophoresis, the gels were viewed using the UV transilluminator (Bio Rad) and photographed.

3.2.5 Genotyping by sequencing of amplified products obtained from primers designed for in-house PCR assay

Primers for the development of an in-house PCR assay aimed at the detection of the single nucleotide polymorphism in codon 617 were designed based on the known DNA sequence of the JAK2 gene (Genbank® accession number NG_009904.1). Forward and reverse primers were designed using the PrimerQuest software [Integrated DNA Technologies manufacturer (IDT), Coralville, Iowa]. The designed primers are shown in Table 3.2. These primers were manufactured by IDT.

Table 3.2: Primers designed for use with the in-house PCR assay for the detection of the JAK2 V617F mutation

Primer	**Sequence (5' → 3')**	**Melting temperature**	**GC content**	**Expected size (bp)**
PrimerF	AGGGACCAAAGCACATTGTAT	54.4 °C	42.9%	320
PrimerR	CCTAGCTGTGATCCTGAAACTG	55.1 °C	50%	

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The primers were analysed for its propensity for formation of hairpins, cross dimers and self-dimers using the Oligo analyser software (IDT). The primers formed internal hairpins with Gibbs free energy (ΔG) of greater than -3 kcal/mole and a 3' end hairpin with ΔG of -2 kcal/mole with less than four bases annealing. Primers also formed self-dimers with a ΔG between -1 kcal/mole and -6 kcal/mole. Forward and reverse primers were analysed for cross dimer formation and the ΔG was shown to be between -1.34 kcal/mole and -5.25 kcal/mole. Apart from the JAK2 gene on chromosome 9, no similar sequences were found on the human genome when NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed using these primers.

3.2.5.1 Genomic DNA amplification and analysis of amplified products obtained from primers designed for in-house PCR assay

To obtain amplified products for sequencing all of the samples (n=60) were processed. Five microliters of genomic DNA (<100 ng) was added to PCR Master Mix (Roche) and 0.4 µM of the designed forward and reverse primers each. Polymerase chain reaction was performed using the DNA Engine Peltier thermo cycler (Bio Rad) under the following conditions: initial denaturation at 94°C for 10 min, followed by 35 cycles of amplification with denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 45 s. Amplification conditions used were recommended by the manufacturer of the PCR Master Mix (Roche). Analysis of amplified products was performed as described in 3.2.4.2.

3.2.5.2 Sequencing of amplified products obtained from primers designed for in-house PCR assay

Sanger sequencing was performed on all products generated by PCR in both directions by Inqaba Biotechnical Industries (Pretoria, South Africa). The same primers used for amplification were also used for sequencing. The CLC main workbench software program v6.0 (CLCBio, Waltham, MA) was used to analyze the sequences against the NCBI reference sequence NG_009904 downloaded from http://www.ncbi.nlm.nih.gov/nuccore/NG_009904.1.

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3.2.6 Data and statistical analysis

Basic descriptive statistics was used to assess diagnostic sensitivity, specificity and the kappa value using STATA 13.1 software. Comparison of the assays was based on the ability of each assay to detect the mutation in samples known to be positive as well as the converse. The term true positive/negative was used to define positive/negative results from each sample tested and confirmed by the comparative assay. For the purpose of this study the AS PCR that was duplicated in the Department of Haematology was considered to be the comparator assay. Results of the three methods were compared. These included: (i) the historic results obtained and provided by the Johannesburg laboratory based on the comparative AS PCR described by Baxter (2005); (ii) the comparative AS PCR that was duplicated in the Department of Haematology and (iii) sequencing results using newly designed in-house primers. The results were used to elucidate agreement between the assays.

Kappa (κ) statistics was used for measuring agreement between methods and was calculated as follows: κ= (Observed-Expected agreement/1-Expected agreement) (De Mast, 2007). For the purpose of assessing agreement, the kappa (κ) statistic was used with the following interpretation cut-offs: $\kappa > 0.75$ indicated excellent agreement, $\kappa = 0.4–0.75$ indicated good agreement and $\kappa < 0.4$ indicated poor agreement (De Mast, 2007).

3.3 Results and Discussion

A total of 59 and 60 DNA samples were amplified using the comparative AS-PCR assay and the newly designed primers for the in-house method, respectively. One sample was insufficient to allow for its use in the comparative AS-PCR method. In addition, amplified product of all of the samples processed by the in-house primers (n=60) were sequenced.

3.3.1 Nucleic acid concentration

Genomic DNA extraction was performed using the Sigma's GenElute™ Blood Genomic DNA Kit. This kit provides a simple and convenient way to isolate pure genomic DNA from fresh or aged whole blood. The kit combines the advantages of silica binding with a micro-spin format and eliminates the need for expensive resins, alcohol precipitation and use of

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hazardous organic compounds such as phenol and chloroform. This extraction kit is suitable for use in a clinical haematology laboratory since it offers good gDNA yields (4-10 µg). The other advantage is that extraction is performed quickly allowing for same day PCR.

The concentration of the DNA was calculated by measuring absorbance at 260, 280 and 320 nm using the NanoDrop 2000c (Thermo Scientific). The ratio of 260/280 for all the samples included in the study ranged from 1.64 to 2.13 with DNA concentrations ranging from 4.3 ng/µL to 63.9 ng/µL (Appendix B). Samples with an A_{260}/A_{280} ratio of less than 1.6 were deemed unsuitable for analysis.

3.3.2 Genotyping by comparative method allele specific PCR

Prior to 2005, no molecular abnormality was described in the literature in patients with PV, ET and PMF (Klco *et al.*, 2010). The discovery of the JAK2 V617F mutation in PV, ET and PMF prompted the introduction of molecular diagnostics in MPN (Langabeer *et al.*, 2015). The detection of the JAK2 V617F mutation has been included in the 2008 WHO diagnostic criteria (Thiele and Kvasnicka, 2009). The identification of the JAK2 V617F mutation is useful for both diagnosis and prognostication; a quick and reliable assay to identify the JAK2 V617F mutation is required (Lay *et al.*, 2006). While Sanger sequencing assay can be used to detect the JAK2 V617F mutation, it has been reported that sequencing assays underestimate the number of patients harbouring the JAK2 V617F mutation (Langabeer *et al.*, 2015). Alternatives to using sequencing include AS-PCR or real time PCR assays (Langabeer *et al.*, 2015).

Numerous AS-PCR methods have been described in literature to investigate the presence of the JAK2 V617F mutation (Baxter *et al.*, 2005; Jones *et al.*, 2005; Benguella-Benmansour *et al.*, 2014). One such method is that of (Baxter *et al.*, 2005). This assay has been implemented unaltered in the diagnostic PCR laboratory at the Charlotte Maxeke Johannesburg laboratory to detect the JAK2 V617F mutation in patients suspected to suffer from MPN. Since this method has been validated in literature and has been adopted as diagnostic test in the reference laboratory it was considered the comparator for the purpose of this study. The authors claimed the analytical sensitivity of the assay to be 3% (Baxter *et al.*, 2005). Specimens and their results were made available by the diagnostic laboratory, which

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included 30 specimens that were positive and 30 specimens that were negative for the mutation. Owing to the volume of one specimen being insufficient, only 59 specimens were performed using this assay.

To confirm the reference laboratory's results, the assay was duplicated in this study. In an attempt to duplicate the method of the reference laboratory in its entirety, PCR Master (Roche) was used as commercial master mix. Only faint bands were yielded around the 364 base pair in some specimens (as shown in Figure 3.1). In addition, non-specific binding or primer dimers resulted in extra bands of between 50 and 100 bp. The presence of primer dimers suggests that the assay requires optimisation. Primer dimers may form under cold-start PCR conditions and may also arise from complementarity of a single nucleotide between primer 3′-ends after 30 cycles of amplification (Brownie *et al.*, 1997). Formation of primer dimers can be reduced by careful primer design, the use of hot-start PCR, the use of touch-down PCR and enzyme formulations, such as AmpliTaq Gold (Thermo Fischer Scientific) (Brownie *et al.*, 1997). The availability of limited funding for this study made it impossible to procure the AmpliTaq Gold master mix.

Figure 3.1: Allele specific PCR gel with Roche master mix showing faint bands and non-specific amplification bands between 50 and 100 bp

A= Ladder, B-= mutant specimen, C-D= wild type specimen, E=mutant specimen F= No template control

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Roche PCR Master was substituted with MyTaq™ Mix (Bioline) which is a ready-to-use 2x mix developed for fast and highly-specific PCR. This specific master mix is believed to exhibit more robust amplification than other commonly used polymerases, delivering a very high yield over a wide range of PCR templates. The substitution improved the quality of the PCR and resulted in bands that could be visualised clearly and consistently on the gels (as shown in Figure 3.2). The expected bands of 364 and 203 bp, which served as internal control and demonstration of the presence of the mutation, respectively, were both generated. Samples with only wild type DNA were expected to yield a single band of about 364 bp. An additional band of about 700 bp was consistently present in the samples harbouring the mutation. The presence of this band has also not been described in the original publication (Baxter *et al.*, 2005). Personal communication with the authors revealed that the 700 bp band was not seen routinely, though some background non-specific bands in 700 bp region were observed.

