INVESTIGATIONS INTO THE MOLECULAR PATHOGENESIS OF ESSENTIAL THROMBOCYTHAEMIA

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Declaration

I, Jonathan Lambert, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date:

Abstract

In order to explore the phenotypic heterogeneity of the myeloproliferative neoplasm essential thrombocythaemia (ET), the role of the *JAK2* mutation V617F in the pathogenesis of the disease was investigated, in particular its relationship to myeloid clonality. The clinical, haematological and molecular characteristics of 133 ET patients were studied. *JAK2* V617F was detected in 55 (41%) patients; a clonal X-chromosome inactivation pattern (XCIP) was found in 24 (39%) of the 62 evaluable female patients. There was no association between *JAK2* mutational status and XCIP status or thrombotic risk, but higher *JAK2* V617F mutant levels were noted in patients who had a thrombosis. A trend towards a higher thrombotic rate was observed in patients whose XCIP was clonal. In 10 untreated *JAK2* V617F-positive ET patients, *JAK2* WT thrombopoiesis was not suppressed despite the presence of a thrombocytosis, suggesting that the regulation of *JAK2* WT thrombopoiesis was abnormal. Eleven patients were screened for the presence of more than one *JAK2* V617F-positive population using an exonic SNP located near the mutation. In ten (91%) of these the mutation appeared to have been independently acquired on at least two occasions. Furthermore, XCIP analysis of *JAK2* V617F-positive erythroid colonies from six ET patients revealed that in one patient the V617F-positive populations were not derived from a single clonal population. An association between the reported *JAK2* haplotype (known as '46/1') and *JAK2* V617F-positive ET patients was observed in the cohort studied. Methylation studies indicated that this haplotype introduced additional methylated sites near to the mutation locus, which may potentially affect conformation of the DNA and mutability of the *JAK2* locus. Together, the studies reported in this thesis suggest that *JAK2* V617F is not the initiating event at least in some cases of ET, and that its presence does not invariably indicate the presence of a monoclonal disorder.

Acknowledgements

I would like to thank Professors Rosemary Gale and David Linch for their guidance and enthusiasm with this project. With their help I have begun to understand how molecular biology may be used to answer fundamental questions in clinical medicine. In addition, I am very grateful to the other members of the laboratory who have helped me throughout these studies, and without whose advice and friendship my research would have been much slower and less enjoyable. I am also indebted to the patients who have provided samples for the tissue bank over the years, and to the doctors who have referred them. Finally, I cannot begin to express my gratitude to my family and friends for their patience, encouragement and support over the past four years.

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Commonly used abbreviations

AD	Autosomal dominant
AML	Acute myeloid leukaemia
AR	Autosomal recessive
bp	Base pairs
BSA	Bovine serum albumin
CML	Chronic myeloid leukaemia
ddH_2O	Double de-ionised water
dHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid c=complementary g=genomic
EEC	Endogeneous erythroid colonies
EPO	Erythropoietin
ET	Essential thrombocythaemia
FCS	Foetal calf serum
Hb	Haemoglobin
HSC	Haemopoietic stem cell
HUMARA	Human androgen receptor assay
JAK2	Janus kinase 2
JH	Janus homology
LD	Linkage disequilibrium
LOH	Loss of heterozygosity
MDS	Myelodysplastic syndrome

MF	Myelofibrosis
MK	Megakaryocyte
MNC	Mononuclear cell
MPL	Myeloproliferative leukaemia virus
MPN	Myeloproliferative neoplasm
PCR	Polymerase chain reaction
PNH	Paroxysmal nocturnal haemoglobinuria
PV	Polycythaemia vera
PVSG	Polycythemia Vera Study Group
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
TET2	Ten-eleven translocation 2
TF	Tissue factor
TPO	Thrombopoietin
WCC	White cell count
WHO	World Health Organisation
WT	Wild-type
XCIP	X-chromosome inactivation pattern

1 Introduction

1.1 Essential thrombocythaemia and the myeloproliferative neoplasms

Essential thrombocythaemia (ET) is a chronic haematological disorder characterised by the sustained overproduction of megakaryocytes (MKs) and platelets in the absence of a recognisable physiological stimulus. It is a rare condition, with an annual incidence of approximately 0.8 new cases per 100,000 population in the UK (McNally *et al,* 1997), though this figure may be as high as 2.5 per 100,000 in some regions (Mesa *et al,* 1999), and there is evidence that these rates are increasing (Girodon *et al,* 2009; Johansson *et al,* 2004). The median age at diagnosis is 65 – 70 years, but it may present at any age of life, and there is a female-to-male preponderance of approximately 2-to-1 (Girodon *et al,* 2009; Jensen *et al,* 2000; Landgren *et al,* 2008).

Although it was initially described in 1934 as a haemorrhagic thrombocythaemia (Epstein and Goedel 1934), its most significant clinical sequelae are thrombotic episodes, which may be life-threatening. Untreated, the rate of thrombotic events in one series was 6.6% per patient-year, though this varied markedly between 1.7% and 31.4% per patient-year depending on a number of risk factors, with a predominance of arterial events (Cortelazzo *et al,* 1990). Haemorrhagic complications are rarer, and are associated with extreme thrombocytosis (platelets greater than $1500x10^9$/L). ET may also transform to the more serious myeloproliferative neoplasm (MPN) myelofibrosis (MF), or to acute myeloid leukaemia (AML), but in the short term the risk of this is small – approximately 5-8% over 10 years, and 1-3% over 10 years, respectively (Cervantes *et al,* 2002; Palandri *et al,* 2009a). For older patients the diagnosis is unlikely to have a significant impact on their life expectancy, but for younger

patients the same may not be true (Bazzan *et al,* 1999; Mesa *et al,* 1999; Passamonti *et al,* 2004; Wolanskyj *et al,* 2006). Cytoreductive therapy reduces the incidence of vascular complications in high-risk patients (Cortelazzo *et al,* 1995) but may increase the risk of malignant transformation (Finazzi *et al,* 2000; Randi *et al,* 2000).

As long ago as 1951, Dameshek recognised the similarities between ET, polycythaemia vera (PV), MF, and chronic myeloid leukaemia (CML), and suggested that they might represent different manifestations of the same 'myeloproliferative syndrome' (Dameshek 1951). He went on to speculate about the existence of a 'myelostimulatory factor', which he believed might be a steroid hormone. Although a number of rarer conditions were later classified as myeloproliferative disorders (renamed MPN by the World Health Organisation, WHO, in 2008), ET, PV, MF and CML still represent the great bulk of this diagnostic category in terms of prevalence and clinical burden.

The finding in 1960 of a recurrent cytogenetic abnormality in most patients with CML – a reciprocal translocation between chromosome 9 and chromosome 22 [t(9;22)(q34.1;q11.2)], known as the Philadelphia chromosome (Nowell and Hungerford 1960) – greatly advanced our understanding of this disorder and led to the recognition that it represents a distinct entity from the other so-called Philadelphia-negative MPNs. It also resulted in significant improvements in the diagnosis and treatment of CML, inspiring intensive efforts to find equivalent abnormalities in ET, PV and MF.

To facilitate research efforts and to help standardise the diagnosis and treatment of patients with MPNs, the Polycythemia Vera Study Group (PVSG) was formed in the 1960s. Its principal role was to coordinate clinical trials of patients with MPNs and, in order to provide consistency between clinical studies, the PVSG introduced formal diagnostic criteria for the three main disorders, which have subsequently been refined by the WHO (Table 1.1). The most recent

Table 1.1 2008 WHO Criteria for the diagnosis of the myeloproliferative neoplasms

		Polycythaemia vera	Essential thrombocythaemia	Myelofibrosis
Major criteria	1	Hb >18.5 g/dL (men) >16.5 g/dL (women) or other evidence of increased red cell volume* *or* elevated red cell mass >25% above mean normal predicted value	Sustained platelet count ≥450x10^9/L	MK proliferation and atypia accompanied by reticulin and/or collagen fibrosis *or* in the absence of reticulin fibrosis, MK changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e. pre-fibrotic PMF)
	2	Presence of *JAK2*V617F or similar mutation (e.g. *JAK2* exon 12)	Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature MKs. No significant increase or left-shift of neutrophil granulopoiesisor erythropoiesis	
	3		Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm	Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
	4		Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of reactive thrombocytosis	Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of reactive marrow fibrosis
Minor criteria	1	Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation		Leukoerythroblastosis
	2	Subnormal serum Epo level		Increased serum LDH
	3	EEC growth		Anaemia
	4			Palpable splenomegaly

Based on Tefferi and Vardiman (2008).

The diagnosis of PV requires fulfilment of either both major criteria and one minor criterion or the first major criterion and 2 minor criteria. The diagnosis of ET requires fulfilment of all 4 major criteria. The diagnosis of PMF requires fulfilment of all 3 major criteria and 2 minor criteria.

* Hb or Hct >99th percentile of reference range for age, sex or altitude of residence; *or* Hb >17 g/dL (men), >15 g/dL (women) if associated with sustained increase of ≥2g/dL from baseline that cannot be attributed to correction of iron deficiency

WHO definition of ET requires all four major diagnostic criteria to be fulfilled: the presence of a sustained thrombocytosis, defined as a platelet count of ≥450x10^9/L; the presence in the bone marrow of megakaryocytic proliferation with increased numbers of mature forms and no increase in immature granulocytic and erythroid precursors; no evidence of another myeloid disorder, i.e. MF, myelodysplasia (MDS), CML or PV; and either the detection of a specific clonal marker or no evidence of a reactive thrombocytosis.

Despite exhaustive studies, cytogenetic analysis of the Philadelphia-negative MPNs has not yielded the same breakthrough as in CML, and so numerous other approaches have been adopted to elucidate the pathogenic processes underlying them. The discovery that PV was characterised by the presence of endogenous erythroid colonies (EECs) – progenitors which proliferate in vitro in the absence of erythropoietin (EPO) – and that endogenous megakaryocytic colonies were present in some cases of ET, raised the possibility that aberrations in the regulation of haemopoiesis might contribute to their pathogenesis (see section 1.3.4). Consequently, the major haemopoietic growth factors, EPO, granulocyte-colony stimulating factor (G-CSF), and thrombopoietin (TPO) were systemically analysed for the presence of genetic mutations, transcriptional defects and disruption of the normal homeostatic mechanisms. Unfortunately, no abnormality proved sufficiently common or specific to an individual disease to offer a major insight into its pathogenesis or be of diagnostic value, with the exception of suppression of EPO in most cases of PV (Messinezy *et al,* 1995). Similarly, the cell surface receptors with which these growth factors interact were not found to harbour recurrent abnormalities in the majority of patients with acquired MPNs.

In the 1980s and 1990s, this failure to discover a common, specific cytogenetic or molecular marker in the Philadelphia-negative MPNs prompted intense interest in the use of clonal markers as an alternative means to identify the haemopoietic population involved in the disease process (see section 1.3.5). Hypothesising that the disorders were initiated by a single, transforming event in a myeloid precursor which then proliferated to generate a population of

phylogenetically related progeny, it was argued that an affected patient should have evidence of clonal myelopoiesis, and it was hoped that this would be of diagnostic use. It soon became apparent, however, that a proportion of patients with bona fide MPNs had polyclonal or oligoclonal myelopoiesis.

The molecular pathogenesis of the Philadelphia-negative MPNs therefore remained obscure for at least forty years after the discovery of the Philadelphia chromosome. Clinically, the absence of a common cytogenetic or molecular marker hindered both diagnostic and therapeutic advances. Distinguishing individuals with ET and PV from those with reactive conditions was frequently a diagnostic challenge, and without a molecular lesion, designing targeted drugs (an approach which proved so successful in CML) was impossible.

For these reasons, the identification in 2005 of a specific point mutation affecting exon 14 of the *JAK2* gene (*JAK2* V617F) in a large proportion of patients with Philadelphia-negative MPNs was greeted by great excitement in the field (section 1.3.7). This finding, it was hoped, would finally explain the pathogenic process which gave rise to these disorders and revolutionise their management, as the discovery of the Philadelphia chromosome had done in CML.

1.2 Physiological megakaryopoiesis and platelet production

1.2.1 MK development and platelet biogenesis

Platelets play a key role in initiating haemostasis following vascular injury, and may also be involved in wound healing, inflammation and the innate immune response. They are small (3.0µm by 0.5µm), discoid anucleate cells, rich in cytoskeletal and signalling proteins, which are produced by budding from their precursor cell, the MK (Hoffbrand *et al,* 2005).

MKs are large (50-100µm diameter) cells, with a polyploid, multilobated nucleus. They arise in the bone marrow from MK progenitor cells (burst-forming unit-MK, BFU-MK, colony-forming unit-MK, CFU-MK, and high-proliferative potential-colony-forming unit-MK). These progenitors are themselves derived from a common MK-erythroid progenitor (MEP) cell, which in turn originates from the haemopoietic stem cell (HSC), via a common myeloid precursor (CMP) (Adolfsson *et al,* 2005; Debili *et al,* 1996; Figure 1.1).

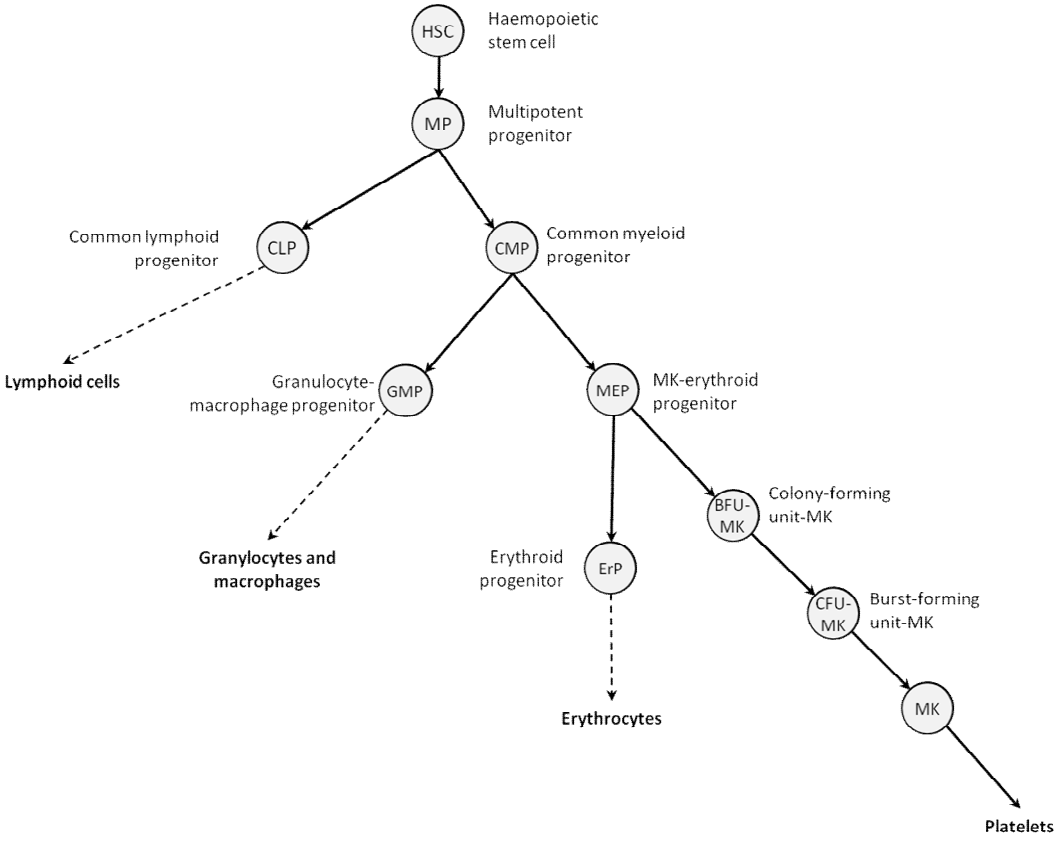


Figure 1.1 Haemopoietic cell differentiation pathway

Schematic representation of the principal stages in MK maturation, with some stages in the maturation pathway of other haemopoietic cells also shown.

During maturation, MKs undergo a number of specific changes which allow effective platelet production. They greatly amplify their DNA content, up to 128N, by a process of endomitosis, which results in cytoplasmic expansion and ribosomal proliferation, in preparation for the rapid production of platelet-specific proteins (Patel *et al,* 2005). MK maturation is also accompanied by the development of extensive internal membrane systems, continuous with the plasma membrane (Behnke 1968; Nakao and Angrist 1968). These internal membrane systems evaginate to form MK pseudopodial extensions called proplatelets, which then fragment to produce platelets (Behnke 1969; Radley and Haller 1982). Before the proplatelets form, large amounts of platelet-specific proteins are synthesised, and either transported to the MK surface or loaded into granules derived from Golgi complexes (Heijnen *et al,* 1998). At the same time, a dense tubular network and a system of channels called the open canalicular network is created to facilitate granule release (Gerrard *et al,* 1976).

Under steady-state conditions MKs represent approximately one in 2000 nucleated bone marrow cells; each one may release 1000-5000 platelets and in total around $2x10^{11}$ platelets are formed every day (Kaushansky 2005; Trowbridge *et al,* 1984). However, in times of increased platelet consumption (e.g. immune thrombocytopenia), the MK mass may increase 3-fold, with a corresponding increase in platelet production (Branehög *et al,* 1975). The regulation of MK proliferation and platelet production is controlled by a complex system of humoral factors, cell surface receptors, intracellular signalling pathways, and nuclear transcription factors.

1.2.2 Hormonal regulation of megakaryopoiesis and thrombopoiesis

Interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF are all important in the early stages of megakaryopoiesis, but the primary humoral regulator of

megakaryopoiesis and thrombopoiesis is undoubtedly TPO, formerly known as the c-Mpl ligand. TPO is critical for all stages of megakaryopoiesis and thrombopoiesis, from the survival of HSCs to platelet formation, and even affects the haemostatic activity of mature platelets (Kojima *et al,* 2001). TPO infusions markedly increase MK ploidy and number, and stimulate platelet production (Kaushansky *et al,* 1994) whereas TPO-knockout mice have dramatically reduced numbers of MK progenitors and are severely thrombocytopenic (de Sauvage *et al,* 1996). MKs cultured *in vitro* in the presence of TPO extend numerous proplatelets, which structurally resemble those seen *in vivo*, and generate platelets which are functionally and structurally similar to normal platelets (Choi *et al,* 1995; Cramer *et al,* 1997).

TPO is encoded by a gene on chromosome 3q26.3-3q27, and is initially synthesised as a 353 amino acid propeptide, which is subsequently modified to a heavily glycosylated 332 amino acid functional protein. Under steady-state conditions, TPO is constitutively produced by the liver and kidneys and secreted into the bloodstream (Lok *et al,* 1994). Following binding to its receptor, c-Mpl on the surface of platelets and MKs, TPO is internalised and degraded (Fielder *et al,* 1996). Consequently, its levels generally vary inversely with platelet and MK mass (Emmons *et al,* 1996; Kuter and Rosenberg 1995). Of note, although endothelial cells also express c-Mpl and have a 100-fold greater surface area than platelets and MKs combined, they seem not to play a significant role in the regulation of serum TPO levels (Cardier and Dempsey 1998; Geddis *et al,* 2006).

In certain conditions, however, TPO levels are not inversely proportional to the platelet count. In immune thrombocytopenia, characterised by immune-mediated platelet and MK destruction, TPO levels may be near-normal (Houwerzijl *et al,* 2005; Kosugi *et al,* 1996), possibly due to its uptake by the increased MK mass and the sequestered platelets. In other circumstances, serum TPO concentrations may be influenced by factors outside this autoregulatory loop: reactive thrombocytosis, commonly seen in inflammatory states, is often accompanied by a rise rather than a fall in TPO levels, due primarily to IL-6 (an acute phase

protein, secreted by macrophages, fibroblasts and endothelial cells in response to bacterial infection and tissue damage) upregulating hepatic TPO synthesis (Hollen *et al,* 1991; Kaser *et al,* 2001; Kishimoto 1989). TPO levels may also be inappropriately normal or elevated in ET (discussed in section 1.3.3). Finally, during severe thrombocytopenia, additional TPO may be expressed by bone marrow stromal cells, though the mechanism for this is poorly understood (Sungaran *et al,* 1997).

1.2.3 Molecular signalling pathways involved in thrombopoiesis

TPO binds to c-Mpl on MKs, other haemopoietic precursors, and platelets, inducing conformational changes in the receptor which initiate signal transduction. This TPO/c-Mpl interaction plays a critical role in the regulation of both megakaryocytic and non-megakaryocytic haemopoietic progenitors: c-Mpl knockout mice have a marked reduction in MKs and are severely thrombocytopenic, but are also severely deficient in progenitors committed to all haemopoietic lineages (Alexander *et al,* 1996). Like other type 1 haemopoietic growth factor receptors, such as the erythropoietin receptor (EPOR) and the granulocyte-colony stimulating factor receptor (G-CSFR), c-Mpl is a single chain molecule without intrinsic tyrosine kinase activity but instead binds and relies on the activity of a non-receptor tyrosine kinase, Janus kinase 2 (JAK2).

In its unbound state, c-Mpl is expressed as a monomer on the cell surface (Horan *et al,* 1996; Alexander *et al,* 1996). Ligand binding induces dimerisation of two adjacent receptors, resulting in mutual cross-activation and activation of the bound JAK2 molecules (Kaushansky 2005). The activated JAK kinases initiate downstream signalling, by phosphorylating tyrosine residues on the receptor itself and on intracellular intermediaries, in particular the signal transducers and activators of transcription (STATs). STATs are critical for the survival and

expansion of haemopoietic progenitors (Kirito *et al,* 2002a; Snow *et al,* 2002), both by inducing transcription of anti-apoptotic genes such Bcl-xL (Kirito *et al,* 2002b), and by interacting with the GATA family of transcription factors (TFs) (Ezoe *et al,* 2005).

In addition, JAK kinases can activate Ras and, by phosphorylating phosphoinositide 3-kinase (PI3K), generate phosphoinositol$_{3,4,5}$ triphosphate (PIP_3). Both PIP_3 and activated Ras inhibit the pro-apoptotic TF forkhead box O (FOXO) 3a, via activation of its negative regulator Akt, and thus promote MK survival (Geddis *et al,* 2001; Miyakawa *et al,* 2001; Nakao *et al,* 2008). Phosphorylated Ras can also activate members of the mitogen-activated protein kinase (MAPK) family, in particular the extracellular signal-regulated kinases (ERK) 1 and 2 which induce MK maturation via activation of the Runt-related TF 1 (RUNX1) (Hamelin *et al,* 2006; Rojnuckarin *et al,* 1999).

The resulting signalling cascade results in MK proliferation, growth and maturation, with increased ploidy, and platelet maturation and release. Modulation of this cascade is provided by simultaneous phosphorylation by JAK kinases of the suppressors of cytoplasmic signalling (SOCS) proteins, and SHP1 and SHIP1 phosphatases, which block cell signalling, primarily by inhibiting the phosphorylating activity of the JAK kinases (Kaushansky 2009; Figure 1.2).

1.2.4 Transcriptional control of megakaryopoiesis and thrombopoiesis

A number of TFs control MK development and thrombopoiesis, of which the best characterised are GATA-1, RUNX1, hypoxia-inducible factor (HIF) and nuclear factor erythroid 2 (NF-E2). GATA-1 is a zinc-finger protein which plays a critical role in all stages of megakaryopoiesis, and MK progenitors from GATA-1-deficient mice show maturation arrest at the promegakaryocyte stage (Shivdasani *et al,* 1997; Vyas *et al,* 1999). The role of GATA-1 in MK development is mediated in part by its interaction with another TF, friend of GATA-1 (FOG-1) and GATA-1

mutations which disrupt this association result in thrombocytopenia due to MK maturation arrest, and anaemia (Muntean and Crispino 2005). GATA-1 also binds the TF RUNX1, resulting in activation of MK promoters such as the αIIb integrin promoter (Elagib *et al,* 2003), and upregulates expression of key genes involved in megakaryopoiesis and platelet assembly, including those encoding the p45 subunit of NF-E2, c-Mpl, platelet factor 4 and a number of platelet glycoproteins (Tsang *et al,* 1997; Vyas *et al,* 1999).

