

**Mutational analysis of the Janus kinase 2 gene in patients with  
Polycythaemia Vera, Essential Thrombocythaemia and Primary  
Myelofibrosis**

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## DECLARATION

I hereby declare that the manuscript “**Mutational analysis of the Janus kinase 2 gene in patients with Polycythaemia Vera, Essential Thrombocythaemia and Primary Myelofibrosis**” submitted for the M.Med.Sc. (Human Molecular Biology) degree at the University of the Free State is my independent effort and has not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.”

All the resources I have made use of in this study are complete and acknowledged in my references.

Quintin Clive Goodyear

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## LIST OF ABBREVIATIONS

%	Percent
°C	Degrees Celcius
μA	MicroAmpere
μm	Micrometers
A	Adenine
ABL	V-abl Abelson murine leukemia viral oncogene homolog
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ASO	Allele Specific Oligonucleotide
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimeters
CML	Chronic Myeloid Leukaemia
Ct	Cycle Threshold
dL	Deciliters
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediamine tetra-acetic acid
Epo	Erythropoietin
EpoR	Erythropoietin-receptor
<i>et al</i>	And others
ET	Essential Thrombocythaemia
FERM	Band four-point-one, ezrin, radixin, moesin

g	Grams
G	Guanine
HRM	High Resolution Melting
JAB	JAK binding protein
JAK	Janus Kinase
JH	JAK homology domain
kDa	kilo Dalton
KV	Kilovolts
L	Liters
M	Molar
mg	Milligrams
min	Minutes
ml	Milliliters
mM	Millimolar
MPD	Myeloproliferative Disorder
MPN	Myeloproliferative Neoplasm
Mpl	Thrombopoietin Receptor
NCBI	National Center for Biotechnology Information
NK	Natural Killer
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PIAS	Protein Inhibitors of Activated STAT's
PMF	Primary Myelofibrosis
pmol/ $\mu$ l	Picomol per Microliter
POP	Performance Optimized Polymer
PV	Polycythaemia Vera
RNA	Ribonucleic Acid
s	Seconds
SH2	Src Homology 2
SLIM	STAT interacting LIM proteins
SNP	Single Nucleotide Polymorphism

SOCS	Suppressors of Cytokine Signaling
STAT	Signal Transducers and Activators of Transcription
T	Thymine
TAE	Tris Acetic Acid EDTA
T <sub>m</sub>	Melting Temperature
TYK2	Tyrosine kinase 2
u/μl	Units per Microliter
μl	Microliter
V2	Variant 2
WHO	World Health Organization
x g	Acceleration times gravity

## CHAPTER 1

### LITERATURE

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#### 1.1 Haematopoiesis

Haematopoiesis refers to the process of blood cell formation (fig 1.1). The primary site of haematopoiesis is in the bone marrow. The process can be divided into three stages. In the first stage, haematopoietic stem cells are formed in the marrow. These stem cells migrate to the liver or stay in the bone marrow where they are exposed to growth factors. The stem cells transform into one of two progenitor cell lineages, either the myeloid or lymphoid progenitor lineage. The lymphoid lineage gives rise to the cells of the adaptive immunity system and includes the B- and the T-lymphocytes, as well as the Natural Killer cells (NK). The B-cells produce antibodies and mature in the bone marrow. The T-cells mature in the thymus and are directly responsible for the disposal of antigens. The Natural Killer cells destroy tumour- and virally infected cells. The myeloid lineage differentiates into erythrocytes, platelets, neutrophils, eosinophils, mast cells, monocytes, macrophages and dendritic cells. The erythrocytes transport oxygen to the organs. Platelets are fragments of megakaryocytes and aid in the repair of damaged blood vessels. The neutrophils have polymorphic nuclei and serve as the body's first line of defence. Eosinophil nuclei have two or three lobes and these cells deal with parasitic infections. Mast cells have large granules which contain histamine and heparin and play a role in the allergic response. The monocytes and macrophages have lobe shaped nuclei and contain many granules. They serve as antigen presenting cells as well as phagocytosing bacterial cells. Finally, the dendritic cells also serve as antigen presenting cells (Hoffbrand *et al.*, 2006).

The aforementioned are all cellular components of blood. The cell counts of the various cells in the blood are closely regulated, however mutations in the various genes coding for the proteins of regulatory pathways can occur, leading to the formation of proliferative neoplasms.

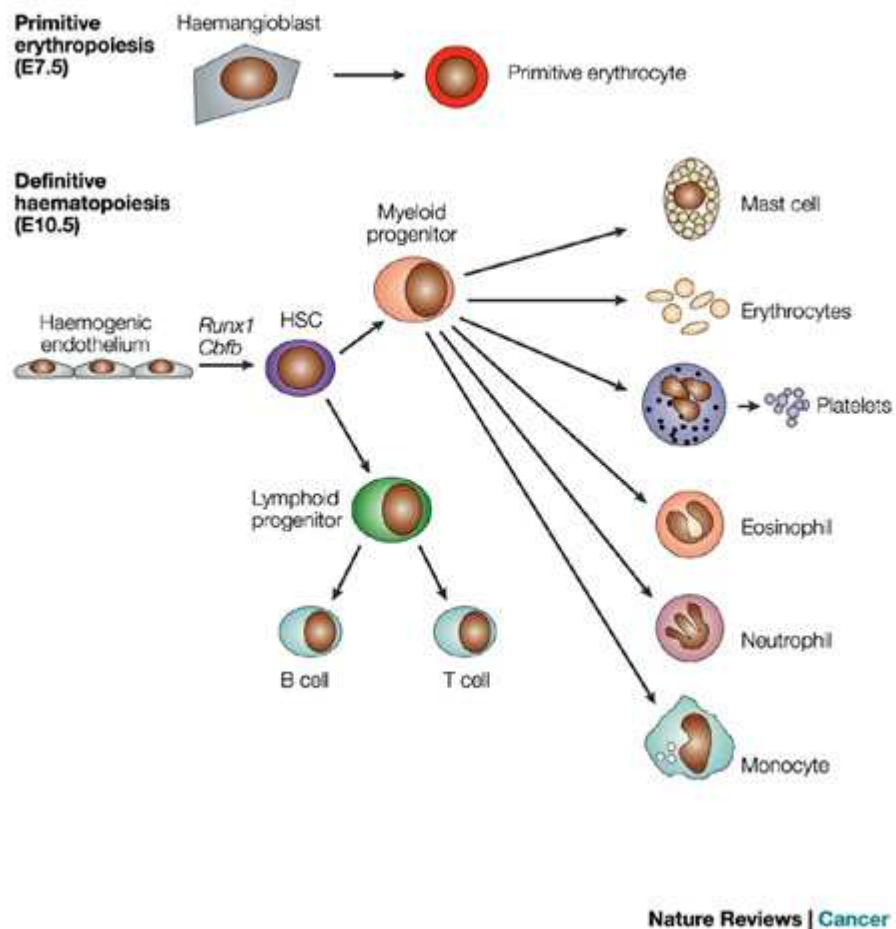


Figure 1.1: The process of haematopoiesis. (Adapted from Speck & Gilliland, 2002).

## 1.2 Myeloproliferative Neoplasms

Myeloproliferative Neoplasms (MPNs) are haematological malignancies characterized by an increase in the proliferation of the myeloid cells. These neoplasms were initially grouped together by Dameshek in 1951 (Dameshek, 1951). He proposed that all these malignancies occur due to uncontrolled clonal proliferation and are closely linked. He termed these conditions, myeloproliferative disorders (MPD). The four classical MPNs are polycythaemia vera (PV), essential thrombocythaemia (ET), primary myelofibrosis (PMF) and chronic myeloid leukaemia (CML).

### 1.2.1 Polycythaemia Vera

Polycythaemia vera (PV) is characterized by an increase in all the myeloid cells, predominantly the red blood cells. Tefferi *et al.* (2008) stated that Louis Henri Vaquez (1982) initially described this disorder in a 40 year old patient in 1892 in his paper “On a special form of cyanosis accompanied by excessive and persistent erythrocytosis”. The patient he examined had presented with several symptoms such as chronic distended veins, palpitations, hepatosplenomegaly and a significant increase in the number of erythrocytes (Stone, 2003). Although Vaquez initially suspected cardiac disease, the patient later died of acute tuberculosis (Stone, 2003). In the autopsy it was discovered that he had a considerably enlarged spleen and liver (Stone, 2003). Vaquez proposed that the cause of this condition was haematopoietic hyperactivity. William Osler continued this work and according to Stone (2003), Osler suggested in his papers that the patients had a distinct clinical picture but that the symptoms were vague. He termed it Vaquez’s disease or polycythemia with cyanosis (Tefferi *et al.*, 2008).

Since the first reported case of PV the criteria to diagnose the neoplasm has become well defined. Currently the World Health Organization (WHO) 2008 criteria is used for the diagnosis of polycythaemia vera. The criteria are divided into major and minor criteria. The diagnosis is confirmed when both major and one minor; or the first major and two minor criteria are met. The criteria to make the diagnosis are summarized in Table 1.1.

Table 1.1: The 2008 WHO criteria used to diagnose PV (Swerdlow *et al.*, 2008).

<b>Major criteria</b>
<ol style="list-style-type: none"> <li>1. Haemoglobin greater than 18.5 g/dL in men and higher than 16.5 g/dL in women.</li> <li>2. Presence of the JAK 2 V617F mutation or other functionally similar mutations such as JAK 2 exon 12 mutations.</li> </ol>
<b>Minor criteria</b>
<ol style="list-style-type: none"> <li>1. Bone Marrow biopsy showing hypercellularity for age with trilineage growth with prominent erythroid, granulocytic and megakaryocytic proliferation.</li> <li>2. Serum erythropoietin level below the normal reference range.</li> <li>3. Endogenous erythroid colony formation <i>in vitro</i>.</li> </ol>

There are several symptoms observed in PV patients and these can vary. High haematocrit and haemoglobin levels are found in PV patients. Other common symptoms include plethora, pruritus, splenomegaly, sweating, weight loss and weakness. Less common symptoms observed include chest pain, epistaxis, erythromelalgia, gout, thrombotic events, Budd-Chairi syndrome and neurological instability (Stuart & Vierra, 2004).

The reported annual incidence of PV varies between different populations. The review undertaken by Johansson (2006) indicated that the incidence ranges from 0.02 to 2.8 per 100,000 annually. The highest incidence (23.5 per 100,000) was reported in the

study of Ania *et al.* (1994) in men aged 70 to 79 years. Polycythaemia vera seems to be more prevalent in men (Gruppo Italiano Studio Policitemia, 1995; Ridell *et al.*, 2000) and the majority of patients are diagnosed after 60 years of age. Conflicting results in regard to gender was observed in a more recent study by Johansson *et al.* (2004) where no link between incidence and gender was found. Very few patients are diagnosed at an age younger than 40 years (Gruppo Italiano Studio Policitemia, 1995) and rare cases of children with PV being diagnosed have been reported (Danish *et al.*, 1980; Poggi *et al.*, 1984). The major risks of polycythaemia vera are venous and arterial thrombosis.

Several different treatments have been used to alleviate and treat the symptoms of polycythaemia vera. Such treatments include but are not limited to phlebotomy, <sup>32</sup>P, chlorambucil, hydroxyurea, interferon alpha and imatinib mesylate (Gleevec™). There is no “golden” treatment for polycythaemia vera and phlebotomy or a combination of the above mentioned are recommended (Campbell & Green 2005). According to Berk *et al.* (1986) patients treated with phlebotomy alone had the highest survival (13.9 years), however there was a high incidence of thrombotic events. Patients given acetylsalicylic acid in conjunction with phlebotomy showed no decrease in thrombotic events and gastrointestinal haemorrhaging occurred (Tartaglia *et al.*, 1986). Hydroxyurea has been shown to be just as effective as phlebotomy and the risk of thrombotic events are less and it is recommended for elderly patients (Fruchtman *et al.*, 1997). The use of interferon alpha has been shown to reduce the need for phlebotomies (Heis *et al.*, 1999), but due to its adverse effects it should be used in younger patients. Substances like chlorambucil and <sup>32</sup>P have been shown to increase the risk of developing into leukaemias (Berk *et al.*, 1986).



## 1.2.2 Essential Thrombocythaemia

Essential thrombocythaemia (ET) is characterized by increased platelet formation ( $>450 \times 10^9/L$ ) and changes in the shape and size of the platelets can also occur (Hoffbrand *et al.*, 2006). According to Sanchez & Ewton (2006) ET was first described by Epstein and Goedel (1934) under a different name of “Hemorrhagic Thrombocytheamia” in 1934. The condition is diagnosed using the WHO 2008 criteria (Table 1.2). In order to diagnose ET all four diagnostic criteria have to be met.

Table 1.2: The WHO 2008 criteria for ET (Swerdlow *et al.*, 2008).

1. Sustained platelet count  $\geq 450 \times 10^9/L$ .
2. Bone marrow biopsy specimen showing proliferation, mostly of the megakaryotic lineage with increased numbers of enlarged, mature megakaryocytes. No significant increase or left-shift of neutrophils, granulopoiesis or erythropoiesis.
3. Not meeting WHO criteria for PV, PMF, BCR-ABL1 positive chronic myelogenous leukaemia, myelodysplastic syndrome or other MPN.
4. Demonstration of JAK 2 V617F or other clonal marker, or in the absence of JAK 2 V617F, no evidence of reactive thrombocytosis.

Fatalities in essential thrombocythaemia are mainly due to three causes; thrombosis, haemorrhaging and possible transformation into a different leukaemia (Barbui & Finazzi, 2005). Thrombosis is a major cause of death and this can occur in several different blood vessels. Thrombosis that occurs in the large arteries is a high cause of mortality and can lead to severe complications in the neurological, cardiac and the lower extremities of the body. Examples of major arterial thrombosis are angina pectoris, myocardial infarction and transient ischemic attacks (Wolanskyj *et al.*, 2006). Major venous thrombotic events can also occur such as pulmonary embolism, deep vein thrombosis and intra-abdominal venous thrombosis (Wolanskyj *et al.*, 2006). Deep vein

thrombosis can arise, which is potentially fatal if it is portal or hepatic thrombosis (Chait *et al.*, 2005). Microvesicles can also become blocked and can cause a variety of symptoms. Aspirin-sensitive erythromelalgia is one of the most distinctive microvesicle disturbances (Brière 2007). Haemorrhaging or bleeding events can also occur in ET patients and can be potentially fatal. These events are often skin deep such as bruises, subcutaneous haematomas and bleeding in the gastrointestinal tract. The haemorrhaging can be fatal after trauma or surgery. Bleeding complications are mostly found in patients with platelet counts exceeding 1000 per  $10^9$  L and higher (Van Genderen *et al.*, 1996; Michiels *et al.*, 2004). Bleeding in these patients is usually due to von Willebrand Syndrome. Patients with ET can present with either thrombosis, haemorrhage or both, however some patients are entirely asymptomatic (Barbui *et al.*, 2004).

The incidence of ET is reported to be 1 to 2.5 per 100,000 (Sanchez & Ewton, 2006) and the median age of diagnosis is between 65 and 70, however this may vary (Brière 2007). The condition is more prevalent in women than men. Literature varies concerning the life expectancy of patients with ET. Some studies indicate that there is no difference in life expectancy of ET patients compared to healthy age and sex matched controls (Barbui *et al.*, 2004). Others indicate that the life expectancy decreases after the first decade of the condition in ET patients to 18.9 years with treatment (Wolanskyj *et al.*, 2006).

Treatment of ET is mainly to alleviate the symptoms and is non-specific. The recommended treatment for ET according to Finazzi & Barbui (2008) is a low dose of acetylsalicylic acid (100 mg) in low risk patients to prevent thrombotic complications and cytoreductive therapy such as hydroxyurea in high risk patients.

### 1.2.3 Primary Myelofibrosis

Primary myelofibrosis (PMF) is characterized by proliferation mostly of megakaryocytes and granulocytes, and in later stages the replacement of bone marrow tissue with fibrous connective tissue. Extramedullary haematopoiesis and hepatic haematopoiesis can also occur. It was first described by Gustav Heuck in 1879 (Tefferi, 2008). The criteria for the diagnosis of PMF are described in Table 1.3. In order to diagnose the condition all three major and two of the minor criteria of the WHO have to be met.

Table 1.3: WHO 2008 Criteria for PMF (Swerdlow *et al.*, 2008).

Major Criteria
<ol style="list-style-type: none"><li>1. Presence of megakaryocyte proliferation and atypia, typically in conjunction with either reticulin and/or collagen fibrosis, or in the absence of significant reticulin fibrosis, the megakaryocyte changes must be accompanied by an increased bone marrow cellularity characterized by granulocytic proliferation and often decreased erythropoiesis.</li><li>2. Not meeting WHO criteria for PV, BCR-ABL1+ chronic myelogenous leukaemia, myelodysplastic syndrome, or other MPNs.</li><li>3. Demonstration of JAK 2 V617F or other clonal marker (eg. MPLW515K/L), or in the absence of a clonal marker, no evidence that the bone marrow fibrosis or other changes are secondary to infection, autoimmune disorder or other chronic inflammatory condition, hairy cell leukaemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.</li></ol>
Minor Criteria
<ol style="list-style-type: none"><li>1. Leukoerythroblastosis.</li><li>2. Increase in serum lactate dehydrogenase level.</li><li>3. Anaemia.</li><li>4. Splenomegaly.</li></ol>

Primary myelofibrosis has two phases, a prefibrotic and a fibrotic phase. In the prefibrotic phase the bone marrow is hypercellular with minimal or absent reticulin fibrosis. The fibrotic phase is characterised by leukoerythroblastosis, splenomegaly, hepatomegaly and droplet shaped cells (Swerdlow *et al.*, 2008).