Figure 3.2: Allele specific PCR gel, the mutation is represented by the presence of a band around 200 bp and another band just before 400 bp. Wild type samples showed only the 364 bp band

A= Ladder, B-F= Wild type samples, G= mutant specimen, H-I= Wild type specimen, J-K mutant specimen, L= wild type, M-Q= mutant samples

Using AS-PCR assay, 26 of 59 samples was confirmed to harbour mutant DNA. The remaining 33 only contained wild type DNA. A summary of the results are contained in Table 3.3. The results obtained from duplicating the comparative AS-PCR method were compared to the historic results as provided by the reference laboratory. Out of 29 samples

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that the reference laboratory classified as positive, only 26 were identified with the mutation when duplicating the method.

Table 3.3: The overall number of samples that tested positive and negative with the AS-PCR and direct sequencing

Assay	**Positive**	**Negative**	**Total**
AS-PCR	26	33	59
Direct sequencing	24	36	60

When analysed statistically, these findings translated in diagnostic sensitivity of 89% and specificity of 100%. The positive predictive value was calculated to be 100% while negative predictive value was 90%. Agreement between the two methods was still acceptable at 94%. The kappa value of 0.89 signified excellent agreement. Causes for the discrepant results found in the three samples were difficult to explain. Traceability of the historic results was virtually impossible owing to a change of laboratory information system years before. Regrettably, it is the researcher's opinion that transcription errors may have contributed to the discrepancy. It is also plausible that the poor quality of the gels with faint bands and non-specific binding as seen in the original method could have hampered interpretation of the results. Because of these unexplained differences a decision was made to replace the historic results obtained from the reference laboratory as comparative method with the duplicated AS-PCR for the purpose of this study.

3.3.2 Genotyping by sequencing of amplicon obtained from primers designed for in-house PCR assay

Part of the aim of this investigation was to develop a diagnostic real time PCR assay to detect the JAK2 V617F mutation that would be quick and easy to perform, while still being sufficiently sensitive and specific. With this in mind, a primer set to amplify the region of interest was designed. Some of the recommendations that had to be kept in mind included that: (i) the ideal length of the primers should be between 18 to 24 bp (Lim *et al.*, 2011); (ii) the GC content of primers should be 30% to 70% (Raymaekers *et al.*, 2009); (ii) 3' end hairpins with ΔG of -2 kcal/mole and internal hairpins with a ΔG of -3 kcal/mole are considered acceptable (Popp and Bauer, 2015); (iv) large negative values for ΔG indicate

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stable binding resulting in undesirable hairpins (Popp and Bauer, 2015) and (v) to avoid primer dimers during PCR it is recommended that 3' end self-dimer should have ΔG of above -5 kcal/mole and internal self-dimer should have ΔG of above -6 kcal/mole (Popp and Bauer, 2015).

The primers that were designed using IDT PrimerQuest software were 21 bp (forward) and 22 bp (reverse) each and had internal hairpins with Gibbs free energy (ΔG) of greater than -3 kcal/mole and a 3' end hairpin with ΔG of -2 kcal/mole with less than four bases annealing. Also, the primers were shown to form self-dimers with a ΔG between -1 kcal/mole and -6 kcal/mole and when forward and reverse primers were analysed for cross dimer formation their ΔG was between -1.34 kcal/mole and -5.25 kcal/mole. These primers were assessed for specificity and upon performing NCBI BLAST no other similar sequences were found on the human genome apart from JAK2 gene. *In silico* assessment suggested that the primer set would be suitable for the intended purpose.

Electrophoresis of the amplicons (n=60) generated by the primers, developed as part of the in-house assay, consistently produced a single, well-defined amplification product of the expected size of about 320 bp (Figure 3.3). No non-specific binding was demonstrable on the gels as shown in Figure 3.3. The use of a commercial master mix and well-designed primers might both have contributed to the optimal performance of the PCR. Further optimisation of the assay was not considered to be necessary.

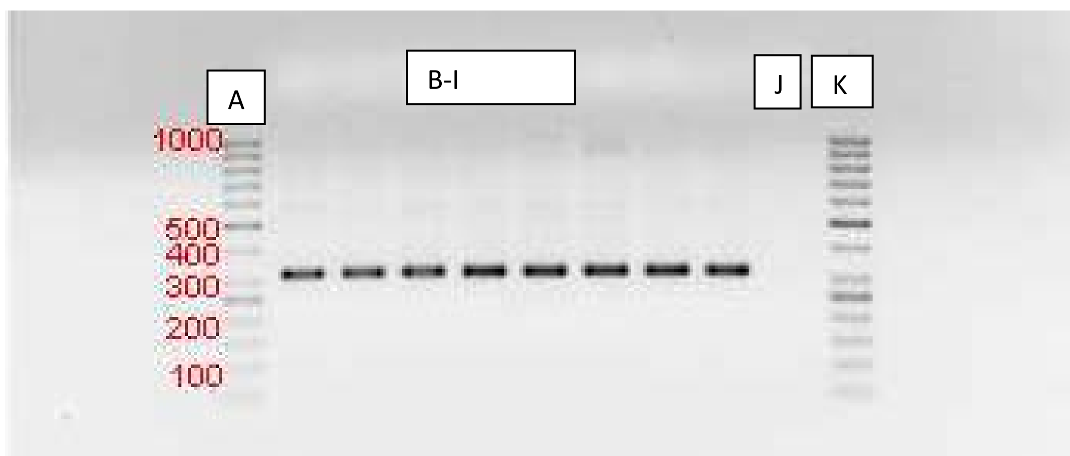

Figure 3.3: In-house PCR assay gel showing a clearly visible band around 300 bp
A= Ladder, B-I= Samples, J= No template control, K= Ladder

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Analysis of the sequenced products revealed the presence of both nucleotides G and T at position 1849 of the JAK2 gene in samples that harboured the mutation. A single peak corresponding to nucleotide G was demonstrated at the same position in all wild type samples. Figure 3.4 illustrates the nucleotide sequences found at the position of the mutation.

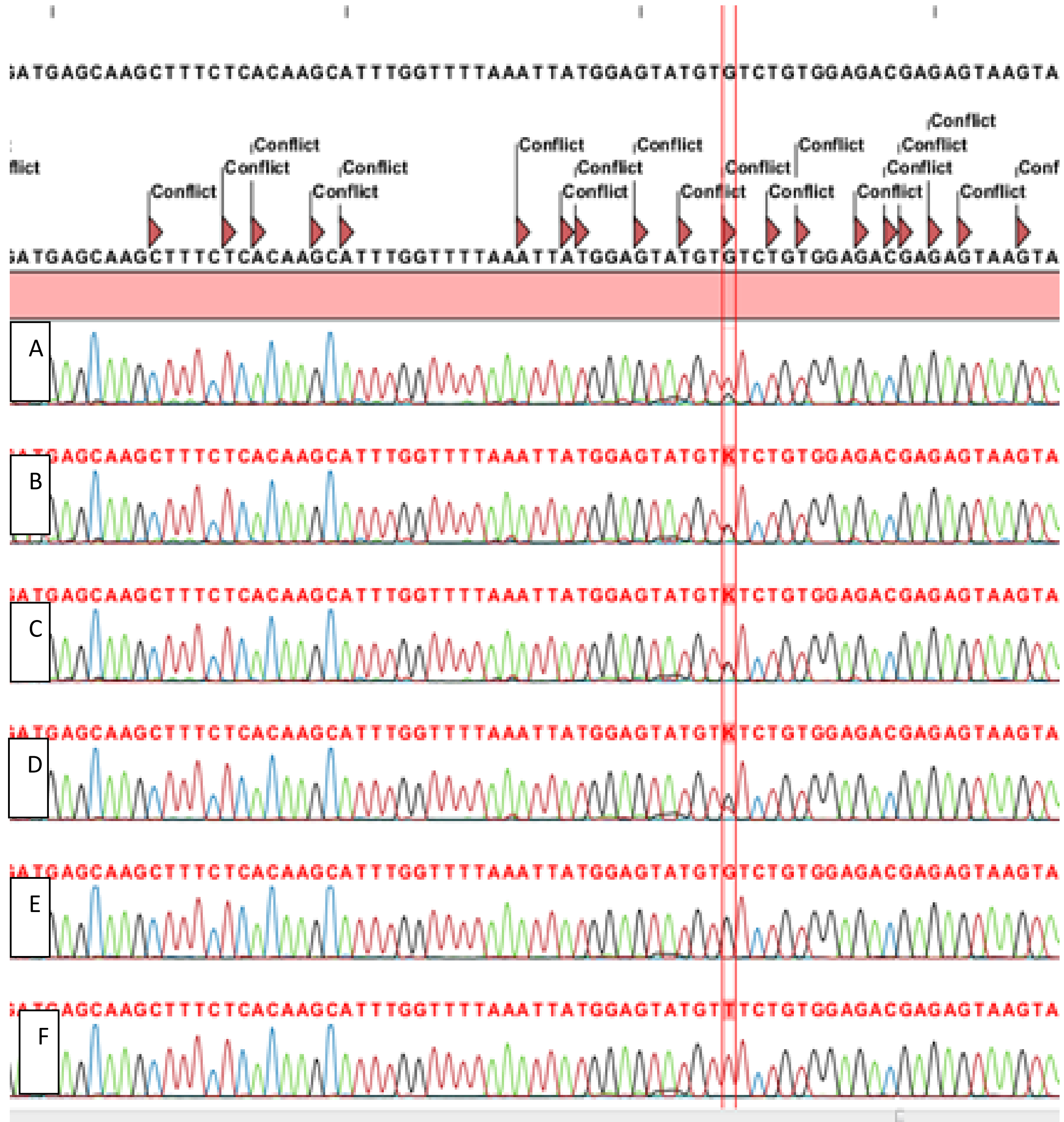

Figure 3.4: Sequencing chromatograms generated by the CLC main workbench software program (CLCBio) showing the presence of four mutant samples, one wild type sample and a homozygous control

A-D Heterozygous mutant samples, E-Wild type samples, F= Homozygous mutant control

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Sequencing of the PCR products in both directions gave consistent results, confirming that the region of interest was indeed being amplified. Sequencing of the homozygous commercial control revealed only nucleotide T. Twenty-four of the 60 samples were shown to have the mutation by sequencing. The remaining 36 contained wild type DNA only. These results showed that six specimens were discordant with the historic Johannesburg laboratory results in that they were found to be negative for the JAK2 V617F mutation by sequencing (Refer to Table 3.3). All of the remainder were concordant with the historic results reported by the Johannesburg laboratory.