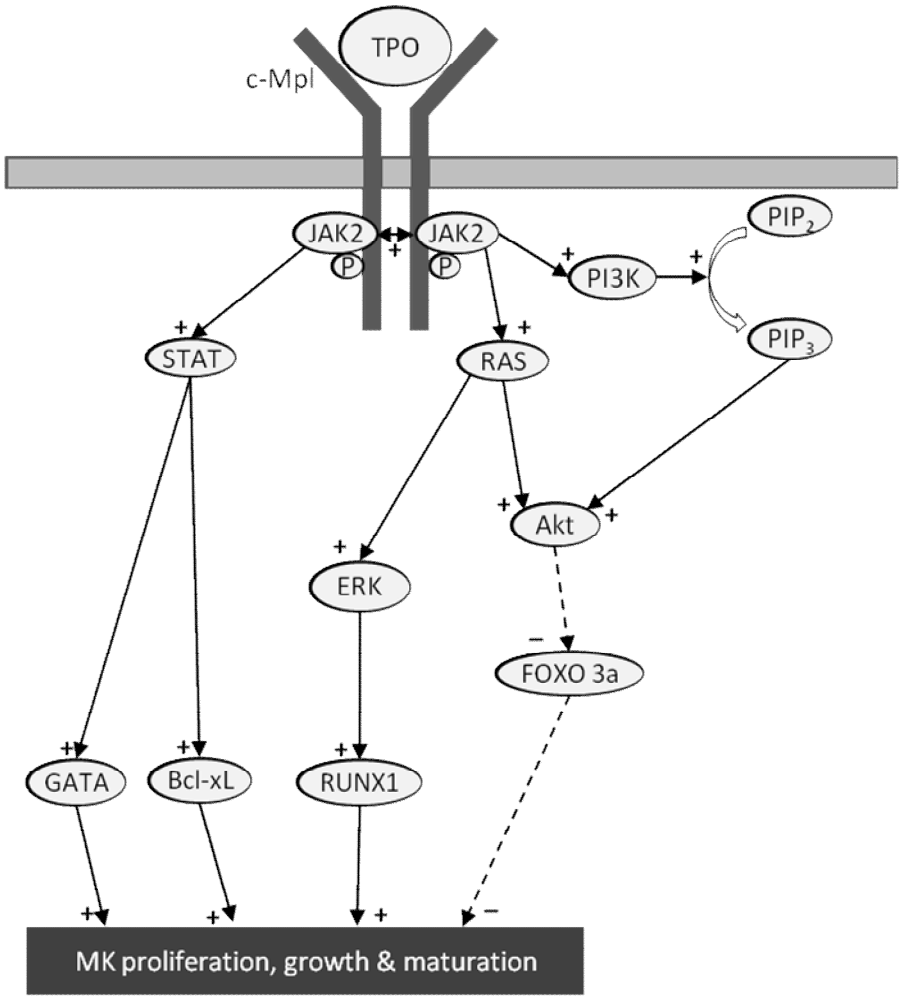


Figure 1.2 Principal JAK2-mediated MK signalling pathways

Following TPO binding, c-Mpl receptor dimerisation occurs, leading to JAK2 cross-activation and activation of downstream signalling proteins, including STAT, RAS and PI3K. This results in MK proliferation, growth and differentiation via induction of transcription factors such as the GATA family, Bcl-xL and RUNX1 and inhibition of the pro-apoptotic transcription factor FOXO 3a.

Like GATA-1, the TF HIF is important both in early and late megakaryopoiesis. It consists of two subunits: HIF-1α, whose expression is regulated by TPO, and HIF-1β, which is stably expressed. By regulating expression of vascular endothelial growth factor (VEGF), which in turn supports the survival of HSCs, HIF-1α is important in the very early stages of megakaryopoiesis (Kirito *et al,* 2005). Further, HIF-1α controls production of stromal cell-derived factor-1 (SDF-1), a trophic factor which induces proliferation of MK progenitors, polyploidisation of MKs, cell migration, and platelet production, and is therefore also involved in the later stages of megakaryopoiesis (Ceradini *et al,* 2004; Lane *et al,* 2000).

The heterodimeric leucine-zipper protein NF-E2 is another TF which plays a crucial role in terminal MK differentiation and platelet release, probably by regulating expression of several genes which encode MK proteins required for thrombopoiesis, including thromboxane synthase, β1-tubulin and Rab27b (Schulze and Shivdasani 2005). Loss of NF-E2 function does not markedly affect early MK differentiation, but results in disorganised internal membranes, severely impaired proplatelet formation and platelet release, and profound thrombocytopenia (Lecine *et al,* 1998).

1.3 ET – pathology and clues to pathogenesis

1.3.1 Cytological and histological changes

Although blood from ET patients usually looks macroscopically normal, using even low-power microscopy the thrombocytosis becomes obvious (Figure 1.3). Platelet anisocytosis may be evident, ranging from tiny forms to giant platelets, but frank morphological abnormalities are rare (Hehlmann *et al,* 1988). Histologically, the bone marrow cellularity varies from normal to

moderately hypercellular for age with a marked proliferation of large and giant MKs, which are characteristically arranged in loose clusters. They typically have deeply lobulated and hyperlobated nuclei, and abundant mature cytoplasm. Expansion of the myeloid and erythroid compartments and reticulin fibrosis are not prominent in ET (Thiele *et al,* 1999).

Ultrastructurally, ET platelets show defective pseudopod development (finger-like projections which appear following platelet activation at the site of vessel injury), reduced concentrations of alpha granules and abnormal tubular systems (Barnhart *et al,* 1980; Tablin *et al,* 1989). Such morphological changes might explain the impairment in platelet aggregation frequently seen in ET patients, who characteristically exhibit a reduced response to adrenaline stimulation (Avram *et al,* 2001; Hehlmann *et al,* 1988; Tsantes *et al,* 2010). Electron microscopy of ET MKs reveals a loss of the cytoplasmic organisation seen in normal MKs. In particular, there is a marked disturbance of the membrane systems, which are greatly increased in amount, disordered, vesiculated, and do not form the clearly-delineated platelet fields required to form normal proplatelets (Tablin *et al,* 1989).

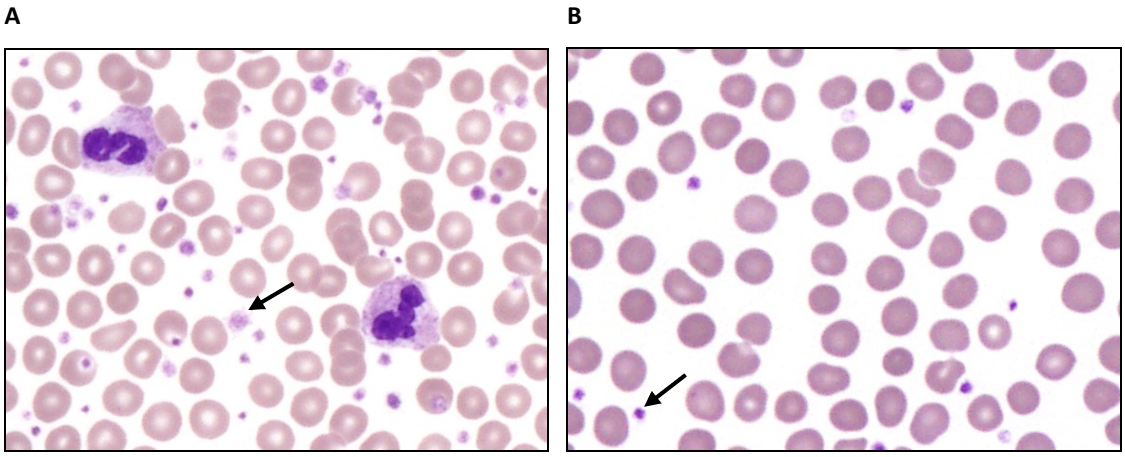


Figure 1.3 Peripheral blood smear from a patient with ET and from a haematologically normal control

A. Patient with ET. B. Haematologically normal control
Arrows indicate platelets

1.3.2 Cytogenetics

Cytogenetic abnormalities are uncommon in ET, present in up to 7% of cases, and none are specific to the disease (Bacher *et al,* 2005; Sessarego *et al,* 1989). The most frequent abnormalities include trisomy 8 and 9, deletions in 13q and 20q (Case Jr 1984; Gangat *et al,* 2009), and abnormalities of chromosome 1 (Gangat *et al,* 2009). Recent single-nucleotide polymorphism (SNP) microarray analysis has also failed to demonstrate the presence of recurrent genomic deletions or amplifications (Kawamata *et al,* 2008; Stegelmann *et al,* 2010), although occasionally acquired loss of heterozygosity affecting the short arm of chromosome 9 (9pLOH) has been identified in ET patients (Kralovics *et al,* 2003; Kralovics *et al,* 2005). The presence of cytogenetic abnormalities appears not to affect prognosis in ET (Gangat *et al,* 2009) and their role in the pathogenesis of the disorder is uncertain.

1.3.3 TPO levels and c-Mpl expression

A number of studies have demonstrated that, in ET, serum TPO levels are similar or elevated compared to normal controls (Table 1.2), which contrasts with the situation in PV, where levels of the principal regulator of erythropoiesis, EPO, are usually suppressed (Johansson *et al,* 2002; Mossuz *et al,* 2004). The reason why TPO is not suppressed in ET has not been convincingly elucidated. In *reactive* thrombocytosis, the increased hepatic TPO synthesis is due to elevated IL-6 levels (Hollen *et al,* 1991; Kaser *et al,* 2001). In ET, however, serum IL-6 levels are either normal (Hollen *et al,* 1991; Panteli *et al,* 2005) or suppressed (Griesshammer *et al,* 1998; Tefferi *et al,* 1994).

One hypothesis is that the c-Mpl-mediated internalisation/degradation mechanism which underpins normal TPO autoregulation is defective in ET. Horikawa *et al* (1997) found that surface c-Mpl expression on platelets from all 17 ET patients studied was dramatically lower

Table 1.2 Studies examining TPO levels in ET

Reference	Normal controls		ET patients		p-value
	n	TPO levels mean±SD /pg.mL^{-1} *	n	TPO levels mean±SD /pg.mL^{-1} *	
(Tahara *et al,* 1996)	50	*0.75 ± 0.29*	6	*2.8 ± 1.55*	<0.05
(Cerutti *et al,* 1997)	32	156.7† (62.2-352.7)	32‡	246.2† (93.5-4596)	0.0006
(Horikawa *et al,* 1997)	21	*0.76 ± 0.21*	17	*1.31 ± 1.64*	NS
(Hirayama *et al,* 1998)	6	22.1 ± 8.2	2	21.1 ± 1.5	NS
(Wang *et al,* 1998)	17	201 ± 112	20	505 ± 459	<0.05
(Griesshammer *et al,* 1998)	117	95.3 ± 54.0	25	545 ± 853	<0.001
(Harrison *et al,* 1999b)	12	122 ± 69	18	162 ± 138	NS
(Li *et al,* 2000)	18	179 ± 112	23	189 ± 131	NS

* Italicised values indicate units are fmol/L

† Median (range)

‡ 12 of these patients had PV

than on normal control samples, with correspondingly lower c-Mpl mRNA expression. Subsequent studies have largely confirmed these findings, but with some variation: Li *et al* (2000) observed a 10-fold reduction in c-Mpl surface expression in ET platelets; Harrison *et al (*1999b) reported a less marked (but still significant) difference, with overlap between ET, other MPNs, and reactive thrombocytosis; Moliterno *et al* (1998), however, found no difference between the ET patients and normal controls. A similar downregulation in surface c-Mpl expression has also been described in ET MKs (Mesa *et al,* 2002). Of note, in the 23 ET patients reported by Li *et al* (2000) whose TPO levels were similar to normal controls, the reduction in platelet c-Mpl expression was accompanied by a four-fold decrease in platelet-mediated TPO clearance. Such a reduction in TPO clearance might explain its elevated levels in some patients with ET, and account for their thrombocytosis. In a murine model, suppression of c-Mpl expression resulted in a dramatic thrombocytosis (Tiedt *et al,* 2009).

Another possible explanation for the elevated TPO levels seen in ET is that transcriptional or translational regulation of TPO synthesis is defective. Mutations in the 5'-untranslated region (5'-UTR) or a splice donor site in intron 3 of the *TPO* gene have been identified in four families with familial thrombocythaemia (FT; Table 1.3A). In haematologically normal individuals, a number of upstream open reading frames (uORFs) located within the 5'-UTR inhibit translation of *TPO* mRNA (Ghilardi *et al,* 1998); the mutations observed in FT reduce this uORF-mediated inhibition, resulting in excessive TPO protein synthesis and thrombocytosis. Equivalent abnormalities in patients with sporadic ET have not, however, been identified (Allen *et al,* 2001; Harrison *et al,* 1998).

Table 1.3 Mutations reported in familial thrombocythaemia

A. Familial thrombocythaemia due to mutations in the *TPO* gene

Reference	Mutation	Location in *TPO* gene	Inheritance*	Number of cases	Number of kindreds	Serum TPO in affected cases (pg/mL)
(Wiestner *et al,* 1998)	IVS3+1G>T	Intron 3 splice donor site	AD	11	1	80-1180
(Jorgensen *et al,* 1998)	IVS3+5A>G	Intron 3 splice donor site	AD	4	Not stated	430-980
(Jorgensen *et al,* 1998)	3252delG	5'-UTR	AD	5	1	422-1820
(Ghilardi *et al,* 1999)	516G>T	5'-UTR	AD	4	1	420-570

B. Familial thrombocythaemia due to mutations in the *MPL* gene

Reference	Mutation	Exon Domain†	Inheritance*	Number of cases	Notes
(Moliterno *et al,* 2004)	K39N (1238G>T)	2 ECD	See note	3 homozygotes 12 heterozygotes	Mild thrombocytosis in heterozygotes, severe in homozygotes. Heterozygote frequency in African-American populations was 7%. Not detected in 250 Caucasian, 40 Hispanic or 39 Asian controls
(Ding *et al,* 2004)	S505N (1073G>A)	10 TMD	AD	8 heterozygotes	All 8 cases in a single kindred
(El-Harith *et al,* 2009)	P106L (317C>T)	3 ECD	See note	8 homozygotes 18 heterozygotes	Affected cases all homozygotes of Arabic ancestry (4 families). Of the 18 heterozygotes, 4 had a mild thrombocytosis. Heterozygote frequency amongst an Arabic population studied was 7%
(Teofili *et al,* 2010)	S505N (1073G>A)	10 TMD	AD	24 heterozygotes	24 cases spread across 8 kindreds. Similar phenotype to sporadic ET (thrombotic and fibrotic risk)

† ECD indicates extracellular domain; TMD indicates transmembrane domain

* AD indicates autosomal dominant

1.3.4 Growth factor hypersensitivity in ET progenitors and possible causes

Another hypothesis put forward to explain excessive platelet production in ET, especially in patients whose TPO levels are not elevated, is that the progenitors are hypersensitive to TPO, or even proliferate in its absence. The existence of EECs in PV is well described (Prchal and Axelrad 1974; Zanjani *et al,* 1977) and represents a minor diagnostic criterion in the latest WHO classification (Swerdlow *et al,* 2008; Table 1.1). Indeed, the presence of EECs has been described in some ET patients (Juvonen *et al,* 1987; Partanen *et al,* 1983), though its significance is unclear. It is possible that it may be an artefact caused by growth factor secretion by contaminating T-cells, or that the majority of patients with a thrombocytosis and EECs actually have PV masked by iron deficiency (Shih and Lee 1994).

A number of studies have demonstrated the presence of endogenous MK colonies (EMCs) in ET (Battegay *et al,* 1989; Juvonen *et al,* 1987), though the rate varies between 63% (Florensa *et al,* 1995) and 100% of cases (Mi *et al,* 2001). Although EMCs have not been reported in reactive thrombocytosis (Dobo *et al,* 2004; Rolovic *et al,* 1995), they have been detected in between 33% and 95% of PV patients (Escoffre-Barbe *et al,* 2006; Mi *et al,* 2001), indicating that they are not specific to ET. Moreover, it has been suggested that components of the 'serum-free media' used to screen for EMCs (e.g. albumin) may in fact have been contaminated by serum, and that the 'spontaneous' megakaryocytic growth actually represented progenitor hypersensitivity to TPO (Axelrad *et al,* 2000). Indeed, Axelrad *et al* (2000) did demonstrate that megakaryocytic progenitors in ET were on average 53-times more sensitive to TPO than were those from normal controls, findings which have been confirmed by subsequent studies (Kawasaki *et al,* 2001; Mi *et al,* 2001). Axelrad *et al* (2000) went on to speculate that progenitor hypersensitivity to regulatory cytokines might exist in every MPN, and that the lineage of the hypersensitive progenitor determined the phenotype.

The observation that transforming factor beta 1 (TGF-β1), a platelet and MK-derived cytokine, was involved in the negative regulation of megakaryopoiesis in mice (Ishibashi *et al,* 1987) prompted speculation that defects in this pathway might be responsible for the presence of EMCs in ET. In keeping with this, Zauli *et al* (1993) demonstrated that megakaryocyte progenitors in ET were less sensitive to TGF-β1 than those from healthy controls. It is possible that this effect is mediated by the TF NFI-B: increased NFI-B has been associated with 9pLOH in some MPN patients (the gene encoding NFI-B is located on chromosome 9p), and in a murine model overexpression of this gene resulted in TGF-β insensitivity (Kralovics *et al,* 2002). A subsequent larger study by the same group, however, refuted the association between 9pLOH and increased NFI-B expression and attributed their earlier findings to chance (Kralovics *et al,* 2003).

Another hypothesis to explain TPO hypersensitivity in ET was that that myeloid progenitors in this disease secreted TPO, generating an autocrine feedback loop. This, however, was refuted by the finding that CD34+ cells only express *TPO* mRNA at very low levels, and that addition of a neutralising antibody to TPO did not affect EMC activity (Taksin *et al,* 1999). The same group and others also investigated the possibility that the activating mutations in c-Mpl observed in FT (Table 1.3B), might also account for the thrombocytosis and autonomous MK proliferation seen in sporadic ET. Two groups therefore sequenced the entire coding region of the gene in a total of 19 patients with non-familial ET, but no mutations were detected (Kiladjian *et al,* 1997; Taksin *et al,* 1999). Subsequent technical advances have allowed larger number of patients to be screened and mutations in the gene encoding c-Mpl have since been identified in approximately 5% of ET patients (see section 1.3.8).

1.3.5 Clonality in ET

Dameshek's original hypothesis for the aetiology of the MPNs suggested that myeloid proliferation occurred in response to a myelostimulatory factor (Dameshek 1951), but he did not speculate on whether the proliferating myeloid cells were derived from a single haemopoietic progenitor or many – i.e. whether it was a monoclonal or polyclonal process. This distinction is of fundamental importance to our understanding of the diseases, since monoclonality is a characteristic feature of neoplastic disorders. Although the Philadelphia chromosome was subsequently detected in most patients with CML (Nowell and Hungerford 1960), in keeping with it being a monoclonal disease, no equivalent abnormality was apparent in the majority of PV and ET patients.

In order to address the issue of clonality in the MPNs, Fialkow *et al* (1967) pioneered the use of X-chromosome inactivation pattern (XCIP) analysis. This technique exploits the cellular mosaicism present in most females (with regard to X-chromosome inactivation) to determine whether a cell population has arisen from a single progenitor without requiring the presence of a specific disease marker. Seminal works by Lyon *et al* (1961) and Beutler *et al* (1962) had shown that in females one X-chromosome is randomly inactivated in every cell during embryogenesis in order to maintain comparable X-linked gene dosage with males. Mohendas *et al* (1981) subsequently demonstrated that the mechanism for this X-chromosome inactivation was methylation. By a process of imprinting, all progeny of these cells inactivate the same X-chromosome as their original progenitor.

In normal polyclonal haemopoiesis one would, therefore, expect some blood cells to inactivate the maternal X-chromosome, and others to inactivate the paternal X-chromosome, since the probability of every stem cell in the original pool inactivating the same X-chromosome by chance is small. By contrast, if a particular population of cells is clonal and has arisen from a single transformed precursor, every cell in that population will inactivate the same X-

chromosome, and the XCIP for the whole population will be 'skewed' if the clonal population is large enough.

In order to determine the XCIP of a tissue or population of cells it is necessary to distinguish between the maternal and the paternal X-chromosome, and between the active and the inactive X-chromosome. The first criterion requires that the individual is heterozygous for an X-linked gene or allelic marker, such as a tandem repeat sequence. The second involves either measuring the relative expression (at the mRNA or protein level) of each X-linked allele for which the individual is heterozygous, or analysing their DNA methylation patterns.

Using this approach, Fialkow *et al* (1967) investigated the clonality of granulocytes from three CML female patients who were heterozygous for isoenzymes of the X-linked gene glucose-6-phosphate dehydrogenase (*G6PD*). In all three patients, only a single G6PD isoenzyme was detectable in their granulocytes whereas both isoenzymes were detected in their fibroblasts, and the authors concluded that this implied that CML was a clonal disease. Whether the same was true for ET was not investigated until 1981, when the same group studied three ET female patients who were also heterozygous for isoenzymes of G6PD. In two patients, only a single G6PD isoenzyme was detectable in all erythrocytes, granulocytes and platelets, whilst in the third patient there was evidence of 95% skewing towards one isoenzyme which had become 100% four months later, a finding which was interpreted as evidence for myeloid clonality in ET. Several other groups subsequently confirmed these observations (Gaetani *et al,* 1982; Singal *et al,* 1983), though patient numbers in these early protein-based studies were limited by the relatively low frequency of G6PD isoenzyme heterozygosity in individuals of non-African ancestry.

Evaluation of much larger cohorts, however, was made feasible by the development of DNA methylation-based approaches to XCIP analysis, allowing approximately 50% of females to be analysed using polymorphic sites within the X-linked genes phosphoglycerate kinase and

hypoxanthine-guanine phosphoribosyltransferase (Vogelstein *et al,* 1985; Vogelstein *et al,* 1987). The proportion of evaluable females was further increased by the analysis of other X-linked loci, such as the highly polymorphic variable number tandem repeat (VNTR) sequences in the DXS255 locus and the human androgen receptor (*HUMAR*) gene, for which more than 90% of females are polymorphic (Allen *et al,* 1992; Boyd and Fraser 1990; Gale *et al,* 1992; Lucas *et al,* 1989; Tsukamoto *et al,* 1994). In addition, PCR rather than Southern blotting-based analysis allowed assessment of smaller amounts of tissue (Gilliland *et al,* 1991).

An alternative approach to the XCIP analysis involves assaying the transcribed mRNA products of an X-linked gene. Early studies exploited a polymorphism in the *G6PD* gene, different from that used in protein-based clonality studies and more frequently informative, to distinguish between the maternal and paternal X-chromosomes (Curnutte *et al,* 1992; Prchal and Guan 1993). Subsequently a number of genes, including iduronate-2-sulfatase, *MPP1* and *FHL-1*, have proved useful in transcription-based clonality analysis, allowing over 95% of females to be evaluated (Gregg *et al,* 2000; Liu *et al,* 2003; Luhovy *et al,* 1995).

Use of XCIP-based clonality analysis in ET expanded rapidly in the 1990s as a result of these technical advances. Initial studies were often interpreted as confirming the results of the earlier protein-based assays – that ET was a clonal disorder – even though some ET patients appeared to have non-clonal myelopoiesis (Anger *et al,* 1990; Kassar *et al,* 1995; Lucas *et al,* 1989; Tsukamoto *et al,* 1994). With increasing use of the technique, however, a number of limitations and caveats became apparent. First, it cannot be used to investigate males, or indeed females who are not heterozygous for an X-linked marker gene. Since about 70% of ET patients are female (Jensen *et al,* 2000), and approximately 90-95% of these are informative for an X-linked marker, at most about two-thirds of ET patients are evaluable using XCIP analysis.

Second, the XCIP only describes the overall clonality pattern for the entire cell population analysed; even if the XCIP of the clonal cells is grossly imbalanced, the presence of non-clonal cells (where the XCIP would be expected to be more balanced) will diminish the degree of skewing of the overall population. To accommodate this, the minimum X-allele inactivation ratio which most studies use to define clonality is 75%:25% – i.e. the degree of inactivation of one allele is at least 3-fold greater than the other (Vogelstein *et al,* 1987). Even so, a small clone within a large polyclonal population may be missed (Levine *et al,* 2006).

Third, since the XCIP is established early in embryogenesis in a small pool of haemopoietic stem cells, extreme skewing may occur by chance (Gale *et al,* 1991; Gale *et al,* 1993). It has been estimated that degree of X-chromosome inactivation commonly used to define clonality (75%:25%) would arise purely by chance in 18% of individuals if XCI occurred in a haemopoietic stem cell pool consisting of eight cells (Gale and Linch 1994). An apparently clonal pattern in myeloid cells may, therefore, reflect an imbalanced constitutional pattern, and would not necessarily indicate the presence of an abnormal population – a phenomenon termed constitutional skewing. T-cells arise from the same haemopoietic stem cell as myeloid cells and are assumed to have the same constitutive XCIP. They are not believed to be involved with the abnormal clone in ET. Using the patient's T-cell XCIP as a control, therefore, allows individuals with constitutional skewing to be identified.

Finally, elderly women may appear to have a clonal XCIP even in the absence of a haematological disorder, a consequence of 'age-related' skewing. Busque *et al* (1996) observed that 38% of haematologically normal elderly females (≥60 years old) had an apparently clonal XCIP, compared to 16% of younger adults, and Gale *et al* (1997) reported equivalent figures of 56% in elderly female controls (≥75 years old) compared to 22% in younger women. T-cell clonality does not correlate well with myeloid clonality in elderly women and a balanced T-cell XCIP does not, therefore, reliably exclude the presence of this phenomenon (Gale *et al,* 1997). Consequently, an imbalanced myeloid XCIP in an elderly

woman does not necessarily imply the presence of a clonal myeloid population and such a result cannot be meaningfully interpreted – a significant limitation in a disease which predominantly affects older people. The finding of a balanced XCIP, however, *does* indicate the presence of polyclonal myelopoiesis, regardless of age.