The symptoms for PMF varies and on average, 30% of PMF patients are asymptomatic at presentation. Common symptoms found include fever, night sweats, weight loss, anaemia, dyspnea and gout. Splenomegaly is found in 90% of patients and hepatomegaly is found in approximately 50% (Ahmed & Chang, 2006). The main cause of death in PMF is transformation to acute myeloid leukaemia, AML (Cervantes & Barosi, 2005).

The reported incidence for PMF ranges between 0.3 and 0.6 per 100,000 (Ridell *et al.*, 2000; Johansson *et al.*, 2004; Girodon *et al.*, 2005; Phekoo *et al.*, 2006; Kutti & Ridell, 2001). Higher incidences have been reported in other studies. Woodliff and Dougan (1976) found the incidence to be 1.8 per 100,000 and the highest incidence of 6.5 was reported by Chaitey *et al.* (1992) in a Jewish population. The incidence of PMF thus varies and is more frequent in elderly patients, median >60 years (Reilly *et al.*, 1997; Cervantes *et al.*, 1997; Dupriez *et al.*, 1996).

Primary myelofibrosis might also occur as a result of transformation of PV or ET into PMF. In the case of PV, one study found that 8 of the 48 (17%) patients being treated with chlorambucil transformed to PMF after 10 years (Najean *et al.*, 1994). This was also observed in the study by Messinezy *et al.* (1985). However, Passamonti *et al.* (2004) found the transformation to be 6% of PV patients over 15 years. Passamonti *et al.* (2004) found the transformation from ET to PMF to be 4% over 15 years. Transformation of ET to PMF was found in 13 cases in the study done by Cervantes *et*

*al.* (2002) and they stated the probability of this is 3% after 5 years of the initial diagnosis, 8% at 10 years and 15% after 15 years.

The only cure for PMF is allogeneic stem cell transplantation; the other treatments only alleviate the symptoms (Cervantes 2004). Treatments include cytoreductive therapy, androgens, splenectomy and radiation therapy. The median survival rate of treated patients with PMF was found to be approximately 5 years (Cervantes *et al.*, 1997). Patients with PMF can be divided into three risk groups based on the disease severity. These groups are low risk, intermediate risk and high risk. The study by Dupriez *et al.* (1996) indicated that the survival rate varied depending on the severity of the condition and found it to be almost 8 years in low, 2.2 in intermediate and 1 year for high risk patients. There are cases however, where PMF patients survive for decades (Cervantes 2004).

#### **1.2.4 Chronic Myeloid Leukaemia**

Chronic myeloid leukaemia (CML) is also a classic myeloproliferative neoplasm and is characterized by uncontrolled production of cells predominantly of the myeloid lineage. The reported incidence of CML is 1 to 2 per 100,000 annually and being more prevalent in men (Frazer *et al.*, 2007).

The cause of CML was identified to be the formation of an oncoprotein tyrosine kinase. The oncoprotein is formed by the fusion of the *BCR* (Breakpoint cluster region) gene on chromosome 22 and the *ABL* (*V-abl* Abelson murine leukemia viral oncogene homolog) gene on chromosome 9. This reciprocal transformation was first identified by Nowell & Hungerford (1960). The SH1 domain of the *ABL* gene is the tyrosine kinase domain and its regulatory domain is lost during the reciprocal translocation. The *BCR* gene

fragment that is fused with the *ABL* SH1 causes the tyrosine kinase to be constitutively active (Frazer *et al.*, 2007). Thus, the activity of the fusion product is not regulated and this results in cell proliferation. The protein formed varies between 185 kDa to 230 kDa depending on the breakpoints of the *BCR* (Sawyers *et al.*, 1999). Studies indicate that the 190 kDa oncoprotein has greater kinase activity than the 210 kDa protein (Lugo *et al.*, 1990, Voncken *et al.*, 1995). The different fusion products are thought to influence the severity of the disease and common symptoms are fatigue, severe weight loss, anaemia, thrombocytosis and splenomegaly (Sawyers *et al.*, 1999).

Due to the discovery of the cause of CML, possible molecular targeted therapies were investigated. The first specific treatment that was developed is the drug imatinib mesylate. Imatinib binds to the adenosine triphosphate (ATP) binding site of the oncoprotein thus inhibiting tyrosine kinase activity by preventing phosphorylation (de Kogel & Schellens, 2007). Since it was discovered that a tyrosine kinase caused the formation of one MPN, research was conducted to determine if other tyrosine kinases could cause other MPNs (James *et al.*, 2005a). This led to the discovery of JAK.

### **1.3 JAK 2 and tyrosine kinases**

The family of Janus kinases (JAKs) consists of four members including JAK 1, JAK 2, JAK 3 and TYK 2 (Ihle *et al.*, 1998). The molecular mass of the JAKs varies between 120 and 140 kDa (Pelligrini & Dusanter-Fourt, 1997) and each is found on a different chromosome. JAK 2 was previously known as “just another kinase” (McLornan *et al.*, 2006) but was later renamed after the Roman god of gates, Janus. JAK 2 is a tyrosine kinase and is found on progenitor cell receptors lacking tyrosine kinase activity such as the erythropoietin- (EpoR) and thrombopoietin-receptors (Mpl).

Tyrosine kinases are a vast group of proteins involved in many cellular functions, such as growth, metabolism and cell differentiation and they are regulated by the tyrosine phosphatases. Tyrosine kinases are enzymes that facilitate the transfer of a  $\gamma$ -phosphate group from an ATP-molecule to the tyrosine residue of a signal transduction molecule (Tefferi & Gilliland, 2005). There are two kinds of tyrosine kinases, receptor tyrosine kinases and non-receptor tyrosine kinases. Receptor tyrosine kinases can autophosphorylate once the ligand is bound. Non-receptor tyrosine kinases such as JAK 2 cause phosphorylation of receptors such as erythropoietin and thrombopoietin once the ligand is bound.

Hormones such as Epo (Erythropoietin) are released upon stimulation, for example when the oxygen carrying capacity in the blood is low, by the kidneys and bind to the receptors of the progenitor cells in the bone marrow. The complex formed between the receptor and its ligand causes dimerization of the receptor. The attached JAK 2 molecule becomes phosphorylated and this “activated” molecule then recruits signal-transducing molecules called STATs (Signal Transducers and Activators of Transcription). STAT molecules, located in the cytosol of the cell, bind to the SH2 domain of the receptor. Once bound the STAT molecules become phosphorylated. The phosphorylated STATs detach and dimerize. The dimerized molecule moves into the nucleus of the cell. Once within the nucleus it recognizes specific DNA sequences within the nucleus and attaches. Once it is bound, it mediates gene transcription and cell proliferation, fig 1.2 (Sandberg *et al.*, 2004). If Epo bound to the receptor the progenitor cell it differentiated into an erythrocyte. The process of erythrocytosis for example, is controlled by JAK 2s function. The pathway is known as the JAK-STAT pathway.

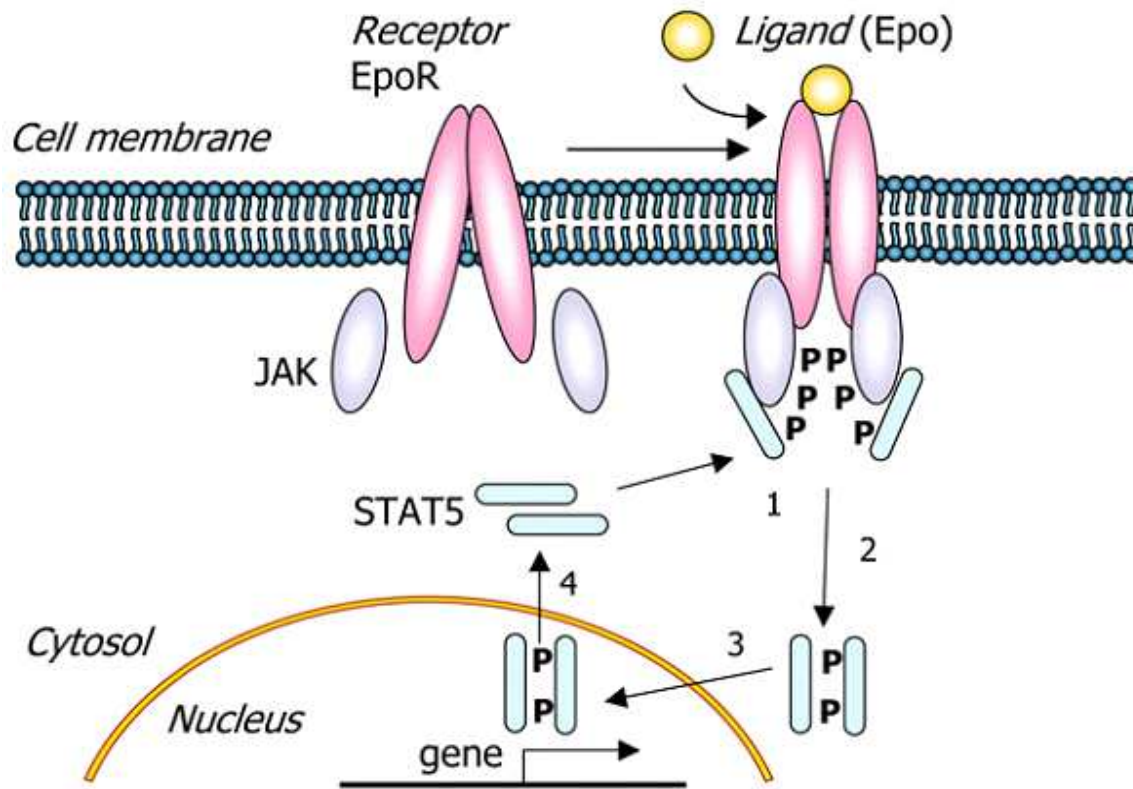


Figure 1.2: A simplified illustration of the JAK/STAT pathway. (Adapted from <http://www.biomedcentral.com>).

#### 1.4 JAK 2 genetic and protein structure

The JAK 2 gene is located on chromosome 9p24 (Yamaoka *et al.*, 2004) and consists of 25 exons (fig 1.3) which are divided into seven homology regions, JH1-JH7 (Sandberg *et al.*, 2004). The JH1 (JAK homology 1) domain is the active kinase domain and is encoded by exons 19 to 25, amino acid numbers, F836-V1123 (Lindauer *et al.*, 2001). The second domain is JH2, also known as the pseudokinase domain, and is encoded by exon 12 to the middle of exon 19, E543-N824 (Lindauer *et al.*, 2001). JH2 regulates the activity of the kinase domain (JH1). JH3 and JH4 are collectively known as the SH2



domain (Src Homology 2), which is responsible for the interaction between the kinase and the regulatory domain. Exons 9 to exon 11 encodes for this domain, L393-P500 (Giordanetto & Kroemer, 2002). The last domain is the FERM (Band four-point-one, ezrin, radixin, moesin) domain (JH4-JH7) and is responsible for the binding of the JAK 2 molecule to the receptor. This is encoded by exons 3 to the middle of exon 9, D36-Y382 (Giordanetto & Kroemer, 2002).

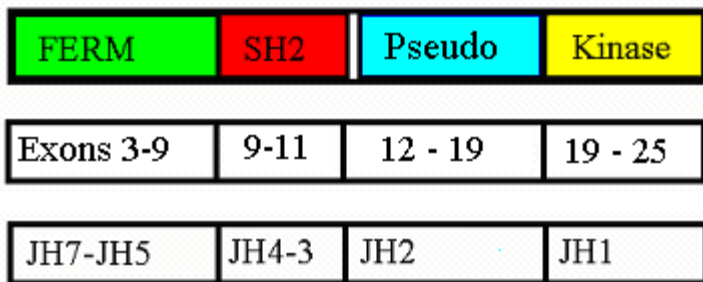


Figure 1.3: Image illustrating the different JAK 2 domains and the exons which encode for the regions (Adapted from James *et al.*, 2005b).

## 1.5 Regulation of JAK 2 Kinase activity

There are several factors involved in the regulation of JAK 2 kinase activity. JAK 2 can regulate its own kinase activity through its domain interactions. One regulator is the FERM domain of JAK 2 which regulates the cell surface expression of the receptor (Huang *et al.*, 2001). The binding of the JAK 2 molecule to the receptor via its FERM domain has also been suggested to initiate the kinase activity (Ihle & Gilliland, 2007). A crucial regulator of the kinase activity is the JH2 domain of JAK 2. Three regions have been identified in the JH2 domain which inhibit the activity of JH1, residues 758-807; 725-757; 619-670 (Saharinen *et al.*, 2003). Deletion of the JH2 domain led to

constitutive tyrosine kinase activity in a previous study (Saharinen *et al.*, 2000), illustrating the auto-inhibitory function of JH2 on JH1 kinase activity. The interaction between the JH1 and JH2 domain also influences the kinase activity. Two main areas of interaction has been identified, the two  $\alpha$ -helices of the JH1 and JH2 domains and between the activation loop of JH1 (D994-E1024) and a loop in JH2, V617-E621 (Lindauer *et al.*, 2001), which alters the catalytic activity of JH1. External factors also influence the JAK-STAT pathway.

Factors external to JAK 2 can influence the JAK-STAT pathway by augmenting or disrupting the phosphorylation. JAK 2 is initially activated once the ligand binds. This active state can be enhanced by additional molecules like Src Homology 2 B Adapter Protein SH2-B $\beta$  (Rui & Carter-Su, 1999). It was found that there was a 20 fold increase in kinase activity when JAK 2 was co-expressed with SH2-B $\beta$ . Phosphorylation of STAT3 and STAT5B was also considerably increased. There are several substances that can also down-regulate the JAK-STAT pathway. One method of regulation is to terminate the phosphorylation. Tyrosine phosphatases such as SHP-1 bind to the phosphotyrosine residue on JAK 2 and inhibit JAK 2 activity (Jiao *et al.*, 1996). Another group of inhibitors are Suppressors of Cytokine Signaling (SOCS). SOCS have several mechanisms by which they suppress JAK 2 function (Sandberg *et al.*, 2004). SOCS 1 and JAK binding protein (JAB) for example have been shown to bind to the tyrosine residue 1007 on JAK 2 and suppress it (Yasukawa *et al.*, 1999). The JAK-STAT pathway is also regulated by Protein Inhibitors of Activated STATs (PIAS). These proteins bind to STAT molecules and influence the extent and duration of JAK 2 phosphorylation (Sandberg *et al.*, 2004). PIAS can alter protein localisation, inter-protein interaction and stability and it influences the transcription of the protein (Khwaja 2006). STAT interacting LIM proteins (SLIM) have been shown to inhibit gene transcription of STATs 1 and 4 (Tanaka *et al.*, 2005).

## **1.6 Mutations in the JAK 2 gene that are associated with MPNs and the detection thereof**

### **1.6.1 The JAK 2 V617F mutation**

The first reported mutation in the JAK 2 gene associated with a MPN was independently identified by five groups in 2005 (Baxter *et al.*, 2005; James *et al.*, 2005a; Kralovics *et al.*, 2005; Zhao *et al.*, 2005; Levine *et al.*, 2005). The V617F mutation was found in exon 14 and is a point mutation where a guanine (G) is substituted by a thymine (T). The mutation results in an amino acid change from a valine to a bulkier phenylalanine. This mutation lies in the activation loop of JH2 and the amino acid change results in a constitutively active tyrosine kinase, regardless of receptor binding. The mutation has been shown to induce hypersensitivity to cytokine receptors and Epo (erythropoietin) (Kralovics *et al.*, 2005). The mutant clone has also been shown to have a survival advantage over the wild type (James *et al.*, 2005a, Kralovics *et al.*, 2005). The mutation causes autophosphorylation of JAK 2 as well as stronger phosphorylation (Zhao *et al.*, 2005). The mutation can either be heterozygous or homozygous. In the study by Kralovics *et al.* (2005) the homozygous JAK 2 V617F mutation led to an increase in the severity of the disease in myeloproliferative patients. The homozygous form of the mutation is most likely due to mitotic recombination (Levine *et al.*, 2005; Kralovics *et al.*, 2005). Patients who harbour the mutation had significantly more complications such as secondary fibrosis, thrombosis and haemorrhages than those MPN patients without the V617F mutation (Kralovics *et al.*, 2005).

Patients with ET harbouring the JAK 2 V617F mutation have been found to have a higher haemoglobin and white cell count with lower platelet counts in comparison to the V617F-negative patients (Campbell *et al.*, 2005). These patients also presented with a higher risk of thrombosis. Homozygosity is rare in patients with ET (Baxter *et al.*, 2005;

James *et al.*, 2005a; Kravolics *et al.*, 2005; Levine *et al.*, 2005; Larsen *et al.*, 2007a; Vannucchi *et al.*, 2007; Antonioli *et al.*, 2008). The thrombotic risk has been found to be 3.9 fold higher in homozygous V617F patients than those homozygous for the wild type allele (Vannucchi *et al.*, 2007a). Transformation from ET to PMF is also higher in JAK 2 V617F positive patients (Vannucchi *et al.*, 2007a). The allele burden of the mutation was found to be higher in patients with splenomegaly and it was also observed in patients with arterial thrombosis at diagnosis (Antonioli *et al.*, 2008). Patients with a high mutational burden also had increased erythrocyte production. This results in a clinical picture similar to PV. The median reported allelic burden is the lowest in ET patients, 7-24% (Antonioli *et al.*, 2008; Larsen *et al.*, 2007a).