The sequencing assay results were subsequently compared to the results of the duplicated AS-PCR assay that were available for 59 specimens. Direct sequencing of the amplicon obtained from primers designed for the in-house PCR assay failed to detect the JAK2 V617F mutation in the gDNA of two specimens that were shown to harbour the mutation by AS-PCR. Apart from these two discrepant results all of the remaining 57 samples yielded comparable results between the two methods. Excellent agreement between the in-house sequencing method and the comparative AS-PCR method was confirmed by a kappa value of 0.89. Compared to the duplicated AS PCR assay, in-house PCR using newly designed primers as assessed by sequencing has a diagnostic sensitivity of 88% and specificity of 100%.

Although the direct sequencing assay offers the advantage of direct visualisation of the nucleotide order, which is of value in screening allelic polymorphisms, it has limited sensitivity (Gut, 2001; Steensma, 2006). In this study, this feature has been confirmed by failure of the sequencing assay to detect the JAK2 V617F mutation in patients who were confirmed to harbour the mutation by AS-PCR assay. Studies confirm that direct sequencing assay is less sensitive as compared to AS-PCR (Baxter *et al.*, 2005; Jones *et al.*, 2005). One study reported that AS-PCR was able to detect the V617F mutation in 97% of PV patients as opposed to sequencing assay that only detected the mutation in 73% (Baxter *et al.*, 2005). Another study reported the analytical sensitivity of AS-PCR to be between 1% and 2% (Jones *et al.*, 2005). Lay *et al.* (2006) reported that a direct sequencing assay failed to detect the JAK2 V617F mutation when the mutation was present in less than 20% of the whole cell population. Other authors reported that direct sequencing assay could not to detect the JAK2 V617F mutation when the mutation was present in less than 5% in a wild type background.

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Unfortunately the analytical sensitivity of the direct sequencing assay was not performed in this study.

This is the first development of an in-house PCR assay in the Department of Haematology. Staff members in the Department of Haematology have limited experience in PCR assays and it was therefore a good learning opportunity for the personnel involved. Experiments to determine the analytical sensitivity of the direct sequencing assay were not performed and, as such, it remains difficult to assess the suitability of the assay to detect the JAK2 V617F mutation in patients with low mutation allele. Although all the guidelines for designing good working primers were followed, it may be possible that the designed primers lack sensitivity leading to discrepant results between the designed direct sequencing assay results and AS-PCR.

3.4 Conclusion

Although acceptable agreement could be demonstrated between detection of the JAK2 V617F mutation by direct sequencing of amplicon, obtained from primers designed for in-house PCR assay, and AS-PCR, the results show that direct sequencing is inferior to the AS-PCR as it failed to detect the JAK2 V617F mutation in some patients. Even though the AS-PCR method has been reported to have an analytical sensitivity of 3%, it is a time consuming assay which requires undesirable post-PCR detection steps (Soheili and Samiei, 2005).

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CHAPTER 4

IDENTIFICATION OF THE JANUS KINASE V617F MUTATION USING REAL TIME POLYMERASE CHAIN REACTION

4.1 Introduction

Myeloproliferative neoplasms (MPNs) are clonal disorders characterised by the increased risk of thrombosis and transformation to acute leukaemia (Chen *et al.*, 2010) with an annual incidence that ranges from 1.15 to 4.99 per 100 000 population (Anderson and McMullin, 2014). In 2005 a recurrent mutation in the JAK2 gene in patients with polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) was reported (Baxter *et al.*, 2005). The mutation occurs at nucleotide 1849 in exon 14 where guanine is substituted by thymine (Delhommeau *et al.*, 2010). It results in the disruption of the auto inhibitory effect of the Janus Homology domain 2 and it activates the Janus tyrosine kinase/ signal transducer and activator of transcription (JAK/STAT) pathway independently of cytokines (Delhommeau *et al.*, 2010). This mutation was shown to be present in 95% of PV and around 50% of ET and PMF patients (Anderson and McMullin, 2014). Discovery of the JAK2V617F mutation has improved the diagnosis of MPNs and also provided the possibility of the development of inhibitory drugs for the management of patients (Kilpivaara and Levine, 2008; Milosevic and Kralovics, 2013). The diagnosis of MPN is based on World Health Organisation (WHO) criteria, which requires the assessment of both clinical and laboratory features (Vardiman *et al.*, 2009). The identification of the JAK2 V617F mutation has become a major criterion in the 2008 WHO diagnostic scheme (Vardiman *et al.*, 2009).

Commercial kits are available for the detection of this JAK2 mutation and could arguably be used in a diagnostic laboratory. One such example is Ipsogen® JAK2 MutaScreen Kit (Qiagen, Hilden, Germany). This assay uses the principle of allelic discrimination where two TaqMan® probes are used in a multiplexed assay. Commercial kits like this one are, however, expensive and from that perspective not suited for routine diagnostic use. With this in mind, cost effective in-house assays are developed for use in diagnostic laboratories. Assays currently used to detect the mutation include direct sequencing, allele specific

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polymerase chain reaction (AS-PCR), PCR- restriction fragment length polymorphism (PCR-RFLP) and real time PCR (Baxter *et al.*, 2005; Lay *et al.*, 2006; Murugesan *et al.*, 2006). Some of the assays described lack sensitivity, are time consuming and cumbersome and others are simply too expensive to be used in the routine diagnostic environment (Qian *et al.*, 2010). Real time PCR has been shown to be a quick and reliable way to detect this mutation with suitable sensitivity (Lay *et al.*, 2006). With real time PCR no post-PCR steps are required and cross contamination of PCR products is eliminated (Lim *et al.*, 2011). One disadvantage of this assay is the high cost of equipment and reagents (Wong and Medrano, 2005). Chemistries that underlie real time PCR are probe based and intercalating dye assays (Gunson *et al.*, 2006).

SYBR Green I is the most widely used DNA intercalating dye for real-time PCR applications that allows the differentiation of PCR products by melting curve analysis (Ponchel *et al.*, 2003; Gudnason *et al.*, 2007). While the use of this dye is cost effective, the disadvantages include non-specific binding to double stranded DNA sequences and the need for extensive optimization of assays (Gudnason *et al.*, 2007). Compared to primer dimers and non-specific PCR products that form part of the fluorescence observed with intercalating dyes, the use of sequence specific probes offer greater specificity (Soheili and Samiei, 2005).

Probe based chemistries include locked nucleic acid (LNA) probes, hydrolysis probes and hybridization probes (Didenko, 2001; Arya *et al.*, 2005; Ballantyne *et al.*, 2008). Locked nucleic acid probes bind to complementary target sequences with very high affinity (Simeonov and Nikiforov, 2002; You *et al.*, 2006) and improve genotyping due to increased duplex stability, specificity and mismatch discrimination (Ballantyne *et al.*, 2008; Owczarzy *et al.*, 2008). This study details the procedural steps to detect the JAK2V617F mutation using LNA and SYBR Green I real time PCR assays.

4.2 Materials and Methods

4.2.1 Study setting

This study was conducted in the Department of Haematology at the Tshwane Academic Division of the National Health Laboratory Service (Pretoria, South Africa). The Faculty of

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Health Sciences Research Ethics Committee of the University of Pretoria approved the study (protocol number 32/2012).

4.2.2 Specimen collection

Sixty specimens of whole blood collected in EDTA were sourced from the reference PCR laboratory at the Charlotte Maxeke Johannesburg Academic Hospital where diagnostic testing has been performed. These specimens originated at the Steve Biko Academic Hospital where clinicians investigated their patients suspected of having MPNs for the presence of the JAK2 V617F mutation.

4.2.3 Genomic DNA extraction

Genomic deoxyribonucleic acid (gDNA) was extracted from peripheral blood using the GenElute™ blood genomic DNA kit (Sigma Aldrich, St Louis, MI) according to the manufacturer's instructions (Appendix A). The gDNA was eluted in 200 µL and stored at -20ºC until analysis.

4.2.4 Genotyping by comparative method allele specific polymerase chain reaction

Allelic discrimination of the JAK2 V617F mutation was performed in an earlier, separate experiment according to a validated method described by Baxter *et al.* (2005). Fifty nine available specimens were evaluated using this AS-PCR assay. The results of this test, which was considered to be the comparative method for the purpose of this study, were used to compare that obtained from the different real time PCR assays described in this method too.

4.2.5 Genotyping by sequencing of amplicon obtained from primers designed for in-house PCR assay

Conventional PCR using the primer set designed for in-house PCR assay shown in Table 4.1, was performed on all 60 samples in a separate experiment. Sanger sequencing was performed on all products generated by this method in both directions by Inqaba Biotechnical Industries (Pretoria, South Africa). The same primers used for amplification were also used

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for sequencing. The CLC main workbench software program (CLCBio, Waltham, MA) was used to analyze the sequences against the NCBI reference sequence NG_009904 downloaded from http://www.ncbi.nlm.nih.gov/nuccore/NG_009904.1.