Taking all these caveats into account, in order to fulfil the criteria of clonal myelopoiesis the patient must be younger than 65 years and have a myeloid XCIP which shows more than 75% inactivation of one allele, which is also at least 20% different from the control (T-cell) XCIP.

As these refinements to the interpretation of XCIP analysis became better understood in the late 1990's, a number of studies were published which suggested that a proportion (between 31% and 57%) of ET patients did *not* have evidence of clonal haemopoiesis (Table 1.4). This finding was of great significance since it contradicted the notion that ET was invariably a clonal, neoplastic disorder. The suggestion that ET was a heterogeneous disease was supported by the observation that the rate of vascular events was influenced by clonality status: of the four studies which examined the clinical associations of myeloid clonality, two found that patients with clonal haemopoiesis appeared more likely to suffer a thrombotic complication during their illness than those without (Chiusolo *et al,* 2001; Harrison *et al,* 1999a), whilst the other two found no significant difference (El-Kassar *et al,* 1997; Shih *et al,* 2002).

Table 1.4 Studies of XCIP in ET

Table includes only those studies which allow the criteria described in Section 1.3.5 to be applied

Reference	Technique	Mean age at diagnosis in years (range)	Definition of clonality	Total patients *n*	Myeloid XCIP			Interpretable patients† (of which clonal, %)	`Comments
					Polyclonal	Clonal			
					Total *n*	Total *n*	≤65 years and polyclonal T-cells *n*		
(El-Kassar *et al,* 1997)	DNA (HUMARA) in T-cells, granulocytes and whole blood RNA (IDS, p55, G6PD) in T-cells, granulocytes, platelets and whole blood	62 (13-77)	Allele ratio <0.20	46	14	32	19	33 (58%)	Two further patients fulfilled the criteria for myeloid clonality if platelets rather than neutrophils were analysed Clonal XCIP associated with increased platelet count at diagnosis and older age (P=0.04 and 0.01 respectively) but no effect on thrombosis
(Harrison *et al,* 1999a)	DNA (HUMARA & PGK) in T-cells and neutrophils. RNA (IDS & G6PD) in T-cells, neutrophils and platelets	53 (11-89)	>75% expression of 1 allele	46	13	33	10	23 (43%)	Thrombotic rate higher in clonal than polyclonal patients (60% vs. 15%; P=0.04) No difference in age or platelet count at presentation, follow-up or bleeding risk between clonal and polyclonal groups. No evidence of megakaryocyte-restricted clonality
(Chiusolo *et al,* 2001)	DNA (HUMARA) in T-cells, granulocytes and	34 (20-63)	>75% expression of	40	15	25	17	32 (53%)	Thrombotic rate higher in clonal than polyclonal patients (41% vs. 7%; P=0.04). The thromboses in the clonal patients were mostly unusual

	CD34+ cells RNA (IDS & p55) in platelets		1 allele						splanchnic events No difference in age or platelet count at presentation, or duration of follow-up. No evidence of megakaryocyte-restricted clonality
(Shih *et al,* 2002)	DNA (HUMARA) in T-cells and granulocytes	55 (18-92)	>75% expression of 1 allele	89	15	54	33	48 (69%)	20 of 89 patients excluded because HUMARA homozygous or XCIP was ambiguous Thrombotic rate not significantly higher in clonal than polyclonal patients (33% vs. 7%; P=0.07)
(Teofili *et al,* 2002)	DNA (HUMARA) in T-cells and granulocytes	49 (24-83)	Not stated	19	4	15	9	13 (69%)	Clinical correlates not reported
(Liu *et al,* 2003)	RNA (MPP1, IDS, G6PD, BTK and FHL1) in T-cells and granulocytes	46 (23-63)	Not stated	12	4	8	8	12 (67%)	T-cell clonality not reported but methods imply that T-cell XCIPs were used to identify cases of constitutional skewing Clinical correlates not reported
Overall				**252**	**65**	**167**	**96**	**161 (60%)**	

Table 1.4 Studies of XCIP in ET – continued

† The interpretable number includes only patients with a polyclonal myeloid XCIP and patients with a clonal myeloid XCIP aged ≤65 years with polyclonal T-cell XCIP (i.e. no evidence of possible age-related skewing or constitutional skewing) where the reported data allows such patients to be identified.

1.3.6 *PRV-1* expression

Whilst XCIP analysis undoubtedly advanced our understanding of the clonal origins of ET, the specific molecular defects which initiated the MPNs remained obscure. In an attempt to identify these abnormalities, Temerinac *et al* (2000) analysed differences in gene expression between MPN and normal cells. Using subtractive mRNA hybridisation in neutrophils from 19 PV and six ET patients, and 21 normal controls, they demonstrated overexpression of a novel gene which they called polycythemia vera-1 (*PRV-1*) in all PV patients and two ET patients. Since *PRV-1* mRNA was not detectable in any normal controls, it was hoped that *PRV-1* analysis would be useful diagnostically. *PRV-1* encodes a granulocyte cell surface receptor – the antigen CD-177 – the function of which is unknown. Surprisingly, despite overexpression of *PRV-1* mRNA, protein expression was not elevated in most PV patients (Klippel *et al,* 2002).

Although a subsequent study found *PRV-1* overexpression in all 37 ET patients investigated (Teofili *et al,* 2002), later studies found lower rates of *PRV-1* overexpression in ET patients – between 17% and 67% (Griesshammer *et al,* 2004; Kralovics *et al,* 2003; Liu *et al,* 2003; Tefferi *et al,* 2004). Taking all the reports together, 69 of 114 (61%) ET patients were *PRV-1* positive (Table 1.5), although the lack of a standard definition for *PRV-1* positivity or overexpression makes direct comparison between the seven studies difficult. Two studies found that *PRV-1* overexpression was strongly associated with EEC formation, usually a hallmark of PV (Griesshammer *et al,* 2004; Kralovics *et al,* 2003). Combined with the observation that 40% of *PRV-1* positive ET patients went on to develop PV, this association led to speculation that *PRV-1* positive ET patients actually had 'masked' PV (Griesshammer *et al,* 2004). Interest in *PRV-1*, however, was soon largely overshadowed by a more significant discovery.

Table 1.5 *PRV-1* studies in ET

Reference	Technique	Definition of *PRV-1* positivity	Number of *PRV-1* positive ET patients	Comments
(Temerinac *et al,* 2000)	Northern blot	Presence of band on gel	2/6 (33%)	1 of the positive patients was borderline (faint band)
(Teofili *et al,* 2002)	Northern blot	Presence of band on gel	37/37 (100%)	All clonal patients expressed *PRV-1*
(Kralovics *et al,* 2003)	Real-time PCR	Increased *PRV-1* expression compared to RPL19*	10/15 (67%)	Noted strong association between *PRV-1* positivity and presence of EECs in individual patients
(Liu *et al,* 2003)	Real-time PCR	Increased *PRV-1* expression compared to GAPDH*	2/12 (17%)	*PRV-1* expression overall similar to haematologically normal controls
(Griesshammer *et al,* 2004)	Northern blot	Presence of band on gel	15/30 (50%)	All 15 *PRV-1* positive patients had EECs and 40% of them developed PV *PRV-1* positive patients more likely to develop thrombotic and microcirculatory complications
(Tefferi *et al,* 2004)	Real-time PCR	Increased *PRV-1* expression compared to GAPDH†	3/14 (21%)	
Overall			69/114 (61%)	

* *PRV-1* expression relative to a housekeeping gene (RPL19 or GAPDH) was determined initially for a cohort of normal controls to define the normal range

1.3.7 *JAK2* V617F

In 2005, five groups independently reported a recurrent G>T transversion in exon 14 of the gene encoding *JAK2*, resulting in a valine-to-phenylalanine substitution in residue 617 (*JAK2* V617F) in patients with MPNs (Table 1.6). Three different routes led the five groups to investigate *JAK2*. James *et al* (2005) had previously observed that impairing *JAK2* activity using a tyrosine kinase inhibitor reduced EEC formation (Ugo *et al,* 2004). Kralovics *et al* (2002; 2003) had reported that approximately 30% of PV patients and one of the 15 ET patients studied had 9pLOH – i.e. acquired uniparental disomy involving the short arm of chromosome 9 as a result of mitotic recombination. In their 2005 study, high-definition mapping identified a 6.3Mbp region on chromosome 9p common to all patients which included *JAK2* (Kralovics *et al,* 2005). The other three groups analysed *JAK2* as part of a systematic tyrosine kinase mutation screen (Baxter *et al,* 2005; Levine *et al,* 2005; Zhao *et al,* 2005).

At least 16 other studies have subsequently examined the frequency of *JAK2* V617F in ET, with rates ranging between 23% and 75%, and an overall mean of 53% (Table 1.7). Some of this variation is due to methodological differences, since direct sequencing is less sensitive than allele-specific polymerase chain reaction (AS-PCR) or PCR-restriction enzyme digestion approaches (Campbell *et al,* 2006b), and some may be due to different stringencies in applying the diagnostic criteria. In most studies which have looked for it, *JAK2* V617F has not been identified in normal controls (Table 1.6), though one study of nearly 4000 Chinese hospitalised patients not known to have an MPN did detect it in about 1% of individuals (Xu *et al,* 2007). This study used nested PCR to increase its sensitivity, however, and it is possible that this increased the risk of false-positivity due to contamination.

Table 1.6 Initial studies reporting *JAK2* V617F

Reference	Background	Technique for mutation detection	PV		ET		MF		Normal controls		Notes
			n	V617F+	*n*	V617F+	*n*	V617F+	*n*	V617F+	
(Baxter *et al,* 2005)	Tyrosine kinase mutation screening	Sequencing confirmed by ARMS PCR	73	71 (97%)	51	29* (57%)	8	16 (50%)	90	0	Mutation identified in multipotent progenitor cells, and present in all EECs in PV. No mutation in T-cells
(James *et al,* 2005)	Role of *JAK2* in PV identified by inhibition and knock-down studies	Sequencing	45	40 (89%)	21	9 (43%)	7	3 (43%)	15†	0	Transfection of V617F into cytokine-dependent cell lines resulted in factor independence. Transplant studies in mice resulted in elevated haematocrit
(Kralovics *et al,* 2005)	Previous identification of 9pLOH in PV and ET patients	Sequencing (neutrophil cDNA)	128	83 (65%)	93	21 (23%)	23	13 (57%)	71‡	0	Higher rate of fibrosis, haemorrhage and thrombosis in V617F-positive patients. Transfection of V617F into BaF3 cells led to cytokine hypersensitivity and IL-3 independence
(Levine *et al,* 2005)	Tyrosine kinase mutation screening	Sequencing	164	121 (74%)	115	37 (32%)	46	16 (35%)	269	0	V617F constitutively activated when expressed in 293T cells
(Zhao *et al,* 2005)	Tyrosine kinase and phosphatase mutation screening	Sequencing	24	20 (83%)	-	-	-	-	12	0	Transfection of V617F into HeLa cells resulted in increased STAT, Akt and ERK phosphorylation

* The majority of these (23/29 = 79%) were only V617F-positive by ARMS analysis, not by sequencing

† 35 secondary erythrocytosis patients were also tested: all were *JAK2* V617F-negative

‡ 9 CML and 11 secondary erythrocytosis patients were also tested: all were *JAK2* V617F-negative

Table 1.7 Frequency of *JAK2* V617F in ET – published studies to date

Reference	n	Mutated (n)	Mutation frequency (%)	Method*
(Levine *et al,* 2005)	21	9	43	PCR sequencing
(Kralovics *et al,* 2005)	93	21	23	PCR sequencing
(Levine *et al,* 2005)	115	37	32	PCR sequencing
(Baxter *et al,* 2005)	51	29	57	AS-PCR
(Jones *et al,* 2005)	59	24	41	ARMS-PCR
(Jelinek *et al,* 2005)	10	3	30	Pyrosequencing
(Goerttler *et al,* 2005)	42	14	33	PCR sequencing (cDNA)
(Campbell *et al,* 2005)	776	411	53	AS-PCR and PCR-enzyme digestion
(Vizmanos *et al,* 2006)	243	151	62	AS-PCR
(Cheung *et al,* 2006)	60	29	48	AS-PCR
(Kiladjian *et al,* 2006)	44	19	43	Real-time AS-PCR
(Lippert *et al,* 2006)	60	45	75	AS- and q-real time-PCR
(Moliterno *et al,* 2006)	84	38	45	qPCR
(Gale *et al,* 2007)	111	43	39	PCR-enzyme digestion
(Larsen *et al,* 2007)	40	21	53	qPCR
(Kittur *et al,* 2007)	176	96	55	AS-qPCR
(Alvarez-Larran *et al,* 2007)	103	44	43	AS-PCR
(Vannucchi *et al,* 2007)	639	382	60	AS-PCR
(Lieu *et al,* 2008)	49	29	59	PCR-enzyme digestion
(Shen *et al,* 2009)	85	53	62	ARMS-PCR
(Palandri *et al,* 2009b)	275	176	64	PCR-enzyme digestion
(Hussein *et al,* 2009)	91	43	47	Pyrosequencing
(Pardanani *et al,* 2010)	226	118	52	AS-PCR
(Wong *et al,* 2010)	102	35	34	PCR-enzyme digestion
Overall	**3555**	**1870**	**53**	

* AS-PCR = allele-specific PCR

ARMS-PCR = amplification refractory mutation system PCR;

qPCR = quantitative PCR

In ET patients whose myeloid cells carry *JAK2* V617F, the mutation is not detectable in constitutional control tissue, such as buccal or hair follicle cells (Baxter *et al,* 2005; Kralovics *et al,* 2005; Levine *et al,* 2005), in keeping with it being a somatically acquired mutation. Further, the majority of studies suggest that such patients' T-lymphocytes are negative for the mutation, implying it is restricted to the myeloid lineage and that it arose in a myeloid progenitor cell rather than a haemopoietic stem cell (Baxter *et al,* 2005).

As described in Section 1.2.3, JAK2 is a non-receptor tyrosine kinase which binds to type 1 haemopoietic growth factor receptors and initiates downstream signalling when ligand binds to the receptor. JAK2 shares a common domain structure with other JAK family members (JAK1, JAK2 and TYK2): a C-terminal kinase domain (JAK homology 1, JH1), adjacent to which is a pseudokinase domain (JH2) which bears significant homology to JH1 but is enzymatically inactive and is predicted to negatively regulate the activity of JH1 in the absence of receptor-bound ligand, an SH2 domain (JH3-4) of unknown function, and an N-terminal FERM domain (JH5-7), which mediates binding to the Box1 domain of the receptor's proximal intracytoplasmic region (Haan *et al,* 2001; Haan *et al,* 2002; Lindauer *et al,* 2001; Pellegrini and Dusanter-Fourt 1997; Richter *et al,* 1998). The V617F mutation is located in the JH2 domain of the molecule, and its acquisition is believed to release the JH1 domains from their JH2-mediated inhibition. Indeed structural modelling published in 2001 had predicted that residue 617 lay within one of two small but critical interaction sites through which the JH2 domain mediated this inhibition (Lindauer *et al,* 2001); replacement of valine with bulky phenyalanine would be expected to disrupt its inhibitory effect.

In keeping with this, transfection of *JAK2* V617F into cytokine-dependent cell lines resulted in cytokine independence, with evidence of JAK2 autophosphorylation and constitutive activation of downstream pathways (James *et al,* 2005; Kralovics *et al,* 2005; Levine *et al,* 2005; Zhao *et*

al, 2005). Transplantation of *JAK2* V617F-transfected bone marrow cells into lethally irradiated C57BL6 mice recapitulated the polycythaemic phenotype, often with leukocytosis, thrombocytosis and some myelofibrotic features (Akada *et al,* 2010; James *et al,* 2005; Wernig *et al,* 2006).

The role of *JAK2* V617F in ET will be discussed in greater detail in Chapters 3, 4, 5 and 6.

1.3.8 Other genetic mutations in ET

In FT, a number of mutations have been described in the genes encoding TPO and c-Mpl (Table 1.3). Attempts to find similar abnormalities in sporadic ET, however, proved fruitless for many years (Allen *et al,* 2001; Harrison *et al,* 1998; Kiladjian *et al,* 1997; Taksin *et al,* 1999), partly because technological constraints limited the number of patients who could be screened. In 2006, Pikman *et al* (2006) sequenced selected exons of the genes encoding c-Mpl, EPOR and G-CSFR in 125 *JAK2* WT MPN patients (66 ET, 45 MF and 14 PV) and observed a *MPL* W515L mutation in four cases of MF. Subsequent studies have demonstrated that *MPL* W515L mutations do also occur in a small proportion of ET patients and other mutations have been reported (Table 1.8). Overall, *MPL* mutations are detectable in approximately 4% of ET patients, of whom 90% are *JAK2* WT. For this reason, the *MPL* mutation frequency is markedly higher in *JAK2* WT than in V617F-positive patients (6% versus 1%). The commonest mutations are W515L and W515K, which account for 70% and 27% respectively of the mutations so far described, with other mutations being S505N (3%) and W515A (1%). Mutations affecting K39 and P106, reported in FT, have not been described in ET, although only two large-scale studies (Beer *et al,* 2008; Pikman *et al,* 2006) have screened for mutations outside residues 505 and 515.

Table 1.8 ***MPL*** **mutations reported in sporadic ET**

Reference	Technique for *MPL* mutation detection	All ET patients		*JAK2* V617F ET patients		*JAK2* WT ET patients		Mutations detected (where stated)*			
		n	*MPL* mutation (% of patients)	*n*	*MPL* mutation (% of patients)	*n*	*MPL* mutation (% of patients)	W515L	W515K	W515A	S505N
(Pardanani *et al,* 2006)	Light-Cycler analysis of W515	318	4 (1%)	182	2 (1%)	136	2 (1%)	4	0	0	NT
(Vannucchi *et al,* 2008a)	Real-time qPCR for *MPL* W515L/K	994	30 (3%)	576	8 (1%)	418	22 (5%)	18	12	NT	NT
(Xu *et al,* 2008)	AS-PCR for *MPL* W515L	102	1 (1%)	43	0 (0%)	59	1 (2%)	1	NT	NT	NT
(Beer *et al,* 2008)	Whole gene sequencing in 18 patients; exon 10 sequencing in 88 patients; AS-PCR for S205N and W515L/K in 670 patients	776	32 (4%)	414	1 (0%)	362	31 (9%)	24	5	0	3
(Schnittger *et al,* 2009)	Light-Cycler analysis of W515	356	26 (7%)	32	0 (0%)	324	26 (8%)	16	9	1	NT
(Ruan *et al,* 2010)	Real-time qPCR for *MPL* W515L/K	199†	7 (4%)	0	0 (0%)	199	7 (3.5%)	6	1	NT	NT

(Patnaik *et al,* 2010)	Light-Cycler analysis of W515	108†	6 (6%)	0	0 (0%)	108	6 (6%)	N/A	N/A	NT	NT
(Glembotsky *et al,* 2010)	Allele-specific PCR for *MPL* S205N and W515L/K	100	1 (1%)	49	0 (0%)	51	1 (2%)	1	0	NT	0
Total		**2953**	**107 (4%)**	**1296**	**11 (1%)**	**1657**	**96 (6%)**	**70**	**27**	**1**	**3**

Table 1.8 MPL mutations reported in sporadic ET – continued

* NT = not tested; N/A = not available

† These studies screened only *JAK2* WT patients.

Residues 505 and 515 lie within the transmembrane domain of c-Mpl, and mutations are believed to disrupt a motif which normally prevents autonomous receptor activation, thereby allowing receptor dimerisation in the absence of ligand (Glembotsky *et al,* 2010). *MPL* W515L-transduced cell lines show cytokine-independent growth, hyper-responsiveness to TPO and constitutive activation of the JAK/STAT signalling pathway (Pikman *et al,* 2006). In a murine bone marrow transplant assay, expression of *MPL* W515L resulted in thrombocytosis, leukocytosis, MK hyperplasia, and splenomegaly due to extramedullary haemopoiesis, a phenotype similar to pre-fibrotic MF (Pikman *et al,* 2006).

Mutations in other genes have been reported infrequently in ET patients. In 2009, Delhommeau *et al* (2009) reported on the presence of mutations in the ten-eleven translocation 2 (*TET2*) gene for in a variety of myeloid disorders. They initially studied six patients with AML who were known to have rearrangements involving chromosome 4q24. FISH analysis revealed a commonly deleted region of chromosome 4 in three of these patients and in a further three MDS patients analysed; this region contained only the *TET2* gene. They then screened five MPN patients for copy number alterations using comparative genomic hybridisation and SNP arrays and in three of them identified a 325kb common region of LOH on chromosome 4q; again this region contained only the *TET2* gene. Direct sequencing of *TET2* in eight of the patients with deletions or LOH at 4q24 revealed mutations in six of them: one frame shift, one in-frame deletion, two nonsense mutations, and two amino acid substitutions.

Delhommeau *et al* (2009) then screened 203 MPN patients (of whom 186 were *JAK2* V617F-positive) for *TET2* abnormalities, and of these 27 (13%) were found to harbour *TET2* alterations. Thirty-one different *TET2* mutations and one whole gene deletion were detected. The mutations were scattered widely throughout the gene, without any apparent 'hotspots'. Twenty-five of the 27 *TET2*-mutated patients were *JAK2* V617F-positive and in these individuals colony analysis suggested that acquisition of *TET2* mutations occurred prior to acquisition of *JAK2* V617F. Five patients had more than one mutation and cloning in one case

confirmed that both *TET2* genes were affected. Delhommeau *et al* (2009) therefore speculated that *TET2* was a tumour suppressor gene – i.e. loss of function due to biallelic mutations or a single allele mutation and deletion of the non-mutated allele resulted in disease. The precise function of *TET2* remains obscure but *TET1*, with which it shares two domains, is known to play a role in the epigenetic regulation of embryonic stem cells (Tahiliani *et al,* 2009).

A subsequent study identified *TET2* mutations in only three of 57 (5%) ET patients (Tefferi *et al,* 2009) and again suggested *TET2* may be associated with *JAK2* V617F. The over-representation of V617F-positive patients in the Delhommeau *et al* (2009) study may therefore help explain the discrepancy in *TET2* mutation frequency between the two reports.

A single study has recently identified mutations in the additional sex combs-like 1 (*ASXL1*) gene, which is thought to be involved in regulating chromatin remodelling and is mutated in approximately 10% of MDS patients and 40% of chronic myelomonocytic leukaemia cases (Carbuccia *et al,* 2009). Of 35 ET patients studied, three had mutations in the gene (all different), one of whom also carried *JAK2* V617F and a *TET2* mutation. *ASXL1* mutations were also present in three patients with MF. Like TET2, the function of ASXL1 is unknown, but the authors suggested it too may be involved in epigenetic regulation and have a tumour suppressor role.

1.4 Aims of the project

The studies reported in this thesis set out to examine the molecular origins of ET, and in particular to investigate the role of *JAK2* V617F in the pathogenesis of the disorder and its relationship to the concept of clonality in ET. One of the major assertions made in relation to the 2008 WHO classification of MPNs has been examined, specifically that ET, like the other MPNs, is a clonal, neoplastic disorder.

The clinical features of a cohort of ET patients and their correlation with a range of molecular characteristics have been analysed (Chapter 3). Regulation of *JAK2* WT and V617F-positive platelets in V617F-positive ET has been examined in Chapter 4, in order to establish whether the V617F-positive platelets expand at the expense of WT platelets, as might be expected in a neoplastic disorder. A further series of experiments, reported in Chapter 5, sought to elicit evidence for more than one clonal population in V617F-positive ET, and thereby determine whether acquisition of *JAK2* V617F occurred more than once. Finally, inherited factors predisposing to the development of V617F-positive ET, specifically the 46/1 haplotype, are examined in Chapter 6.

2 Materials and Methods

2.1 Sample preparation

2.1.1 Reagents

- Ammonium chloride (Sigma-Aldrich, Gillingham, UK)
- Calcium- and magnesium-free phosphate buffered solution (PBS) (Invitrogen, Paisley, UK)
- Chloroform (VWR International Ltd, Lutterworth, UK)
- Double de-ionised water (ddH_20)
- DTAB (dodecyl-trimethyl-ammonium bromide) (Sigma-Aldrich, Gillingham, UK)
- Dynal CD3 beads (Invitrogen, Paisley, UK)
- EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, Gillingham, UK)
- Ethanol 100% (VWR International Ltd, Lutterworth, UK)
- Ficoll-Paque™ PLUS (GE Healthcare, Buckinghamshire, UK)
- Foetal calf serum (FCS), sterile filtered (Invitrogen, Paisley, UK)
- Giemsa stain 0.4% w/v in buffered methanol solution, pH 6.9 (Sigma-Aldrich, Gillingham, UK)
- May-Grünwald stain 0.25% w/v in methanol (Sigma-Aldrich, Gillingham, UK)

- Plastic whole blood tube with spray-coated K_2EDTA, 4mL (BD, Oxford, UK)
- RPMI-1640 medium with L-glutamine (Invitrogen, Paisley, UK)
- Sodium chloride (VWR International Ltd, Lutterworth, UK)
- Tris (Hydroxymethyl) methylamine (VWR International Ltd, Lutterworth, UK)
- TRIzol™ (Invitrogen, Paisley, UK)

2.1.2 Buffers

- DNA lysis buffer: 20g DTAB (final concentration 8%), 22g NaCl (final concentration 1.5 M), 25 mLs of 1M Tris Cl pH 7.8 (100 mM). Made up to 250mL with ddH_20.