There is a spectrum of individuals that are heterozygous, homozygous mutant or homozygous wildtype for the V617F mutation within the PV affected population. The prevalence of homozygosity for the V617F mutation in the PV population is between 20% to 30% (Levine *et al.*, 2005; Baxter *et al.*, 2005; Kravolics *et al.*, 2005; Tefferi *et al.*, 2006; Larsen *et al.*, 2007a; Vannucchi *et al.*, 2007a). In the study by Larsen *et al.* (2007a) it was observed that patients who were homozygous for the V617F mutation had a significantly higher incidence of splenomegaly, lower platelets levels, higher leukocyte concentrations and higher CD34 counts. Low haematocrit levels as well as low haemoglobin levels were also observed. These findings were confirmed by Vannucchi *et al.* (2007a); however they found a higher haematocrit level in homozygous patients. No correlation between cardiovascular events and the state of the mutation was found. Patients who were homozygous for the V617F mutation required more frequent chemotherapy. The homozygous mutant PV patients had a higher probability to transform into PMF (Tefferi *et al.*, 2006; Vannucchi *et al.*, 2007a). The mutational load is higher in patients with PV in comparison to ET (Passamonti *et al.*, 2006; Larsen *et al.*, 2007a; Vannucchi *et al.*, 2007b). The range of the mean allelic burden varies greatly; one study reports the mean of the allelic burden in PV patients to be as high as 52% (Vannucchi *et al.*, 2007b). Patients with a load of 65% or greater are more prone to splenomegaly (Vannucchi *et al.*, 2007b; Larsen *et al.*, 2007a). Patients with a load

greater than 75% have a 3.56 fold higher risk of thrombosis compared to the reference population (Vannucchi *et al.*, 2007b). Patients with such a high mutational load of the V617F mutation required more chemotherapy.

Primary Myelofibrosis patients harbouring the V617F mutation have been shown to have a poorer survival rate (Tefferi *et al.*, 2005; Campbell *et al.*, 2006) as well as a higher number of white blood cells and neutrophils (Campbell *et al.*, 2006). The reported frequencies for the homozygous mutation is between 5.7% and 27.6% (Barosi *et al.*, 2007; Kravolics *et al.*, 2005; Tefferi *et al.*, 2005) however it has also been reported at 85% (Larsen *et al.*, 2007a). Heterozygosity for V617F is in the region of 30% to 45% (Barosi *et al.*, 2007; Kravolics *et al.*, 2005; Tefferi *et al.*, 2005). In the study by Barosi *et al.* (2007) it was found that the rate of transformation from heterozygous to homozygous was 10 per 100 patient-years. The homozygous patients tend to have a greater risk of splenomegaly, higher white blood cell counts, and higher frequency of pruritus than the heterozygous or wild-type patients (Barosi *et al.*, 2007). Higher platelet counts were observed in patients who were heterozygous for the mutation (Barosi *et al.*, 2007). The mean allelic burden found in PMF patients is 54% to 67% (Guglielmelli *et al.*, 2007; Larsen *et al.*, 2007a). Of note is that patients with a low allelic burden of V617F (1-25%) were found to have a poorer survival rate (Guglielmelli *et al.*, 2007).

Patients with ET have the lowest allelic burden of V617F and the lowest prevalence of homozygotes. PV has an intermediate range of both whereas PMF has the highest allelic burden of V617F and highest prevalence of homozygotes. This is indicative that these three disorders might be one disorder in various stages, which might explain how one mutation is found in all three MPNs.

### **1.6.2 Prevalence of the JAK 2 V617F mutation**

The prevalence of the V617F mutation in the different MPNs varies. In PV patients, the mutation is found in 95% of individuals whereas its prevalence is at 60% in individuals suffering from ET or PMF (Vannucchi *et al.*, 2009). The V617F mutation has been found in other disorders as well. Examples thereof are chronic neutrophilic leukaemia (McLornan *et al.*, 2005; Jones *et al.*, 2005; Steensma *et al.*, 2005), idiopathic splenic vein thrombosis (Regina *et al.*, 2007), hyper eosinophilic syndrome (Jones *et al.*, 2005), systemic mastocytosis (Steensma *et al.*, 2005) and refractory anemia (Steensma *et al.*, 2005; Szpurka *et al.*, 2006)

### **1.6.3 JAK 2 in the “normal” healthy individuals and the JAK 2 46/1 haplotype**

Several studies have investigated the prevalence of the JAK 2 V617F mutation in seemingly healthy individuals. A study performed by Xu *et al.* (2006) found the V617F mutation in 37 of the 3,935 control samples. However these samples were collected from patients in a hospital and this raises the question of how healthy these individuals were. Sidon *et al.* (2006) confirmed that the V617F mutation is found in healthy controls, however they examined a small cohort and the mutation was found at a low level (below 0.01% mutated alleles) which might not be clinically significant. On the contrary McClure *et al.* (2006) did not find the V617F mutation in their tested healthy population. In a more recent study 198 samples were tested from blood donors and patients with non-haematological conditions. The mean percentage of the V617F clone in regard to wild type was 0.0071% and all the samples fell below the 1% limit of clinical relevance (Martinaud *et al.*, 2010). Thus the JAK 2 V617F mutation is found in normal controls but the levels are below that of clinical importance.

Recently haplotypes have been identified that may predispose patients to acquire the V617F mutation. Kilpivaara *et al.* (2009) identified a germline SNP allele in the JAK 2 locus (rs10974944) that predisposes the patient to the formation of a MPN. It was found in conjunction with the V617F mutation and also increased the familial risk of MPN formation. The SNP is found on two haplotypes of JAK 2 namely 46 and 1. Jones *et al.* (2009) found that the JAK haplotype consisting of type 46 and type 1 in MPN patients is a factor for the development of JAK 2 V617F MPNs. This haplotype also has been reported to predispose patients for thrombopoietin receptor (Mpl) mutations (Jones *et al.*, 2010) and inferior survival has also been implicated in PMF patients (Teferi *et al.*, 2010). A different haplotype GGCC was identified by Olcaydu *et al.* (2009). It was found in 88% of their cases and was found to be more frequent in JAK 2 V617F patients. They suggest that the haplotype may increase susceptibility to DNA damage or replication errors and lead to hyper-mutability.

#### **1.6.4 JAK 2 exon 12 Mutations**

As stated before the V617F mutation is found in 95% of the patients with PV. The JAK 2 gene was screened for other mutations in the remaining 5% whom are negative for V617F (Scott *et al.*, 2007). This led to the identification of mutations in exon 12. Initially Scott *et al.* (2007) found 4 mutations in exon 12 and linked it to an increase in red blood cell production, low platelet levels, low leukocytes as well as low levels of erythropoietin in serum. More than 18 mutations have since been identified in exon 12 (Butcher *et al.*, 2008; Williams *et al.*, 2007; Pardanani *et al.*, 2007; Martínez-Aviléz *et al.*, 2007; Pietra *et al.*, 2008; Percy *et al.*, 2007; Li *et al.*, 2008; Schnittger *et al.*, 2009; Ma *et al.*, 2009; Bernadi *et al.*, 2009; Kouroupi *et al.*, 2008; Colaizzo *et al.*, 2007; Jones *et al.*, 2008). The most frequent reported mutation in exon 12 is the N542-E543del (Scott *et al.*, 2007; Pardanani *et al.*, 2007; Percy *et al.*, 2007; Pietra *et al.*, 2008; Kouroupi *et al.*, 2008; Schnittger *et al.*, 2009). The majority of the reported mutations are heterozygous, however homozygosity has been reported (Percy *et al.*, 2007; Pietra *et al.*, 2008,

Schnittger *et al.*, 2009). Of note was that a mutation in exon 12 was found in conjunction with the V617F mutation only once before and only in one patient (Li *et al.*, 2008). The prevalence of exon 12 mutations in V617F-negative polycythaemia vera patients varies from 10-100% (Ormazábal *et al.*, 2008; Scott *et al.*, 2007; Butcher *et al.*, 2008; Pietra *et al.*, 2008; Williams *et al.*, 2007; Martínez-Aviléz *et al.*, 2007; Pardanani *et al.*, 2007; Schnittger *et al.*, 2009).

### **1.6.5 Other JAK 2 mutations in MPNs**

Several studies have investigated JAK 2 exons 12 and 14 for mutations in MPN patients however few studies have reported mutations in other regions of the gene. In PV patients, silent mutations have been described in exon 19 where a cytosine (C) is replaced by a thymine (T) and similarly in exon 25 (C-T) by Ormazábal *et al.* (2008). A more recent study done by Ma *et al.* (2009) found 18 mutations in exon 12, 12 in exon 13, seven in exon 14 and two in exon 15. A total of 19 mutations in that study were novel. From the literature, it appears that exons 12 to 15 in the JAK 2 gene, is a mutational hotspot (Ma *et al.*, 2009) and this region encodes for the regulatory domain and has the potential to influence the kinase activity.

### **1.6.6 Detection of the JAK 2 gene mutations**

Mutations in the JAK 2 gene can be the cause of several MPNs and has even been included in the diagnosis of these disorders. Accurate methods of detection such as allele specific- PCR, sequencing and high resolution melting curve analysis are needed to detect these mutations. Allele specific PCR is the most sensitive method used, however it has the limitation of detecting only known specific mutations (Jones *et al.*, 2008). Sequencing is also used and has the advantage that it can detect any mutations



in a specific sequence, thus it has a wider application than conventional PCR, but the disadvantage is that it is less sensitive. Sequencing can determine the exact base that is mutated (Jones *et al.*, 2008). More recently melting curve analysis has been used to identify the presence of mutations. The advantages thereof is that it is rapid, sensitive and more cost effective than sequencing however it has the limitation that it only detects the presence of a mutation, but does not identify it (Lay *et al.*, 2006). It uses either probes or dyes to monitor the accumulation of amplification product as it is formed. The dyes bind to double-stranded DNA and as this dissociates due to an increment increase in temperature, fluorescence is released. This fluorescence at these increments are measured and plotted as a curve (Reja *et al.*, 2010). The profile formed by the melting curve is dependent on the fragment length as well as the sequence composition. A wild-type profile is generated from a sample containing no mutations to establish a reference. The samples are melted as well and the plotted curves are compared to the reference. If there is a SNP in the sequence, the melting profile changes, indicating a difference compared to the reference. Samples with a similar profile are grouped together. An example of mutation shift is illustrated in the figure 1.4 below.

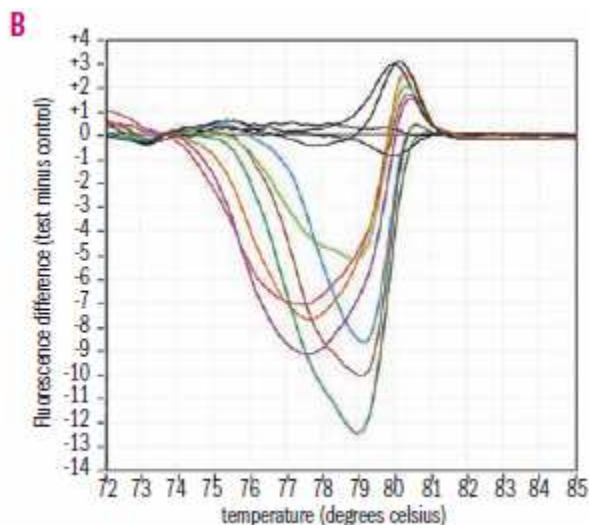


Figure 1.4: A difference plot of several samples screened for exon 12 mutations (Jones *et al.*, 2008).

## **1.7 Rationale**

Several studies have been performed on exons 12 and 14 of the JAK 2 gene in MPN patients however; very few have examined the remaining exons. Some researchers have identified possible mutations in the other exons of JAK 2 and they indicate that it may be the cause of a MPN. In addition several patients have a MPN but are negative for mutations in exons 12 and 14.

## **1.8 Aim of the study**

The aim of the study was to detect reported and novel SNPs in exons 3 to 25 of the JAK 2 gene. Each patient's granulocytes cDNA was compared against their own T-lymphocytes cDNA to determine if it could be used as an internal control. High resolution melting curve analysis was used for the screening of the patient's exon and sequencing was used for confirmation and identification.

## CHAPTER 2

### MATERIALS AND METHODS

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#### 2.1 Study Design

The study design was an observational descriptive study. The JAK 2 gene was screened for mutations using a combination of high resolution melting curve analysis and sequencing. The prevalence of these mutations in various MPNs was recorded.

#### 2.2 Sampling

##### **Samples**

Based on the results of a local study (data not shown) on exons 12 and 14 of the JAK 2 gene, patients were selected who were diagnosed using the WHO 2008 criteria. Five tubes of EDTA (Ethylenediamine tetra-acetic acid) blood were collected from the patients who fulfilled the inclusion criteria, from the Haematology Clinic at Universitas Hospital, Kimberly, and Bethlehem. Ethics approval was granted by the ethics committee of the Faculty of Health Sciences, University of the Free State (ETOVS NR15/08). The cohort consisted of 15 patients, six PV, five ET and four MF (Table 2.1). Informed consent was obtained before phlebotomy. The patient's granulocytes were used as samples.

**Inclusion criteria:** Patients were diagnosed with PV, ET or PMF by a clinician using the WHO 2008 Criteria (Swerdlow *et al*, 2008).

**Exclusion criteria:** Not diagnosed with the above mentioned MPNs

**Controls:**

Each patient's own T-lymphocytes were used as an internal control. T-lymphocytes were isolated with magnetic beads.. T-Cells from each subject were processed to establish a reference sequence. This in turn was compared to the reference sequence for the JAK 2 gene ([http://www.ensembl.org/Homo\\_sapiens/Transcript-/Sequence\\_cDNA?db=core;g=ENSG00000096968;r=9:49850335128183;t=ENST00000381652](http://www.ensembl.org/Homo_sapiens/Transcript-/Sequence_cDNA?db=core;g=ENSG00000096968;r=9:49850335128183;t=ENST00000381652), accessed on 31-Oct-2011).

**Inclusion Criteria:** The majority of the cells consisted of T-lymphocytes.

**Exclusion Criteria:** If the population contained less than 70% T-lymphocytes the sample was discarded.

Table 2.1: A summary of the patients used in the study together with their diagnosis. The patients with the same MPN are grouped together.

<b>Patient no</b>	<b>Myeloproliferative Neoplasm</b>
Patient 1	Polycythaemia vera
Patient 2	Polycythaemia vera
Patient 4	Polycythaemia vera
Patient 5	Polycythaemia vera
Patient 10	Polycythaemia vera
Patient 11	Polycythaemia vera
Patient 3	Essential thrombocythaemia
Patient 7	Essential thrombocythaemia
Patient 8	Essential thrombocythaemia
Patient 9	Essential thrombocythaemia
Patient 12	Essential thrombocythaemia
Patient 6	Primary myelofibrosis
Patient 13	Primary myelofibrosis
Patient 14	Primary myelofibrosis
Patient 15	Primary myelofibrosis

### **2.3 Isolation of granulocytes from whole blood**

Five tubes of 5 ml EDTA (7.2 mg) blood were collected from the patients and layered onto 3 ml of Histopaque 1077® (Sigma-Aldrich, Steinheim, Germany) in 15 ml Falcon tubes, ensuring that the layers did not mix. After centrifugation at 700 x g for 30 min, the plasma layer was discarded and the lymphocyte layer was transferred to a separately labelled tube for T-cell enrichment. The buffy layer containing the granulocytes was transferred to a separate tube. The cell suspensions were washed with a PBS (Phosphate Buffer Saline) solution ,concentration X1, and resuspended in 4 ml of PBS.

## **2.4 T-cell enrichment**

The lymphocyte layer still contained some myeloid cells; therefore it was treated with magnetic T-cell fluorobeads (One Lambda, Canoga Park, USA). A volume of 100  $\mu$ l of the beads were added to the lymphocytes and kept on ice for three minutes with constant rotation. Afterwards the tube was placed on a magnet for three minutes to allow separation from the rest of the cells. The supernatant was discarded before the tube was removed from the magnet and the cells were resuspended in 1-2 ml of PBS. The suspended cells were homogenized in TRI-Reagent® (Applied Biosystems, Foster City, USA).

## **2.5 Removal of T-lymphocytes from the granulocytes**

A 100  $\mu$ l volume of the T-cell fluorobeads was added to the tube containing the granulocytes and erythrocytes. This was kept on ice for three minutes followed by placing it on a magnet for an additional three minutes. The supernatant was transferred to a separate tube and stabilized in TRI-Reagent®. The two cell populations (granulocytes and lymphocytes) after isolation were analysed on the Sysmex XE-2100 (Sysmex House, Garamonde Drive, Wymbush, Milton Keynes) to determine the purity of the cells by means of a full blood count.

## **2.6 TRI-Reagent® homogenization of granulocytes and lymphocytes**

As erythrocytes were still present in the cell population it was lysed before stabilization. A volume of 50 ml red cell lysis buffer (100 ml of 0.114M  $\text{NH}_4\text{Cl}$  pH 7.4, 100 ml of 0.01M  $\text{NH}_4\text{HCO}_3$  and 800 ml of sterile deionized  $\text{H}_2\text{O}$ ) was added to both enriched T-lymphocyte and granulocyte tubes for 10 minutes and mixed occasionally. The tubes were centrifuged at 3,000 x g for 10 minutes and the

supernatant was discarded. This was performed twice and 3.2 ml of TRI-Reagent® was added to the cell pellets. The pellets were dissolved by pipetting and aliquots of the homogenate were frozen at -70°C for later RNA extraction.

## **2.7 RNA extraction**

The TRI-Reagent® homogenates were thawed for 5 minutes at 37°C and 15 µl Proteinase K, 20.2mg/ml, (Fermentas, Burlington, Ontario, Canada) was added to each sample. This was incubated at 65°C for 20 minutes and 350 µl of chloroform was added. The samples were placed on ice for three minutes and afterwards were centrifuged at 12,000 x g for 15 minutes. The RNA in the upper phase was retained. A volume of 350 µl absolute isopropanol was added to each sample after which they were incubated on ice for 30 minutes. After the incubation the samples were centrifuged at 12,000 x g for 10 minutes and the supernatant was discarded. The RNA pellet was washed twice with 1 ml of 70% EtOH and the pellet was left to dry in a fume cabinet for 10 minutes. The pellet was rehydrated by adding 40 µl of DEPC treated water and left to dissolve for 15 min at 55°C. The extracted RNA of the samples was electrophoresed on a 3% agarose ethidium bromide stained gel to determine the presence. The absorbance was measured at 260 using the GeneQuant Pro DNA/RNA Calculator (Biochrom, 22 Cambridge Science Park, Milton Road, Cambridge, UK) to determine the RNA concentration. The extracted RNA was stored at -80°C for later use.