4.2.6 Locked nucleic acid probe real time PCR

Real time PCR with LNA probes was performed on 60 samples to detect the JAK2 V617F mutation. The results generated by the real time PCR assay were compared to the AS-PCR and direct sequencing assay results.

4.2.6.1 Primer and probe design

Primers for the detection of the single nucleotide polymorphism (SNP) in codon 617 were designed based on the known DNA sequence of the JAK2 gene (Genbank® accession number NG_009904.1). Both forward and reverse primers were designed using the Integrated DNA Technologies (IDT) PrimerQuest software (Coralville, Iowa). The primer sequences are shown in Table 4.1.

Table 4.1: Primers and probes used for detection of the JAK2 V617F mutation using LNA probe real time PCR

Primer	**Sequence (5' → 3')**
Forward primer	AGGGACCAAAGCACATTGTAT
Reverse primer	CCTAGCTGTGATCCTGAAACTG
Wild type probe	HEX GATG+T+G+T+CT+G+TGG /laBkFQ
Mutant probe	6FAM ATG+T+T+T+CT+GT+G+GAG /laBkFQ

+: LNA base; laBkFQ: Lower black fluorescence quencher

In addition to conforming to the recommendations for primer design, the primers were assessed to be suitable when analysed for the possibility of forming hairpins, cross dimers and self-dimers using the Oligo analyser software (IDT). Performance of the primers was evaluated in a separate experiment that confirmed their ability to amplify the region of interest yielding a 320 bp product with sequences flanking the position of the SNP.

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Wild type and mutant probes were designed, with the help of a consultant from IDT, to be complimentary to the wild type and mutant nucleotide sequences, respectively. The wild type probe comprised 12 bases and included six LNAs of which two were present on the bases surrounding the SNP of interest. The wild type probe was labelled with Hexacloro-Fluorescein (HEX) at the 5' end. The mutant probe consisted of 14 bases and was designed to contain seven LNAs of which two flanked the SNP of interest. The mutant probe was labelled with 6-Carboxy Fluorescein (FAM). Lower black FQ quencher was attached at the 3' end of both probes. The designed primers and probes were manufactured by IDT. The sequences of the wild type and mutant probes are shown in Table 4.1.

4.2.6.2 Genomic DNA amplification using LNA probe real time PCR

All 60 samples were processed to investigate the diagnostic utility of the assay. In addition, 30 of the 60 patient samples were randomly selected and processed blindly. The investigator was blinded for the mutational status of the latter samples. Real time PCR was performed in a final volume of 20 µL using the Cepheid SmartCycler II platform (Maurens-Scopont, France). Optical channel one and two were used. Channel one on the Cepheid SmartCycler II is calibrated for FAM and channel two is calibrated for Cyanine (Cy3) and Alexa Fluor 532, which are excited between 500 and 550 nm. The maximum excitation of the HEX dye is 535 nm; channel two was suitable for detection of HEX.

The reaction mixture contained 0.8 µL of 0.4 µM of the forward and reverse primers each, 0.4 µL of 0.2 µM of mutant and 0.4 µL of 0.05 µM wild type probes, 10 µL of 2X Qiagen QuantiNova™ probe PCR master mix, 2.6 µL RNase free water (Qiagen) and 5 µL of (<100 ng) template gDNA. Polymerase chain reaction was performed under the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of amplification with denaturation at 95°C for 10 s, combined annealing and extension at 60°C for 30 s as per recommendations of the manufacturer of the master mix. Genomic gBlocks® Gene Fragments (IDT) consisting of mutant and wild type sequences each together with no template control were included with each run.

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4.2.6.3 Determining analytical sensitivity

The analytical sensitivity of the LNA real time PCR was determined using wild type gBlocks® gene fragments mixed with that of homozygous mutant gBlocks® gene fragments in different concentrations (100% mutant, 50% mutant, 20% mutant, 10% mutant, 5% mutant, 2% mutant, 1% mutant and 0.1% mutant). The concentration of the wild type and mutant probe was 0.05 µM and 0.4 µM, respectively. The dilutions were prepared fresh and subjected to PCR in three replicate experiments to ensure reproducibility of the assay. The limit of detection was defined as the lowest dilution of mutant DNA in which all the three replicates resulted in positive amplification (Broeders *et al.*, 2014).

4.2.7 SYBR Green real time polymerase chain reaction

4.2.7.1 Primer design

Primers for the detection of the SNP in codon 617 using SYBR Green were designed based on the known DNA sequence of the JAK2 gene (Genbank® accession number NG_009904.1). Refer to Table 4.1 for primer sets.

4.2.7.2 Genomic DNA amplification

Real time PCR was performed in a final volume of 20 µL using the Cepheid SmartCycler II system. The reaction mixture contained 0.8 µL of 0.4 µM of the forward and reverse primers each, 10 µL of 2X SensiFast™ SYBR No-ROX master mix (Bioline, Taunton, MA) , 3.4 µL sterile water and 5 µL of (<100 ng) template DNA. Polymerase chain reaction was performed following manufacture`s recommendations under the following conditions: polymerase activation at 95°C for 3 min, followed by 40 cycles of amplification with denaturation at 95°C for 5 s, annealing at 55°C for 10 s and extension at 72°C for 20 s. Melt curve analysis was performed at a ramp rate of 0.2°C/second from 40°C to 95°C.

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4.2.8 Data analysis

Two different real time PCR assays were compared to the comparative conventional PCR assay to assess their usefulness in allelic discrimination of the JAK2 V617F mutation. In addition, both assays were also compared to sequencing results that were previously obtained using the designed in-house primers. The results were used to elucidate agreement between the assays and calculate the positive and negative predictive values of the method. Basic descriptive statistics was used to assess diagnostic sensitivity, specificity and the kappa value (κ) using STATA 13.1 data analysis software.

Kappa value was used for assessing agreement between methods and was calculated as follows: κ= (observed-Expected agreement/1-Expected agreement) (De Mast, 2007). For the purpose of assessing agreement, κ was used with the following interpretation cut-offs: $\kappa > 0.75$ indicated excellent agreement, $\kappa = 0.4–0.75$ indicated good agreement and $\kappa < 0.4$ indicated poor agreement (De Mast, 2007).

4.3 Results

4.3.1 Nucleic acid concentration

The concentration of gDNA as calculated by measuring absorbance using the NanoDrop 2000c (Thermo Scientific, Waltham, MA) platform ranged from 4.3 ng/µl to 63.9 ng/µl. Samples with an A_{260}/A_{280} ratio of less than 1.6 were not processed. The results are presented in Appendix B.

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4.3.2 Locked nucleic acid probe real time PCR

Real time PCR with locked nucleic acid probes generated amplification curves as shown in Figure 4.1. In wild type samples only the probe labelled with HEX was detected as shown by the amplification curve in Figure 4.2, whereas in heterozygous samples, both probes were detected as shown by the presence of two amplification curves corresponding to HEX and FAM in Figure 4.3.

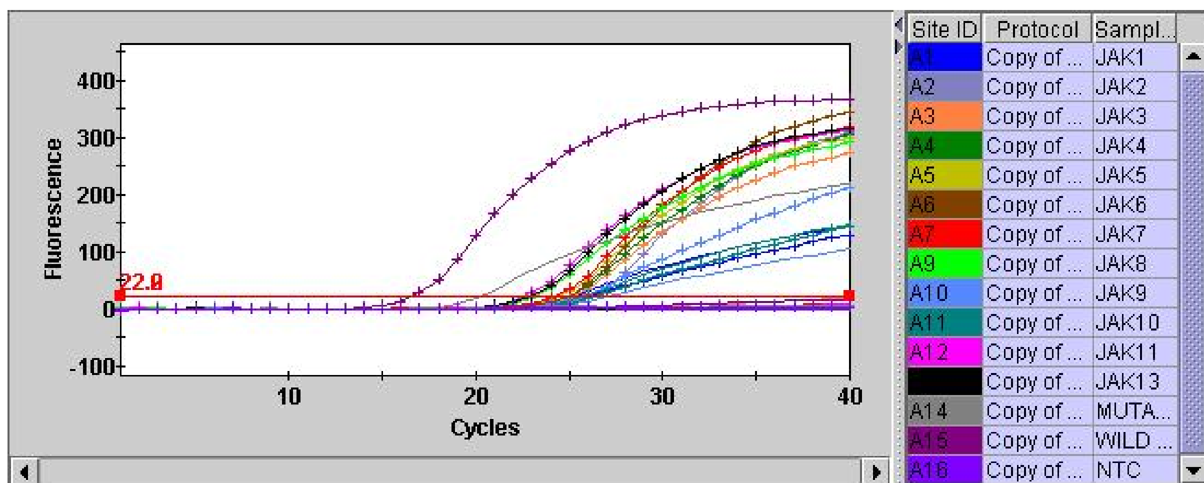

Figure 4.1: An example of amplification curves generated by the Cepheid SmartCycler II system when LNA probe real time PCR was performed