2.1.3 Purification of neutrophils, platelets and T-lymphocytes

Neutrophils, platelets and T-lymphocytes were purified from peripheral blood prior to DNA and RNA extraction (Figure 2.1). Peripheral blood was collected into 4mL tubes containing EDTA and centrifuged for 15 minutes at 200g at room temperature. The upper three-quarters of the plasma layer was harvested as platelet-rich plasma, pooled and spun for 15 minutes at 2000g. The supernatant was discarded and the pellet washed in 10mL calcium- and magnesium-free PBS containing 10mmol/L EDTA and centrifuged for 15 minutes at 2000g at room temperature. The washing process was repeated once more and the resulting platelet suspension counted using a Sysmex KX21N whole blood cell analyser (Sysmex, Milton Keynes UK). Aliquots of $5x10^9$ platelets were then centrifuged for 15 minutes at 2000g at room

temperature and the supernatant was carefully removed. The pellet was resuspended in 1mL TRIzol reagent by repeated pipetting and stored at -80°C for RNA extraction.

The sample remaining in each tube after removal of the platelet rich plasma, comprised mostly of erythrocytes and leukocytes, was made up to 4mL with calcium- and magnesium-free PBS containing 10mmol/L EDTA, mixed and centrifuged for 5 minutes at 2000g. The buffy coat, comprised mostly of leukocytes, was recovered from each tube and pooled. The samples remaining in the 4mL tubes were re-mixed, spun again for 5 minutes at 2000g, and any additional buffy coat recovered and pooled with the first harvest. The resulting buffy coat suspension was made up to 14mL with calcium- and magnesium-free PBS containing 10mmol/L EDTA, layered onto 12mL Ficoll-Paque™ PLUS and centrifuged for 20 minutes at 800g at room temperature, with minimum acceleration and braking. Mononuclear cells were then harvested from the interface, resuspended in 15mL RPMI containing 2% FCS (RF2%), and spun for 5 minutes at 800g at room temperature. The supernatant was carefully removed and the pellet resuspended in 10mL RF2%.

The suspension was counted using the Sysmex analyser to obtain the total leukocyte count. T-lymphocytes were then purified from the mononuclear cell suspension using anti-CD3-coated magnetic beads ($4x10^5$ beads/µL suspended in PBS containing 0.1% bovine serum albumin, BSA, and 0.02% sodium azide). For every $1x10^6$ leukocytes in the mononuclear cell suspension, 2.5µL ($1x10^6$) of magnetic beads were used. These were first washed by adding them to 10mL RF2% in a reaction tube, shaking the mixture, and placing the tube against a magnet for 3 minutes. Without removing it from the magnet, the tube was inverted to discard the supernatant, leaving only the washed beads in the tube which was then removed from the magnet. The mononuclear cell suspension was added to the washed beads, the mixture shaken, and incubated for one hour at 4°C with end-over-end rotation to allow the beads to bind the CD3-positive T-lymphocytes. Following this, the CD3-negative fraction was removed by placing the tube against the magnet for 3 minutes then pouring off the supernatant by

inverting the tube whilst holding it against the magnet. The CD3-positive cells and beads remained in the tube and were resuspended in 10mL fresh RF2% and the process repeated twice, to produce a suspension of purified T-lymphocytes (bound to the beads) in 10mL RF2%.

To allow morphological analysis of their purity, aliquots of approximately 50,000 T-lymphocytes were diluted to a total volume of 300µL with RF2% and used to prepare cytospins. The cytospin slides were stained using the May-Grünwald Giemsa technique: slides were placed in May-Grünwald stain for 5 minutes at room temperature, transferred to dilute Giemsa stain (1:20 in ddH_20) for 20 minutes at room temperature, then rinsed with ddH_20 and allowed to air dry.

For RNA extraction, aliquots of $5x10^6$ T-lymphocytes were spun for 5 minutes at 300g at 4°C, the supernatant removed, the pellet resuspended in 1mL TRIzol reagent by repeated pipetting, and stored at -80°C. For DNA extraction, aliquots of $15x10^6$ T-lymphocytes were spun for 5 minutes at 800g at room temperature, the supernatant removed, the pellet resuspended in 2.5mL RF2%, and 5mL DTAB lysis buffer added. The mixture was shaken and incubated for 5 minutes at 68°C then stored at -20°C for at least 12 hours prior to DNA extraction.

To obtain purified neutrophils, residual Ficoll-Paque™ PLUS was removed after the MNCs had been harvested, the pellet disrupted by vortexing, resuspended in 2.5mL PBS and 22.5mL 0.8% ammonium choride solution added to lyse red blood cells. The suspension was vortexed and incubated on ice for 15 minutes, then spun for 5 minutes at 800g, the supernatant removed, and the pellet resuspended in 10mL RF2%. The neutrophil suspension was counted using the Sysmex analyser.

Cytospin slides of the purified neutrophils were prepared as described above for T-lymphocytes. The purified neutrophils were stored in TRIzol reagent for RNA extraction and in DTAB lysis buffer for DNA extraction, as described above.

2.1.4 RNA extraction

Samples stored in TRIzol reagent were thawed at room temperature and 0.2mL chloroform was added. The mixture was shaken vigorously, incubated at room temperature for 3 minutes, and spun for 15 minutes at 12,000g at 4°C. The aqueous upper phase of the resulting mixture was transferred to a tube containing 0.5mL isopropyl alcohol, shaken, incubated at room temperature for 10 minutes, then spun for 10 minutes at 12,000g at 4°C to precipitate the RNA. The supernatant was carefully removed and 1mL 75% ethanol added. The mixture was vortexed, spun for 5 minutes at 7,500g at 4°C, the supernatant removed, and the RNA pellet allowed to air-dry for 10 minutes then suspended in 500µL ddH_20. RNA samples were stored at -80°C.

2.1.5 Genomic DNA extraction

Samples stored in DTAB were thawed at room temperature and 7.2mL chloroform was added. The mixture was shaken vigorously and spun for 15 minutes at 3000g at room temperature. The aqueous upper phase of the resulting mixture was transferred to a tube containing 8mL absolute ethanol, shaken, then spun for 5 minutes at 1000g at room temperature to precipitate the DNA. The supernatant was carefully removed and 2mL 75% ethanol added. The mixture was shaken, spun for 5 minutes at 1000g at room temperature, and the supernatant removed. The DNA pellet was allowed to air dry for 15-20 minutes, then suspended in 50-300µL ddH_2O depending on the size of the pellet and incubated overnight at 4°C with rotation. The DNA concentration was measured using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and working dilutions of 50ng/µL made. All DNA

samples were stored at 4°C.

2.2 Molecular biology

2.2.1 Reagents

- Acetonitrile (Transgenomic Ltd, Glasgow, UK)
- Agarose (Bioline, London, UK)
- 10x Bioline ammonium buffer (Bioline, London, UK)
- Bioline magnesium chloride 50mM (Bioline, London, UK)
- BIOTAQ™ DNA polymerase 5u/µL (Bioline, London, UK)
- Bovine serum albumin (BSA) 10mg/mL (New England Biolabs, Hitchin, UK)
- Bromophenol blue (VWR International Ltd, Lutterworth, UK)
- Carbenicillin (Invitrogen, Paisley, UK)
- N,N-Dimethylformamide ≥99.8% (Sigma Aldrich, Gillingham, UK)
- DNA size standard – PA400 (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- dNTPs 100mM each (Bioline, London, UK)
- Ethidium bromide 10mg/mL (Invitrogen, Paisley, UK)
- Glycogen 20mg/mL (Sigma Aldrich, Gillingham, UK)
- GoTaq® DNA polymerase 5u/µL (Promega, Madison, USA)

- GoTaq® Flexibuffer (Promega, Madison, USA)
- GoTaq® magnesium chloride 25mM (Promega, Madison, USA)
- Luria-Bertani (LB) broth tablets (Sigma Aldrich, Gillingham, UK)
- Nusieve low melting point agarose (VWR International Ltd, Lutterworth, UK)
- Olignucleotide primers (Integrated DNA Technologies, Leuven, Belgium)
- Optimase® reaction buffer (Transgenomic Ltd, Glasgow, UK)
- Optimase® magnesium sulphate 25mM (Transgenomic Ltd, Glasgow, UK)
- Optimase® DNA polymerase (Transgenomic Ltd, Glasgow, UK)
- Orthoboric acid (VWR International Ltd, Lutterworth, UK)
- peqGOLD MicroSpin Cycle-Pure PCR Purification Kit (Peqlab, Sarisbury Green, UK)
- 5x Phusion® HF buffer containing $MgCl_2$ 7.5mM (New England Biolabs, Hitchin, UK)
- Phusion® Hot Start DNA Polymerase 2u/µL (New England Biolabs, Hitchin, UK)
- QIAquick™ Gel Extraction Kit (Qiagen, Crawley, UK)
- Restriction enzymes and buffers (New England Biolabs, Hitchin, UK)
- Sample loading solution (SLS) (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- SDS (sodium dodecyl sulphate) (Sigma Aldrich, Gillingham, UK)
- 3M sodium acetate pH 5.2 (Sigma-Aldrich, Gillingham, UK)
- Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Paisley, UK)

- TOPO TA Cloning® Kit with One Shot® MAX Efficiency™ DH5α-T1R E. coli competent cells (Invitrogen, Paisley, UK)
- Tri-ethylene ammonium acetate (TEAA) (Transgenomic Ltd, Glasgow, UK)
- Tris (Hydroxymethyl) methylamine (VWR International Ltd, Lutterworth, UK)
- X-Gal (Sigma Aldrich, Gillingham, UK)

2.2.2 Buffers

- 10x TBE (pH8.3), for 1 litre: Tris 108.9g, orthoboric acid 55.7g, EDTA 7.4g
- Loading buffer: 30% glycerol, 0.025% bromophenol blue in 1xTBE

2.2.3 Polymerase chain reaction (PCR)

Four different DNA polymerases were used in the PCR experiments reported in this thesis. The general reaction conditions for each polymerase are described below, and variations from these for specific situations are described in the appropriate chapters. For every DNA polymerase, a master mix containing all necessary reagents except DNA template was prepared and aliquoted according to the number of samples prior to the addition of DNA template. Thermal cycling was performed using a DNA Engine Tetrad 2 Peltier Thermal Cycler (BioRad Laboratories, Hemel Hempstead, UK) using a ramping speed of 1.0°C/S unless otherwise specified.

All oligonucleotide primer sequences are listed in Appendix 1.

2.2.3.1 BIOTAQ™ PCR

BIOTAQ™, a non-proofreading Taq polymerase, was used for the majority of the experiments reported in this thesis which did not require an enzyme with 3' to 5' exonuclease proofreading properties.

A master mix was prepared containing 1x reaction buffer [final concentrations: 16mM $(NH_4)_2SO_4$, 67mM Tris-HCl, 0.01% Tween-20], 1mM $MgCl_2$, 0.5µM each oligonucleotide primer, 0.2mM each dNTP, 0.05unit/µL polymerase, and made up to the desired volume with ddH_20. To this mix, 2.5ng/µL template DNA was added. Cycling conditions were 30 seconds denaturation at 95°C, 30 seconds primer annealing at a temperature specific to the primers used, and 30 seconds primer extension at 72°C, repeated between 22 and 40 times, depending on the nature of the PCR (see specific methods). A final step of 5 minutes at 72°C was included to ensure complete extension of the PCR products.

2.2.3.2 GoTaq® Hot Start PCR

GoTaq® is also a non-proofreading Taq polymerase, but differs from BIOTAQ™ polymerase in that it is bound to a proprietary antibody which inactivates it until the reaction mixture is heated to 94°C or above for 2 minutes, whereupon its activity is restored ('hot start' activity). This minimizes non-specific DNA amplification at lower temperatures. GoTaq® polymerase was therefore used when BIOTAQ™ polymerase generated multiple non-specific PCR products.

A master mix was prepared containing 1x Promega flexibuffer (proprietary formulation), 2mM $MgCl_2$, 0.05unit/µL GoTaq® Hot Start polymerase, 0.5µM each oligonucleotide primer, 0.2mM each dNTP, and made up to the desired volume with ddH_20. To this mix, 2.5ng/µL template DNA was added. Following an initial incubation for 2 minutes at 95°C to activate the polymerase enzyme, between 22 and 35 cycles of PCR were performed, each cycle comprising a 30 second denaturation step at 95°C, a 30 second primer annealing step at a temperature

specific to the primers used, and a 45 second primer extension step at 72°C. After the last cycle, a final step of 5 minutes at 72°C was included to ensure complete extension of the primers.

2.2.3.3 Optimase® PCR

Optimase® is a DNA polymerase with 3' to 5' exonuclease proofreading activity which was used when high-fidelity PCR amplification was required, most commonly to generate products for analysis by denaturing high-performance liquid chromatography (dHPLC).

A master mix was prepared containing 1x reaction buffer (proprietary formulation), 1.5mM $MgSO_4$, 0.05unit/µL Optimase® polymerase, 0.5µM each oligonucleotide primer, 0.2mM each dNTP, and made up to the desired volume with ddH_20. To this mix, 2.5ng/µL template DNA was added. Following an initial denaturation step of 5 minutes at 95°C, between 22 and 35 cycles of PCR were performed, each cycle comprising a 30 second denaturation step at 95°C, a 30 second primer annealing step at a temperature specific to the primers used, and a 60 second primer extension step at 72°C. After the last cycle, a final step of 5 minutes at 72°C was included to ensure complete extension of the primers.

2.2.3.4 Phusion® PCR

Phusion® was used to generate PCR products from very low concentrations of template DNA or cDNA (e.g. from old or degraded samples), especially when longer PCR products (>500bp) were required. Like Optimase®, it is a proofreading polymerase, making it suitable for applications such as dHPLC, and like GoTaq®, it has 'hot start' activity. It was generally used when no satisfactory products could be obtained using one of the other DNA polymerase enzymes.

A master mix was prepared containing 1x HF buffer (proprietary formulation; contains $MgCl_2$ at a final concentration of 1.5mM), 0.02unit/µL Phusion® polymerase, 0.5µM each oligonucleotide primer, 0.2mM each dNTP and made up to the desired volume with ddH_20. To this mix, 2.5ng/µL template DNA was added. Following an initial denaturation/activation step of 2 minutes at 98°C, between 32 and 40 cycles of PCR were performed, each cycle comprising a 42 second denaturation step at 98°C, a 42 second primer annealing step at a temperature specific to the primers used, and a 60 second primer extension step at 72°C. After the last cycle, a final step of 5 minutes at 72°C was included to ensure complete extension of the primers.

2.2.4 Restriction enzyme digestion

Restriction enzymes were obtained from New England Biolabs. Genomic DNA or PCR products were added to a mixture of the appropriate 1x reaction buffer, restriction enzyme, ddH_20 and, if necessary, 100µg/ml BSA, according to the manufacturer's instructions, and incubated at the recommended temperature for 2-12 hours, according to manufacturer's guidance.

2.2.5 Reverse-transcription PCR (RT-PCR)

Complementary DNA (cDNA) was generated from RNA by reverse transcription, using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit. RNA concentration was measured using the NanoDrop ND-1000 UV-Vis spectrophotometer, and 100ng-1µg of RNA was used as template. The RNA sample was made up to 8µL with ddH_2O and added to a mastermix containing 2µL RT enzyme mix (a proprietary formulation of SuperScript™ III reverse transcriptase and RNaseOUT™ recombinant ribonuclease inhibitor) and 10µL 2x RT

reaction mix (final concentration 5mM $MgCl_2$, 1.25ng/µL random hexamers, 0.5mM dNTPs, 1.25µM oligo$(dT)_{20}$ primers). The reaction mixture was mixed gently and incubated for 10 minutes at 25°C, followed by 30 minutes at 50°C, 5 minutes at 85°C, then chilled on ice. Following the addition of 1µL *E. coli* RNaseH (2U/µL), the mixture was incubated for 20 minutes at 37°C. The resulting cDNA was stored at -80°C until needed and used in a 1 in 4 dilution as the template for PCR reactions.

2.2.6 Agarose gel electrophoresis

PCR products were visualised using agarose gels (1.5%-3.5%). Agarose (0.53-1.23g, depending on desired gel concentration) was added to 35mL 1x TBE, dissolved by heating in a microwave oven, and 3.5µL 1mg/mL ethidium bromide added. The gel was cooled, poured into a mould, allowed to set and immersed in 1x TBE containing 100ng/mL ethidium bromide running buffer. PCR product or restriction enzyme digest was mixed with loading buffer (30% glycerol, 0.025% bromophenol blue in 1xTBE), loaded into the wells and electrophoresed by the application of a potential difference across the gel. The use of a DNA ladder allowed estimation of the PCR product size. The gel was then placed on a UV transilluminator to visualise PCR products, and digital images made.

2.2.7 Fragment analysis of PCR products and restriction enzyme digestion products

Fragment analysis was used to quantify the relative amount of multiple products generated by PCR or following restriction enzyme digestion.

PCR was performed using one fluorescently-labelled primer and one unlabelled primer. The DNA polymerase used varied according to the exact application, as did the number of cycles

and the final concentration of each primer. Specific details are described in the appropriate chapter. Where appropriate, PCR products underwent restriction enzyme digestion as described in Section 2.2.4.

To perform fragment analysis, 2µL of the PCR or restriction enzyme digestion product were mixed with 0.5µL size standard and 38µL sample loading solution (deionised formamide), and run on the Beckman Coulter CEQ 8000 DNA Genetic Analysis System (Beckman Coulter UK Ltd., Buckinghamshire, UK). The instrument denatured the sample and separated the different PCR products according to fragment length by capillary electrophoresis. Elution of each product was detected by laser-induced excitation of its fluorescent label, and indicated by a peak at the appropriate elution time on a graph generated by the supplied software. By reference to the size standards, each product's size was estimated by the software based on its elution time. The area under the curve for each peak reflected the relative amount of each product, derived by the software from the amplitude and duration of its fluorescence. The relative amount of each product was calculated by expressing the area under the appropriate peak as a proportion of the total area under all the peaks.

2.2.8 DNA sequencing

For DNA sequencing, PCR products were prepared and then purified using the peqGold micro-spin cycle-pure kit. One volume PCR product was mixed with 10 volumes buffer CP (proprietary formulation) then vortexed and the mixture applied to a PerfectBind DNA spin-column assembled in a 2mL collection tube and spun for 2 minutes at 10,000g at room temperature. The spin-column was transferred to a clean 1.5mL collection tube, 20µL DNA Elution Buffer (proprietary formulation) applied to the column matrix and incubated for 5 minutes at room temperature, then spun for 1 minute at 8,000g at room temperature to elute the purified PCR

products into the collection tube. These were sequenced by the UCL Wolfson Institute for Biomedical Research DNA sequencing service using an Applied Biosystems 3730*xl* 96-capillary DNA analyser (Life Technologies, California, USA) with BigDye® 3.1 chemistry.

2.2.9 LB-carbenicillin culture medium and plates

For LB-carbenicillin culture medium, 12 LB medium capsules were dissolved in 500mL ddH_20 and autoclaved, then cooled to approximately 60°C, and carbenicillin added to a final concentration of 100µg/mL. For LB-carbenicillin-agar plates, 7.5g agar was added to the mix prior to autoclaving (final concentration 1.5% w/v); after the addition of carbenicillin the medium was poured into Petri dishes, allowed to set for one hour, and stored at 4°C. Prior to bacterial spreading, plates were incubated at 37°C for 30 minutes to dry, and spread with 40µL X-gal solution (40mg/mL X-gal in dimethylformamide), then incubated at 37°C for a further 30 minutes.

2.2.10 TA cloning

TA cloning allows individual PCR products to be analysed by ligating them into a plasmid which is then transfected into competent bacteria and these are then cultured on LB-carbenicillin-agar plates. Multiple, discrete bacterial clones are generated, each containing a PCR product.

The cloning studies reported in this thesis used the Invitrogen TOPO TA Cloning® Kit with One Shot® MAX Efficiency™ DH5α-T1R *E. coli*. The plasmid vector in this kit has single 3'-thymidine (T) overhangs, bound covalently to topoisomerase I, which allow efficient ligation of PCR products generated using non-proofreading Taq polymerases as these add a single deoxyadenosine (A) to the 3' end of PCR products. Cloning was performed according to

manufacturers' instructions. Briefly, 1 to 4µL of fresh PCR product were added to 1µL salt solution and 1µL TOPO vector, made up with de-ionised water to 6µL, and incubated for 30 minutes at room temperature. A vial of One Shot® Chemically Competent *E. coli* was thawed on ice, 2µL of the ligated PCR product added, and incubated on ice for 30 minutes. Following incubation at 42°C for 30 seconds without shaking, the vial was transferred to ice, 250µL room temperature SOC medium added, and incubated at 37°C for 1 hour with shaking (200rpm). Between 5µL and 50µL cell suspension was then spread on pre-warmed LB-agarose-carbenicillin plates coated with X-gal (section 2.2.9), and the plates incubated at 37°C overnight. Transformed colonies containing an inserted PCR product were identified by their white or light-blue colour due to disruption of the lacZα gene in the pCR®2.1-TOPO® plasmid. Individual colonies were picked and expanded by incubation in LB-carbenicillin medium at 37°C for 12 hours.

3 Clinical and molecular characteristics of the ET patient cohort

3.1 Introduction

Despite substantial improvements in our understanding of ET since it was first described over 75 years ago (Epstein and Goedel 1934), the diagnosis and management of the condition remain challenging. Distinguishing between ET and other causes of thrombocytosis, such as other MPNs, infection, bleeding, malignancy, iron deficiency, autoimmune inflammatory disorders and hyposplenism, is often difficult and in many cases remains a process of exclusion. Similarly, prognostication in ET can be challenging since ET is a heterogeneous disorder and patients vary markedly in their clinical course. For some, ET represents a relatively benign condition with a comparatively low risk of thrombosis and exerts little, if any, effect on their life expectancy. For others, the disorder is associated with major morbidity – such as life-threatening arterial and venous thromboses or haemorrhage – and premature death. Since, in general, more aggressive disease justifies more intensive treatment, estimating at diagnosis the risk of later complications is an essential part of deciding on appropriate therapy. Considerable efforts have therefore been made to improve the diagnostic approach to individuals with thrombocytosis, and then, in those with ET, to identify clinical and pathological features which predict outcome.

3.1.1 Diagnostic criteria for ET

Criteria for the diagnosis of ET were first proposed in 1975 by the PVSG (Berlin 1975). These required the presence of a sustained platelet count exceeding $1000x10^9$/L, a bone marrow showing megakaryocytic hyperplasia without significant fibrosis or myelodysplasia, and the absence of polycythaemia, CML, iron deficiency or a reactive cause for the thrombocytosis such as infection, malignancy or an inflammatory process. Subsequent revisions of the PVSG criteria have reduced the platelet threshold to $600x10^9$/L (Table 3.1; Murphy *et al,* 1986; Murphy *et al,* 1997). Despite claims that a threshold of $400x10^9$/L would allow more ET patients to be detected and treated (Sacchi *et al,* 2000), this cut-off was rejected, largely due to concerns that some of the many patients with a reactive thrombocytosis of between $400x10^9$/L and $600x10^9$/L would be erroneously diagnosed with ET in the absence of a robust means of distinguishing between the two conditions. Consequently the initial WHO diagnostic criteria stipulated that patients must have a minimum platelet count of $600x10^9$/L to be diagnosed with ET (Jaffe 2001).