## **2.8 Complementary DNA Synthesis**

The RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada) was used for complementary DNA (cDNA) synthesis. The reagents used are summarized in Table 2.2. The RNA, random hexamer primers and DEPC

treated water was mixed to a volume of 12  $\mu\text{l}$  and denatured for 5 minutes at 65°C. Afterwards the reaction buffer, RNase inhibitor, dNTP's and the RevertAid™ enzyme were added and incubated for 5 minutes at 25°C followed by a 60 minute step at 42°C. The reaction was terminated by heating at 70°C for 5 minutes.

Table 2.2: A list of the reagents used in cDNA synthesis and the amount required.

<b>Reagent</b>	<b>Volume (<math>\mu\text{l}</math>)</b>
Template RNA	10
Random Heximer Primers (0.2 $\mu\text{g}/\mu\text{l}$ )	1
DEPC-treated water	1
5X Reaction Buffer	4
RiboLock™ RNase Inhibitor (20u/ $\mu\text{l}$ )	1
10mM dNTP mix	2
RevertAid™ M-MuLV Reverse Transcriptase (200u/ $\mu\text{l}$ )	1
<b>Total reaction volume</b>	<b>20</b>



## 2.9 Polymerase Chain Reaction

The cDNA was used as a template for the polymerase chain reaction (PCR) reaction. A manual Hot Start PCR reaction was used with a total reaction volume of 27.5  $\mu$ l. A mastermix was made of the reagents in Table 2.3. A volume of 27.5  $\mu$ l was aliquoted into each tube and 2  $\mu$ l of cDNA was added. The samples were amplified on the thermal cycler starting with an initial denaturing phase of 95°C for 10 minutes. The run consisted of 40 cycles, containing a step at 95°C for 15 seconds, T<sub>m</sub> (Melting temperature) -5°C for 15 seconds and 72°C for 45 seconds step. The samples were held at 72°C for 7 minutes afterwards. The PCR product was stored at 4°C until it was loaded on an agarose gel. T<sub>m</sub> was calculated using the nearest neighbour method. Exons 1 and 2 were not included in the study as it is non-coding.

Table 2.3: The reagents needed the PCR of one sample.

Reagent	Volume ( $\mu$ l)
5X Buffer (Clear)	5
MgCl <sub>2</sub> (25mM)	2
dNTP's (10mM)	0.5
Forward primer (100pmol/ $\mu$ l) <sup>1,2</sup>	0.2
Reverse primer (100pmol/ $\mu$ l) <sup>1,2</sup>	0.2
GoTaq (5u/ $\mu$ l) (Promega, 2800 Woods Hollow Road, Madison, USA)	0.2
Nuclease free water	19.4
<b>Total reaction volume</b>	<b>27.5</b>

1. The primers differ for the different exons.
2. The primers were designed using Light-Cycler Probe Design 2™ software (Roche, 68298 Mannheim, Germany).

Table 2.4: List of the primers used for the study as well as their Tms. The primers sequences below is written 5' to 3'.

Exon	Forward	Reverse	Tm Average (Design program)
3	CTCTTCCTCCTCCTCCC	CACAGGTGTGATACCACAA	60°C
4	CCATCTGGGGAGTATGT	CATGCCGATAGGCTCT	59.9°C
5	CAACCAGGCATAATGTACT	TCTAACACTGCCATCCC	60°C
6	GTGCTGAAGCTCCTCT	CTGCGAAATCTGTACCTTATT	59.8°C
7A	TGGGATGGCAGTGTTAG	TCTCTGTGTAGAAGGCAG	59.7°C
7B	CCAATGCAAAGCCACT	TGCTTGCTTAATACTGACATCAA	59.3°C
8	TTCAGTGGTCAAGAGGGA	ACACGAAAGACAAAGCT	59.4°C
9	GCCGAGTTGTAACATCCA	CCAGTCTGATTACCTGCT	60.3°C
10	GCAGATGCACATCATTACC	CTTCTTTGTCCCCTGAGG	60.5°C
11	TGTATGTACTTCGATGCAGT	GCCTCTGTAATGTTGGTG	59.8°C
12	AACTGTTGCTCGAGACA	AGTTGACCGTAGTCTCCTA	59.8°C
13	CACCAACATTACAGAGGC	AACCAAATGCTTGTGAGAA	59.9°C
14	GACTACGGTCAACTGCA	AGCAACTTCAAGTTTCCATAAT	59.9°C
15	ATGTGTCTGTGGAGACG	TGCCAGGATCACTAAGTT	60.2°C
16	ATTATGGAACTTGAAGTTGCT	TTTTTAGGATTTTCAATGCATTCAG	59.7°C
17	AACTTAGTGATCCTGGCAT	GGTTTGCTAATTCTGCCC	59.9°C
18	ACCTCTAAGTGCTCTGG	CAGGGCACCTATCCTCA	59.5°C
19	GCCTTCTTTCAGAGCCA	ACATCTCCACACTCCC	59.9°C
20	GGGTTTTCTGGTGCCT	GTGATCTATCCGTTCTTTTATGT	59.9°C
21	CCCTACAGCATGACAAC	GCCAGATCCCTGTGGA	59.8°C
22	GTTTACGAGACTATCTTCAAAAACA	CCAAACATCTGAGGCCA	60°C
23	CACAGGGATCTGGCAAC	CAAATGGAACACGATCATCT	60.1°C
24	AGCTTTGGAGTGGTTCT	TCCCTAAAGGAGGGGC	60.1°C
25	GCAATGACAAACAAGGACAG	AGTCCACAGCAATGTGAA	60.3°C

## **2.10 Gel Electrophoresis**

After the PCR was completed the samples were electrophoresed on an agarose gel to confirm whether or not the PCR was successful and to determine if the amplicon was the correct size. The correct amount of agarose was weighed off to make a 3% gel and TAE X1 buffer was added. The solution was heated in a microwave oven on high for 2 minutes or until the agarose was fully dissolved and 3 µl of ethidium bromide was added after slight cooling. The gel was poured into the casting tray and left for 20 minutes to solidify. Afterwards the gel was submerged in 1X TAE buffer and 10 µl aliquots of the loading buffer and the PCR product were loaded onto the gel as well as a 100 bp molecular weight marker (Promega, Madison, USA). The gel was electrophoresed at 150 V for approximately 30 minutes. A picture was taken of the gel and the results were interpreted.

## **2.11 Clean-up of the PCR product**

The amplicon length differed between the exons and the correct size fragment (confirmed with a 100 bp molecular weight marker) was excised from the gel using a 1 ml pipette tip. The excised product was placed in 100 µl nuclease free water and left overnight to diffuse out of the agarose. This supernatant was used as template for the high resolution melting curve and the sequencing reactions.

## **2.12 High Resolution Melting Curve PCR (HRM-PCR)**

HRM was performed to screen for mutations using MeltDoctor™ (Applied Biosystems, Foster City, USA). The initial hot start PCR product was used as

template for the reaction. A mastermix was made of the reagents listed in Table 2.5. The melting curve reaction was performed on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The samples were amplified again before melting. The amplification was performed under the following conditions: Initial phase of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 58°C for 1 minute. After amplification was completed the product was melted under the following conditions: 95°C for 10 seconds, 60°C for 1 minute, 95°C for 15 seconds and 60°C for 15 seconds. The ramp rate started at 60°C rising by 0.03°C per second until it reached 95°C. After determination of an aberrant HRM profile, sequencing of the PCR product was performed. Each patient's granulocyte profile was compared to the lymphocyte profile. A shift from the lymphocyte profile indicated the presence of a change in the sequence.

Table 2.5: The reagents used and the amount required per melting curve reaction

<b>Reagent</b>	<b>Required Amount (µl)</b>
MeltDoctor™ Master Mix	10
H <sub>2</sub> O	8.6
Template PCR product	1
Forward Primer (100pmol/µl)	0.2
Reverse Primer (100pmol/µl)	0.2
<b>Total Reaction Volume</b>	<b>20</b>

### 2.13 Sequencing

Sequencing of PCR products was performed to confirm the presence of a mutation and determine its position in the exon. Sequencing of the purified PCR products was performed using the ABI Big Dye Terminator Ready reaction kit v3.1 (Applied Biosystems, Foster City, USA). The parameters for the sequencing reaction were 25 cycles of denaturation at 96°C for 10 seconds, a annealing at  $T_m$ °C for 5 seconds and extension at 60°C for 30 seconds. A mastermix of each primer was made for the reaction and the reagents are listed in Table 2.6

Table 2.6: The reagents for the sequencing cocktail and the amounts necessary.

Reagents	Required per sample (µl)
2.5 Big Dye Terminator	4
5X Sequencing Buffer	2
Primer (1pmol/µl)	3.2
Purified PCR Product	8
H <sub>2</sub> O	2.8
<b>Total</b>	<b>20</b>

### 2.14 Purification of sequencing product

Purification of the sequence reaction products was performed using ethanol/sodium acetate precipitation in micro centrifuge tubes according to ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit protocol which was slightly

modified. A mixture was made containing 3  $\mu$ l of 3M sodium acetate (pH 5 and 4°C), 63  $\mu$ l of ice-cold 95% ethanol and 14.5  $\mu$ l sterile distilled water. A volume of 10  $\mu$ l of the sequencing product was added. This was vortexed for 30 seconds and centrifuged at 12, 700 x g for 15 seconds. Incubation in the dark at room temperature for 30 minutes followed and the samples centrifuged for 30 minutes at 12, 700 x g. The supernatants were aspirated and 250  $\mu$ l of 70% ethanol (-20°C) was added. The samples were vortexed for 2 minutes and were centrifuged again for 10 minutes at 12, 700 x g. The supernatants were aspirated again and the samples were incubated for 1 minute at 90°C to allow the pellets to dry.

### **2.15 Resuspension of the samples for automated sequencing with POP-7**

After the pellets were dried, 25  $\mu$ l of Hi-di formamide (Applied Biosystems, Foster City, USA) was added to resuspend the samples. This was vortexed for 1 minute and centrifuged for 30 seconds. Afterwards the samples were denatured at 95°C for 2 minutes and placed on ice for 5 minutes. The samples were vortexed for 15 seconds and centrifuged at 12, 700 x g for 30 seconds. The resuspended samples were stored at 4°C in the dark until they were loaded on the sequencer.

### **2.16 Capillary electrophoresis**

The resuspended samples were electrophoresed on the ABI Prism 3130 Genetic Analyser (Applied Biosystems, Foster City, USA). The separation was performed in a coated capillary, 36 cm by 50 $\mu$ m internal diameter, filled with POP-7 polymer (Performance Optimized Polymer 7, Applied Biosystems, Foster City, USA). The samples were electro-kinetically injected for 12.5 s at 1.2 KV and electrophoresed for approx 90 minutes at 8.5 volts/cm, 5  $\mu$ A and 60°C.

## **2.17 Data analysis**

After completion of the capillary electrophoresis the sequencing results were analysed using Sequencing Analyses v5.3.1 (Applied Biosystems, Foster City, USA). The settings for analysis were SeqPop7Vs3 protocol and the instrument protocol was LongRapidSeqPop7 using the Z: bigDyeV3 Dye Set. The data obtained from the analysis was checked using Chromas lite ([http://www.technelysium.com.au/-chromas\\_lite.html](http://www.technelysium.com.au/-chromas_lite.html), accessed on 5 May 2008) and compared with reported sequence data. The sequences were aligned using Lalign ([http://www.ch.embnet.org/-software/LALIGN\\_form.html](http://www.ch.embnet.org/-software/LALIGN_form.html) accessed 2009-2011).

## **2.18 Data capture and statistics**

The data obtained was recorded in an Excel spreadsheet. This sheet contained information regarding each patient's JAK 2 gene sequence and the mutations, if present. A biostatistician was not needed as it was basic statistics such as calculating frequencies that were used in the study.

## CHAPTER 3

### RESULTS AND DISCUSSION

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#### 3.1 Sampling

Blood samples were collected from 15 MPN patients and cell separation was performed as previously discussed. A full count was performed on the two isolated cell populations (granulocytes and lymphocytes) using the Sysmex XE-2100 (Sysmex House, Garamonde Drive, Wymbush, Milton Keynes) to determine the cell purity. The full blood count results are summarized in Table 3.1. The average granulocyte population consisted of 96.6% myeloid cells and the controls (T-lymphocyte population) consisted of 70.4% lymphocytes. There was thus considerable contamination within the controls as there were other cells present from the myeloid lineage. The fluorobeads used in the cell separation are magnetic and coated with a monoclonal antibody specific for the CD2 marker on T-lymphocytes. (Product insert :Fluorobeads®-T and Fluorobeads-T Developer, One Lambda, 21001 Kittridge Street, Canoga Park, USA). The T-lymphocytes bind to the magnetic beads and are separated by use of a magnet. However, neutrophils and monocytes are phagocytic cells and may engulf the beads as it is seen as a foreign body. These cells can then get separated together with the T-lymphocytes (Dynabeads® M-450 Technical handbook). This is a possible reason why the controls consisted of almost 30% myeloid cells. Both cell populations of the 15 patients were stabilized with Tri-Reagent®.



Table 3.1: The purity of the separated granulocyte and lymphocyte populations as determined by means of a full blood count.

	<b>Granulocytes</b>	<b>Lymphocytes</b>
Cell Type	Percentage of cells	Percentage of cells
Neutrophils	86.9	7.7
Monocytes	2.1	21.9
Eosinophils	7.6	0.0
Lymphocytes	3.4	70.4

### 3.2 RNA Extraction and cDNA Synthesis

RNA was successfully extracted from the Tri-Reagent® stabilized samples (data not shown). The lowest concentration of the total extracted RNA was 25.6 ng/µl. Complementary DNA was successfully synthesized from the granulocyte as well as the lymphocyte RNA. The yield of the extracted RNA was low in some of the samples (data not shown) and this might be due to degradation.

RNA is very susceptible to degradation by RNAses and incorrect preparation could lead to degradation. RNA is also vulnerable at low temperatures (Holland *et al*, 2003). The results obtained from degraded samples cannot be compared to that of intact samples. RNA integrity can influence the expression pattern of a gene and the expression can vary between different tissues (Pérez-Novo *et al*, 2005). A recent study by Kang *et al* (2011) also illustrated that stabilization of samples in substances like TRIzol® can preserve the RNA quality for a long period of time when frozen. The half-life of RNA is minutes to days (Vennemann & Koppelkamm, 2010). The RNA was immediately transcribed to cDNA as it is more stable. cDNA was successfully synthesized from all the samples (data not shown). The cDNA was used as a template for the PCR reaction.

### 3.3 PCR and HRM Optimization

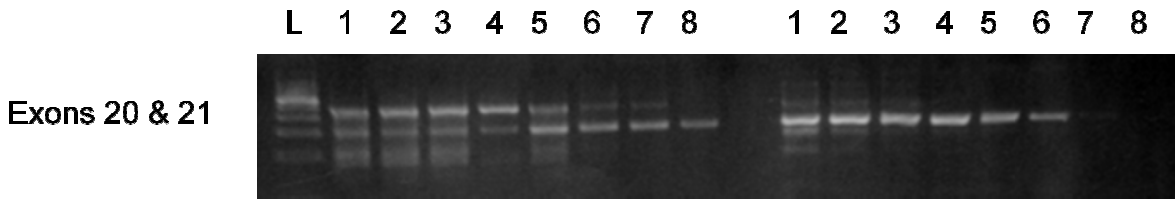
The PCR for the different exons of the cDNA of a healthy control was setup and amplified in a thermal cycler using the gradient function with the annealing temperature set between 55°C and 65°C. The optimal annealing temperatures for the primers are summarized in Table 3.2. The PCR product in the majority of the exons amplified optimally at 61.6°C. However, complications were observed during the optimization (fig 3.1). Multiple size amplicons were observed for some of the exons. This was investigated and is discussed later in this chapter.

Fragment excision was used to remove the PCR products from the agarose gels and purified. This excised stabbed product was used as template for the high resolution melting curve analysis. The Ct value for optimal high resolution melting curve analysis must be in the range of 20-25 cycles (A guide to High Resolution Melting (HRM) Analysis). A dilution series consisting of undiluted, 1:20; 1:1,000; 1:10,000; 1:100,000 and 1:1 000, 000 was setup. At the 1:1 000, 000 dilution the Ct value was 24.18 for the granulocytes and 25.98 for the lymphocytes. Therefore the excised amplicons of the samples were diluted 1:1000, 000 in all the melting curve reactions. Diluting the product also minimizes the effects of possible inhibitors. The analyses of the individual exons are discussed in the next sections.

Table 3.2: A summary of the optimal annealing temperatures for the different primer pairs.

<b>Exon</b>	<b>Optimal annealing temperature (°C)</b>
3	61.6
5	61.6
6	61.6
7a	61.6
7b	61.6
9	61.6
10	61.6
12	61.6
17	61.6
21	61.6
25	61.6
20	62.6
22	62.6
23	62.6
4	59.7
8	59.7
11	59.7
14	59.7
15	59.7
16	59.7
19	59.7
13	58.3
18	58.3
24	58.3

A.



B.