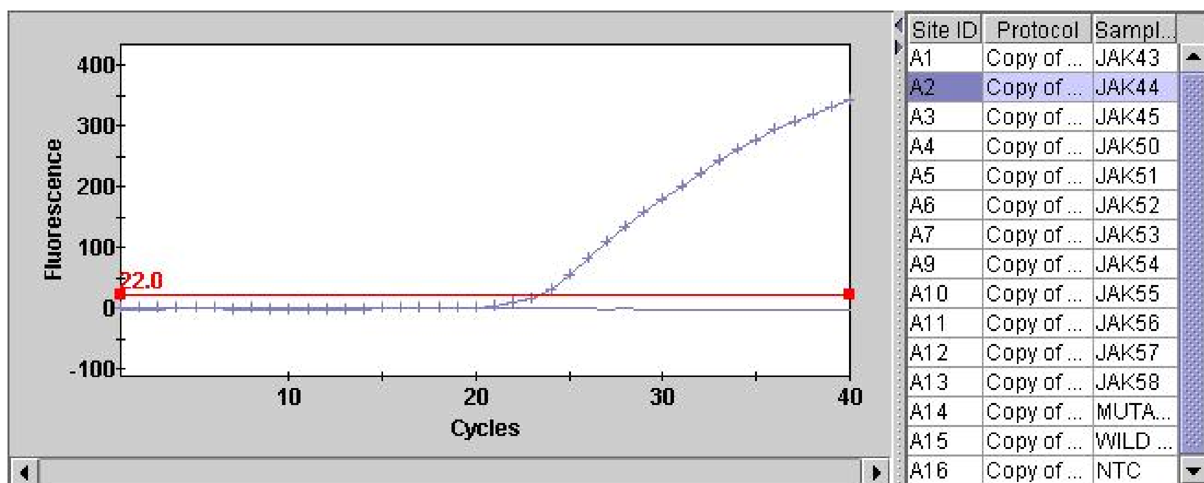

Figure 4.2: An example of wild type amplification represented by the presence of a single positive amplification curve

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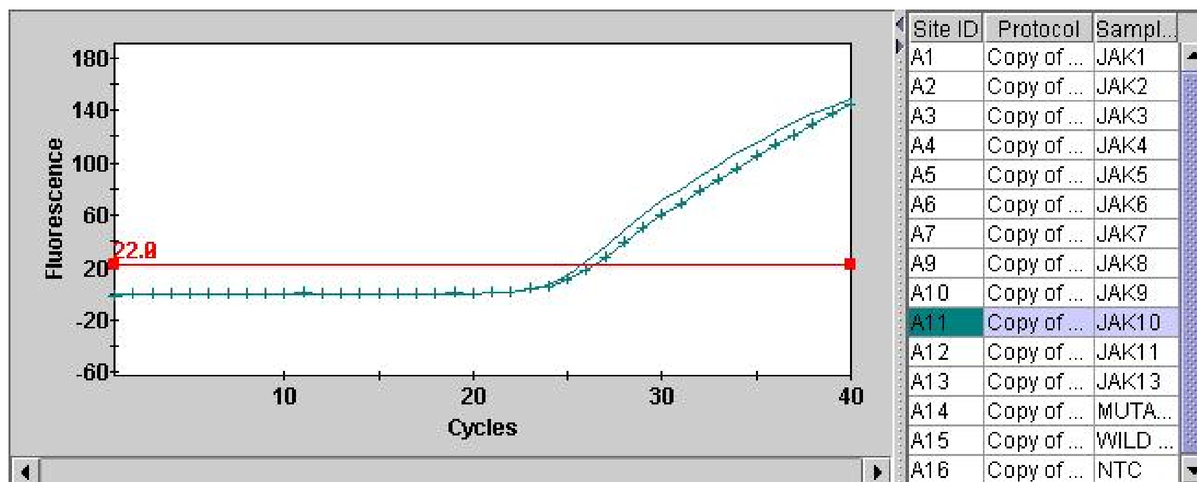

Figure 4.3: A heterozygous sample represented by the presence of two amplification curves. One curve indicates the presence of the mutant allele and the other that of the wild type allele

A total of 60 DNA samples were amplified using this method. The mutation was successfully detected in 24 of the 60 samples (40%) (Refer to Appendix C for a full set of results). Thirty-six samples were shown to only contain wild type DNA using this method. The threshold cycles of all the samples processed ranged between 23.12 and 29.05 with FAM, whereas with HEX it ranged between 21.28 and 33.62 as shown in Appendix D.

Since one specimen was insufficient for analysis using the comparative method, the results of only 59 specimens obtained by this real time method could be compared to the reference method. When the results using this method were compared to that obtained from the comparative AS-PCR method, all but two of the samples shown to have the mutation by the reference method were identified by the real time assay. The ability to identify the mutation using the real time method was identical to that of Sanger sequencing when all 60 samples were evaluated, in that the same 24 samples were shown to harbour the mutation by both methods indicating 100% agreement. Of the 30 randomly selected samples for which the investigator was blinded, 11 (36%) were identified as positive for the mutant allele and 19 contained wild type DNA only. Results of the samples that were processed blindly were 100% concordant with the initial LNA real time PCR results.

Real time PCR had a diagnostic sensitivity of 88% with a specificity of 100% when compared to the AS-PCR method. The positive predictive value of the real time PCR method was 100% and the negative predictive value 91%. Agreement of the real time PCR assay

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with the AS-PCR method was 94% with a kappa value of 0.89. There was complete agreement between sequencing and real time PCR (100%).

4.2.3 Determining the analytical sensitivity of the LNA probe-based PCR

Using gBlock® mixtures with decreased proportions of mutant DNA, a positive amplification curve was reproducibly detected at 0.1% of mutant DNA. The limit of detection of this assay is 0.1% in wild type DNA background. Figure 4.4 shows the analytical sensitivity amplification curves.

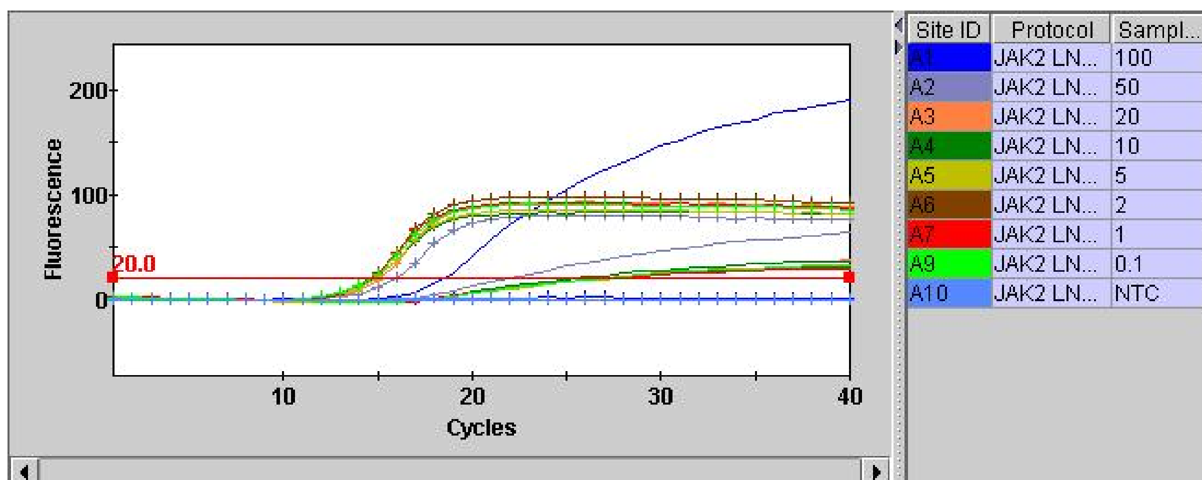

Figure 4.4: Analytical sensitivity curve showing positive amplification of the different dilutions as generated by the Cepheid SmartCycler II system

4.3.4 SYBR Green real time PCR assay results

A real time PCR assay with SYBR Green and melting curve analysis, using the designed primers was unable to differentiate between mutant and wild type alleles. Both the mutant and wild type alleles yielded melting peaks at a temperature of about 81ºC as shown in Figure 4.5.

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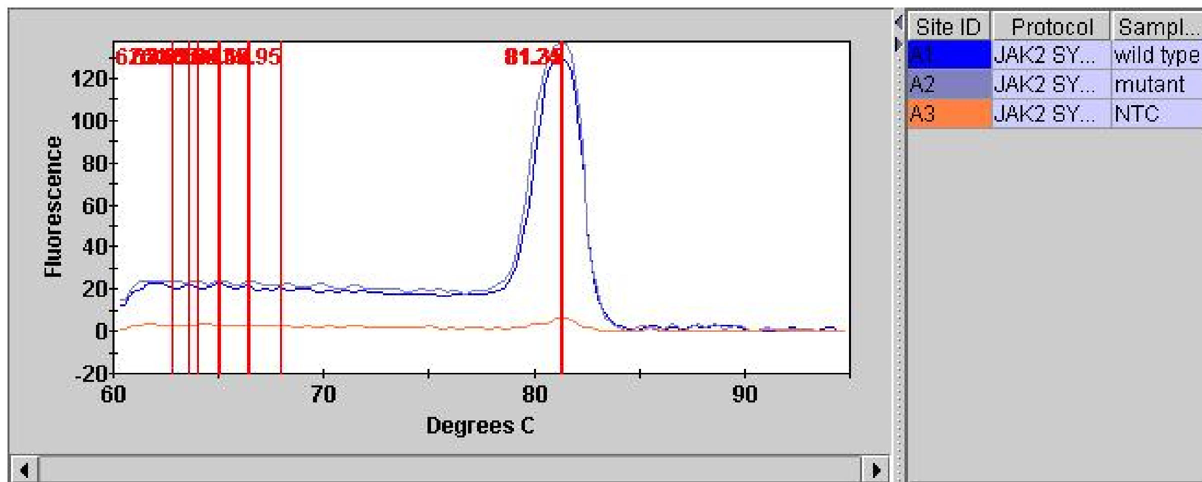

Figure 4.5: SYBR green melting curve results of mutant and wild type controls as generated by the Cepheid SmartCycler II system. The melting curve for mutant and wild type controls could not be distinguished from each other

4.4 Discussion

The detection of the JAK2 V617F mutation is essential for the diagnosis of MPNs and its detection has been included in the 2008 WHO diagnostic criteria (Thiele and Kvasnicka, 2009). Detection of the JAK2 V617F mutation is also important as directed therapies targeted at the inhibition of the JAK2 kinase activity are currently being developed (Levine and Gilliland, 2008; Quintás-Cardama *et al.*, 2010). Assays that are currently used for this purpose include direct sequencing, AS-PCR, PCR-RFLP and real time PCR assays (Greiner, 2006). Direct sequencing is a gold standard for mutation analysis but limited by low sensitivity and high cost (Liu *et al.*, 2014).