Table 3.1 PVSG Criteria for the diagnosis of ET

All seven criteria must be fulfilled to make a diagnosis of ET

1	Platelet count greater than 600 x 10^9/L
2	Haematocrit less than 40% or normal RBC mass
3	Stainable iron in the marrow or normal RBC mean corpuscular volume (If these measurements suggest iron deficiency, PV cannot be excluded unless a trial of iron therapy fails to increase the RBC mass into the polycythaemic range.)
4	No Philadelphia chromosome or BCR-ABL gene rearrangement
5	Collagen fibrosis of the bone marrow absent or less than one third of the biopsy area without both marked splenomegaly and a leukoerythroblastic blood film
6	No cytogenetic or morphologic evidence for a myelodysplastic syndrome
7	No cause for a reactive thrombocytosis

From Murphy *et al* (1997)

The discovery of *JAK2* V617F and *MPL* mutations in approximately 50% and 5% respectively of ET patients (Tables 1.7 and 1.8) had a significant impact on the diagnosis of ET. They both facilitated the distinction between reactive thrombocytosis and ET and enabled identification of a population of individuals with a mild thrombocytosis (below $600x10^9$/L) who carried these mutations and suffered the same thrombotic complications as ET patients with higher platelet counts (Michiels *et al,* 2007). As a result, a reduction of the platelet threshold was once again proposed (Tefferi *et al,* 2007) and on this occasion a lower value of $450x10^9$/L was adopted by the WHO in their revised 2008 criteria, shown in Table 1.1 (Swerdlow *et al,* 2008). The 2008 WHO classification requires the presence of MK hyperproliferation in the bone marrow, with large, mature MKs and normal erythroid and myeloid precursors, and the exclusion of other MPNs. Unlike previous criteria, it does not stipulate that reactive causes of a thrombocytosis must be systematically excluded if a molecular marker (e.g. *JAK2* V617F) is detected. The 2008 WHO classification is likely to become the most widely accepted diagnostic criteria for the diagnosis of ET, and in the UK it has recently been incorporated into the British Committee for Standards in Haematology guideline for the investigation of individuals presenting with a thrombocytosis, although the diagnostic criteria in this guideline are subtly different from the WHO ones in that they do not require that a bone marrow biopsy be performed if a molecular marker is detected (Harrison *et al,* 2010).

3.1.2 Impact of clinical features at presentation on prognosis

In the 2008 WHO monograph, ET, along with the other conditions hitherto called myeloproliferative *disorders*, was reclassified as a neoplasm (Swerdlow *et al,* 2008). Nonetheless, in many patients – particularly older ones who comprise the majority of affected individuals – the diagnosis of ET has little or no effect on life expectancy (Table 3.2). Bazzan *et al* (1999) found that the life expectancy of older ET patients (>55 years at diagnosis) was

similar to that of age-matched healthy controls, whereas that of younger patients was shortened compared to controls. Although Mesa *et al* (1999) reported that the overall survival of a small cohort of ET patients was poorer than that of age-matched controls, Passamonti *et al* (2004) found that ET patients of all ages had a similar life expectancy to healthy controls. Certainly, in the first 10 years following diagnosis, survival appears to be equivalent to controls although it may be worse thereafter (Wolanskyj *et al,* 2006). The different findings of these groups probably reflect differences in the composition of the patient cohorts they studied, and a number of biological characteristics at diagnosis have now been identified which are associated with inferior survival in ET (Table 3.2).

The most frequent complications observed in individuals with ET are thromboembolic, with a historical rate of approximately 7% per patient-year following diagnosis (Cortelazzo *et al,* 1990), and a 10-year cumulative incidence (CI) of thrombosis of up to 42% (Wolanskyj *et al,* 2006), although this varies widely (see below). Treatment with cytoreductive agents has been shown to reduce the rate of thromboembolic events in ET patients (see Section 3.1.4), but there is concern that some of these agents may also increase the risk of leukaemic transformation. For this reason, considerable efforts have been made to predict the relative risks of thrombotic complications in order to select those patients who are most likely to benefit from cytoreductive medications, and conversely to identify patients for whom such treatment is not indicated (Table 3.3).

Two independent clinical risk factors – increasing age and prior history of thrombosis – are now widely accepted to be associated with an increased rate of thromboembolic complications in ET patients, although several other factors have also been proposed. In their retrospective study of 100 ET patients, some of whom were treated with busulphan, Cortelazzo *et al* (1990) observed that the average thrombotic rate in events per patient-year varied markedly according to age: in individuals younger than 40 years it was 1.7%; in those aged 40-60 years it was 6.3%; and in those older than 60 years it was 15.1%. The equivalent

Table 3.2 Recent studies analysing life expectancy in ET

Reference	*n*	Median follow up (years)	Median survival (years)	Risk factors associated with inferior survival	Overall survival compared to aged-matched controls
(Bazzan *et al,* 1999)	187	4.1	Not reported	None identified	Significantly worse for patients <55 years old at diagnosis: mortality rate was 4.2x that of matched controls, mostly due to thrombosis. Note only 58% of patients were on anti-platelet agents Life expectancy similar to controls for patients > 55 years old at diagnosis
(Passamonti *et al,* 2004)	435	9.3	22.6	Prior thrombosis Male sex	Similar regardless of age
(Chim *et al,* 2005)	231	Not reported	13	Age >60 at diagnosis Cigarette smoking	Not reported
(Wolanskyj *et al,* 2006)	322	13.6	18.9	Age >60 at diagnosis Prior thrombosis Diabetes mellitus Cigarette smoking WCC >15 at diagnosis	Similar to controls in the first decade after diagnosis, but significantly worse thereafter
(Dan *et al,* 2006)	381	4.3	Not reported	Age >60 at diagnosis Prior thrombosis	Similar to controls
(Gangat *et al,* 2007)	605	7	18	Age >60 at diagnosis Prior thrombosis Diabetes mellitus Cigarette smoking WCC >15 at diagnosis Anaemia	Not reported

Table 3.3 Studies assessing conventional risk factors for thrombosis in ET

Reference	*n*	P-value for association between specified risk factor and post-diagnosis thrombotic rate*								Comments
		Age > 60 years	Prior thrombosis	Diabetes mellitus	Hyper-tension	Hyper-lipidaemia	Cigarette smoking	Platelet count	White cell count	
(Cortelazzo *et al,* 1990)	100	<0.001	<0.0005	–	NS	NS	NS	–	–	P values for comparison against 200 normal controls
(Colombi *et al,* 1991a)	103	NS	<0.001	–	–	–	–	NS	–	Age analysed as a continuous variable
(Besses *et al,* 1999)	148	0.0007	<0.0001	NS	0.01	0.0002	NS	NS	–	Platelet count analysed as < or > $1000x10^9$/L In multivariate analysis, risk of thrombosis was predicted by: age >60 (P=0.002), prior thrombosis (P=0.001), and hyperlipidaemia (P<0.001)
(Jantunen *et al,* 2001)	132	NS	–	NS	NS	NS	0.02	NS	–	Age analysed as a continuous variable. Male sex also associated with an increased thrombotic risk (P=0.0001)
(Shih *et al,* 2002)	89	<0.001	–	NS	0.002	NS	–	NS	NS	Age analysed as a continuous variable In multivariate analysis, only increasing age associated with an increased thrombotic risk (P=0.03)
(Chim *et al,* 2005)	231	NS	NS	–	–	–	NS	NS	NS	WCC categorised as < or > $11x10^9$/L Platelet count categorised as < or >

										1200x10^9/L Splenomegaly at diagnosis associated with a reduced thrombotic risk
(Wolanskyj *et al,* 2006)	322	0.002	<0.001	0.03	0.02	NS	NS	–	0.005	Prior thrombotic complications included only prior arterial events WCC was categorised as < or > 15x10^9/L In multivariate analysis thrombotic risk was predicted by age >60 (P<0.001) and WCC > 15x10^9/L (P=0.02)
(Carobbio *et al,* 2007)	439	0.02	0.02	–	–	–	–	NS	0.01	WCC was categorised as < or > 8.7x10^9/L In multivariate analysis thrombotic risk was predicted by age > 60 (P=0.004), prior thrombotic event (P=0.004) and WCC ≥ 8.7x10^9/L (P=0.001)
(Carobbio *et al,* 2008a)	657	0.008†	0.008†	–	–	–	–	NS	0.04-0.008‡	†Age > 60 and/or prior thrombotic event combined as 'high-risk' category ‡P value depended on WCC cut-off value (P=0.04 for 7.2x10^9/L, 0.02 for 8.7x10^9/L, and 0.008 for 10.4x10^9/L)

Table 3.3 Studies assessing conventional risk factors for thrombosis in ET – continued

* P value is for univariate comparison against ET patients in the same study without the specified risk factor unless stated otherwise in the comments column.

Dash (–) indicates analysis not performed. NS = not significant.

rate in a cohort of 200 haematologically normal controls was 1.2%. The thrombotic risk was significantly higher than the controls in the oldest age group whereas in the two younger categories the difference was not significant. A number of later reports also found that increasing age was associated with an increased thrombotic risk (Besses *et al,* 1999; Carobbio *et al,* 2007; Carobbio *et al,* 2008a; Shih *et al,* 2002; Wolanskyj *et al,* 2006) although none of these had control arms and instead compared older ET patients with younger ones. Cortelazzo *et al* (1990) also noted that ET patients who had had a prior thrombotic event were at significantly higher risk of a subsequent thrombosis than those who had never had a thrombosis (OR = 13, $P < 0.0005$), a finding corroborated by several other studies (Besses *et al,* 1999; Carobbio *et al,* 2007; Colombi *et al,* 1991b; Wolanskyj *et al,* 2006).

The role of vascular risk factors such as hypertension, diabetes mellitus (DM), hyperlipidaemia and cigarette smoking in predicting the thrombotic risk in ET patients is uncertain. Whilst Besses *et al* (1999) found that hypertension and hyperlipidaemia, but not DM or cigarette smoking, were associated with an increased thrombotic rate, other studies have reported contradictory results. Some have noted an increased thrombotic risk associated with cigarette smoking alone (Jantunen *et al,* 2001), hypertension alone (Shih *et al,* 2002), or hypertension and DM (Wolanskyj *et al,* 2006). Cortelazzo *et al* (1990) did not detect any association between vascular risk factors and thrombotic risk.

Surprisingly, patients with the highest platelet counts seem not be at greatest risk of thrombotic complications (Table 3.3). Indeed a recent study has suggested that moderately elevated platelet counts ($650\text{-}1000 \times 10^9$/L) are associated with the highest risk of thrombosis (Carrobbio Blood 08), whereas patients with extreme thrombocytosis are most likely to suffer haemorrhagic complications due to consumption of higher molecular-weight multimers of von Willebrand factor (Budde *et al,* 1984; van Genderen *et al,* 1994; vanGenderen *et al,* 1996). Recently, leukocytosis has been identified in a number of retrospective studies as a risk factor for thrombosis (Barbui *et al,* 2009; Carobbio *et al,* 2008a; Carobbio *et al,* 2008b; Landolfi *et al,*

2007; Wolanskyj *et al,* 2006), although other studies have suggested it is of more limited prognostic value, being predictive only in younger patients (De Stefano *et al,* 2010), or of no value at all (Gangat *et al,* 2009). Other proposed risk factors for thrombosis include leukocyte activation and elevated haematocrit (Falanga *et al,* 2000; Wolanskyj *et al,* 2005).

Patients with ET are at increased risk of developing MF and AML compared to healthy controls, athough the absolute risk remains low. The largest long-term follow-up study observed 435 patients (Passamonti *et al,* 2004) and found that the 15-year CI of developing MF and AML was 4% and 2% respectively, markedly lower than the incidence of thrombosis and solid tumours (17% and 8% respectively). In a study of 386 patients, Palandri *et al* (2009a) reported the 10-year CI of fibrotic and leukaemic transformation as 5% and 3% respectively, and Wolanskyj *et al* (2006) reported equivalent figures of 4% and 1% in their study of 322 patients. Owing to the rarity of these events, there is little data relating to the risk of fibrotic or leukaemic transformation, although one study found that increasing age, anaemia, and a platelet count exceeding $1000x10^9$/L at diagnosis were associated with a higher incidence of leukaemic transformation (Gangat *et al,* 2007).

3.1.3 Impact of molecular characteristics on prognosis

The role of molecular markers in predicting outcome in ET is less well-defined than in many other haematological disorders. Nonetheless, a number have been proposed for use in prognostication, specifically the presence of myeloid clonality by XCIP analysis, and *JAK2* V617F and *MPL* W505 mutational status.

XCIP analysis exploits the cellular mosaicism present in most females with regard to X-chromosome inactivation to determine whether a cell population has arisen from a single progenitor – i.e. whether the population is monoclonal (see Section 1.3.5). If all the constituent

cells have inactivated the same X-chromosome, this is suggestive of a monoclonal population, assuming there is no evidence of constitutional or age-related skewing. By contrast, if there is a mix of inactivated X-chromosomes, this is suggestive of a polyclonal population. The XCIP may also be predictive of outcome. Of the four studies which reported on the clinical significance of the presence of a clonal myeloid population (Table 1.4), two observed that it was associated with an increased thrombotic risk (Chiusolo *et al,* 2001; Harrison *et al,* 1999a). It is worth noting, however, that in total these two studies reported 16 patients who suffered thrombotic events, but in 14 of these (88%) the first event occurred at or prior to diagnosis; consequently, these patients would be deemed high risk on clinical grounds alone, so whether XCIP status provides additional useful prognostic information is uncertain.

Approximately 50% of ET patients carry the *JAK2* V617F mutation (see Section 1.3.7), a finding which has both facilitated the diagnosis of ET and prompted comparison between those patients who harbour the mutation and those who do not. A number of studies have shown that, compared to *JAK2* WT ET patients, those who are V617F-positive tend to have higher haemoglobin concentrations and lower platelet counts (Table 3.4). In these regards they more closely resemble PV patients, who are almost invariably V617F-positive, than *JAK2* WT ET patients. It has, therefore, been argued that V617F-positive ET might represent a *forme fruste* of PV. Loss of the full PV phenotype may be due to modifiers such as sex, iron stores, erythropoietin levels, and the V617F mutant level (Campbell and Green 2006). PV is generally believed to confer a higher thrombotic rate and poorer prognosis than ET (Cervantes *et al,* 2008; Passamonti *et al,* 2004) and the suggestion that V617-positive ET resembled it immediately led to speculation that mutant-positive ET patients may have a worse outcome than WT ones. Despite extensive research, it remains uncertain whether this is truly the case (Table 3.5)

Table 3.4 Published studies reporting presenting characteristics of ET patients according to *JAK2* V617F status

A. Sex and age

Reference	Total *n*	*JAK2* WT *n* (%)	V617F+ *n* (%)	Female sex *n* (%)				Median age at diagnosis *years*			
				Overall	*JAK2* WT	V617F+	P	Overall	*JAK2* WT	V617F+	P
(Baxter *et al,* 2005)	51	22 (43%)	29 (57%)	27 (53%)	12 (55%)	15 (52%)	NS	55	54	55	NS
(Campbell *et al,* 2005)	776	362 (47%)	414 (53%)	464 (60%)	206 (57%)	258 62%)	0.1	56	52	60	<0.0001
(Cheung *et al,* 2006)	60	31 (52%)	29 (48%)	36 (60%)	17 (55%)	19 (65%)	NS	44	41	46	NS
(Kittur *et al,* 2007)	176	80 (45%)	96 (55%)	110 (63%)	47 (59%)	63 (66%)	0.35	57	48	62	0.03
(Vannucchi *et al,* 2007)	624*	257 (41%)	368 (59%)	428 (69%)	169 (66%)	259 (70%)	NS	50	47	52	<0.05
(Palandri *et al,* 2009b)	275	100 (36%)	175 (64%)	115 (42%)	41 (41%)	74 (42%)	NS	60	55	63	0.0015
(Pardanani *et al,* 2010)	226	108 (48%)	118 (52%)	145 (64%)	60 (56%)	85 (72%)	0.01	56	50	61	0.008
Overall†	**2263**	**977 (43%)**	**1271 (56%)**	**1325 (59%)**	**552 (56%)**	**773 (61%)**	**[1/7]**	**54**	**50**	**58**	**[5/7]**

* A further 14 who were 'homozyous' for *JAK2* V617F (mutant level > 50%) were not included in the authors' main analysis and are not shown here

† Values shown are means for the whole series, weighted according to the size of the study; values shown under the P-value for each parameter indicate the number of studies reporting a significant association with *JAK2* mutation status out of the total studies shown

Table 3.4 Published studies reporting presenting characteristics of ET patients according to *JAK2* V617F status – continued

B. Full blood counts

Reference	Total *n*	*JAK2* WT *n*	V617F+ *n*	White cell count at diagnosis (10^9/L)				Haemoglobin at diagnosis (10^9/L)				Platelet count at diagnosis (10^9/L)			
				Overall*	*JAK2*WT	V617F+	P	Overall*	*JAK2*WT	V617F+	P	Overall*	*JAK2*WT	V617F+	P
(Baxter *et al,* 2005)	51	22 (43%)	29 (57%)	*10.3*	9.4	10.9	NS	*14.1*	14.1	14.1	NS	*968*	1024	926	NS
(Campbell *et al,* 2005)	776	362 (47%)	414 (53%)	*10.0*	9.3	10.6	<0.0001	14.0	13.5	14.5	<0.0001	*961*	1030	902	<0.0001
(Cheung *et al,* 2006)	60	31 (52%)	29 (48%)	9.0	7.9	10.1	NS	13.8	13.0	14.2	0.04	872	954	867	NS
(Kittur *et al,* 2007)	60	80 (45%)	96 (55%)	*10.1*	9.4	10.4	NS	*13.7*	13.1	14.0	0.04	*840*	977	785	0.01
(Vannucchi *et al,* 2007)	176	257 (41%)	368 (59%)	10.0	8.9	10.2	0.008	13.9	13.2	14.2	<0.0001	1000	1130	975	0.001
(Palandri *et al,* 2009b)	624†	100 (36%)	175 (64%)	*8.9*	8.4	9.3	NS	Not reported‡				*850*	939	787	NS
(Pardanani *et al,* 2010)	275	108 (48%)	118 (52%)	8.7	8.3	9.0	NS	14.2	13.6	14.7	<0.0001	744	825	712	<0.0001
(Baxter *et al,* 2005)	226	22 (43%)	29 (57%)	9.7	8.6	10.1	<0.0001	13.8	13.2	14.1	0.0001	1000	1109	997	0.03
Overall§	**2263**	**977 (43%)**	**1271 (56%)**	**9.5**	**8.8**	**9.9**	**[3/8]**	**14.0**	**13.4**	**14.4**	**[6/7]**	**905**	**999**	**869**	**[5/8]**

* In four studies (italicised), overall values were not reported, so they have been calculated here as means based on the medians reported for *JAK2* WT and V617F patients, weighted according to the size of each group

† A further 14 who were 'homozyous' for *JAK2* V617F (mutant level > 50%) were not included in the authors' main analysis and are not shown here

‡ Haematocrit was similar in the *JAK2* WT and V617F-positive patients (0.41 vs. 0.43, P>0.05)

§ Values shown are means for the whole series, weighted according to the size of the study; values shown in square brackets under the P-value for each parameter indicate the number of studies reporting a significant association with *JAK2* mutation status out of the total studies shown

Table 3.5 Studies addressing the association between *JAK2* V617F and thrombosis (venous and arterial)

Reference	n†	Patient group	%*JAK2* V617F+	Median f/up (mo)	Association between *JAK2* V617F and thrombosis?‡	Details	Confounding factors associated with *JAK2* V617F status	Independent association between *JAK2* V617F and thrombosis?	Comments
(Kralovics *et al,* 2005)	244	128 PV, 93 ET, 23 IMF	48%	NR	Yes	Higher overall§ rate of thrombosis in V617F+ group (26% vs 15%)*	Age, disease duration	ND	No separate analysis for ET subgroup
(Wolanskyj *et al,* 2005)	150	ET	49%	137	No	No difference in rate of thrombosis at any time (45% vs 38%) or after diagnosis (33% vs 29%).	Age Hb, WCC	No	Not powered to detect small differences in thrombotic rate (eg. 5% and 15%)
(Campbell *et al,* 2005)	776	ET	53%	NR	Yes	Higher rate of VTE *prior* to diagnosis in V617F+ group (11 vs 2 events)*. Trend towards higher rate of VTE after diagnosis (12 vs 4 events). No difference in arterial events.	Age, Hb, neutrophil count	ND	Patients mostly derived from MRC PT-1 trial
(Cheung *et al,* 2006)	60	ET	48%	NR	Yes	Higher overall rate of thrombosis in V617F+ group (62% vs 26%)**.	Hb	ND	
(Heller *et al,* 2006)	50	ET	48%	79	Yes	Higher overall rate of thrombosis in V617F+ group (11 vs 1 events)**	Age, Hb,	Yes	Relatively young cohort (78% ≤60 years). Most thrombotic events occurred prior to diagnosis of ET
(Finazzi *et al,* 2007)	179	ET	58%	66	Yes	Higher overall rate of thrombosis in V617F+ group (33% vs 17%)*	Hb, WCC	ND	77 further patients had PV, in whom the thrombotic rate was 43% (not significantly different to V617F+ ET group)
(Ohyashiki *et al,* 2007)	49	ET	63%	NR	Yes	Higher overall rate of thrombosis in V617F+ group (29% & vs 6%)	Hb, WCC	ND	Also analysed 34 PV patients: no association between homo- or heterozygosity for *JAK2* V617F and thrombotic risk
(Carobbio *et al,* 2007)	277	ET	55%	NR	No	No difference in post-diagnosis rate of thrombosis (hazard ratio = 1.4, 95% CI 0.7 to 3.0)	Hb, WCC	No	.

(Kittur *et al,* 2007)	176	ET	55%	59	Yes	Higher rate of VTE after diagnosis in V617F+ group (11% vs 3%)*. No difference in pre-diagnosis VTE (11% vs 6%), or arterial events at any time (38% vs 29%).	Age, Hb, WCC	Yes[2]	Significance on multivariable analysis was lost if history of prior thrombosis was included.
(Alvarez-Larran *et al,* 2007)	103	ET <40 years old	43%	120	No	No difference in post-diagnosis rate of thrombosis (9 vs 13 events).	Hb	No	Major thrombotic events only included
(Vannucchi *et al,* 2007)	962	323 PV, 639 ET	73%	60	Yes	Increased rate of arterial and venous thrombosis in ET patients with mutant level >50% (n=14) compared with all other ET patients, at diagnosis and during follow-up*.	Age, Hct	Yes	
(Hsiao *et al,* 2007)	53	ET	66%	35	Yes	Higher overall rate of thrombosis in V617F+ group (43% vs 11%)*	Hb, WCC	ND	Those with thrombosis were significantly older and had significantly higher WCC than those without
Carobbio *et al,* 2009	1282	867 ET 415 PV	57%	49	Yes	Thrombotic risk highest in PV, intermediate in V617F-positive ET, lowest in WT ET (RR 2.3, 1.5 and 1.0 respectively).	Age, WCC, Hb	Yes (see comments)	Thrombotic risk at 5+ years post-diagnosis associated with mutant level >25%. At equivalent mutant levels, the thrombotic risk was similar for ET and PV
(De Stefano *et al,* 2009)	132	ET	63%	NR	Yes	Higher overall rate of thrombosis in V617F+ group (OR 2.2; 95%CI 1.1-4.4), due to markedly higher rate in patients <60 years old and those with mutant level >50% (n=8)	Hb, WCC, splenomegaly	ND	
(Chim *et al,* 2010)	141	ET	57%	NR	Yes		Hb, WCC	No	Independent thrombotic risk factors were haemoglobin and age

Table 3.5 Studies addressing the association between *JAK2* V617F and thrombosis (venous and arterial) – continued

Table 3.5 Studies addressing the association between *JAK2* V617F and thrombosis (venous and arterial) – preceding pages

* p<0.05; **p<0.005; 'OR' denotes odds ratio; 'CI' denotes confidence intervals; 'ND' denotes not done; 'NR' denotes not reported.

† number of evaluable patients in whom *JAK2* V617F status was known and clinical data was available

‡ on univariate analysis

§ 'overall' refers to thrombosis at presentation or follow-up

A number of studies have suggested that, in ET, the presence of *JAK2* V617F is associated with an increased risk of thrombotic complications, either at diagnosis (Campbell *et al,* 2005), after diagnosis (Kittur *et al,* 2007), or at any point (Cheung *et al,* 2006; Finazzi *et al,* 2007; Heller *et al,* 2006; Ohyashiki *et al,* 2007) although in some reports the increased risk was confined to younger patients (De Stefano *et al,* 2009) or those with mutant levels greater than 50%, which are found in 2-6% of ET patients (De Stefano *et al,* 2009; Vannucchi *et al,* 2007). In the majority of these studies, however, there was no evidence of an independent association between *JAK2* mutation status and thrombotic risk once adjustment was made in multivariate analysis for confounding factors, typically greater age and white cell count which are both associated with V617F-positivity. Further, several studies have failed to find any association whatsoever between *JAK2* V617F-positivity and increased thrombotic complications (Alvarez-Larran *et al,* 2007; Caramazza *et al,* 2009; Wolanskyj *et al,* 2005).

A recent meta-analysis of 21 studies with 3150 ET patients concluded that the overall thrombotic rate was higher in V617F-positive patients (OR 1.92, 95% CI 1.45-2.53), but did not adjust for confounding factors, or distinguish between thrombotic events occurring prior to presentation and those occurring thereafter (Lussana *et al,* 2009). Clearly, it is the risk of future events which are most germane to management decisions at diagnosis, and whether *JAK2* V617F status influences this remains uncertain.