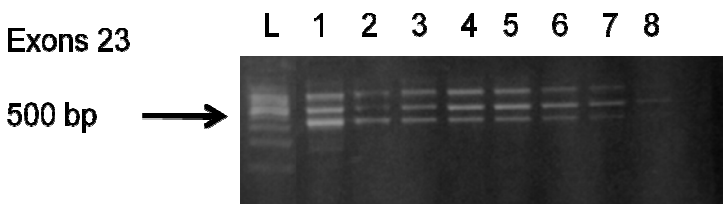


Figure 3.1: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the gradient PCR products for exons 20, 21 and 23 of the cDNA of a healthy control. Figure A shows the multiple amplicons formed at the different annealing temperatures for exons 20 and 21 and figure B illustrates those for 23. Lane L: 100 bp molecular weight marker. Lane 1: T<sub>m</sub> 55.1°C. Lane 2: T<sub>m</sub> 55.4°C. Lane 3: T<sub>m</sub> 56°C. Lane 4: T<sub>m</sub> 57°C. Lane 5: T<sub>m</sub> 58.3°C. Lane 6: T<sub>m</sub> 59.7°C. Lane 7: T<sub>m</sub> 61.6°C. Lane 8: T<sub>m</sub> 62.6°C.

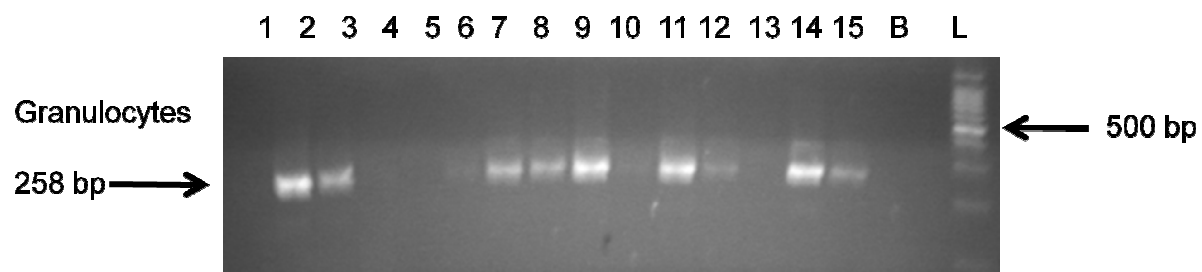
### 3.4 Analysis of Exon 14 of the JAK 2 gene

#### 3.4.1 The PCR of Exon 14

Exon 14 was the region examined first as the well described V617F mutation lies within this exon. This exon also forms part of the regulatory domain of the JAK 2 gene. A PCR was setup using the conditions stated earlier and the product was electrophoresed on a gel (fig 3.2). Some of the samples did not amplify. The PCR was repeated using the amplicon of the first reaction as template and the same set of primers (Double

PCR). The second PCR was successful in amplification of all the samples, however faint additional fragments were observed with the granulocyte samples (fig 3.3). The technique has been shown to increase the sensitivity of the assay. In addition it has also been shown to overcome issues such as PCR inhibition (Mayer & Palmer, 1996). This is a well established technique and variations thereof have been used in haplotyping (Eitan & Kashi, 2002). The double PCR increased the sensitivity and was used for all the exons examined further. The expected size amplicons were excised from the gel, purified and diluted (1:1 000,000) for use in the high resolution melting curve analysis.

A.



B.

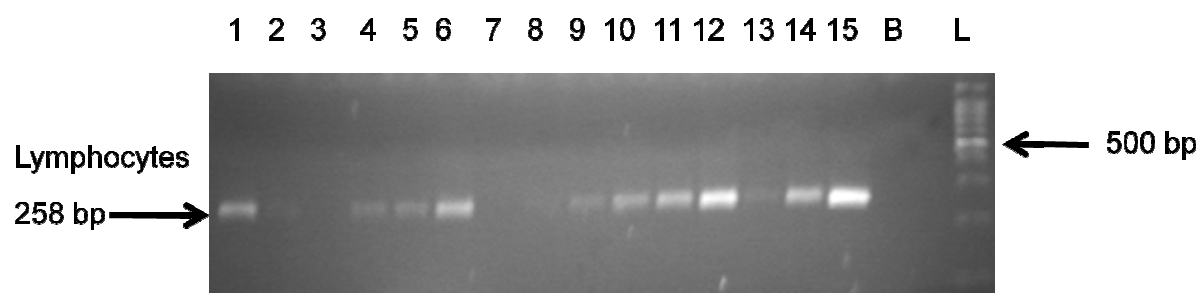
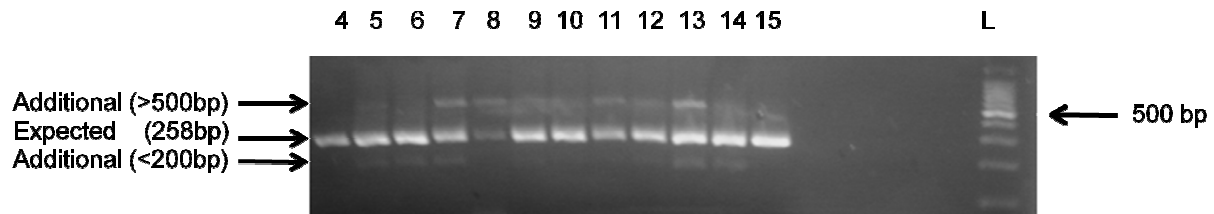


Figure 3.2: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the initial PCR of the cDNA of exon 14 of all the samples for the granulocytes (A) and the lymphocytes (B). The samples that failed initial amplification were subjected to a second PCR using the initial PCR product as template. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

A.



B.

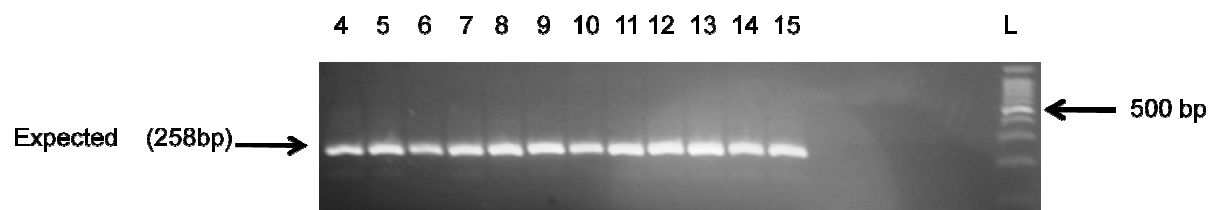


Figure 3.3: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the products of the double PCR of exon 14 for the granulocytes (A) and lymphocytes (B) for all the samples. Lanes 4-15: Patients 4-15. Lane L: 100 bp molecular weight marker.

### 3.4.2 Melting Curve Reaction of Exon 14

All 30 samples (15 granulocytes, 15 lymphocytes) were processed together using the conditions stated in Table 2.5. Multiple peaks were observed (fig 3.4) in the derivative melting curve plot. The melting curve product was loaded onto a 3% ethidium bromide stained agarose gel to determine whether multiple sized products were present. The gel illustrated that in most of the samples in both lineages three fragments were present (fig 3.5). The sizes of the three products were: 160 bp for the smallest one (termed V2), 258 bp for the expected amplicon and ~500 bp for the larger fragment (termed V1). The three different sized amplicons for each sample were excised, purified and used for sequencing.

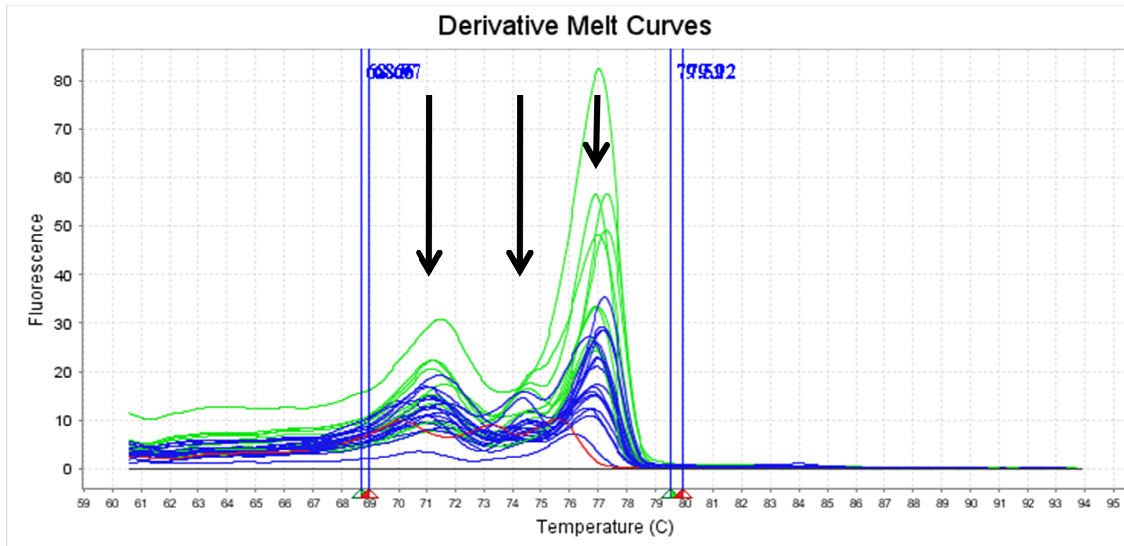
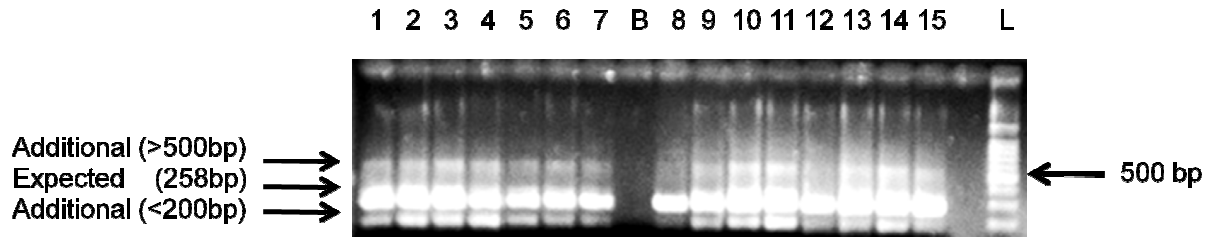


Figure 3.4: The derivative melt curve plot of the high resolution melting curve reaction of exon 14 for the granulocytes as well as the lymphocytes of all the samples. Released fluorescence is plotted against the melting temperature of the products. Three variants (different colours) as well as three peaks (indicated by the arrows) were observed indicating the presence of multiple sized fragments.

A.



B.

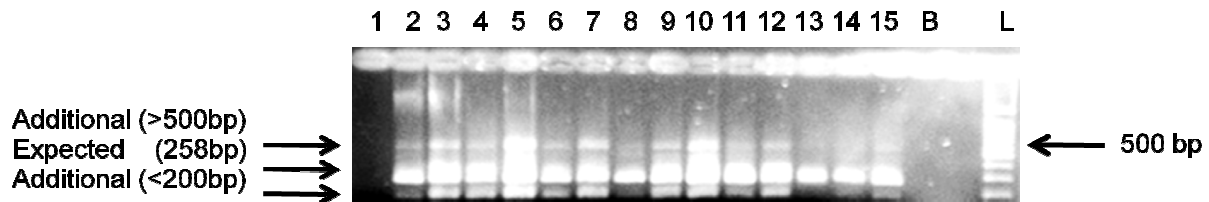


Figure 3.5: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the melting curve products of exon 14 of the granulocytes (A) and lymphocytes (B) of all the samples. At least three different sized amplicons were observed in both lineages in all of the samples. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

### 3.4.3 Sequencing Analysis of the Expected 258 bp Amplicon of Exon 14

The expected fragment was successfully sequenced in all 30 samples. The G-T, V617F mutation was successfully detected by sequencing in some of the samples. This mutation however was also found in the lymphocytes of some of the samples. Literature varies whether the V617F mutation is strictly found only in the myeloid cells. Several studies have indicated that the V617F mutation is not found in mature B- and T-lymphocytes (Baxter *et al.*, 2005; James *et al.*, 2005b; James *et al.*, 2005a Scott *et al.*, 2007; Lasho *et al.*, 2005; Martinez-Avilés *et al.*, 2007; Pietra *et al.*, 2008; Li *et al.*, 2008;



Butcher *et al.*, 2007). Bogani *et al.* (2007) however, using similar separation methods, found the V617F mutation in the T-lymphocytes, B-lymphocytes and NK cells in approximately half of their PMF patients. The sample size in their study was small and only consisted of 12 patients and their cell purity was 93%, indicating that approximately 7% of the cells were non-myeloid. Larsen *et al.* (2007b) used the FACSVantage to separate the cell types and used a very sensitive quantitative real-time PCR. The V617F mutation was found in the T-lymphocytes of nine of the 13 patients included in their study. The mutation load in two of these patients was above 60%. The cell population in their study also was not absolutely pure and in addition real-time PCR was used with a sensitivity of 1:1,000. Therefore, it is possible that the results obtained might be due to granulocyte contamination of the lymphocyte subset.

In a study by Ishii *et al.* (2006) it was found that the V617F mutation was present in the B-lymphocytes of two patients and in the T-lymphocytes of one patient. They used a three step purification process to isolate the cells and the purity of the isolated B- and T-lymphocytes was found to be equal and greater than 99%. Of note is that the patient in their study who had the V617F mutation in the T-lymphocytes was homozygous for the mutation in the other myeloid cells, as well as the CD34+/Stem cells. They propose that the V617F mutation is found in the T-lymphocytes of a subgroup of patients with PV. Delhommeau *et al.* (2007) also used a three stage purification process and had a cell purity greater than 98%. In their study only two of the patients with PV had the mutation in their B-lymphocytes and NK cells. It was not observed in the T-lymphocytes of the PV patients; however it was found in the T-lymphocytes of two PMF patients at a very low level. Whether the V617F mutation is found only in the myeloid lineage is still debatable, however if it is present in the lymphoid cells it might originate from a very early precursor cell type.

In our study, the V617F mutation was found in the T-lymphocytes in 10 out of 15 patients. The mutation was heterozygous in all of these samples. The remaining five patients were negative for this mutation in their lymphocytes. Eight patients were heterozygous, two homozygous and five were negative for this mutation in their

granulocytes. Patients 4 (PV) and 13 (post PV PMF) showed homozygosity for the V617F mutation. The minor lymphocyte contamination of the granulocyte fraction did not seem to have an effect on the sequencing data of exon 14. Two patients were found to be homozygous for the mutation and thus the wild type of the lymphocytes was not detected. The electropherograms in figure 3.6 indicate the sequences for the three states of the V617F mutation (homozygous, heterozygous and wild-type). Homozygosity has been reported previously in PMF and PV patients as discussed earlier. Of note however, is that patient 4 had a neutrophil count three times higher than the normal reference range, which has not been reported previously in the literature. Five of the six PV (83.4%), three of the five ET (60%) and two of the four PMF (50%) patients had the V617F mutation. This correlates well with the frequencies reported in the literature.

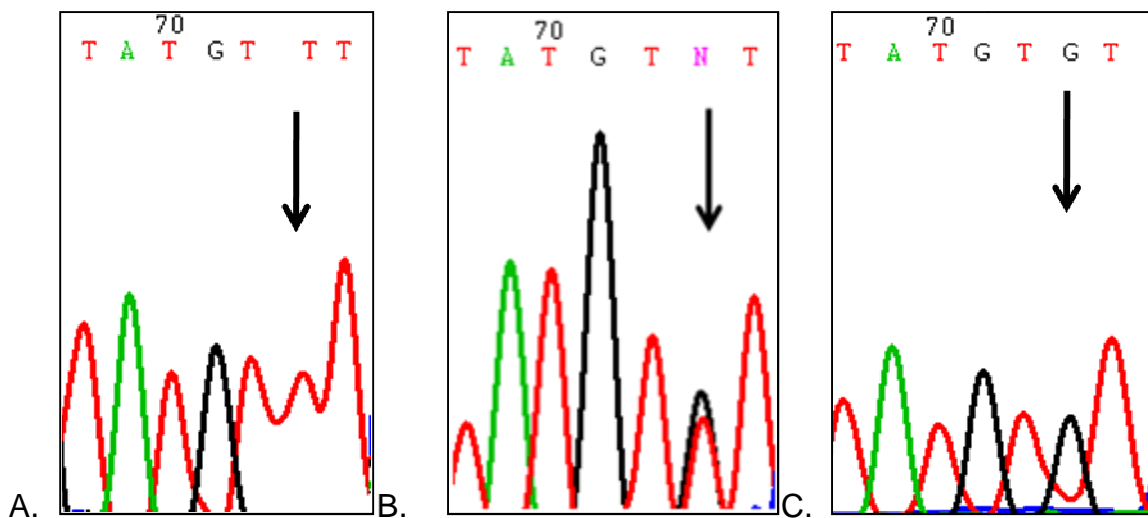


Figure 3.6: Three sequencing electropherograms of sections of exon 14, indicating the three states of the V617F mutation in the study. Figure A indicates the homozygous mutant form of V617F in patient 4. Figure B illustrates the heterozygous form and figure C indicates the wild type.

All the patients that were positive for the V617F mutation in their granulocyte population were also positive in their T-lymphocyte population. The reason for this most probably is that the cell purity of the T-lymphocytes was only at 70% and the mutation was thus detected due to granulocyte contamination. The presence of the V617F mutation in both lineages of the patients is tabulated in Table 3.3. Due to the fact that the T-lymphocyte population might not be pure enough, it was decided to only use the granulocyte cDNA of the patients for the analysis of the other exons.

Table 3.3: The presence and zygosity of the V617F mutation in the granulocyte and lymphocyte populations of all the samples. The patients with the same MPN are grouped together and X indicates the presence of this mutation. Patients 5, 8, 12, 6 and 15 are negative for the mutation.

Patient no	MPN	Granulocyte		Lymphocyte	
		Homozygous	Heterozygous	Homozygous	Heterozygous
Patient 1	PV		X		X
Patient 2	PV		X		X
Patient 4	PV	X			X
Patient 5	PV				
Patient 10	PV		X		X
Patient 11	PV		X		X
Patient 3	ET		X		X
Patient 7	ET		X		X
Patient 8	ET				
Patient 9	ET		X		X
Patient 12	ET				
Patient 6	PMF				
Patient 13	PMF	X			X
Patient 14	PMF		X		X
Patient 15	PMF				

### 3.4.4 Sequencing Analysis of the Variant Amplicons of Exon 14

In addition to the expected sized amplicon, two other fragments were also observed; the larger (Variant 1, V1, approx 500 bp) and the smaller (Variant 2, V2, 160 bp) fragments. These fragments were excised from the gel, purified and sequenced.