In this study a LNA probe based real time PCR assay was developed and evaluated for its ability to detect the JAK2 V617F mutation. Locked nucleic acid is an analogue of nucleotides that contains an internal 2′-O, 4′-C methylene bridge, which locks the ribose ring into a C3′-endo conformation (Denys *et al.*, 2010). Introduction of LNAs into probes increases thermal stability of the probe with + 3ºC to + 8°C per modification (Denys *et al.*, 2010). Another advantage of LNA probes is that they bind to complementary target sequences with very high affinity, which allows for the use of very short probes (Simeonov and Nikiforov, 2002; You *et al.*, 2006). In this study the wild type probe contained six LNAs and the mutant probe seven LNAs. Increasing the number of LNAs in the mutant probe was

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necessary to increase the melting temperature (T_m) of the probe to greater than that of the primers and thus increasing specificity of the probe. During the experiment it was noted that the standard 1:1 wild type probe to mutant probe ratio did not provide optimal results. The wild type probe binds with high affinity, thus reducing the intensity of the mutant probe seemingly competing with the mutant probe in this multiplexed assay. In an attempt to optimise the assay's performance, the wild type probe concentration was adjusted in a separate experiment by making a serial dilution of the wild type probe in nuclease free water and assessing its performance (data not shown here). The concentration of the wild type probe that gave optimal results was found to be 0.05 µM. This concentration of wild type probe was used together with 0.4 µM mutant probe in the PCR assay with satisfactory results. The Cepheid SmartCycler II system is programed with a default baseline of five to 40 cycles. To optimise the experiment, the background subtraction was adjusted from cycle three to 20. Cycle threshold was set at 20 as determined by analysing the second derivative curve. The threshold cycles of all the samples processed ranged between 23.12 and 29.05 with FAM, and between 21.28 and 33.62 with HEX. The variability of threshold cycles could not be explained by the concentration of template DNA alone since poor correlation was noted. It is conceivable that the allele burden may influence the threshold cycles.

The real time PCR assay with LNA probes was performed and results compared to AS-PCR and sequencing assays. A total number of 59 samples were processed using AS-PCR and of those 26 specimens tested positive. Sixty samples were analysed using LNA real time PCR and sequencing assays. The LNA real time PCR assay and sequencing both identified 24 samples as positive. All the samples that were positive by the sequencing and LNA assays were also positive by the AS-PCR assays. Compared to the reference method, sequencing and real time PCR assays both failed to classify three samples as positive. It should be stated that the AS-PCR assay was used as comparator only by virtue of this assay being the diagnostic assay employed in the reference laboratory at the time of planning the study. It is also conceivable that the developed real time PCR is more accurate than the comparative AS-PCR. With this in mind, the most plausible explanation for the discordant results could perhaps be false positive results generated by the AS-PCR assay. According to Ma *et al.,* (2009) the risk of false positives with AS-PCR is very high when the level of the JAK2 V617F mutant allele is low whereas the risk of false positive results is minimal when sequencing is used (Ma *et al.*, 2009).

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Assays with adequate analytical sensitivity are required for the detection of JAK2V617F, since the mutation may be present in a small fraction of the cells (Gong *et al.*, 2013). Consistent positive amplification was obtained down to the 0.1% dilution. The acceptable sensitivity of a qualitative test should be equal to or below 20% (Broeders *et al.*, 2014). It is recommended that the assay for the detection of the JAK2 V617F mutation in a clinical setting should have an analytical sensitivity of at least 1% to ensure that more than 90% of cases are detected (Gong *et al.*, 2013).

Both sequencing and real time PCR had a diagnostic sensitivity of 88% with a specificity of 100% as compared to the AS-PCR method. The positive predictive value of the real time PCR assay was 100% and the negative predictive value 91%. The agreement between the real time PCR assay and sequencing was 100%. Agreement between the real time PCR assay and the comparative AS-PCR assay was calculated to be 94% with kappa value of 0.89. A kappa value of greater than 0.75 signifies excellent agreement and it can be concluded that the developed real time PCR assay performs favourably when assessed against the comparative AS-PCR method.

The developed real time PCR assay has been shown to have good analytical sensitivity and as such it is suitable to be used as a diagnostic assay. It is important to note that an assay that detects less than 0.1% of mutant allele is more likely to give false positive results in a diagnostic setting (Gong *et al.*, 2013). To assess the robustness of a real time PCR assay different experimental conditions, such as annealing temperatures, the use of different instruments and operators can be introduced (Broeders *et al.*, 2014). This aspect of assay evaluation was not explored in this study. However, to show that the assay were able to perform equally well on another platform, a PCR run was processed on the Qiagen Rotor-Gene Q 2Plex system. Satisfactory results were obtained that appeared to be similar to that seen using the Cepheid SmartCycler II system. Amplification efficiency is an important consideration when performing real time quantitative PCR (Wong and Medrano, 2005). The purpose of the developed assay was to provide qualitative results and literature suggests less stringent requirements for amplification efficiency when a qualitative assay is evaluated (Broeders *et al.,* 2014).

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This study focussed on developing a qualitative assay for JAK2 V617F mutation detection. Recently the importance of quantitative JAK2 V617F detection assays has been shown (Takahashi *et al.*, 2013). Quantitative assays for this mutation are not widely used but may be valuable for long-term monitoring of JAK2 V617F allele burden as a marker of minimal residual disease (Takahashi *et al.*, 2013). Sensitive and accurate quantification assays are needed for monitoring of the allele burden in response to JAK2 inhibitors (Denys *et al.*, 2010). Quantitative Amplification refractory mutation system real time PCR and allele-specific wild-type-blocking PCR have been shown to be sensitive assays for monitoring minimal residual disease in patients with JAK2 V617F positive disease (Lange *et al.*, 2013). The main aim of this study was to develop a qualitative assay to be used as a diagnostic tool but future work will focus on developing a quantitative assay that can be used to monitor response to therapy once this treatment modality becomes available to patients in South Africa.

Part of the intent of this investigation was to develop a cost-effective real time PCR assay that can be used to detect the JAK2 V617F mutation in a diagnostic setting. In an attempt to investigate whether a real time PCR assay with an intercalating dye and melt curve analysis would be feasible, SYBR green dye was used. The use of SYBR green with melting curve analysis could not distinguish the mutant and the wild type alleles from one another. Both alleles yielded a melting peak at about 81ºC. The results showed that a real time PCR assay with melt curve analysis using the Cepheid SmartCycler II platform and SYBR Green as instrument and reagent combination cannot be used to detect the JAK2 V617F mutation.

One possible solution could be to employ High resolution melt curve (HRM) analysis to detect the mutation. This assay offers an advantage over the normal melt curve analysis in that it is able to detect SNPs with higher sensitivity including one base pair substitutions (Rapado *et al.*, 2009). Saturating fluorescent dyes, such as EvaGreen, LC Green, SYTO Dye and Chromofy are frequently used to monitor denaturation of PCR products in HRM analysis (Sharma *et al.*, 2013). High resolution melt curve analysis with the LightScanner™ and LightCycler™ platforms in the detection of JAK2 V617F mutation has been shown to have a sensitivity of 5% (Er *et al.*, 2009; Qian *et al.*, 2010). It also offers benefits, such as lower workforce, saving of time and a decrease in the risk of post PCR contamination (Er *et al.*, 2009). Regrettably the Cepheid SmartCycler II platform does not have HRM analysis

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capability and limited funding made it impossible to procure reagents to perform HRM on the Qiagen Rotor-Gene Q 2Plex system. However, future work will include the evaluation of HRM as a cost-effective alternative.

Complete method validation requires assessment of amplification efficiency, robustness, linearity, analytical sensitivity and specificity, diagnostic sensitivity and specificity, accuracy, precision, repeatability and reproducibility before this assay could be introduced into the diagnostic laboratory. Precision, reproducibility and robustness of the assay in this study were not performed and as such the assay cannot be incorporated into the routine diagnostic laboratory. The Cepheid SmartCycler II system cannot perform HRM and this assay could not be explored further at this time. Traceability of the historic results was virtually impossible owing to the change of the laboratory information system the year before. It would have been ideal to compare the performance of the developed real time PCR to a commercial kit but costs prohibited this possibility..

4.5 Conclusion

The developed LNA probe real time PCR assay is a suitable diagnostic method for detecting the JAK2 V617F in the studied clinical setting. It has good sensitivity; it is easy to set up and has rapid turnaround times. The developed in-house assay also appears to be cost effective and it provides choice of reagent quality over commercial real-time PCR assay.