The clinical significance of the presence of *MPL* W515 mutations is difficult to evaluate due to the small numbers of ET patients in whom they have been detected. The largest published study found that, amongst 994 ET patients, 30 (3%) carried *MPL* W515 mutations and these patients had an increased rate of arterial events at diagnosis and of microcirculatory disturbance, but multivariate analysis was not attempted (Vannucchi *et al,* 2008a). Another major study detected 32 patients with *MPL* mutations in a cohort of 776 patients (4%) and reported an association between *MPL* mutations and an increased risk of venous events after

entry into the study and death compared to *JAK2* WT/ *MPL* WT patients but these associations were lost after adjusting for age and prior history of thrombosis (Beer *et al,* 2008).

3.1.4 Risk stratification and therapy for ET patients

All patients with ET are routinely prescribed low-dose aspirin, which has been shown to reduce the incidence of non-fatal thrombosis in PV (Landolfi *et al,* 2004), although it has never been prospectively studied in ET. Aspirin may also relieve the vasomotor symptoms associated with ET such as headaches and erythromelalgia (erythaema and a burning sensation of the hands or feet). Other therapeutic interventions in ET include addressing concomitant vascular risk factors such as hypertension, hyperlipidaemia, diabetes mellitus, obesity and smoking.

Cytoreductive medication is generally reserved for those patients at high risk of thrombotic or haemorrhagic complications. Most national or international guidelines define high risk for thrombosis as age greater than 60 years or a history of thrombotic events (Barbui *et al,* 2004; Harrison *et al,* 2010; Tefferi 2008). Thrombotic events are generally deemed to include myocardial infarction, angina pectoris, stroke, transient ischaemic attack, deep venous thrombosis, pulmonary embolism, peripheral arterial thrombosis and retinal artery occlusion, although severe erythromelalgia unresponsive to anti-platelet therapy may also be included (Barbui *et al,* 2004). Patients at high risk of bleeding are those with a history of major haemorrhage or who have extreme thrombocytosis, although the platelet threshold used for implementation of cytoreduction varies between 1000 $x10^9$/L and 1500 $x10^9$/L (Barbui *et al,* 2004; Harrison *et al,* 2005; Harrison *et al,* 2010; Tefferi 2008).

It is unclear whether any ET patients who are younger than 60 years old and do not have a prior history of thrombotic events or an extreme thrombocytosis would also benefit from cytoreduction. The ‘intermediate risk’ arm of the MRC Primary Thrombocythaemia 1 (PT-1)

Study is currently investigating the role of cytoreduction in patients aged 40-60 years without high-risk features. Some experts have recommended that patients within this age group with additional vascular risk factors such as diabetes or hypertension requiring therapy, familial thrombophilic defects and leukocytosis should also be considered for cytoreductive therapy (Barbui *et al,* 2004; Carobbio *et al,* 2008a; Harrison *et al,* 2005).

3.1.5 Choice of cytoreductive agent

The first-line of cytoreductive therapy is usually hydroxycarbamide (formerly called hydroxyurea), which exerts its cytotoxic effects by inhibition of ribonucleotide reductase and hence DNA synthesis, and has been shown to reduce the incidence of thrombotic complications in 'high-risk' ET patients (Cortelazzo *et al,* 1995). Anagrelide, a newer agent, is a phosphodiesterase inhibitor which selectively blocks megakaryocyte maturation without affecting neutrophil production. It is licensed in the EU for the treatment of ET patients who are intolerant of, or resistant to, hydroxycarbamide and is most commonly used when the dose of hydroxycarbamide required to control the patients' platelet counts renders them neutropenic. Interferon alpha (IFNα) inhibits TPO-induced megakaryopoiesis by inducing SOCS-1 expression, thereby suppressing proliferation of CFU-MKs and the more immature pluripotent haemopoietic progenitors (Lengfelder *et al,* 1996), resulting in reduction of platelet counts. Since it is not believed to be teratogenic, IFNα is the preferred treatment for ET patients requiring cytoreduction in pregnancy. Alkylating agents such as chlorambucil and busulphan and radio-isotopes such as ^{32}Phosphorus also have cytoreductive properties but are now rarely used in the treatment of ET as they increase the rate of progression to acute leukaemia, especially if used in combination with hydroxycarbamide (Finazzi *et al,* 2000; Passamonti *et al,* 2004; Sterkers *et al,* 1998). By contrast, no intervention in ET has been shown to reduce the risk of leukaemic transformation.

The high risk arm of the PT-1 study compared cytoreductive agents in 809 ET patients at high risk of thrombosis and found that hydroxycarbamide was superior to anagrelide in preventing arterial events but less effective in preventing venous events. Hydroxycarbamide was also associated with a lower incidence of myelofibrotic transformation, intolerable side effects and serious bleeding (Harrison *et al,* 2005). For this reason, some experts recommend hydroxycarbamide as the first-line cytoreductive agent in high-risk ET patients (Harrison *et al,* 2010; Tefferi 2008), although (unproven) concerns about the long-term leukaemogenic potential of hydroxycarbamide mean that others favour anagrelide or IFNα for younger patients (Barbui *et al,* 2004; Emadi and Spivak 2009).

3.1.6 Aims

In this chapter, the clinical, haematological and molecular characteristics at disease presentation of the cohort of ET patients used in subsequent studies have been analysed and the results correlated with their clinical outcome.

3.2 Patients, materials and methods

3.2.1 Patient recruitment and sample collection

Patients were recruited from the University College Hospitals London Essential Thrombocythaemia clinic and from haematology clinics in the UK between May 1996 and January 2010, and provided written, informed consent in keeping with the Declaration of

Helsinki. Prior to 2008, the 1986 PVSG Criteria (Murphy *et al,* 1986) were used for the diagnosis of ET; from 2008 onwards, the WHO Criteria were used (Swerdlow *et al,* 2008).

Peripheral blood samples (approximately 25mL) were collected into EDTA and cell purification performed as described in Section 2.1.1. For the majority of patients, neutrophil DNA and RNA, T-lymphocyte DNA and RNA, and platelet RNA samples were prepared and stored in a tissue bank. At the start of the research reported in this thesis, this tissue bank contained samples from 114 ET patients. During the period of the research, samples from 19 new patients and follow-up samples from 8 existing patients were added to the bank.

3.2.2 Clinical and demographic characteristics

Patients were assessed at diagnosis for the presence of splenomegaly, bleeding and thrombotic manifestations (as defined in Section 3.1.4), and their blood counts recorded. Where possible, patients were followed up to record the use of cytoreductive and anti-platelet agent(s), the development of haemorrhagic or thrombotic complications, the onset of myelofibrosis or acute myeloid leukaemia, or death.

Clinical and blood count data was obtained from a departmental clinical database created prior to the start of this research for 59 patients, although in some cases the data here was incomplete, and from medical case notes for 62 patients. For 12 patients no presentation clinical or blood count data was available.

3.2.3 *JAK2* V617F status

Patient neutrophil DNA was screened for the presence of *JAK2* V617F using PCR to amplify exon 14 of the *JAK2* gene followed by restriction enzyme digestion with *Afl*III to distinguish between wild-type and mutant alleles (Figure 3.1) as previously published (Gale *et al,* 2007). All oligonucleotide primer sequences are listed in Appendix 1.

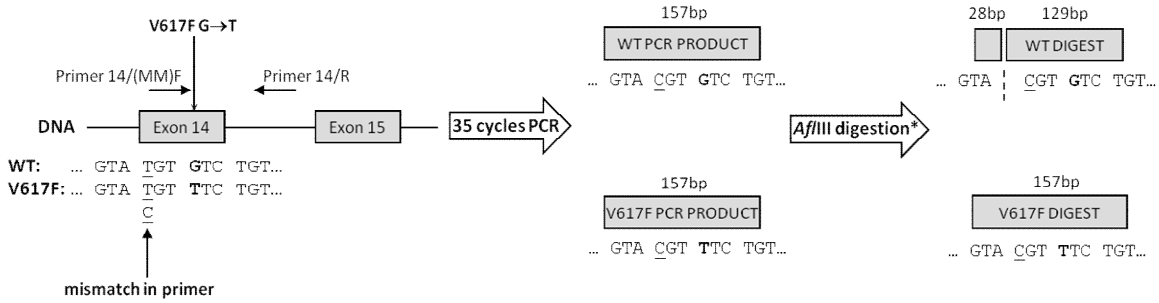


Figure 3.1 Method for PCR of JAK2 exon 14 followed by AflIII digestion to distinguish between *JAK2* WT and V617F alleles

Bold indicates V617F mutation site, underlining indicates location of mismatch (T>C) introduced by primer. **Afl*III recognition site is ACGTG

BIOLINE™ PCR was performed as described in Section 2.2.3.1 using primers flanking the *JAK2* V617F mutation site in exon 14. Since no restriction enzymes were available which could discriminate between WT and mutant-positive PCR products directly, a mismatched forward primer, *JAK2*ex14/(MM)F, was designed. This introduced a mismatch immediately upstream of the mutation site to create a recognition site for *Afl*III which would cut the WT allele whilst leaving the mutant allele uncut. Using this mismatched forward primer and an intronic reverse primer, *JAK2*ex14/R, a 157bp PCR product spanning the mutation site in exon 14 was amplified. Thirty-five cycles of PCR were performed with an annealing temperature of 60°C,

and PCR products were separated by agarose gel electrophoresis (2% gel) and visualised by UV transillumination.

A digestion mix was then prepared containing 5 units *Afl*III enzyme, 1x manufacturer's buffer 3 and 100µg/mL bovine serum albumin (BSA), made up to 3µL with ddH_2O, and to this 7µL PCR product was added and incubated for 6 hours at 37°C. Following 3.5% agarose gel electrophoresis, digestion products were visualised by UV transillumination. *Afl*III cut the wild-type allele to 129bp and 28bp but did not cut the mutant allele (157bp).

Screening for *JAK2* V617F had already been performed for 114 patients at the start of the research reported in this thesis, and was performed for a further 19 patients during the course of this research.

3.2.4 *JAK2* V617F mutant level quantification in neutrophil gDNA

JAK2 exon 14 PCR and *Afl*III digestion was performed as described in Section 3.2.3, except that a fluorescently-labelled exonic reverse primer (*JAK2*ex14/RF) was used. Only 30 cycles of PCR were performed in order to minimise the formation of heteroduplex products, since they would not be cut by *Afl*III and would therefore mimic homoduplex V617F-positive products. Denaturation, annealing and extension times were 60 seconds in each cycle. Following digestion with *Afl*III, 2µL of each product underwent fragment analysis as described in Section 2.2.7. The wild-type and the mutant peaks were at 129bp and 157bp respectively. Mutant allele level was calculated by expressing the area under the 157bp peak as a proportion of the total area under both peaks. Each sample was analysed three times and the mean value of the three results calculated.

JAK2 mutant level analysis had already been performed for 42 patients prior to the start of the research reported in this thesis, and was performed for a further six patients during the course of this research.

3.2.5 X-chromosome inactivation pattern (XCIP) analysis

In order to determine an XCIP, it is necessary to distinguish between the two X-chromosomes and between the active and the inactivated X-chromosome (Figure 3.2). To distinguish between the two X-chromosomes, the subject must be heterozygous for a polymorphic X-chromosome gene which is known to be differentially methylated on the active and inactive X-chromosomes. In these studies, the human androgen receptor gene, *HUMAR*, was studied, since it is heterozygous in 90% of females (Allen *et al,* 1992), with different alleles distinguishable on the basis of size. This is due to the presence of a highly polymorphic variable number tandem repeat (VNTR) sequence of 11-31 trinucleotide (CAG) repeats in the first exon of the *HUMAR* gene.

The active and inactive chromosomes are distinguished on the basis of differential methylation using a methylation-sensitive restriction enzyme (*Hpa*II) followed by PCR. Since the inactivated *HUMAR* allele is methylated, subjecting the DNA to digestion with *Hpa*II will cut the active (unmethylated) allele just upstream of the VNTR region. If PCR is then performed using primers located outside the cutting site, only the intact inactive (methylated) allele will be amplified. If some cells in a population inactivate one X-chromosome and others inactivate the other X-chromosome, two different-sized PCR products will be generated, assuming the individual is heterozygous at the *HUMAR* locus. The relative amount of the two products is proportional to the relative degree of methylation of the two alleles within the cell population, and can be quantified by fragment analysis by calculating the areas under the corresponding peaks.

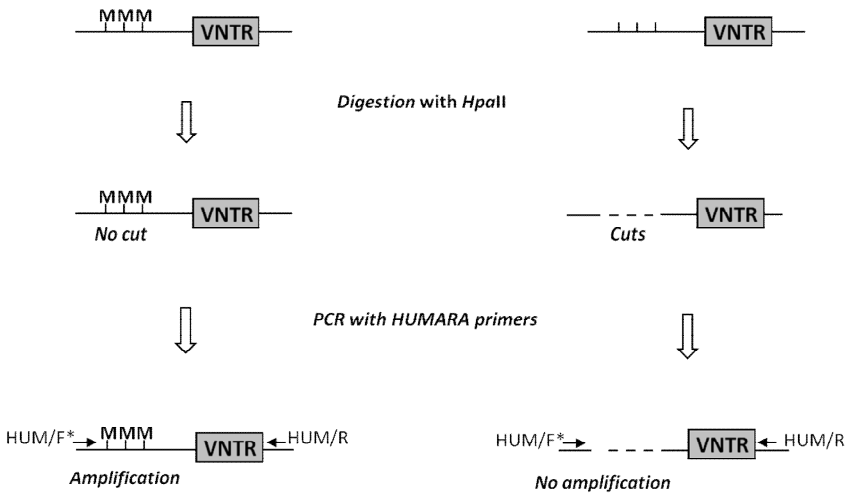


Figure 3.2 Principle of XCIP analysis using HUMARA

The *HUMAR* allele on the left is methylated (indicated by M), whereas the *HUMAR* allele on the right is not. Methylation-sensitive digestion with *Hpa*II leaves the methylated allele intact (left) but cuts the unmethylated allele (right). PCR with primers which anneal outside the methylation sites allows efficient amplification of the intact allele (left), whereas the cut allele is not amplified (right).

VNTR indicates variable number tandem repeat

However, the relative amounts of the final PCR products does not depend solely on the amount of each allele present in the template DNA following *Hpa*II digestion. If equal amounts of two different-sized alleles are used as template in the same PCR, the smaller allele may be preferentially amplified if a large number of cycles is used (Walsh *et al,* 1992). For this reason, only 22 cycles of PCR were performed (Gale *et al,* 1996). Furthermore, in order to identify and control for any preferential amplification of the smaller allele due to its size rather than differential methylation, the PCR was performed twice for every DNA sample: in one, *Hpa*II-digested DNA was used as template, in the other non-*Hpa*II digested DNA was used. In the latter, equal amounts of the two PCR products were expected. The results from the two PCRs were then compared and the effect of the *Hpa*II digestion was determined. In this way the

degree of methylation of each *HUMAR* allele in the original sample was calculated. This analysis was performed on both neutrophil and T-cell DNA to evaluate methylation in myeloid cells and lymphoid cells respectively. The latter are not believed to be involved in MPNs and were therefore used to reflect the patient's constitutional XCIP.

Methylation-sensitive digestion: Two reactions were set up, one with and one without *HpaII*. For the former, 4μL gDNA was added to a reaction mixture containing 2.5 units *RsaI,* 12.5 units *HpaII,* 1x manufacturer's buffer 1, in a total reaction volume of 5μL. For the second reaction, 2μL gDNA was added to a reaction mixture containing 2.5 units *RsaI,* 1x manufacturer's buffer 1, and made up to a total reaction volume of 5μL with ddH_2O. *RsaI* was included in both reactions as it cut the genomic DNA outside the primer annealing sites, regardless of methylation status, and thereby improved PCR efficiency. Both reactions were incubated at 37°C overnight.

PCR: A PCR reaction mix was prepared containing 1x Promega buffer, 0.2mM each dNTP, 1ng/μL each *HUMAR* primer (*HUMAR/*U, a fluorescently-labelled forward primer, and *HUMAR/*D, an unlabelled reverse primer), and made up to 14μL with ddH_2O. This was added to the digest products, followed by a drop of mineral oil and incubated at 95°C for 5 minutes. Samples were then held at 85°C whilst Taq polymerase mixture (1 unit Promega aTaq made up to 1μL with ddH_2O) was added, then 22 cycles PCR performed (45 seconds 95°C, 45 seconds 68°C, 30 seconds 72°C) followed by a final extension of 15 minutes at 72°C. PCR products (2μL each) were then analysed by fragment analysis as described in Section 2.2.7.

Calculation of X-chromosome inactivation status: For polymorphic individuals, two major peaks were expected – one which represented the shorter *HUMAR* allele, A, and one which represented the longer *HUMAR* allele, B (Figure 3.3). In addition, each major peak frequently had one or more 'slippage' peaks, each being smaller than the major peak in 3bp increments, caused by Taq slippage during the PCR, and the area of the largest slippage peak was added to

the corresponding major peak. Using the fragment analysis software, the total area of alleles A and B was calculated for the reaction without *Hpa*II (A^- and B^- respectively) and for the reaction containing *Hpa*II (A^+ and B^+ respectively). The relative percentage inactivation of allele A (%A) and allele B (%B) is given by the following equations:

$$\%A = \frac{(A^+ / A^-)}{(A^+ / A^-) + (B^+ / B^-)} \quad \text{and} \quad \%B = \frac{(B^+ / B^-)}{(A^+ / A^-) + (B^+ / B^-)}$$

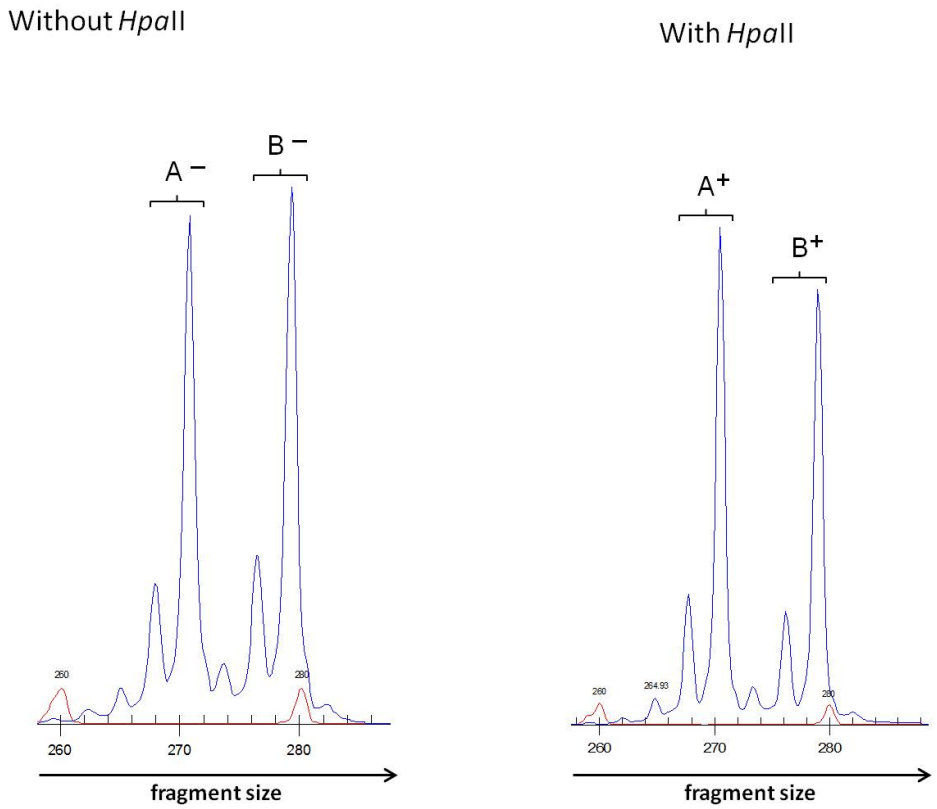


Figure 3.3 Fragment analysis chromatogram of HUMARA PCR products from a single patient

Chromatograms of PCR products from neutrophil gDNA which has undergone restriction enzyme digestion in the absence (left) and presence (right) of *Hpa*II.
In each chromatogram, the major peaks representing the shorter and longer *HUMAR* alleles are labelled A and B respectively. A number of smaller slippage peaks are visible to the left of the corresponding major peak. For each allele the area of the largest slippage peak was added to that of the corresponding major peak (as bracketed) for use in the HUMARA calculation.

XCIP analysis data was already available for 94 patients at the start of the research reported in this thesis, and was performed for a further 11 patients during the course of this research.

3.2.6 *MPL* mutation screening

Mutations in *MPL* most commonly occur in exon 10 and affect residue W515. *MPL* exon 10 was therefore screened for the presence of mutations by denaturing HPLC.

3.2.6.1 Generation of a MPL W515L-positive control by site-directed mutagenesis

A mutant-positive control sample was generated by site-directed mutagenesis (Figure 3.4). Two short-length W515L-positive products were generated from normal control gDNA template using mismatched primers *MPL*W515L/F and *MPL*W515L/R (Figure 3.4A). A 149bp *MPL* W515L-positive PCR product was amplified using primers *MPL*ex10/F, which annealed just upstream of *MPL* exon 10, and *MPL*W515L/R, and a 130bp *MPL* W515L-positive PCR product was amplified using primers *MPL*ex10/R2, which annealed just downstream of *MPL* exon 10, and *MPL*W515L/F (Figure 3.4B). These two products were generated in separate reactions, using 35 cycles of BIOTAQ™ PCR as described in Section 2.2.3.1, and an annealing temperature of 60°C. The two PCR products were electrophoresed on a 1.5% low-melting point agarose gel, visualised by UV transillumination, cut out from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) as per manufacturers' instructions.

Equal volumes of each purified PCR product were then mixed together. A 252bp W515L-positive PCR product was amplified by BIOTAQ™ PCR using 1μL of the PCR product mixture as template in a 20μL total reaction volume with primers *MPL*ex10/F and *MPL*ex10/R2, an annealing temperature 60°C and 25 cycles of PCR. Other conditions were as described in Section 2.2.3.1. The 252bp W515L-positive PCR product was then purified using the PEQLab

A

B

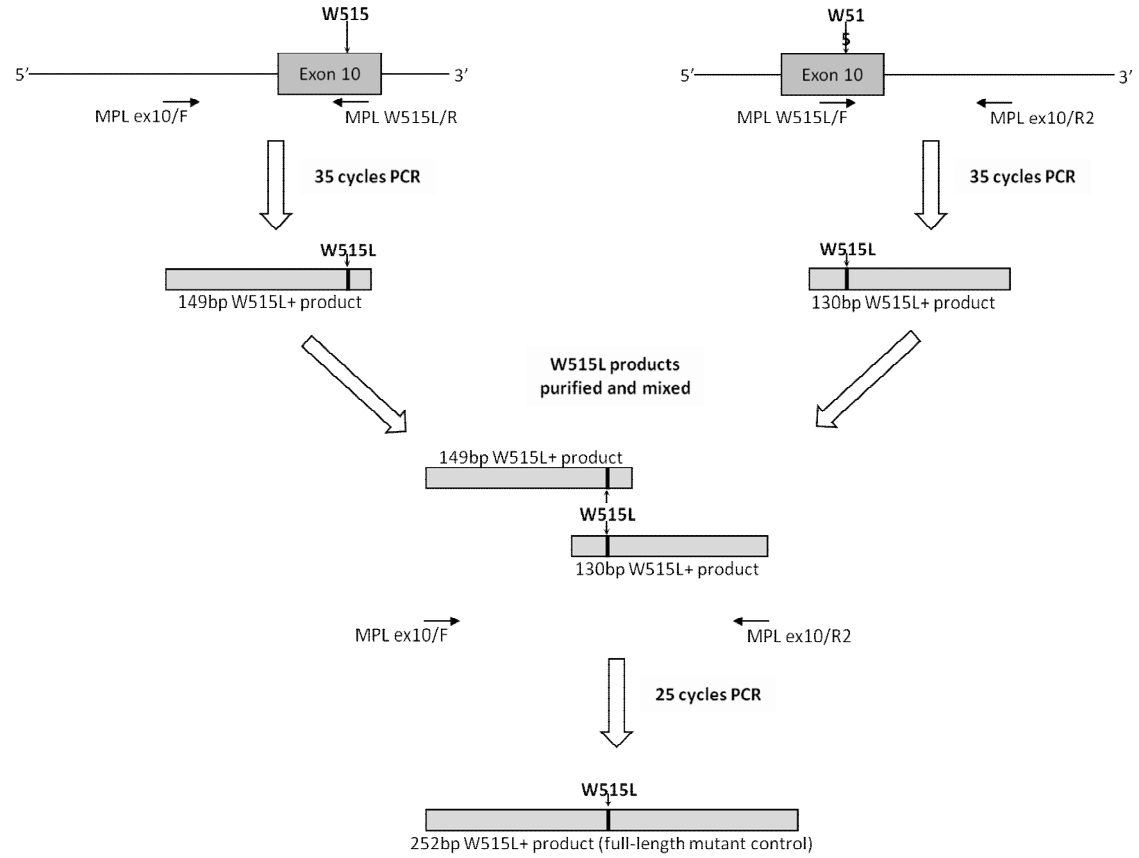


Figure 3.4 Site-directed mutagenesis to create a *MPL* W515L-mutant positive control

A. Genomic sequence of part of *MPL* exon 10 (codon 515 in bold), showing WT forward and reverse strands, and the two mismatched primers which introduce the G>T substitution (mismatch underlined)

B. Generation of mutant-positive product

Gold PCR purification kit, according to manufacturers' instructions, and the presence of the W515L mutation confirmed by direct sequencing.