The primers used to amplify the cDNA of exon 14 lie within exon 13 and exon 15. Thus the expected exon 14 amplicon contains the last 72 bases of exon 13, all 88 bases of exon 14 and the first 98 bases of exon 15 (fig 3.7). Sequencing of fragment V2 indicated that it is shorter than expected due to the deletion of the entire exon 14 (fig 3.8). Thus V2 consists of exon 13 and exon 15. This has been reported before in a study by Ma *et al.* (2009). In their study they also used first strand cDNA synthesis from extracted RNA, PCR and sequencing. They proposed that this is most likely a splice variant as it was not detected in genomic DNA. Ma *et al.* (2010) did a follow up study on the exon 14 deletion and found that it was only detected in MPN and suspected MPN patients. Furthermore they found that the deletion leads to a truncated JAK 2 protein.

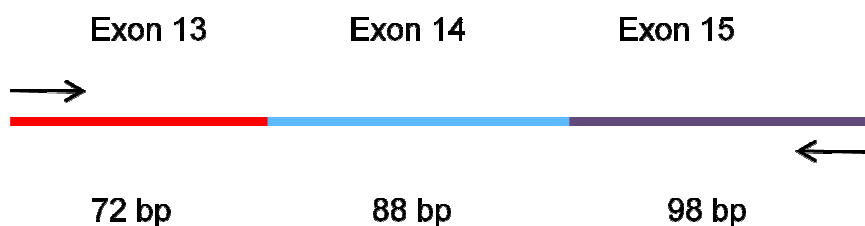


Figure 3.7: A depiction of the expected amplicon of exon 14. The forward primer lies in exon 13 and the reverse primer in exon 15. The total amplicon size is 258 bp and consists of 72 bp fragment of exon 13, the 88 bp of exon 14 and 98 bp fragment of exon 15.

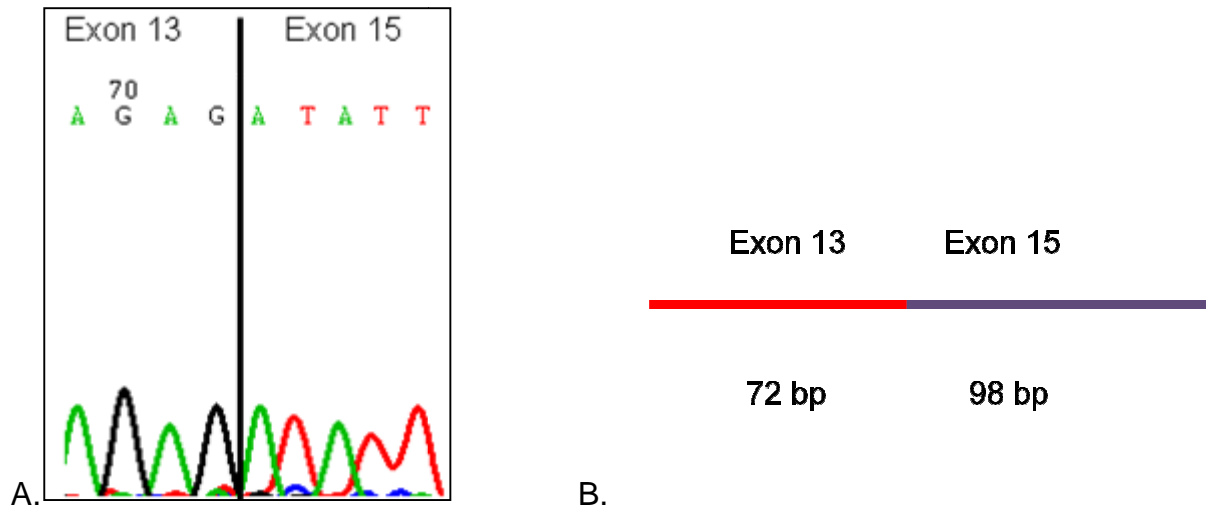


Figure 3.8: A section of the sequencing electropherogram showing the composition of the smaller fragment (V2) found after the amplification of exon 14. Figure A depicts that exon 13 is immediately followed by exon 15. Figure B is a schematic of the size and conformation of the V2 fragment.

The sequencing results of the larger variant (V1) was more complex. After several attempts the larger V1 fragment could only be sequenced in the reverse direction. The sequencing indicated a section which had a single sequence, followed by a section of overlapping sequence and ending again in a single sequence (fig 3.9). As this was the reverse sequence the initial single sequence observed was a section of exon 15, followed by the overlapping sequence and lastly a single sequence again. After analysis the overlapping sequence was shown to be a fragment of exon 13 overlapping the expected exon 14 fragment. It is thus possible that the overlapping observed sequence might be the V2 fragment (containing exon 13 and 15) and the expected fragment (containing exon 14) overlapping one another (fig 3.10). The size of the observed overlapping sequence did not correlate with the size of the fragment observed on the agarose gel. After examining the reverse sequence data further, another faint sequence was observed following the expected exon 13 fragment. This extra sequence could successfully be analyzed for approximately 60 bases. It was found to be an

inversion of exon 13 followed by an inverted section of exon 14. All of the above might explain the composition of the larger fragment V1 (fig 3.11). Of concern is that the forward reactions were not successful and therefore it is possible that other splice variants or possible presence of SNPs might affect the primer binding site in exon 13.

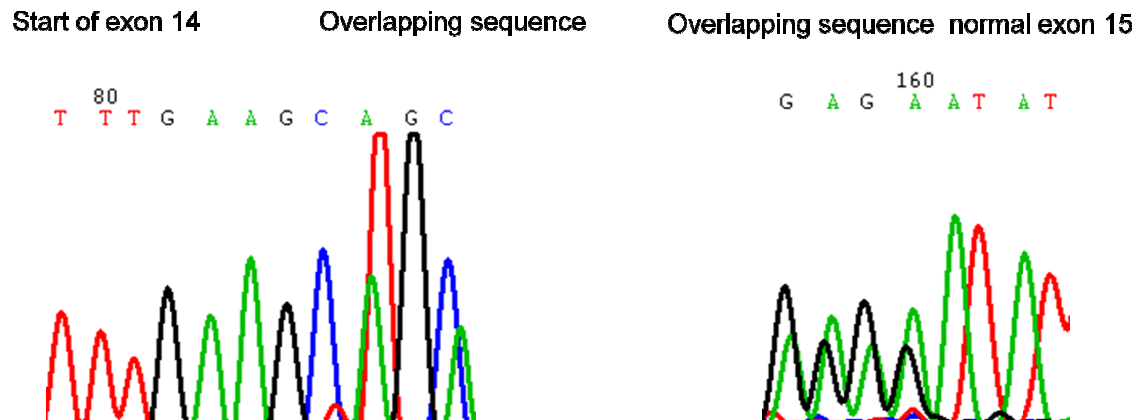


Figure 3.9: The reverse sequencing electropherogram obtained from the larger V1 fragment of exon 14. The sequence starts with a single sequence (exon 15) for approximately 50 bases. This is followed by a overlapping sequence of 72 bp (overlap of exons 13 and 14) and ends again with a single sequence containing 16 bases of exon 14 followed by 72 bp of exon 13.

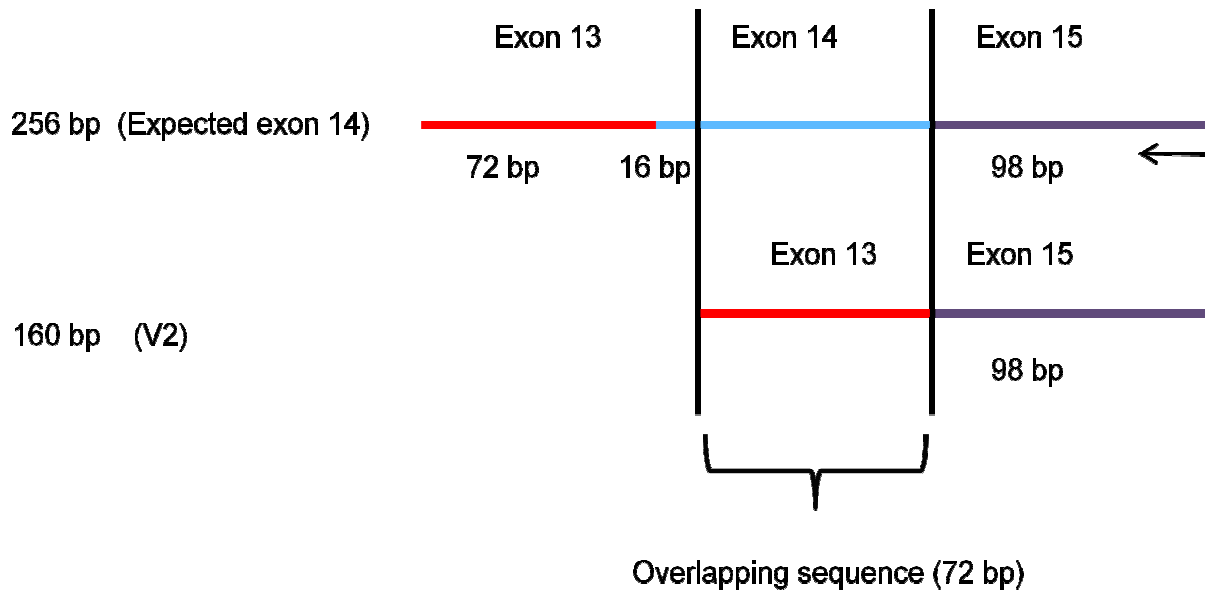


Figure 3.10: A schematic illustrating the possible composition of the double sequence observed in the larger exon 14 fragment V1. There might be two clones present. As this is the reverse sequence, the length of exon 15 is identical in both fragments. After exon 15 is the double sequence which might be a section consisting of an overlap of exons 13 and 14. The last section is single sequence again and consists of 16 bp of exon 14 and 72 bp of exon 13.

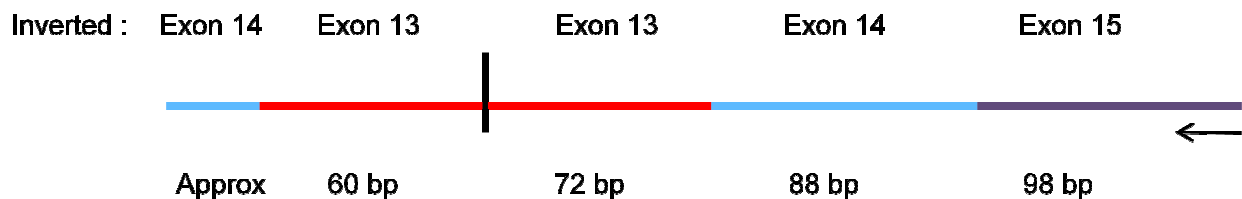


Figure 3.11: A schematic presentation of the possible composition of the larger exon 14 fragment (V1) including the faint sequence at the end. The expected 258 bp fragment consisting of exon 13, 14 and 15 is present. In addition an inverted segment consisting of 60 bp of exon 13 and a section of exon 14 follows the normal exon 13. The total fragment size therefore is greater than 300 bp.

### **3.4.5 Summary of Exon 14**

The V617F mutation was found in exon 14 of both cell lineages and the frequency thereof in the different MPNs correlate well with the literature. The reason why it was found in the T-lymphocytes is most probably due to granulocyte contamination. Apart from this mutation in exon 14, two other amplicon variants were also observed. The smaller variant (V2) has been described in literature before as a possible splice variant. The larger variant (V1) appears to contain an inverse duplication of the region and further research is needed. The high resolution melting curves for exon 14 in the study did not correlate with the sequencing data, however similar studies have shown successful HRM analysis of exon 14 (Murugesan *et al.*, 2006; Lay *et al.*, 2006).

## **3.5 Analysis of Exon 12 of the JAK 2 gene**

### **3.5.1 PCR of Exon 12**

Exon 12 was amplified using primers which attach in exon 11 and exon 13. Double PCR was performed as previously discussed and was successful for all the samples. No additional fragments were observed. The amplicon was excised and diluted for HRM analysis.

### **3.5.2 High Resolution Melting Curve Analysis of Exon 12**

High resolution melting curve analysis was performed on the 15 granulocyte samples. Three different variants were observed and one sample had a significant deviation from the wild type on the difference plot (fig 3.12). The PCR product was excised, purified and used as template for sequencing.



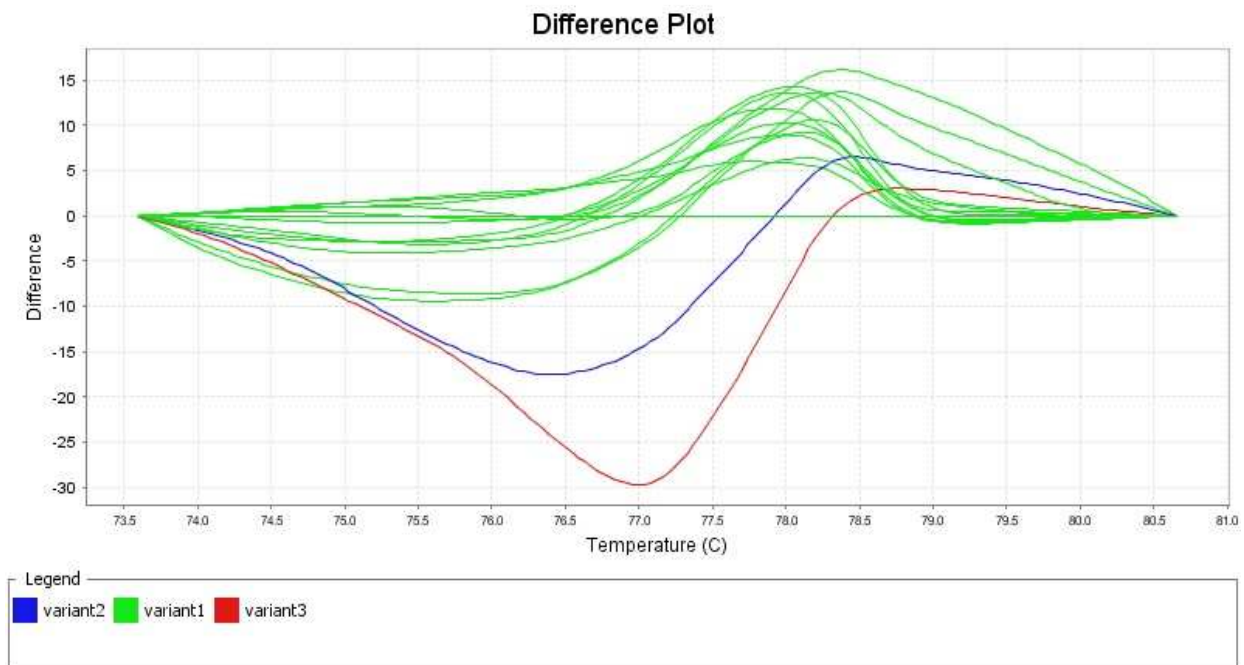


Figure 3.12: The HRM difference plot of exon 12 for the granulocytes of all the samples. Three different variants were observed (different colours). One sample (red) had 30 points difference on the plot in comparison to the wild-type (green).

### 3.5.3 Sequencing Analysis of Exon 12

All the samples of exon 12 were sequenced and showed no mutations or SNPs in the exon. The primers used for the amplification of this exon lie in exon 11 and exon 13. Thus the product consists of 62 bp of exon 11, 128 bp of exon 12 and the first 77 bp of exon 13 (fig 3.13). In patient 5 a novel heterozygous mutation was found at codon 550 (exon 13). This is a single base mutation of a C to A. This leads to an amino acid change of a serine to arginine. Further research on the effect of this SNP, we term S550R, is needed. Unfortunately the HRM analysis of exon 12 and the sequencing did

not correlate. The sample of patient 5 (containing S550R) grouped together with the wild type in the HRM difference plot according to the HRM analysis software. The sequence of the samples that deviated from the wild type in the HRM difference plot (blue and red), were identical to that of the wild type sequence (green) and the samples that grouped with it.

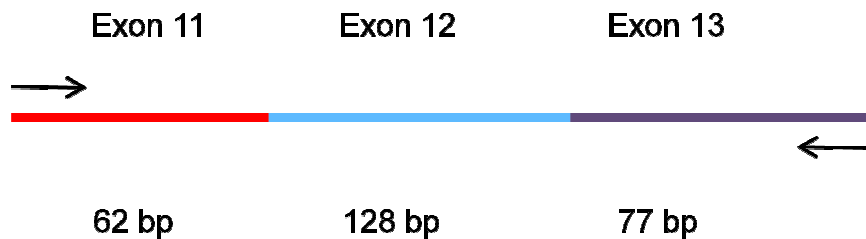


Figure 3.13: A schematic of the expected PCR fragment after amplification of the cDNA for exon 12. The amplicon consists of the last 62 bases of exon 11, all the bases of exon 12 and the first 77 bases of exon 13.

### 3.5.4 Summary of Exon 12

More than 30 mutations have been described in exon 12. These mutations are summarized in a recent review by Scott (2011). High resolution melting curve analysis has also been shown to detect and differentiate between various mutations in other studies (Jones *et al.*, 2008; Schnittger *et al.*, 2009; Rapado *et al.*, 2009). Three variants were observed with high resolution melting curve analysis in this study; however no mutations were identified in exon 12. High resolution melting curve analysis is more sensitive than sequencing and the study by Jones *et al.* (2008) found that in some cases the mutation could not be detected with sequencing because the copy number was too low. In the study by Lay *et al.* (2006) the V617F mutation in exon 14 could be detected by melting curve analysis and not with sequencing if the clonal load was below 20%. In exon 12 Butcher *et al.* (2008) found exon 12 mutations in isolated endogenous

erythroid burst-forming units and this suggests that detection of these mutations with sequencing can be difficult. ASO-PCR has been shown to detect the mutation in cases where sequencing could not (Kouroupi *et al.*, 2008). Thus it is possible that there were mutations in exon 12 as shown in the HRM difference plot, but the copy number might have been too low to be detected with sequencing.