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CHAPTER 5
CONCLUSION

5.1 Concluding remarks

The detection of the JAK2 V617F mutation is of importance in the diagnosis of MPN (Langabeer *et al.*, 2015). Molecular diagnostic algorithms have been designed to allow identification of clonal markers in patients suffering from these disorders (Langabeer *et al.*, 2015). Selection of molecular diagnostic assays requires consideration of technical aspects such as reliability, reproducibility and analytical sensitivity (Langabeer *et al.*, 2015).

The primary aim of this study was to develop real time PCR assays that can be used as diagnostic assays in a routine setting. Real time PCR is a highly sensitive assay (Lim *et al.*, 2011). Studies have reported analytical sensitivity of 0.01% with Taqman and LNA probes (Sidon *et al.*, 2006; Hammond *et al.*, 2007). In this study, LNA probe based real time PCR was developed for the detection of the JAK2 V617F mutation. Initially primers were developed to amplify the region of interest. To confirm the specificity of primers, conventional PCR was performed followed by sequencing of PCR products which confirmed that the intended product was being amplified. Probes, designed to be complimentary to the wild type and mutant template DNA, were subsequently designed and combined in a real time PCR assay.

Evaluation of AS-PCR, sequencing and LNA probe real time PCR revealed that these three methods yielded concordant results in more than 80% of the cases. The developed LNA probe real time PCR assay was shown to have acceptable diagnostic sensitivity and specificity as well as suitable limit of detection. In addition, the assay is easy to set up and has rapid turnaround times. Overall, the developed LNA probe real time PCR assay is considered to be a suitable qualitative diagnostic assay for detecting the JAK2 V617F mutation in a clinical setting. It is acknowledged that complete method validation also requires assessment of amplification efficiency, robustness, linearity, analytical sensitivity and specificity, diagnostic sensitivity and specificity, accuracy, precision, repeatability and

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reproducibility before this assay could be introduced into the diagnostic laboratory. In addition, cost analysis of this assay is recommended before commercial use.

Part of the intent of this study was to determine whether real time PCR using SYBR green dye with melt curve analysis could be used to detect the JAK2 V617F mutation reliably. Results of the experiments conducted showed that SYBR green dye with melt curve could not distinguish between mutant and wild type allele. This indicates that SYBR green dye cannot gainfully be utilised on the Cepheid SmartCycler II platform to detect the JAK2 V617F mutation. Further exploration of the use of intercalating dyes in an assay of this nature is anticipated.

5.2 Future research

The present study focussed on developing a qualitative real time PCR for the detection of the V617F mutation. Currently, clinical research is focussing on the development of drugs that can modify the disease's natural history and the development of prognostic biomarkers (Tefferi, 2015). Future studies should focus on developing of a quantitative real time PCR assay to detect and quantify mutant alleles. Quantitative assays for this mutation are valuable tools for long-term monitoring of JAK2 V617F allele burden to monitor response to therapy (Takahashi *et al.*, 2013).

Next generation sequencing is increasingly being used in laboratories to assess targeted gene panels (Gong *et al.*, 2013). The use of NGS allows broad multi-gene coverage in a single assay; this may help in detecting various mutations of interest simultaneously. Sequencing of the exome can facilitate the identification of disease-causing mutations in pathogenic presentations where the exact genetic cause is not known (Grada and Weinbrecht, 2013). This assay may also be used to quantify the percentage of V617F mutant allele and to detect leukemic transformation (Langabeer *et al.*, 2015). The feasibility of using NGS in a routine clinical setting should also be evaluated in future studies.

Quantitation of the target allele may be performed by digital PCR (Langabeer *et al.*, 2015). Digital PCR is an emerging approach for absolute quantitation of the target allele without the requirement for standard curve construction or comparison to a reference gene (Kinz *et al.*,

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2015). This is achieved by partition of the template DNA into multiple PCR reactions so that each contains a single copy or no copies of the target of interest (Kinz *et al.*, 2015). This approach results in improved sensitivity and precision with minimal requirements for validation and standardisation (Kinz *et al.*, 2015). Digital PCR has been validated for detection of the JAK2 V617F mutation (Langabeer *et al.*, 2015). Future research should also assess the feasibility of using digital PCR in a routine diagnostic laboratory.

Proteomics which refers to the study of the whole set of proteins present in a cell at a given time has been employed in the field of haematology to investigate proteome modifications associated with haematological neoplasms (Mossuz, 2008). Proteomics has been used to identify therapy related proteasomes and predict clinical behaviour in adult acute lymphocytic leukaemia (Mossuz, 2008). In MPN, proteomics has been used to identify and correlate serum biomarkers to percentage of JAK2 V617 alleles (Mossuz *et al.*, 2008). The correlation of serum biomarkers to the proportion of mutated alleles provide a useful tool for characterisation of PV patients at diagnosis (Mossuz *et al.*, 2007). Mass spectrometry methods have been used to identify serum proteins in JAK2 V617F positive PV patients and Apolipoprotein–Al was identified as useful serum marker (Mossuz *et al.*, 2007). Levels of Apo-A1 were mostly increased in PV patients with high levels of mutated alleles (Mossuz *et al.*, 2007). The JAK2 V617F mutation dosage impacts on serum protein expression in PV patients (Mossuz *et al.*, 2007).

In this study, SYBR green dye with melt curve analysis was used to investigate if it will be feasible to use an intercalating dye to detect the mutation. With SYBR green dye unable to distinguish the mutant and the wild type alleles from one another on our routine PCR platform, it would be ideal to evaluate HRM capability to detect the mutation on other analysers in our setting. Future work should also evaluate the use of HRM as a cost-effective alternative for detecting the V617F mutation.

With the integration of CALR and MPL mutations into the 2016 WHO diagnostic criteria for MPN, developing assays for the detection of exon 12 mutations, CARL and MPL will be beneficial in a routine clinical setting. Detecting CALR and MPL mutations as clonal markers will especially benefit patients with ET and PMF who do not harbour the JAK2 V617F mutation. The presence of CALR, JAK2 V617F and MPL mutations results in

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different prognosis to patients. Detecting the three mutations in a clinical laboratory will help to stratify prognostic implications to the treating clinician.

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APPENDIX A: Equipment and reagents

Resuspension solution

Lysis solution C

Column preparation solution

Prewash solution concentrate

Elution solution (10mM Tris-HCl, 0.5 mM EDTA, pH 9.0)

Proteinase K

97% Ethanol

Distilled water

GenElute Miniprep binding columns in tube

Collection tubes

55ºC heating block

Pipette tips

Microcentrifuge tubes

Genomic DNA extraction method

Deoxyribonucleic acid was extracted from peripheral blood using the GenElute™ blood genomic DNA kit (Sigma Aldrich) as follows:

- A volume of 20 µl of Proteinase K was placed into a 1.5ml microcentrifuge tube and 200 µl of peripheral blood added
- A volume of 200 µl of the Lysis solution C was added and the mixture was vortexed for 15 sec
- The mixture was incubated at 55ºC heating block for 10 min

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- A volume of 500 µl of the column preparation solution was added to tubes containing GenElute Miniprep binding columns and centrifuged at 12000 rpm for 1 min using Hettich Mikro 200R centrifuge (USA)
- The flow through liquid was discarded
- After 10 min of incubation, 200 µl of 97% ethanol was added to the lysate and mixed by vortexing
- The entire contents of the tube were transferred into treated GenElute Miniprep binding columns column and centrifuged at 6500 rpm for 1 min
- A volume of 500 µl of Prewash solution was added to the column and centrifuged for 1 min
- The collection tube containing flow through liquid was discarded and the column was placed on a new collection tube
- A volume of 500 µl of Wash solution was added to the column and centrifuged for 3 min at 12000 rpm to dry the column
- A volume of 200 µl of the Elution solution was added directly into the centre of the column and centrifuged at 6500 rpm for 1 min to elute the DNA
- The eluted gDNA was stored at -20ºC

Determination of DNA concentration using the NanoDrop 2000c spectrophotometer (Thermo Scientific)

The concentration of DNA was determined by pipetting 2 µl of DNA sample onto the NanoDrop 2000c spectrophotometer sensor (Thermo Scientific). The NanoDrop 2000c spectrophotometer uses the Beer Lambert equation (C=A/CE*b) to determine the concentration of DNA. The C is the nucleic acid in ng/µl, A is the absorbance in AU, E is the wavelength dependant extinction coefficient in ng-cm/µl and b is the path length in cm. In DNA concentration determination, the absorbance peak is at 260 nm.

Preparation of working TBE buffer

The 1X working TBE buffer was prepared by adding 100 ml of the stock 10X (Bio Rad, Germany) solution into 900 ml distilled water.

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Agarose gel preparation

The 2% (m/v) agarose was prepared by dissolving 2 g of molecular grade agarose powder (Bio Rad, Germany) in 100 ml of the 1X TBE (Bio Rad, Germany) working buffer solution. Agarose powder was dissolved by heating in a microwave. After cooling, 5µl ethidium bromide (10mg/ml) (Bio Rad, Germany) was added. The gel was poured into a casting tray with a comb to form wells of the gel.