Mutant levels are reported to be relatively low in ET, with a median value of approximately 15-25% for both *JAK2* V617F (Vannucchi 08; Girodon 08) and *MPL* W515K/L (Beer 08; Jones 10). In order to ensure that the screening technique was sufficiently sensitive to identify the majority of mutant-positive samples, a positive control with a low *MPL* W515L level (approximately 10%) was included with every run. This control was produced by mixing nine volumes of WT gDNA with one volume of the 252bp W515L-positive PCR product of an equal concentration. In order to equalise the concentrations of the W515L-positive PCR product and the WT gDNA, the former was diluted in ddH_2O (1 in 10, 1 in 100, 1 in 1000, 1 in 10,000). The dilution which was closest in concentration to the WT gDNA was identified by using each one, as well as WT gDNA, as template in a *MPL* exon 10 PCR. An Optimase® PCR was performed using conditions as specified in Section 2.2.3.3, with primers spanning *MPL* exon 10, *MPL*ex10/F3 (forward) and *MPL*ex10/R2 (reverse). A 'touchdown' PCR technique was used, based on manufacturer's recommendations, which involved using an initial annealing temperature 7°C above the mean melting temperature of the two primers (65°C), and gradually reducing the annealing temperature with successive cycles: two cycles, T_a=72°C; two cycles, T_a=71°C; two cycles, T_a=70°C; three cycles, T_a=69°C; 13 cycles, T_a=68°C; 13 cycles, T_a=66°C.

Visual inspection of the PCR products by UV transillumination following agarose gel electrophoresis revealed that the 1 in 10,000 dilution of the W515L-positive PCR product and the WT gDNA generated similar strength products. WT gDNA and the 1 in 10,000 dilution of the W515L-positive product were therefore mixed together in a 9:1 ratio to generate the 10% (approximately) *MPL* W515L-positive control.

3.2.6.2 Screening for MPL exon 10 mutations using dHPLC

The Transgenomic WAVE® system (Transgenomic Ltd, Glasgow, UK) of dHPLC was used to screen for mutations of *MPL* exon 10. This technique exploits differences in the physical properties of heteroduplex and homoduplex PCR products to detect the presence of somatic mutations. Heteroduplex products are generated in a PCR when the template DNA contains a mixture of WT and mutant alleles. One strand of the heteroduplex product contains the WT allele and the other contains the mutant allele (or rather its complement). This mismatch introduces instability into the PCR product, and consequently the two strands are less strongly bound together at this site than at the equivalent site in homoduplex PCR product which is composed of two perfectly complementary DNA strands, either both WT or both harbouring the same mutation. Denaturing the PCR product by heating it to 95°C then allowing it to re-anneal at progressively lower temperatures maximises heteroduplex formation.

In dHPLC, the PCR products are pumped through a polystyrene-divinylbenzene copolymer column at a temperature which has been calculated to partially denature them. The denaturation profile of a PCR product depends on its sequence and can be predicted using the manufacturer's Navigator software. The partially-denatured products bind to the column and are eluted off by progressively increasing concentrations of acetonitrile. Since the DNA strands of the heteroduplex product are less strongly bound than those of the homoduplex product, they will be elute off the column earlier. A UV detector generates a chromatogram based on the timing and intensity of DNA elution. The presence of heteroduplex products are therefore detectable as a peak arising before the main homoduplex peak.

In order to screen the cohort of ET patients for *MPL* exon 10 mutations, a 226bp fragment spanning *MPL* exon 10 was amplified using an Optimase® PCR as described in Section 3.2.6.1 and analysed by dHPLC. PCR products were electrophoresed on a 2% agarose gel and visualised by UV transillumination, then denatured by incubation for 5 minutes at 95°C and

slowly cooled: 1 minute at 93.5°C, and then 1 minute at progressively lower temperatures in 1.5°C increments down to 39.5°C. Denatured products were run on the Transgenomic WAVE® instrument at 62.5°C and 65.8°C, which were the optimal column temperatures for partial denaturation of the amplicon, calculated using Transgenomic Navigator software (Figure 3.5).

Samples with abnormal WAVE patterns were further analysed by direct sequencing of *MPL* exon 10. *MPL* exon 10 PCR products were generated as described in Section 3.2.6.1, purified using the peqGold micro-spin cycle-pure kit, and sequenced by the UCL Wolfson Institute for Biomedical Research DNA sequencing service (Section 2.2.8) using primers *MPL*ex10/3F and *MPL*ex10/R2.

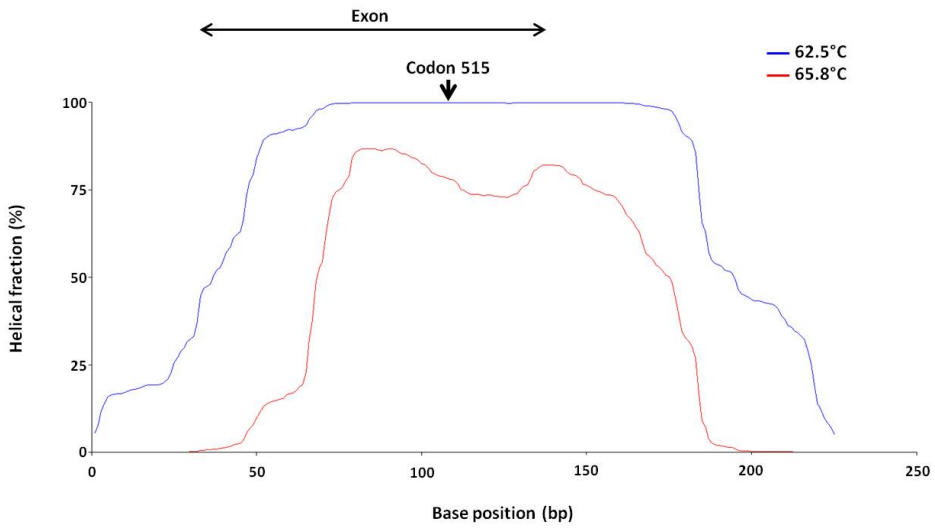


Figure 3.5 Theoretical melting curves of *MPL* exon 10 PCR product generated by Transgenomic Navigator software

A 226bp PCR product amplified from gDNA using primers *MPL*ex10/F3 (forward) and *MPL*ex10/R2 (reverse) is shown, with the predicted helical fraction after running on the dHPLC instrument at 62.5°C and 65.8°C.

3.3 Results

3.3.1 Overall results and analysis by clinical risk factors at presentation

The clinical and demographic characteristics of the 133 patients studied are shown in Table 3.6. Of the 133 patients, 28 (21%) were male. At diagnosis, the median age was 48 years (range 8-84 years), 34 patients were aged 60 or over, and the median platelet count was $805x10^9$/L (range 453-4875 $x10^9$/L). At the time of analysis (February 2010), the median time since diagnosis was 12.6 years (range 0.7-31.4). Follow-up data was available on 104 patients and 29 patients were lost to follow-up. The median patient age at the time of analysis was 62.8 years (range 24.6-97.8). Eighty-two patients (78% of those evaluable) received cytoreductive medication during follow-up.

Overall, 37 patients (36% of those evaluable) had a thrombotic episode, of whom 20 had suffered a thrombosis prior to diagnosis, 11 post diagnosis, and six suffered thromboses both pre and post diagnosis. The 37 patients who had a thrombosis (at any stage) were significantly older at diagnosis than those who did not (53 versus 44 years; P=0.03, t-test), but had similar presenting blood counts: median platelets 819 versus $802x10^9$/L; haemoglobin 13.6 versus 13.9g/dL; white cell count 9.5 versus 9.2 $x10^9$/L (Table 3.7). There was no significant association between suffering a thrombosis prior to or at diagnosis and suffering one after diagnosis (P=0.10, Fisher's exact test). Six patients (6%) had haemorrhagic complications. Six patients (6%) transformed to MF, of whom one later developed AML and died, one underwent a stem cell transplant, one was awaiting a transplant at the time of analysis and three were lost to follow up. Six patients died (6%), one due to AML, one pneumonitis, one gliobastoma multiforme, one dementia, and two unknown causes.

Table 3.6 Clinical and demographic characteristics of ET patients included in study

	Data available *n*	Value
Total patients		133
Sex, *n* (% of total*)	133	
Male		28 (21%)
Female		105 (79%)
Median age at presentation, years (95% CI)	121	48.0 (43.4-50.0)
Median platelet count at presentation, x 10^9/L (95% CI)	117	805 (874-1073)
Median haemoglobin at presentation, g/dL (95% CI)	77	13.7 (13.3-14.0)
Median white cell count at presentation, x 10^6/L (95% CI)	50	9.4 (9.3-11.63)
Median time since diagnosis, years (95% CI)	121	12.6 (12.1-14.7)
Palpable splenomegaly, n (% of total*)	80	7 (9%)
Cytoreductive therapy†, n (% of total*)	104	
None		22 (21%)
Hydroxycarbamide		70 (67%)
Anagrelide		20 (19%)
Interferon-alpha		12 (12%)
Other (^{32}P, chlorambucil or busulphan)		7 (7%)
Complications, n (% of total*)	104	
Thrombosis prior to or at presentation		26 (25%)
Thrombosis post-presentation		17 (16%)
Thrombosis (at any stage)‡		37 (36%)
Bleeding (at any stage)		6 (6%)
Death (all causes)		6 (6%)
Myelofibrotic transformation		6 (6%)
Leukaemic transformation		1 (1%)

* Where percentages of the total are shown, these are the percentage of the number of patients for whom data on that parameter was available.

† Patients are counted for each therapy used and so may appear more than once.

‡ Six patients had thromboses both pre- and post diagnosis

Table 3.7 Effect of presenting age and blood counts on occurrence of thrombosis at any stage

Presenting variable*	No thrombosis		Thrombosis		P-value (t-test)
	n	Median (95% CI)	*n*	Median (95% CI)	
Age, years	84	44 (40.0-48.6)	37	53 (47.8-56.5)	0.03
Haemoglobin, g/dL	45	13.9 (13.3-14.3)	32	13.6 (12.8-14.0)	0.3
White cell count, $x10^9$/L	29	9.2 (8.9-11.2)	21	9.5 (8.7-13.4)	0.4
Platelet count, $x10^9$/L	81	802 (870-1143)	37	819 (796-1011)	0.3

* The presenting age and blood counts were only available for a proportion of patients (see Table 3.6)

3.3.2 Analysis by XCIP status

During the research reported in this thesis, XCIP analysis was performed on samples from 10 patients, of whom two were uninterpretable due to constitutional skewing, and two were not informative at the *HUMAR* locus. Of the six patients with interpretable XCIPs, three (50%) had clonal myelopoiesis (i.e. greater than 75% expression of one allele, a difference between neutrophil and T-cell XCIP of greater than 20%, and patient age less than 65 years) and three (50%) had polyclonal myelopoiesis. Data was available for a further 92 patients from analysis performed prior to the start of the research reported in this thesis. Overall, therefore, XCIP results were available for 102 of the 105 female patients in the study. No PCR products were obtained in the remaining three patients.

In 36 of the 102 patients, XCIP results were not interpretable, either due to age (*n*=21) or constitutional skewing (*n*=15), and in four more patients they were not informative at the

HUMAR locus, leaving 62 patients with evaluable XCIPs. Of these, 38 (61%) were polyclonal, and 24 (39%) were clonal (Table 3.8). Clonal patients tended to be younger at presentation than polyclonal patients, 35.5 versus 43.0 years, although the difference was not quite significant (P=0.06, t-test). The median presentation platelet count was higher in clonal patients than polyclonal patients, 950 versus 780x10^9/L, and this difference just reached statistical significance (P=0.05, t-test). The median presenting haemoglobin, 12.6 versus 13.7g/dL, was similar in the two groups (P=0.51, t-test).

Table 3.8 Clinical and demographic characteristics of patients studied according to their XCIP status

	Clonal	Polyclonal	P-value
Total patients, n (% of evaluable patients)	24 (39%)	38 (61%)	-
Median age at presentation, years (95% CI)	35.5 (29.4-43.1)	43.0 (39.0-50.9)	0.06
Median platelet count at presentation, x 10^9/L (95% CI)	950 (804-1617)	780 (717-965)	0.05
Median haemoglobin at presentation, g/dL (95% CI)	12.6 (11.8-14.2)	13.7 (12.7-14.1)	0.5
Median time since diagnosis, years (95% CI)	13.4 (11.0-17.5)	11.7 (10.3-14.0)	0.2
Cytoreductive therapy, n (% of evaluable patients*)			
No	2 (12%)	5 (17%)	1.00
Yes	15 (88%)	24 (83%)	
Complications, n (% of evaluable patients*)			
Thrombosis prior to or at presentation	7 (47%)	6 (21%)	0.09
Thrombosis post-presentation	3 (20%)	3 (10%)	0.4
Thrombosis (at any stage)†	8 (53%)	8 (28%)	0.1
Bleeding (at any stage)	0	1	
Death (all causes)	1	1	
Myelofibrotic transformation	3	0	
Leukaemic transformation	1	0	

* Clinical data was available for 15 clonal patient and 29 polyclonal patients

† Three patients had thromboses both pre- and post-presentation (two with clonal XCIP and one with polyclonal XCIP)

The rate of thrombotic events was higher in clonal patients than in polyclonal patients, both prior to presentation and overall, but this difference did not reach statistical significance (47% versus 21%, P=0.09; and 53% versus 28%, P=0.11, Fisher's exact test). There was no difference in the rate of thrombosis after presentation, 20% versus 10% (P= 0.39, Fisher's exact test).

3.3.3 Analysis of *JAK2* V617F status

During the research reported in this thesis, 20 ET patients were screened for the presence of the *JAK2* V617F mutation, of whom ten (50%) were mutant-positive. There was no difference in median age at presentation in mutant-positive patients compared to WT ones (53 years versus 48 years, P=0.43, t-test), in the median presenting platelet count (734x10^9/L versus 788 x10^9/L, P=0.51, t-test), or the rate of thrombotic complications (50% versus 30%, P=0.65, Fisher's exact test). The median mutant level in the V617F-positive patients was 15% (range 5%-21%).

JAK2 V617F screening had been performed for a further 113 patients prior to the start of the research described in this thesis. In total, therefore, data was available for 133 ET patients, of whom 55 (41%) were mutant-positive (Table 3.9). *JAK2* mutant levels were available in 48 V617F-positive patients (in the other seven patients no PCR products were obtained), and in these the median mutant level was 17% (range 5-100%). Patients who harboured the *JAK2* V617F mutation were significantly older than those who were *JAK2* WT, median age 53.0 versus 39.5 years (P<0.0001, t-test). Mutant-positive patients also had a significantly lower presenting platelet count, median 772 versus 950x10^9/L, (P = 0.011, t-test) and a significantly higher presenting haemoglobin, 14.2g/dL versus 13.0g/dL (P=0.0002, t-test).

Table 3.9 Clinical and demographic characteristics studied according to *JAK2* status

	Data available *n* (*JAK2* WT : V617F)	*JAK2* WT*	*JAK2* V617F†	P-value
Total patients	133	78 (58%)	55 (41%)	-
Sex	133 (78 : 55)			
Male		16 (21%)	12 (22%)	1.00
Female		62 (79%)	43 (78%)	1.00
Median age at presentation, years	121 (69 : 52)	39.5 (36.4-45.0)	53.0 (50.4-59.0)	<0.0001
Median platelet count at presentation, x 10^9/L	117 (68 : 49)	950 (937-1264)	772 (747-871)	0.011
Median haemoglobin at presentation, g/dL	77 (35 : 42)	13.0 (12.4-13.4)	14.2 (13.8-14.7)	0.0002
Median white cell count at presentation, x 10^6/L	50 (18 : 32)	10.0 (8.9-13.1)	9.0 (8.8-11.6)	0.52
Median time since diagnosis, years	121 (69 : 52)	12.5 (11.6-14.9)	12.7 (11.6-15.6)	0.770
Palpable splenomegaly	80 (36 : 44)	3 (9%)	4 (10%)	1.00
Cytoreductive therapy	104 (53 : 51)			
None		14 (26%)	8 (16%)	
Hydroxycarbamide		30 (57%)	40 (78%)	
Anagrelide		14 (26%)	6 (12%)	
Interferon alpha-2a		4 (8%)	8 (16%)	
Other (^{32}P, busulphan or chlorambucil)		5 (9%)	2 (4%)	
Cytoreductive therapy	104 (53 : 51)			
No		14 (26%)	8 (16%)	0.231
Yes		39 (74%)	43 (84%)	

Continuous variables are shown using median (95% confidence intervals), P-values using the t-test. Categorical variables are shown using *n* (percentage of total), P-values using Fisher's exact test.

* Where percentages are given, these represent the percentage of the total number of *JAK2* WT patients for whom data for that parameter was available (shown in second column)

† Where percentages are given, these represent the percentage of the total number of *JAK2* V617F patients for whom data for that parameter was available (shown in second column)

Table 3.9 Clinical and demographic characteristics studied according to JAK2 status – continued

Complications	104 (53 : 51)			
Thrombosis prior to or at presentation		10 (19%)	16 (31%)	0.256
Thrombosis post-presentation		6 (11%)	11 (22%)	0.191
Thrombosis (at any stage)‡		14 (26%)	23 (45%)	0.099
Bleeding (at any stage)		6 (11%)	2 (4%)	
Death (all causes)		0 (0%)	6 (12%)	
Myelofibrotic transformation		3 (6%)	3 (6%)	
Leukaemic transformation		0 (0%)	1 (2%)	

‡ Four *JAK2* V617F-positive patients and two *JAK2* WT had thromboses pre- and post diagnosis

There was no difference in sex-distribution, median follow up, prevalence of splenomegaly or usage of cytoreduction between the two groups. There was no difference in the incidence of thrombotic complications pre- or post-presentation. Although, the incidence of thrombosis at any time did appear higher in the V617F-positive group than in the *JAK2* WT group, 45% versus 26%, the difference did not reach statistical significance (P= 0.1, Fisher's exact test). Patients who had had a thrombosis (at any time) did, however, have a significantly higher mean mutant level than those who did not have a thrombosis, 11% versus 5% (P=0.02; t-test).

Mutant levels greater than 25% have been associated with a particularly high thrombotic risk (Antonioli *et al,* 2008; Carobbio *et al,* 2009). Of the 37 patients who had a thrombosis, six (16%) had mutant levels greater than 25%, compared to three (5%) of the 67 patients who did not have a thrombosis (P<0.0001, Fisher's exact test).

All six patients who died were *JAK2* V617F-positive, resulting in a significantly higher mortality rate in the V617F-positive group, 12% versus 0% (P=0.01, Fisher's exact test).

3.3.4 Correlation between XCIP status and *JAK2* mutational status

Of the 22 *JAK2* V617F-positive patients with an interpretable XCIP, 16 (73%) had a polyclonal XCIP and six (27%) had a clonal XCIP. Of the 40 *JAK2* WT patients with an interpretable XCIP, 22 (55%) had a polyclonal XCIP and 18 (45%) had a clonal XCIP. There was no association between XCIP status and *JAK2* genotype (P=0.19, Fisher's exact test).

An analysis was performed to determine whether, in female V617F-positive patients, the size of the mutant-positive population could account for the difference between the T-cell and neutrophil XCIP. This analysis was based on the assumption that the mutant-positive population was clonal and all of its member cells therefore shared the same inactivated X-chromosome carrying either allele A or B. Scott *et al* (2006) have reported that in ET, mutant-positive cells are heterozygous for the mutation. Therefore, if a patient has a particular mutant level (x%), the percentage of mutant-positive cells (y%) will be double this and the remainder will be WT (z%). Thus y = 2x, and z = 100-2x.

Using the constitutive (i.e. T-cell) XCIP of A_T% : B_T%, the predicted neutrophil XCIP is given by one of two possibilities depending on whether allele A or B is inactivated in the mutant-positive population. If allele A is inactivated, the predicted neutrophil XCIP (A%:B%) = [(y/100) x 100] + [(z/100) x %A_T] : [(z/100) x %B_T]. If allele B is inactivated, the predicted neutrophil XCIP (A%:B%) = [(z/100) x %A_T] : [(y/100) x 100] + [(z/100) x %B_T].

Using this calculation, the two possible neutrophil XCIPs were predicted for each patient, allowing for the inactivation of either the A or the B-allele in the mutant-positive population. The two predicted neutrophil XCIPs were then compared to the actual neutrophil XCIP observed in each patient, and the one which was closer to the observed value was identified to allow the likely inactivated X-chromosome of the mutant-positive clone to be determined.

This analysis has already been published for 20 of the patients reported in this thesis (Gale *et al,* 2007) and demonstrated that the difference between the observed neutrophil XCIP and the closer of the two predicted ones ranged between 0% and 17% – i.e. in every case the difference was within the limit of technical variation (20%) for this technique, indicating that the size of the mutant-positive population could indeed account for the difference between the T-cell and neutrophil XCIP. This analysis was then performed for the two V617F-positive female patients included in this thesis who had not previously been reported and revealed that difference between the observed and the predicted neutrophil XCIP was 14% in one patient and 27% in the other (Table 3.10).

Table 3.10 *JAK2* V617F-positive patients with an interpretable XCIP: the effect of mutant level on neutrophil clonality status

Patient	Clonality Status*	Neutrophil XCIP		T-cell XCIP		% V617F†	% V617F+ cells‡	Predicted Neutrophil XCIP§				% Difference (observed – predicted)
								if A clone		if B clone		
		A%	B%	A%	B%			A%	B%	A%	B%	
1	C	100	0	69	31	7%	14%	**73**	**27**	59	41	27
2	P	73	27	72	28	26%	52%	**87**	**13**	35	65	14

* C indicates clonal, P indicates polyclonal

† In neutrophil DNA

‡ Assumes heterozygous mutant status in all cells

§ Calculated using 'y' to represent the percentage of mutant-positive cells and 'z' to represent the percentage of WT cells, based on a T-cell XCIP of A_T%:B_T%, and the following equations: if the A allele is inactivated in the clone the predicted neutrophil XCIP (A%:B%) = [(y/100) x 100] + [(z/100) x %A_T] : [(z/100) x %B_T]; if the B allele is inactivated in the clone the predicted neutrophil XCIP (A%:B%) = [(z/100) x %A_T] : [(y/100) x 100] + [(z/100) x %B_T].

3.3.5 *MPL* exon 10 mutation screening

PCR for *MPL* exon 10 was performed in 133 patients. PCR products were obtained for 131 of these and analysed by dHPLC. The 10% W515L-positive control was distinguishable from the wild-type control using a column temperature of both 62.5°C and 65.8°C (Figure 3.6). Interpretable WAVE chromatograms were obtained for 126 patients. Clearly abnormal chromatograms were observed in three (2%) of these patients– two females and one male. All three cases were *JAK2* WT and represented 4% of *JAK2* WT patients. Direct sequencing of these samples demonstrated mutations in *MPL* exon 10 in all three cases. One case of *MPL* W515K and two of *MPL* W515L were detected and in all three cases the height of mutant peak was smaller than that of the WT one on the sequencing chromatogram (Figure 3.7). For two further patients the WAVE chromatogram was equivocal but in these cases there was no evidence of mutations in *MPL* exon 10 on sequencing.