### 3.6 Analysis of Exon 13 of the JAK 2 gene

#### 3.6.1 PCR of Exon 13

Double PCR was performed on the cDNA of the patients granulocytes as previously discussed in section 3.4.1. Multiple size fragments were observed in the gel and it differed among the patients (fig 3.14). The cause of the multiple fragments was examined.

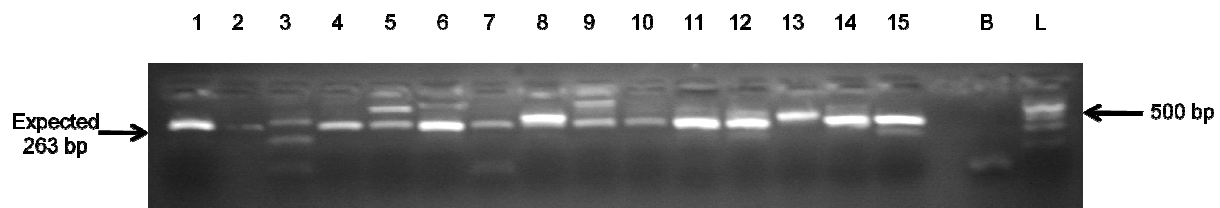


Figure 3.14: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the amplification of the granulocyte cDNA for exon 13. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

The possibility of non-specific amplification due to faulty enzymatic activity was investigated. In the reaction above GoTaq® (Promega, 2800 Woods Hollow Road, Madison, USA) was used, but it has limited proof reading ability. TaqGold® (Applied

Biosystem, Foster City, USA) was used in a second run to determine if using an enzyme with better proof reading ability can get rid of the multimers. No difference was observed on the gel between the two enzymes (data not shown). The possibility of secondary structures causing the multiple fragments was examined.

Secondary structures can cause synthesis errors from the polymerase (Viswanathan *et al*, 1999). Non-specific amplification can be especially problematic where the template has a high GC content (Varadaraj & Skinner, 1994; McDowell *et al*, 1998). Addition of substances like low molecular weight sulfones have shown to enhance the PCR (Chakrabarti & Schutt (2001). Dimethyl sulfoxide (DMSO) was used in the study as it disrupts base pairing (Frackman *et al*, 1998). DMSO was used at five percent of the total reaction volume. No difference was observed between the DMSO at 5% (fig 3.15) and the conventional PCR. Exon 13 could not be sequenced because of the multiple amplicons.

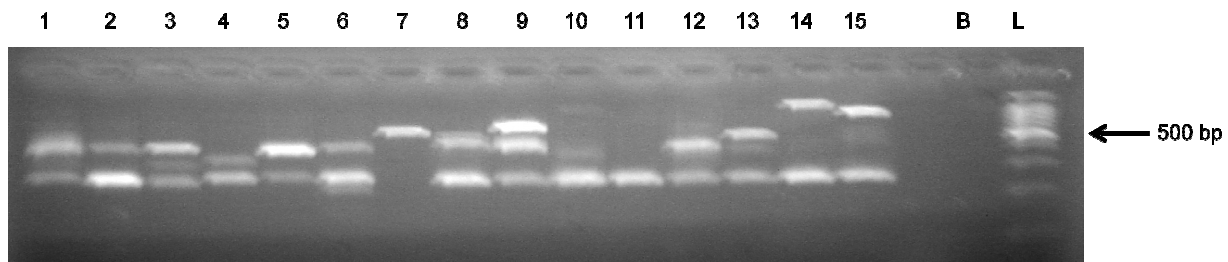


Figure 3.15: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the amplification of the granulocyte cDNA for exon 13 using 5% DMSO. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

The primers used in the study are overlapping, thus sequence data can be obtained for exon 13 by looking at the forward sequence used for exon 12 and the reverse sequence

used for exon 14. The reverse sequence of exon 14 indicated that after the expected exon 13 fragment an inverted exon 13 and possibly inverted exon 14 was present. With the forward sequencing reaction of exon 12, after the expected exon 13 fragment, an inverted exon 13 was also observed at a low intensity. It is possible that a duplication and inversion of the regions flanking exon 13 occurred (fig 3.16). This might explain why multiple amplicons were observed as the primers used might have more than one binding site on the cDNA. This could lead to multiple fragments forming due to different primer binding combinations. More research is needed to elucidate the exact cDNA sequence of the samples between exons 12 and 14.

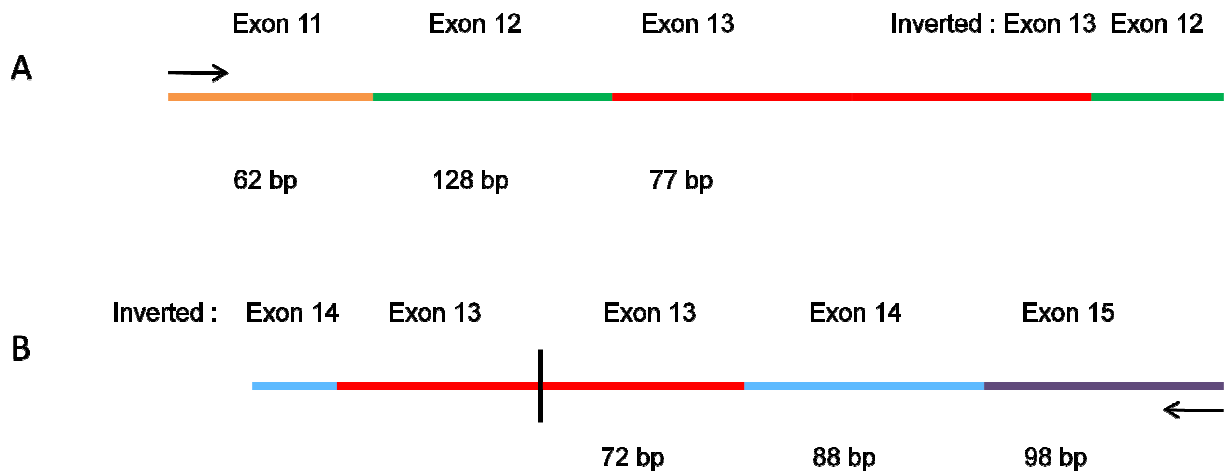


Figure 3.16: A schematic of the forward sequencing reaction obtained for exon 12 (A) and the reverse sequencing reaction for exon 14 (B). It was possible to use these sequences to construct the sequence of exon 13. It indicates that exon 13 becomes duplicated and inverted.

### 3.6.2 Summary of Exon 13

Exon 13 could not be sequenced due to the multiple sized amplicons observed. A heterozygous novel mutation was found in one patient in this exon by examining the amplicon of exon 12. This base change was found in a PV patient negative for the V617F mutation and this might be the cause for the formation of the MPN. Further research is needed to determine the impact of the S550R (fig 3.17) base change. The reason for the multiple size amplicons might be due to duplication and inversion of exon 13 and its surrounding regions. Further research is needed to determine the exact composition of this inversion duplication. No HRM analysis could be performed on the amplicons for exon 13.

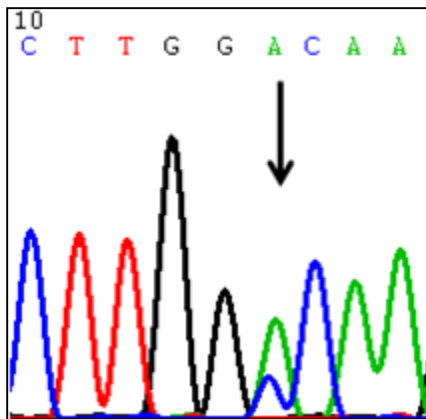


Figure 3.17: The sequencing electropherogram indicating the novel heterozygous exon 13 SNP. A cytosine is substituted for an adenine. The effect thereof is uncertain.

Multiple sized amplicons were also observed in the PCR products of 12 of the other exons of JAK 2 and it was decided to focus only on the region between exon 10 and exon 17.

### 3.7 Analysis of Exon 11 of the JAK 2 gene

#### 3.7.1 PCR of Exon 11

The PCR was performed on the cDNA of the granulocytes of all 15 patients and run on a 3% agarose gel. The PCR was successful with all the samples; faint additional fragments were observed however (fig 3.18). The expected amplicon as well the additional fragments were excised from the gel and purified for sequencing.

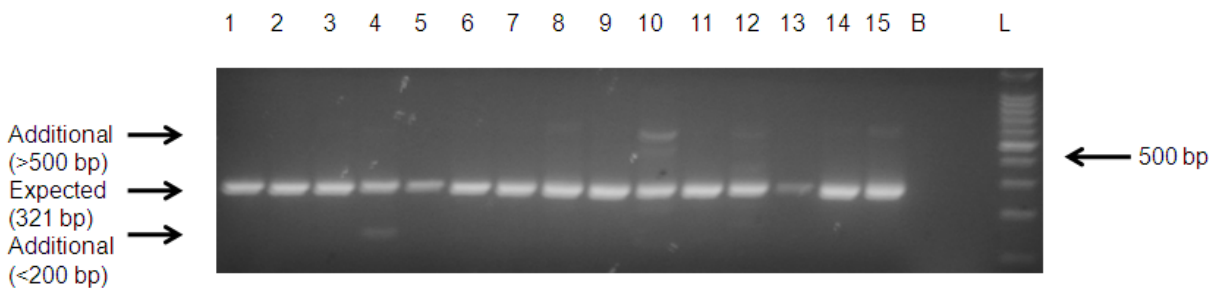


Figure 3.18: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the cDNA of the granulocytes for exon 11. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

#### 3.7.2 Sequencing Analysis of Exon 11

All the expected amplicons were sequenced successfully, however the faint additional fragments such as is found in patient 4 failed to be sequenced. Fourteen of the fifteen patients had no mutations in exon 11. A homozygous SNP was found in patient 3 at codon 486. It is a base change from a thymine to a guanine (GTT – GTG), both codons encode for a valine (fig 3.19). This SNP is found in the SNP database (rs10119726) of

NCBI [<http://www.ncbi.nlm.nih.gov/projects/SNP/>] and the clinical significance is uncertain.

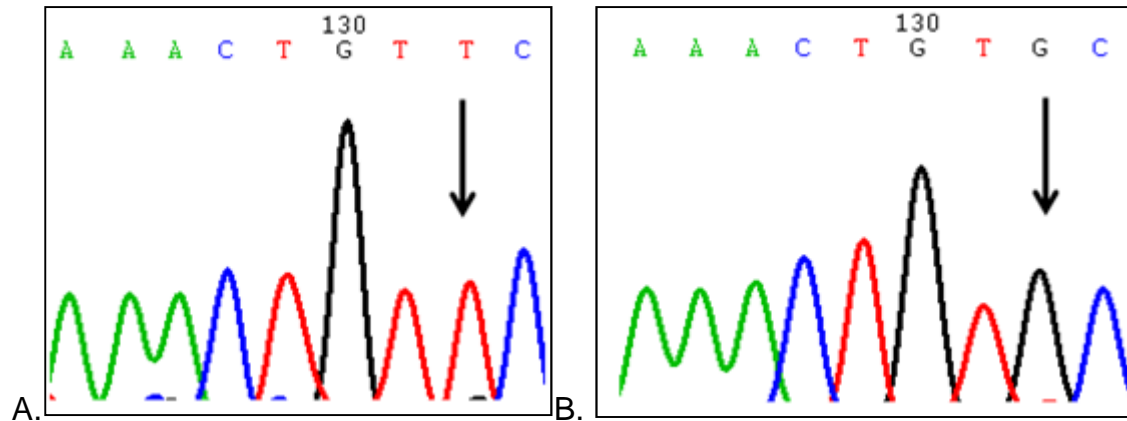


Figure 3.19: Two sequencing electropherograms of granulocyte cDNA samples sequenced for exon 11. Figure A indicates the wild type sequence and figure B the sequence that has a homozygous SNP of a T to a G as found in patient 3.

### 3.8 Analysis of Exon 15 the JAK 2

#### 3.8.1 PCR of Exon 15

The PCR was performed on the cDNA of the granulocytes of the 15 patients and were electrophoresed on a 3% agarose gel (fig 3.20). The amplicon observed in patient 4 was smaller than the expected amplicon. The expected and additional fragment was excised, purified and sequenced.



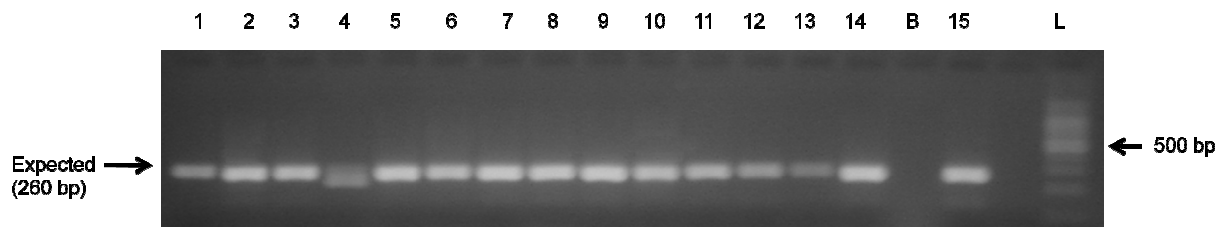


Figure 3.20: A photo of the 3% agarose ethidium bromide stained gel electrophoresis of the granulocyte cDNA amplicons of exon 15. The amplicon of patient 4 was identified to be smaller than the expected 260 bp fragment. Lanes 1-15: Patients 1-15. Lane B: Blank. Lane L: 100 bp molecular weight marker.

### 3.8.2 Sequencing Analysis of Exon 15

Thirteen of the 15 patients tested showed no deviation in sequence from the wild type. Patient 4 had an additional amplicon that was smaller than the expected size and it was successfully sequenced. The sequencing data showed a normal electropherogram (fig 3.21) however when it was compared to the reference sequence its alignment score was at 30%. The sequence was investigated further using BLAST (Basic Local Alignment Search Tool) on two nucleotide sequence databases (Ensembl <http://www.ensembl.org/Multi/blastview>) and NCBI <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and was found to have a 100% homology with a section of the glypican-5 precursor gene on chromosome 13. The primer sequences were analysed and it was found that the last nine bases on the 3' end of both primers were similar for the JAK 2 gene and the glypican-5 precursor gene. This was not found in any of the other patients and even when the reaction was repeated it still showed the same result. This might be a result of a SNP causing mismatch priming.

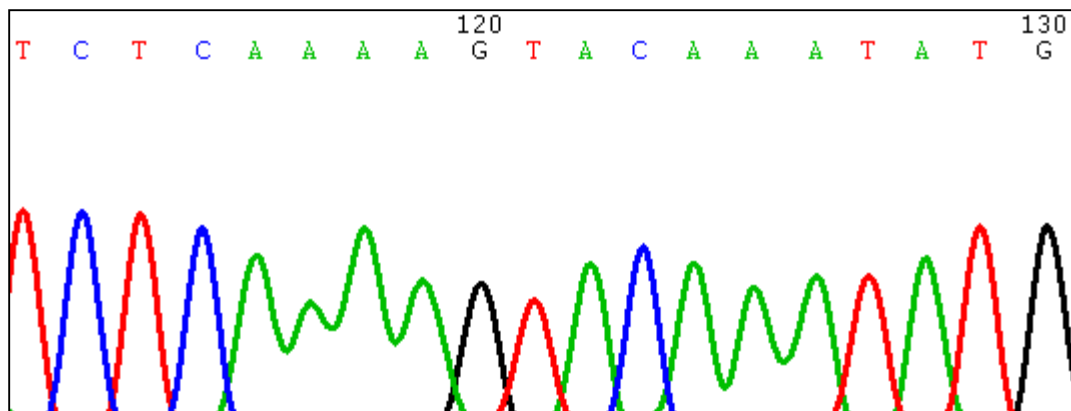


Figure 3.21: The sequence electropherogram found when the smaller exon 15 amplicon of patient 4 was sequenced. The sequence represented a section of the glypican-5 precursor gene instead of exon 15 of the JAK 2 gene.

Studies have shown that the occurrence of SNPs varies from 1 per 350 to 1300 bp (Cargill *et al.*, 1999; Wang *et al.*, 1998; Altshuler *et al.*, 2000). The distribution of SNPs also varies among as well as within chromosomes (Wang *et al.*, 1998). These SNPs might occur at primer binding sites leading to mispriming. Mispriming is where the primers anneal to partially complementary sequences (Boyle *et al.*, 2009). It has been shown that a SNP at the 3' end of the primer has a greater effect on quantification than it does at the 5' end using real-time PCR (Boyle *et al.*, 2009). It is possible that for patient 4 there is a SNP in the 5' region of the primer binding site which gives the glypican-5 precursor preferential amplification over the JAK 2 gene.

One patient was identified to have a heterozygous SNP at codon 643 (fig 3.22). This is a single base change from a thymine to a cytosine. This variant has also been reported on the SNP data base (rs2230728). The clinical significance of this variant is also currently unknown.