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APPENDIX B: Nucleic acid concentration as measured by the Nanodrop 2000C spectrophotometer (Thermo Scientific)

Sample number	Nucleic acid Concentration	260/280
JAK1	14.1	1.75
JAK2	16.4	1.75
JAK3	5.7	1.81
JAK4	8.6	1.77
JAK5	10.7	1.84
JAK6	9.2	1.9
JAK7	11.7	1.9
JAK8	31.3	1.79
JAK9	15	1.9
JAK10	25	1.87
JAK11	50.8	1.89
JAK13	11.7	1.8
JAK14	19.4	1.85
JAK16	11.2	1.84
JAK17	11.7	1.69
JAK18	5.1	1.7
JAK20	14.5	1.88
JAK21	4.3	1.8
JAK22	18.4	1.76

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APPENDIX B: Nucleic acid concentration as measured by the Nanodrop 2000C spectrophotometer (Thermo Scientific) (Continued)

Sample number	Nucleic acid Concentration	260/280
JAK23	14.8	1.8
JAK25	8.2	1.78
JAK26	6.8	1.8
JAK27	10.4	1.72
JAK28	8.6	2.06
JAK29	5.2	2.13
JAK30	8.5	1.89
JAK32	12.9	1.83
JAK34	10.4	1.75
JAK35	8.7	1.8
JAK36	9.1	1.65
JAK37	37.7	1.71
JAK38	9	1.74
JAK39	23.9	1.83
JAK40	30.2	1.77
JAK41	13.6	1.78
JAK42	63.5	1.78
JAK43	22.2	1.76
JAK44	18.1	1.75
JAK45	37.9	1.85
JAK50	39.4	1.83

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APPENDIX B: Nucleic acid concentration as measured by Nanodrop 2000C spectrophotometer (Thermo Scientific) (Continued)

Sample number	Nucleic acid Concentration	260/280
JAK51	42.5	1.77
JAK52	22	1.65
JAK53	63.9	1.77
JAK54	8.5	1.78
JAK55	37.7	1.89
JAK56	34.2	1.85
JAK57	18.1	1.7
JAK58	31.3	1.74
JAK60	10.7	1.78
JAK61	16.4	1.8
JAK62	14.1	1.77
JAK64	11.2	1.71
JAK65	14.8	1.83
JAK66	13.6	1.85
JAK69	22.2	1.64
JAK70	8.7	1.72
JAK71	10.5	1.83
JAK72	37.4	1.7
JAK73	31.7	1.89
JAK74	18.7	1.87

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APPENDIX C: Results of the three molecular assays

Specimen number	**Sequencing**	**Duplicate ASPCR**	**Real time**	**Historic**
JAK1	Positive	Positive	Positive	Positive
JAK 2	Negative	Negative	Negative	Negative
JAK 3	Negative	Positive	Negative	Positive
JAK 4	Negative	Negative	Negative	Negative
JAK 5	Negative	Negative	Negative	Negative
JAK 6	Negative	Negative	Negative	Positive
JAK 7	Negative	Negative	Negative	Negative
JAK 8	Negative	Negative	Negative	Negative
JAK 9	Positive	Positive	Positive	Positive
JAK 10	Positive	Positive	Positive	Positive
JAK 11	Negative	Negative	Negative	Negative
JAK 13	Negative	Negative	Negative	Negative
JAK 14	Negative	Negative	Negative	Negative
JAK 16	Negative	Negative	Negative	Negative
JAK 17	Negative	Negative	Negative	Negative
JAK 18	Positive	Positive	Positive	Positive
JAK 20	Negative	Negative	Negative	Positive
JAK 21	Negative	Negative	Negative	Negative
JAK 22	Negative	Negative	Negative	Negative

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APPENDIX C: Results of the three molecular assays (Continued)

Specimen number	Sequencing	Duplicate ASPCR	Real time	Historic
JAK 23	Positive	Positive	Positive	Positive
JAK 25	Negative	Negative	Negative	Negative
JAK 26	Negative	Negative	Negative	Negative
JAK 27	Negative	Negative	Negative	Negative
JAK 28	Positive	Positive	Positive	Positive
JAK 29	Positive	Positive	Positive	Positive
JAK 30	Negative	Negative	Negative	Negative
JAK 32	Negative	Negative	Negative	Negative
JAK 34	Negative	Negative	Negative	Negative
JAK 35	Positive	Positive	Positive	Positive
JAK36	Negative	Negative	Negative	Negative
JAK 37	Negative	Negative	Negative	Negative
JAK 38	Positive	Positive	Positive	Positive
JAK 39	Negative	Negative	Negative	Negative
JAK 40	Negative	Negative	Negative	Negative
JAK 41	Positive	Positive	Positive	Positive
JAK 42	Negative	Negative	Negative	Negative
JAK 43	Positive	Positive	Positive	Positive
JAK 44	Negative	Negative	Negative	Negative

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APPENDIX C: Results of the three molecular assays (Continued)

Specimen number	Sequencing	Duplicate ASPCR	Real time	Historic
JAK 45	Negative	Negative	Negative	Negative
JAK 50	Positive	Positive	Positive	Positive
JAK 51	Positive	Positive	Positive	Positive
JAK 52	Negative	Negative	Negative	Positive
JAK 53	Negative	Positive	Negative	Positive
JAK 54	Positive	Positive	Positive	Positive
JAK 55	Positive	Positive	Positive	Positive
JAK 56	Positive	Positive	Positive	Positive
JAK 57	Positive	Positive	Positive	Positive
JAK 58	Positive	No result	Positive	Positive
JAK 60	Positive	Positive	Positive	Positive
JAK 61	Positive	Positive	Positive	Positive
JAK 62	Positive	Positive	Positive	Positive
JAK 64	Negative	Positive	Negative	Positive
JAK 65	Positive	Positive	Positive	Positive
JAK 66	Positive	Positive	Positive	Positive
JAK 69	Positive	Positive	Positive	Positive
JAK 70	Negative	Negative	Negative	Negative
JAK 71	Negative	Negative	Negative	Negative
JAK 72	Negative	Negative	Negative	Negative
JAK 73	Negative	Negative	Negative	Negative
JAK 74	Negative	Negative	Negative	Negative

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APPENDIX D: Threshold cycle values of the LNA probe real time PCR as generated by the Cepheid SmartCycler II system

Specimen number	FAM	HEX	Diagnosis
JAK1	25.27	25.92	Heterozygous mutant
JAK2	0	26.05	Homozygous wild type
JAK3	0	25.14	Homozygous wild type
JAK4	0	25.02	Homozygous wild type
JAK5	0	24.42	Homozygous wild type
JAK6	0	24.91	Homozygous wild type
JAK7	0	24.27	Homozygous wild type
JAK8	0	23.14	Homozygous wild type
JAK9	27.05	25.73	Heterozygous mutant
JAK10	25.77	26.41	Heterozygous mutant
JAK11	0	22.64	Homozygous wild type
JAK13	0	23.07	Homozygous wild type
JAK14	0	24.34	Homozygous wild type
JAK16	0	25.22	Homozygous wild type
JAK17	0	24.04	Homozygous wild type
JAK18	26.93	28.77	Heterozygous mutant
JAK20	0	24.39	Homozygous wild type
JAK21	0	23.97	Homozygous wild type
JAK22	0	24.8	Homozygous wild type
JAK23	26.19	25.9	Heterozygous mutant
JAK25	0	24.41	Homozygous wild type

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APPENDIX D: Threshold cycle values of the LNA probe real time PCR as generated by the Cepheid SmartCycler II system (Continued)

Specimen number	FAM	HEX	Diagnosis
JAK26	0	24.3	Homozygous wild type
JAK27	0	24.5	Homozygous wild type
JAK28	26.2	25.22	Heterozygous mutant
JAK29	28.7	26.08	Heterozygous mutant
JAK30	0	23.97	Homozygous wild type
JAK32	0	24.08	Homozygous wild type
JAK34	0	24.75	Homozygous wild type
JAK35	25.49	33.62	Heterozygous mutant
JAK36	0	24.2	Homozygous wild type
JAK37	0	23.51	Homozygous wild type
JAK38	28.56	23.42	Heterozygous mutant
JAK39	0	23.4	Homozygous wild type
JAK40	0	22.95	Homozygous wild type
JAK41	29.05	24.99	Heterozygous mutant
JAK42	0	22.42	Homozygous wild type
JAK43	24.39	26.13	Heterozygous mutant
JAK44	0	23.29	Homozygous wild type
JAK45	0	23.57	Homozygous wild type
JAK50	24.78	23.16	Heterozygous mutant
JAK51	24.63	23.21	Heterozygous mutant

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APPENDIX D: Threshold cycle values of the LNA probe real time PCR as generated by the Cepheid SmartCycler II system (Continued)

Specimen number	FAM	HEX	Diagnosis
JAK52	0	21.28	Homozygous wild type
JAK43	0	25.54	Homozygous wild type
JAK54	27.4	23.52	Heterozygous mutant
JAK55	24.49	21.37	Heterozygous mutant
JAK56	23.38	26.13	Heterozygous mutant
JAK57	24.18	26.03	Heterozygous mutant
JAK58	26.58	23.19	Heterozygous mutant
JAK60	24.59	21.62	Heterozygous mutant
JAK61	23.6	23.85	Heterozygous mutant
JAK62	23.74	24.13	Heterozygous mutant
JAK64	0	22.12	Homozygous wild type
JAK65	24.93	23.84	Heterozygous mutant
JAK66	23.93	22.19	Heterozygous mutant
JAK69	26.94	23.89	Heterozygous mutant
JAK70	0	22.82	Homozygous wild type
JAK71	0	22.64	Homozygous wild type
JAK72	0	22.87	Homozygous wild type
JAK73	0	25.91	Homozygous wild type
JAK74	0	24.76	Homozygous wild type

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