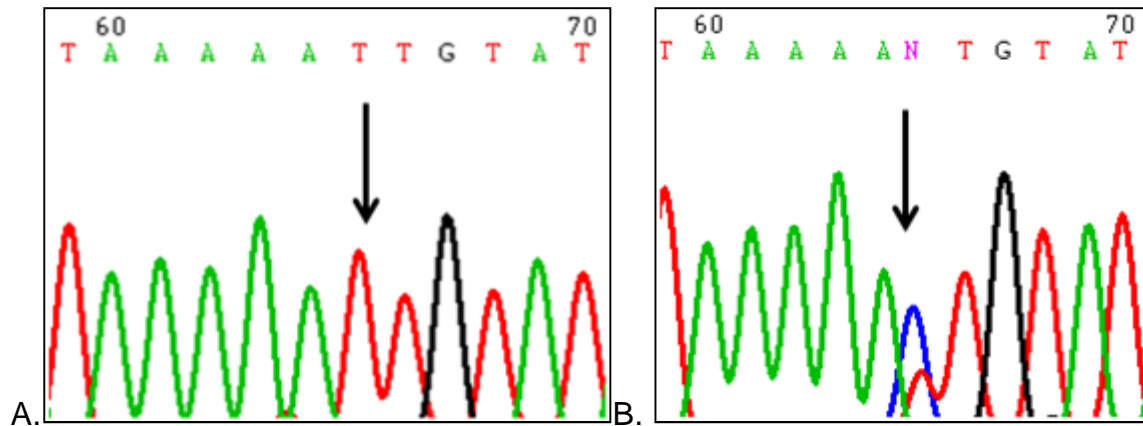


Figure 3.22: Two electropherograms of the sequencing results exon 15 in two of the samples. Figure A indicates the wild type and figure B indicates the heterozygous variant at codon 643.

### 3.8.3 Summary of Exon 15

In exon 15 thirteen of the 15 sequences were identical to the wild-type sequence. The amplicon of patient 4 was found to be from an entirely different gene. This might be due to a primer binding site SNP. One patient had a heterozygous SNP of a T to a C, the clinical relevance is uncertain.

### 3.9 Other Exons of the JAK 2 Gene

Exons 10, 16 and 17 were also successfully screened using sequencing (results not shown). No mutations or SNPs were identified in these exons. Little research has been performed on these exons and very few single nucleotide polymorphisms are reported in the database ([http://www.ensembl.org/Homo\\_sapiens/Transcript/Sequence\\_cDNA?db=core;g=ENSG00000096968;r=9:49850335128183;t=ENST00000381652](http://www.ensembl.org/Homo_sapiens/Transcript/Sequence_cDNA?db=core;g=ENSG00000096968;r=9:49850335128183;t=ENST00000381652) accessed on 31-Oct-2011). In other studies of exon 16 a deletion of 5 amino acids in Down's

syndrome patients (Malinge *et al.*, 2007) as well as an arginine to a glycine mutation (Kearney *et al.*, 2009) was described. Only two variants are reported in exon 10 and only 1 in exon 17. It is possible that these regions are not as prone to mutations as the regions between exon 11 and 15.

## CHAPTER 4

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### 4.1 Conclusion

Myeloproliferative neoplasms are malignancies where there is an increase in the production of myeloid cells. The four classical myeloproliferative neoplasms are polycythaemia vera, essential thrombocythaemia vera, primary myelofibrosis and chronic myeloid leukaemia. A mutation in the JAK 2 gene termed V617F was found to be the cause of some of the myeloproliferative neoplasms. Some patients however suffer from a myeloproliferative neoplasm but are negative for the V617F mutation.

The aim of the study was to detect other possible SNPs in the JAK 2 gene using high resolution melting curve analysis and confirmation thereof with sequencing. Unfortunately the melting curve analysis data did not correlate with the sequencing as was seen in the screening of exons 12 and 14, further research is needed to try and optimize the HRM, perhaps using different primers.

Initially it was attempted to separate two cell lineages of each patient. This was done so that each patient could serve as its own internal control. The separation method used in the study however was not sensitive enough and there was cell cross-contamination. Future studies attempting cell separation should add a third isolation step using FACSVantage. cDNA was used in the study as it represents the transcribed protein. Seven exons of JAK 2 were successfully screened using sequencing.

The V617F mutation was found in exon 14 and the prevalence thereof correlated well with that described in literature. Two variants were also observed. The one was a

deletion of the entire exon 14 and the other indicated a duplication variant which had not previously been described. Further research is needed to determine the conformation of this variant.

Previously reported SNPs were found in exons 11 and 15. The clinical significance of these SNPs is uncertain and the patient with the SNP in exon 15 did not have the V617F mutation. This might be a cause for the MPN in this patient. A novel SNP was identified in exon 13 (S550R). The effect of this SNP is still uncertain and further studies are needed. The PCR of exon 13 indicated the formation of several amplicons of different size. The reason for this is might be due to rearrangements in the region. Primer mismatching might be the cause resulting from a SNP. The primers for exon 15 of the JAK 2 gene bound to the glypican-5 precursor gene in this patient.

No other SNPs were identified in exons 10, 16 and 17. Very few variants have been reported in this region and those reported occur in Down's syndrome patients. The remaining exons of JAK 2 could not be screened in this study mostly due to time constraints and the formation of different sized amplicons.

Exons 14 and 12 are frequently examined in the literature and several mutations have been reported in these exons. Studies examining the whole JAK 2 gene are limited. In this study we attempted to screen all the exons, however this could not be achieved. This indicates that careful design and optimization should be done for studies on large genes. The results obtained from this study agree with the conclusion of other similar studies that the region between JAK 2 exons 11-15 is a mutational hotspot.

The diagnosis of the myeloproliferative neoplasms is challenging, thus more and more molecular markers are used to aid in the diagnosis. JAK 2 mutations, especially the

V617F mutation has been identified as the cause for some of the MPNs. In this study several SNPs and two deletion/duplication variants were observed. The significance thereof is still uncertain but it might be the cause of the condition in patients without the V617F mutation. The role of JAK 2 in MPN formation needs to be examined further.

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### 4.3 Summary

All the cells of blood arise from two lineages, the myeloid and the lymphoid lineage. The various cells of blood perform vital functions in the body. These cell counts are closely regulated by regulatory pathways. Mutations within genes that encode for the proteins involved in these pathways can occur. These mutations can cause uncontrolled proliferation of the cells. Myeloproliferative neoplasms are malignancies where there is an uncontrolled increase in the formation of the myeloid cells. The four classical neoplasms are polycythaemia vera, essential thrombocythaemia, primary myelofibrosis and chronic myeloid leukaemia.

A mutation (V617F) in the tyrosine kinase, Janus kinase 2, has been found to be the cause of at least three of the classical MPNs. The mutation lies in the domain of the protein that controls its tyrosine kinase activity. The tyrosine kinase thus is constitutively active and causes proliferation of the myeloid cells. The V617F mutation lies in exon 14 and more recently several mutations have been described in the neighbouring exons encoding for the regulatory domain of the gene. Very few studies have been done on the other exons of the JAK 2 gene.

In the study it was attempted to screen 15 MPN patients for mutations in the JAK 2 gene. Two different cell populations (lymphocytes and granulocytes) of each patient were screened. It was found that the cell purity was not sufficient in the study and better separation techniques are required for future studies. Only the granulocytes were used for the remainder of the study. High resolution melting curve analysis was used to screen the patients for mutations, however the data did not correlate with the sequencing results and it was decided to proceed with sequencing of all the samples.

Seven of the 25 exons of the JAK 2 gene were successfully sequenced. The remaining exons could not be screened due to time constraints and complications such as multiple amplicon formation. Two previously reported single nucleotide polymorphisms were found in exons 11 and 15 in two patients. The clinical significance thereof is uncertain however, the patient whom had the SNP in exon 15 was negative for the V617F mutation and had a MPN. In exon 14 the V617F mutation was identified and the prevalence thereof correlates to that reported in literature. A novel SNP was found in exon 13 of a PV patient negative for the V617F mutation and the significance thereof is also uncertain. Additionally a novel inverse duplication consisting of at least of exon 13 was also identified. No mutations were identified in exons 10, 12, 16 and 17 of the JAK 2 gene.

This was, to our knowledge, the first report in South Africa that found the prevalence of the V617F mutation in MPN patients correlating to the prevalence reported in literature. A novel SNP was identified in exon 13 and further studies are needed on the possible effect thereof. The previously reported SNPs found in exons 11 and 15 might be the cause of the formation of a MPN, however further research is needed. A novel duplication variant was also identified and this might be a possible splice variant. The study showed that the region between exons 10 and 15 in the JAK 2 gene is a mutational hotspot and further studies are needed to elucidate the effect thereof.

#### 4.4 Opsomming

Al die selle van die bloed ontstaan vanaf twee sellyne, die myeloïede en die limfoïede lyn. Die verskillende selle van die bloed verrig noodsaaklike funksies in die liggaam. Hierdie seltellings word streng gereguleer deur verskeie regulatoriese paaie. Mutasies kan voorkom in die gene wat kodeer vir die proteïene wat betrokke is in die regulatoriese paaie. Hierdie mutasies kan veroorsaak dat daar onbeheerde vermeerdering is van die selle. Myeloproliferatiewe neoplasmas is maligniteite waar daar 'n onbeheerde toename is in die vorming van die myeloïde selle. Daar is vier klassieke neoplasmas naamlik polysitemie vera, essensiële trombositemie, primêre myelofibrose en chroniese myeloïede leukemie.

'n Mutasie (V617F) in die tirosien kinase, Janus kinase 2, is gevind as die oorsaak van ten minste drie van die klassieke MPNs. Die mutasie kom voor in die domein van die proteïen, wat verantwoordelik is vir die beheer oor die tirosien kinase aktiwiteit. Die tirosien kinase is dus voortdurend aktief en dit veroorsaak die vermeerdering van die myeloïede selle. Die V617F mutasie lê in ekson 14 van die geen en meer onlangs is daar mutasies beskryf in die naburige eksone wat kodeer vir die regulerende domein. Baie min studies is al gedoen op die ander eksone van die JAK 2 geen.

In die studie het ons gepoog om 15 MPN pasiënte te ondersoek vir mutasies in die JAK 2 geen. Twee verskillende sel lyne (limfosiete en granulose) van elke pasiënt is geïsoleer. Daar is vasgestel dat die suiwerheid nie voldoende was nie en beter sel skeidings tegnieke moet toegepas word vir toekomstige studies soos byvoeging van sortering met vloeisitometrie. Slegs die granulose is gebruik vir die res van die studie. Hoë resolusie smeltings kurwe analise is gebruik om die pasiënte vir mutasies te ondersoek, maar die data het nie ooreengestem met die basis volgorde resultate nie.

Daar is dus besluit om voort te gaan deur slegs basis volgorde analise te gebruik vir al die monsters.

Sewe van die 25 eksone van die JAK 2 geen is suksesvol ondersoek. Die oorblywende eksone kon nie ondersoek word nie as gevolg van tydsbeperkings en komplikasies, soos die vorming van verskeie grootte amplicone. Twee enkel nukleotied polimorfismes (SNP) wat voorheen beskryf is, is gevind in eksone 11 en 15 in twee pasiënte. Die kliniese belang daarvan is onseker, maar die pasiënt wat die SNP in ekson 15 het, is negatief vir die V617F mutasie en dus mag die SNP die oorsaak wees vir die MPN. In Ekson 14 is die V617F mutasie geïdentifiseer en die voorkoms daarvan stem ooreen met dit wat in die literatuur gerapporteer word. 'n Unieke SNP is gevind in ekson 13 van 'n PV pasiënt wat negatief is vir die V617F mutasie en die betekenis daarvan is ook onseker. 'n Unieke inverse duplikasie is ook geïdentifiseer in ekson 13. Geen mutasies is in eksone 10, 12, 16 en 17 van die JAK 2 geen geïdentifiseer nie.

Sover ons kennis strek is hierdie die eerste verslag in Suid-Afrika wat die voorkoms van die V617F mutasie in MPN pasiënte korreleer met dit wat in die literatuur gerapporteer word. Verdere studies word beoog om die moontlike effek van die unieke SNP in ekson 13 te bepaal. Die voorheen gerapporteerde SNPs wat gevind is in eksone 11 en 15 mag moontlik die oorsaak wees vir die ontwikkeling na 'n MPN, maar verdere navorsing is egter nodig. Die unieke duplisering variant wat ook geïdentifiseer is kan 'n moontlike splytings variant wees. Die studie het getoon dat die area tussen eksone 10 en 15 in die JAK 2 'n brandpunt vir mutasies is en verdere studies word benodig om die effek daarvan te bepaal.

## 4.5 Appendix

### **CONSENT DOCUMENT**

#### **CONSENT TO PARTICIPATE IN RESEARCH**

You have been asked to participate in a research study.

You have been informed about the study by Dr André de Kock.

You may contact Dr André de Kock at 051-4053283 any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation.

If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.



The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

\_\_\_\_\_  
Signature of Participant

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Study Leader  
*(Where applicable)*

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Witness  
*(Where applicable)*

\_\_\_\_\_  
Date

## INFORMATION DOCUMENT

Study title: Mutational analysis of the Janus Kinase 2 gene in patients with Polycythaemia Vera, Essential Thrombocythaemia and Primary Myelofibrosis

Greeting:

Introduction:

I, Dr André de Kock., am doing research on mutations found in MPD's. Research is just the process to learn the answer to a question. In this study we want to learn more about the mutations that occur in MPD'S and if we have novel mutations that occur in South African individuals. This study involves research and will not influence your treatment. The study could have future spin-offs in the diagnosis and treatment of MDP's.

**Invitation to participate:** We are asking/inviting you to participate in a research study *(or asking for your permission to include your child in a research study)*.

**What is involved in the study** – We will collect 5 – 10 ml of your blood. The genetic material will be taken from your blood cells for further study. The study involves searching for changes that may have occurred in your genetic material. These changes may be involved in the development and progression of your disease. After the study the material obtained from your blood will be destroyed. About 50 individuals from South Africa will be invited to take part in this study.

**Risks** of being involved in the study: There are no risks involved for you in this study; only the discomfort of having a blood sample taken from you by a professional nurse or doctor.

**Benefits** of being in the study. The information we will gather from the analysis of your blood sample will provide us with genetic information on MPD's as well as information of the genetic make-up of the local populations.

**Alternative procedures** or courses of treatment that might benefit the subject. The information gathered in this study may influence future diagnosis and treatment of patients with MPD's.

***The subject will be given pertinent information on the study while involved in the project and after the results are available.***

**Participation is voluntary**, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

**Reimbursements:** There is no reimbursement for this study.

**Confidentiality:** Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research and the Medicines Control Council. If results are published, this may lead to individual/cohort identification.

**Contact details of researcher(s)** – for further information/reporting of study-related adverse events. Dr André de Kock, 051-4053283

**Contact details of REC Secretariat and Chair** – for reporting of complaints/problems. Ms / Me H Strauss, Research Division / Navorsingsafdeling. Internal Post Box / Interne Posbus G40. E-mail address / E-posadres: [gndkhs.md@mail.uovs.ac.za](mailto:gndkhs.md@mail.uovs.ac.za). ☎(051) 4052812

**The following must be included in the consent form when applicable:**

- a. A statement that the particular treatment or procedure may involve risks to the subject that are currently unforeseeable (*or to the embryo or foetus, if the subject is or may become pregnant*).
- b. Anticipated circumstances under which the subject's participation may be terminated by the investigator without the subject's consent.
- c. Any additional costs to the subject that may result from participation in the research.
- d. The consequences of a subject's decision to withdraw from the research and procedures for orderly termination of participation by the subject.
- e. A statement will be provided to the subject that significant new findings developed during the course of the research which may relate to the subject's willingness to continue participation.
- f. Where genetic tests are to be performed, a separate information sheet and consent form will be made available.
- g. A statement that specimens will be stored for future research pertaining to the specific research question being studied. Specify how long specimens will be stored for, where they will be stored, and whether these will be made anonymous. If stored for future genetic testing, a further consent form will be signed.

## INFORMATION DOCUMENT FOR GENETICS RESEARCH

### Some information on the study

- We are planning a research project on Myeloproliferative Disorders (MPD) and request your permission to use some of your tissue for laboratory tests/further laboratory tests.
- The tests will involve an analysis of the genetic composition of the JAK2 gene and are aimed at increasing the understanding of the causes and behaviour of your condition.
- Genes are what you inherit from you parents. They are found in every part of your body and therefore they will be present in any of your tissue and blood which we remove.
- The findings of this study will not have direct bearing on your management.
- The findings may benefit/eventually benefit others in terms of prevention or treatment of the condition.
- You are free to refuse consent and you do not have to give reasons for doing so. *(Researchers are to be aware that for some genetic research, individual's participation may be requested by, and may primarily serve interests of other family members, and individuals may feel duty bound to agree to participation).*

### Privacy and Confidentiality

- The following arrangements have been made to ensure privacy and confidentiality of your genetic information:
  - Your name will not appear on any of the blood samples or DNA samples, only a number.
  - This number will only be known to the principle researcher.

- At no time during the study will your name be connected to the samples or results.
- The only connection will be between your specific condition and the samples.
  
- Your genetic material and information will be used in an unidentifiable form.
  
- Because your material or information is to be made unidentifiable, it will not be possible to provide you with personal research results.

### **Results of research**

- Researchers will endeavour to provide information about the outcome of the research.
  
- It is not intended to provide feedback because the link between your name and your sample will be broken.

### **Family members**

- Information about family members, in addition to that provided by you, is not required for the research.
  
- Your material and information will not be released for other uses without consent, unless required by law.

### **Storage**

- We will not retain some of the same tissue in storage for possible future research related to the present research question.

- Your genetic material and information will be disposed of at the end of the study, once the sample storage and record-keeping requirements of good research practice have been met.
- Do you have any sensitivity on how your tissues should be disposed of? If so, what are they? These will be recorded and taken into account at the time of disposal.
- We will not be able to dispose of your tissue after the research has started, as it will be stored in an unidentifiable form.

### **Voluntary Participation**

- You do not have to agree to take part in this research and you are free to withdraw from the research at any time. Your routine medical treatment will not be compromised in any way if you do not participate.

### **Community Consent**

- None needed

### **Recruitment of Relatives**

- None intended.