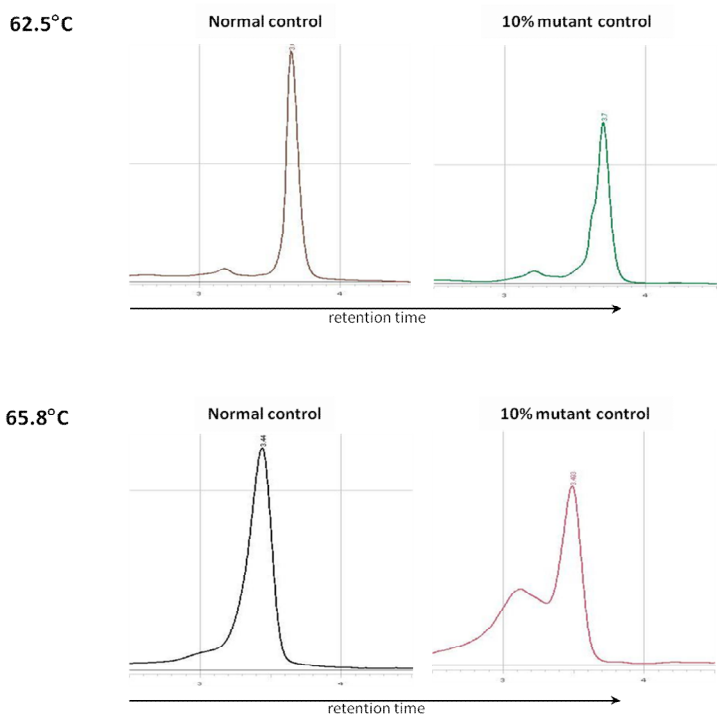


Figure 3.6 dHPLC chromatogram traces of *MPL* exon 10 PCR products amplified using normal control DNA and a 10% W515L-positive control as template

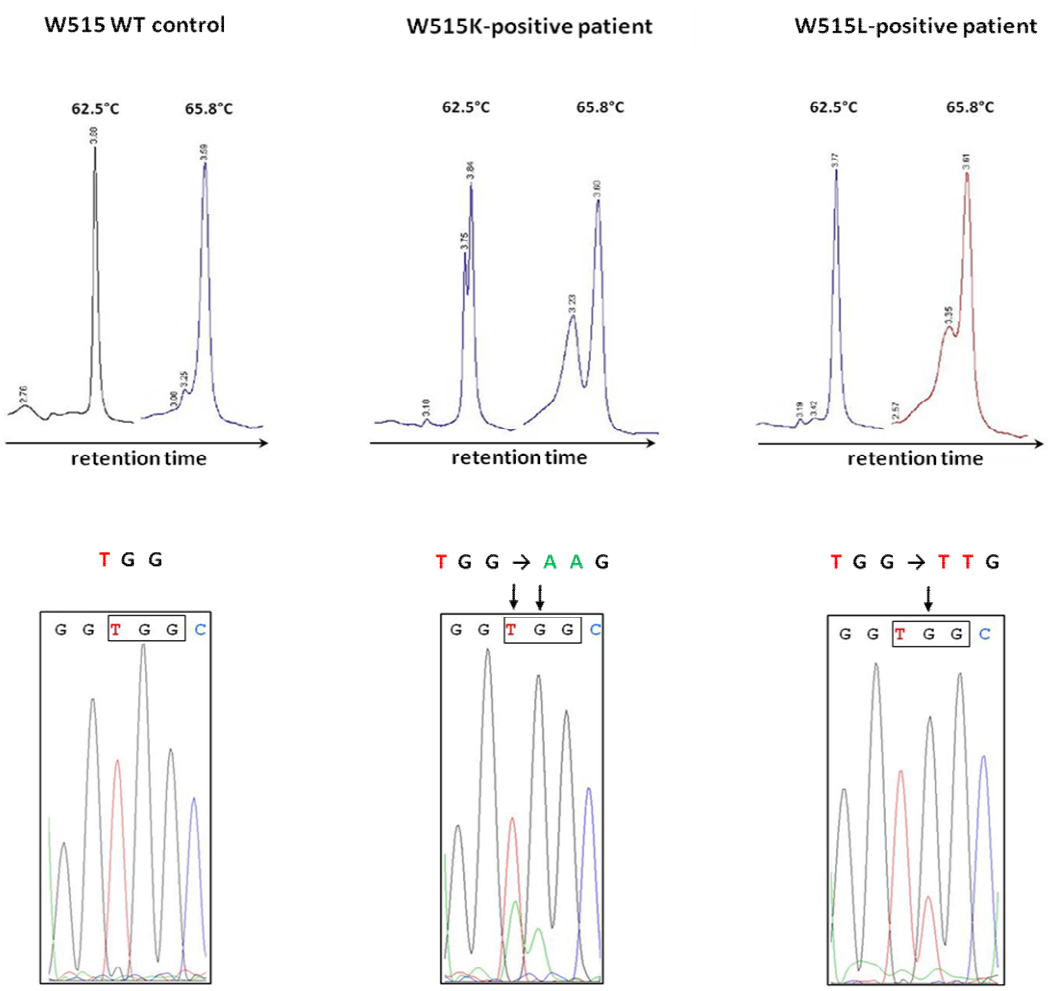


Figure 3.7 dHPLC chromatogram traces and corresponding direct sequencing chromatogram traces of samples with *MPL* W515 mutations

Codon 515 is boxed on the sequencing traces; vertical arrows indication loci of point mutations

3.4 Discussion

The data in this chapter address the clinical, laboratory and molecular characteristics of a cohort of 133 ET patients studied in our institution, and their relationship to outcome. Overall, the cohort of patients reported in this thesis was broadly similar to those reported by most published studies in terms of presenting characteristics and clinical course. The median age at

presentation, at 48 years, was slightly lower than the average reported in recent studies which is 54 years (Table 3.4), possibly as a result of referral bias to UCH which provides tertiary level specialist care for patients with haematological disorders. Consequently surrounding district hospitals may have preferentially referred younger patients who might be perceived to benefit most from expert management. Females represented 79% of the ET patients; this female preponderance is typical of most published series, although it is perhaps more marked than generally reported (59% of patients; Table 3.4) and it is likely that this reflected the active recruitment by the department of female patients specifically for XCIP analysis. The median presenting haemoglobin and white cell count in our cohort were similar to those reported by most other studies (13.7g/dL versus 14.0g/dL and 9.4 $x10^9$/L versus 9.5$x10^9$/L respectively), but the median platelet count was lower than that generally observed (805$x10^9$/L versus 905$x10^9$/L). The reason for this is unclear.

The incidence of thrombotic complications at or prior to presentation (25%) was similar to that reported by other groups: 22% in Bellucci *et al* (1986) and 19% in Passamonti *et al* (2004). The median duration since diagnosis for the group was 12.6 years, during which time 16% of patients suffered thrombotic complications. This is compatible with the 15-year cumulative thrombotic risk of 17% reported by Passamonti *et al* (2004) and the 18% rate of thrombosis observed by Tefferi *et al* (2001) during a median follow-up of 9.2 years. In the cohort studied here, patients who suffered a thrombosis were on average older at diagnosis than those who did not (53 versus 44 years old; Table 3.7) but both groups had similar presenting platelet counts, which is in keeping with the majority of published studies (Table 3.3).

The rates of leukaemic and myelofibrotic transformation during follow-up (1% and 5% respectively) were also similar to published data. Passamonti *et al* (2004) reported the rates of leukaemic and myelofibrotic transformation were both 5% at 15 years post-diagnosis, whilst equivalent figures at 10 years post-diagnosis were reported by Palandri *et al* (2009a) as 1% and 4% respectively, and by Wolanskyj *et al* (2006) as 3% and 5% respectively.

Sixty-two of the 105 female patients in the study had an interpretable XCIP, and of these 24 (39%) were clonal (Table 3.8), a slightly lower proportion than that reported in most published studies (range 43% to 69%, overall mean 62%; Table 1.4). It is notable, however, that with one exception (Teofili *et al,* 2002) all the studies which reported a clonal XCIP rate greater than 55% did not control for the possibility of constitutional skewing by analysing T-cell XCIPs. Consequently, some of the patients they classified as having acquired myeloid skewing might have had constitutional skewing, meaning the true figure may have been lower.

The finding that patients with clonal myelopoiesis had significantly higher presenting platelet counts than those with polyclonal myelopoiesis (median $950x10^9$/L versus $780x10^9$/L; P=0.05) has been previously reported (El-Kassar *et al,* 1997), although it was not observed by Chiusolo *et al* (2001) or Shih *et al* (2002). Of the 44 patients with interpretable XCIPs and clinical follow-up data studied in this thesis, 21 have previously been reported by Harrison *et al* (1999a), who did not find any association between presenting platelet count and XCIP (median $722x10^9$/L in clonal patients versus $778x10^9$/L in polyclonal patients, P=0.92). This discrepancy presumably reflects the different characteristics of the 23 patients reported here who were not included in the 1999 study.

A near-significant trend to younger age in clonal patients compared to polyclonal ones (35.5 versus 43.0 years, P=0.06) was noted in the cohort reported in this thesis. It is possible, however, that this trend may simply have reflected the exclusion from the analysis of 21 women with clonal XCIPs who were older than 65 on the grounds of possible age-related skewing. If these 21 patients are grouped together with the 24 women with clonal XCIPs who were 65 or younger, the median age of this combined group was no different to that of the polyclonal group (56 versus 43 years, P=0.25). As discussed above, Harrison *et al* (1999a) have previously reported on nearly half of the evaluable patients studied in this thesis and did not identify any difference in age between the clonal and polyclonal patients (48 versus 40 years, P=0.55). The other three studies which examined this issue produced conflicting data: Shih *et*

al (2002) reported that patients with a clonal XCIP were significantly younger at presentation than those with a polyclonal XCIP, whereas el-Kassar *et al* (1997) observed the reverse, and Chiusolo *et al* (2001) did not identify any association between XCIP and age at diagnosis. There does not appear to be, therefore, a clear relationship between XCIP and age at presentation.

In the cohort examined in this thesis, the overall rate of thrombotic events was higher in patients with a clonal myeloid XCIP than those whose XCIP was polyclonal, but this was not statistically significant (53% versus 28%, P=0.11). Harrison *et al* (1999a) had previously reported on many of the patients included in this thesis and found that clonal myelopoiesis was associated with a significantly higher thrombotic rate than polyclonal myelopoiesis (60% versus 15%, P=0.04). Whilst the results from these two analyses appear contradictory, the difference between them was of borderline statistical significance (P=0.05, Fisher's exact test). This slight difference may have been due to the relatively high thrombotic rate in the polyclonal patient group reported in this thesis compared to that reported in any of the other comparable studies (Table 3.11), which may have occurred by chance. It may also reflect minor methodological variations between the studies. For instance, angina was regarded in this thesis as a thrombotic episode, in keeping with current standard practice (Barbui *et al,* 2004; Harrison *et al,* 2005), but in some of the previous studies it was not.

Chiusolo *et al* (2001) observed a significantly higher thrombotic rate amongst clonal patients than polyclonal ones (41% versus 7%, P=0.04), and Shih *et al* (2002) reported a non-significant trend towards an increased thrombotic rate in patients with a clonal XCIP (33% versus 7%, P=0.07). By contrast, el-Kassar *et al* (1997) found no such association (32% versus 21%, P=0.70), although they grouped haemorrhagic and thrombotic complications together, making direct comparison with the other studies difficult. Individually these studies are too small to convincingly demonstrate an association between myeloid XCIP status and thrombotic risk. However, if the data from the four studies is combined (excluding the data from Harrison *et al*, 1999a), then there is more robust evidence that thrombotic risk is significantly higher in

patients with clonal myelopoiesis (38% for patients with a clonal XCIP versus 18% for patients with a polyclonal XCIP, P=0.008; Table 3.11).

Table 3.11 Summary of studies examining association between myeloid XCIP status and thrombotic rate

Reference	Median follow-up, months	Polyclonal XCIP		Clonal XCIP		Significant association between XCIP and thrombosis?
		Total *n*	Thrombotic complications *n* (% of total)	Total *n*	Thrombotic complications *n* (% of total)	
(El-Kassar *et al,* 1997)	45	14	3 (21%)*	19	6 (32%)*	No P=0.70
(Harrison *et al,* 1999a)	73	13	2 (15%)	10	6 (60%)	Yes P=0.04
(Chiusolo *et al,* 2001)	36	15	1 (7%)	17	7 (41%)	Yes P=0.04
(Shih *et al,* 2002)	45	15	1 (7%)	33	11 (33%)	No P=0.07
Reported in this thesis	151	29	8 (28%)	15	8 (53%)	No P=0.11
Overall †	**84‡**	**73**	**13 (18%)**	**84**	**32 (38%)**	**Yes P=0.008**

Includes only the four studies which allow the criteria described in Section 1.3.5 to be applied and which included data on thrombotic complications. All P-values derived using Fisher's exact test

* Includes both thrombotic and haemorrhagic complications (this study did not distinguish between them)

† Data from Harrison *et al* (1999a) is excluded from the overall analysis since all the patients reported in this study are included in the cohort reported in this thesis

‡ Weighted according to size of study

Of the 133 patients in the cohort, 55 (41%) were *JAK2* V617F-positive, with a median mutant level of 17%. Both these findings are in keeping with those reported by the majority of published studies, in which the overall prevalence of *JAK2* V617F is 53% and the median mutant level is 22% (Tables 1.7 and 3.12). At presentation, *JAK2* V617F-positive patients had

Table 3.12 *JAK2* V617F mutant levels in the MPNs

Reference	Cells analysed	Quantification method	ET		PV		MF		P-value (ET vs. PV)	P-value (ET vs. MF)
			n	Mutant level %*	*n*	Mutant level %*	*n*	Mutant level %*		
(Passamonti *et al,* 2006)	Neut	qRT-PCR	10	15 (2-35)	23	32 (2-90)	10	27 (12-93)	0.01	NR
(Moliterno *et al,* 2006)	Neut	AS-RTPCR	36	47 (10-63)	77	67 (35-100)	8	100 (48-100)	<0.001	NR
(Lippert *et al,* 2006)	Neut	qPCR	42	19 (3-50)	58	61 (8-98)			<0.001	
(Dupont *et al,* 2007)	Neut†	qRT-PCR	77	30 (2-85)	93	50 (10-100)			<0.001	
(Bellosillo *et al,* 2007)	Neut	AS RTPCR	43	10 ±7§						
(Gale *et al,* 2007)	Neut	PCR/restriction digest	43	18 (5-100)						
(Larsen *et al,* 2007)	leuk	qPCR	21	7 (1-39)	90	23 (1-92)	13	67 (37-99)	0.001	<0.00001
(Pemmaraju *et al,* 2007)‡	Neut	AS-RTPCR	37	43 (11-64)						
(Kittur *et al,* 2007)	NR	AS qPCR	96	6 (1-100)						
(Theocharides *et al,* 2008)	Neut	NR	15	23 ±15§	33	63 ±25§			<0.005	
(Vannucchi *et al,* 2008b)	Neut	qRT-PCR	382	26 (4-52)	297	48 (7-97)	168	46 (14-100)	<0.001	
(Girodon *et al,* 2008)	Neut	AS qPCR	127	18 ±12§	94	54 ±24§				

(Hussein *et al,* 2009)	BM	Pyrosequencing	43	24 (5-40)	138	47 (NR)	78	44 (6-89)	<0.001	<0.01
(Pardanani *et al,* 2010)	BM	AS qPCR	118	7 (1-68)						
(Antonioli *et al,* 2010)	Neut	qRT-PCR	68	32 ±18[§]	104	50 ±26[§]			<0.0001	
Overall			**1158**	**22**	**1007**	**49**	**277**	**47**		

Table 3.12 *JAK2* V617F mutant levels in the MPNs – continued

Neut indicates Neutrophils, leuk indicates leukocytes, NR indicates not reported

* Medians and ranges quoted unless otherwise stated

† Also quantified V617F levels in total BM MNCs in ET & PV patients: results similar to those for Neut

‡ May include some of the patients reported by Moliterno *et al* (2006)

§ Mean ±SD

lower median platelet counts than *JAK2* WT ET patients (772x10^9/L versus 950x10^9/L, P=0.011), higher median haemoglobin concentrations (14.2g/dL versus 13.0g/dL, P=0.0002), and were older (53.0 versus 39.5 years, P<0.0001). These associations between *JAK2* mutant status and presenting characteristics have also been reported by most published studies (Table 3.4), and have led to the hypothesis that mutant-positive ET patients have a phenotype that lies somewhere between *JAK2* WT ET and PV (Campbell and Green 2006). Some studies have suggested that *JAK2* V617F-positive patients have higher presenting white cell counts than WT patients (Campbell *et al,* 2005; Kittur *et al,* 2007; Pardanani *et al,* 2010), but this was not observed in the cohort reported here (9.0 versus 10.0, P=0.52), nor was there any difference in the prevalence of splenomegaly.

In the cohort studied here, those patients who were *JAK2* V617F-positive did not have a greater thrombotic rate than WT ones, whether at diagnosis (31% versus 19%, P=0.26), during follow-up (22% versus 11%, P=0.19), or overall (45% versus 26%, P=0.10). The published data are conflicting and suggest that any effects of *JAK2* V617F on thrombotic risk are modest (Table 3.5) and this cohort may, therefore, have been too small to detect them. Nonetheless, it is noteworthy that patients who suffered a thrombosis had a higher mean mutant level than those who did not (11% versus 5%, P=0.02). In absolute terms, however, this difference (6%) is small and may not indicate a true biological difference between the two groups. It may, instead, reflect the presence of a few individuals with high mutant levels in the group of patients who had a thrombosis. According to a number of recent studies, patients with mutant levels greater than 50%, seen in 2%-6% of ET patients, have a significantly increased thrombotic risk compared to all other ET patients (De Stefano *et al,* 2009; Vannucchi *et al,* 2007), and two groups have observed the same phenomenon in patients with mutant levels above 25% (Antonioli *et al,* 2008; Carobbio *et al,* 2009). Of the 104 patients reported in this thesis for whom clinical follow-up data was available, the proportion of patients with a mutant level greater than 25% was significantly higher in the 37 patients who had a thrombosis than in

the 67 who did not (16% versus 5%, P<0.0001). It appears, therefore, that the thrombotic risk may be related to the presence of high mutant levels rather than simply the presence or absence of *JAK2* V617F, and this may account for the lower thrombotic rate in ET patients compared to PV patients, who more frequently harbour high mutant levels. One study has even suggested that at equivalent mutant levels the thrombotic risk is similar in the two disorders (Carobbio *et al,* 2009).

The mortality rate was significantly higher in the V617F-positive group, but since the majority of deaths were not related to haematological causes, this is likely to have been coincidence. No published studies have reported any similar association between *JAK2* mutational status and mortality, though Vannucchi *et al* (2007) did find that the 2% of ET patient who had a V617F mutant level greater than 50% did have an increased rate of myelofibrotic transformation.

MPL W515 mutations were detected in only three of the patients reported in this thesis, accounting for 2% of all patients and 4% of *JAK2* WT patients, figures similar to those of other studies (Table 1.8). None of them suffered thrombotic complications either prior to or following presentation, but the small patient numbers precluded meaningful interpretation of these findings. Of the three *MPL* W515-positive patients, one had a clonal neutrophil XCIP, one was male, and one was uninterpretable on the grounds of constitutive skewing.

No association between *JAK2* V617F and XCIP clonality was observed. This is surprising since, if V617F-positive cells are clonal, a higher rate of XCIP clonality might be expected in V617F-positive patients than in WT patients. In fact the opposite was true, although the difference was not significant: the proportion of patients with clonal myelopoiesis was 27% in the V617F-positive group compared to 45% in the *JAK2* WT one, P=0.19. These findings are in keeping with those of Levine *et al* (2006) who compared XCIP clonality and V617F mutant levels in PV, ET and MF. Whereas a correlation between clonality and mutant level was demonstrated in

PV, no such correlation was detectable in ET and MF. The apparent absence of correlation between mutant level and clonality in ET may relate the lower mutant levels usually reported in ET compared to PV (Table 3.12). The smaller mutant-positive population in ET would, therefore, be expected to exert a more modest effect on the overall myeloid clonality than in PV. Since the XCIP approach to clonality analysis has a limit of technical variation of 20%, it may not be sufficiently sensitive to detect such subtle effects.

An earlier study from our group has examined the relationship between the size of the mutant-positive population and T-cell XCIP in determining the neutrophil XCIP (Gale *et al,* 2007). Using the assumptions that mutant-positive cells are heterozygous for V617F and clonal, and therefore inactivate the same X-chromosome, this study calculated two possible resulting neutrophil XCIP values, depending on whether the clonal population was assumed to inactivate the 'A' or 'B' *HUMAR* alleles. In all 20 patients studied, one of the two calculated neutrophil XCIPs was within the limit of technical variation for the assay (20%) of the observed neutrophil XCIP. In the studies reported in this thesis, this analysis was performed for a further two patients: in one the closer of the two calculated neutrophil XCIPs was 14% different than the observed value, in the other it was 27% (Table 3.10). In the second individual, the observed neutrophil XCIP was 100%:0%, whereas the closer of the calculated values was 73%:27%, based on a T-cell XCIP of 69%:31% and the assumption that the mutant-positive population, which represented 14% of myeloid cells, inactivated the A-allele. In this patient the V617F-positive population could not account for the difference between the T-cell and neutrophil XCIPs, implying that the clonal population was larger than mutant-positive one. One possible explanation for this is that the V617F-positive clonal population is derived from a larger, *JAK2* WT one, in keeping with a pre-*JAK2* event in this individual.

Overall, in 21 of the 22 patients, the neutrophil XCIP could be predicted from their mutant level and T-cell XCIP, within the technical limits of the assay. It therefore appears that in the majority of ET patients, the size of the mutant-positive population can account for the

difference between the T-cell and neutrophil XCIP, even if the mutant-positive population is too small to achieve a neutrophil XCIP which fulfils the criteria for clonality. The size of the mutant-positive population required to achieve a clonal neutrophil XCIP depends on the T-cell XCIP and the X-chromosome which is inactivated in the mutant-positive population. For instance, in a patient whose T-cell XCIP is 60%:40%, if the mutant-positive population inactivates the A allele, a mutant level of 25% is needed to generate a clonal neutrophil XCIP (80%:20%) based on the calculation described in Section 3.3.4. However, if it inactivates the B allele a level of 33% is required for a clonal neutrophil XCIP (20%:80%). In the cohort reported in this thesis, five patients with interpretable XCIPs had mutant levels of at least 25%, of whom four had evidence of clonal myelopoiesis. The fifth patient (Table 3.10, patient 2) in this group had a mutant level of 26%, yet her T-cell and neutrophil XCIPs were very similar (72%:28% and 73%:27% respectively).

It is notable that the majority of patients with a clonal XCIP studied in this thesis were *JAK2* WT (18 out of 24 patients, 75%). This would indicate that these 18 individuals harboured a myeloid clone, large enough to affect the overall neutrophil XCIP, which was due to a somatic mutation other than *JAK2* V617F. One of these harboured a *MPL* W515K mutation, but in the other 17 no mutations were detected.

In summary, the majority of the clinical and laboratory characteristics of the cohort of patients studied in this thesis were similar to those reported in most published studies, as were the observed differences between *JAK2* V617F-positive and *JAK2* WT patients with regard to haemoglobin level, platelet count and age. XCIP status appeared not to predict the incidence of vascular complications, though when the data was combined with that from other studies a significant association was observed. Whilst *JAK2* status was not associated with thrombotic risk, the *JAK2* mutant level was. There was no association between the presence of *JAK2* V617F and clonal myelopoiesis as determined by XCIP analysis. This observation may have been due

to the XCIP technique's lack of sensitivity to small clones, and conversely to the presence of V617F-negative patients with clonal myelopoiesis who are likely to harbour other mutation.

4 *JAK2* V617F-positive and *JAK2* WT thrombopoiesis in ET

4.1 Introduction

Although *JAK2* V617F is present in a large proportion of patients with the three major MPNs, the mutant level has consistently been reported to be lower in ET than in PV or MF (Table 3.12). A number of studies have suggested that, in ET, V617F -positive neutrophils represent only a minority of the total neutrophil population and that this proportion remains stable over many years (Antonioli *et al,* 2010; Besses *et al,* 2010; Gale *et al,* 2007; Girodon *et al,* 2008; Theocharides *et al,* 2008). This implies that *JAK2* V617F-positive and *JAK2* WT granulopoiesis stably co-exist in ET patients for prolonged periods, which differs from erythropoiesis in PV, where low EPO levels lead to suppression of *JAK2* WT erythroid cells and selection of EPO-hypersensitive V617F-positive erythroid cells (Dupont *et al,* 2007).

The stable co-existence of mutant-positive and *JAK2* WT granulopoiesis in ET is not typical of a neoplastic disorder and may simply be a feature of analysing neutrophils, rather than the more biologically-relevant platelets. The nature of this relationship between V617F-positive and *JAK2* thrombopoiesis is, therefore, unclear and will be investigated in this chapter.

4.1.1 The long-term stability of the V617F-positive population in ET

Three of the seminal studies which first reported on the occurrence of *JAK2* V617F in MPNs observed that transfection of Ba/F3 cells with the mutant *JAK2* gene resulted in these cells becoming hypersensitive to EPO and IL-3 (James *et al,* 2005; Kralovics *et al,* 2005; Levine *et al,*

2005). Kralovics *et al* (2005) went on to demonstrate that, in the presence of serum, V617F-transfected cells showed increased survival and proliferation compared to *JAK2* WT-transfected ones. In view of this, the V617F-positive myeloid cells in patients with ET might be expected to outgrow the *JAK2* WT ones and eventually to represent the majority of the myeloid population, as they do in PV and Philadelphia-positive cells do in CML (Dubé *et al,* 1984aa; Dubé *et al,* 1984b). There is convincing evidence, however, that this is not the case.

A number of studies have examined the V617F mutant level in the three major MPNs (Table 3.12). Although the mutant level varies widely in individual ET patients, the overall median level for all the published studies is only 22% (range of medians 6%-47%), and significantly lower than that observed in either PV or MF (overall medians 49% and 47% respectively). In ET the percentage of myeloid cells carrying the mutation is likely to be double the mutant level, based on the data from Scott *et al* (2006). They genotyped haemopoietic colonies from 17 V617F-positive ET patients and found that in every case a mix of *JAK2* WT-homozygous and V617F-heterozygous colonies was present, but no patient harboured V617F-homozygous colonies. Although Dupont *et al* (2007) and Pardanani *et al* (07 2007) subsequently reported occasional V617F-homozygous haemopoietic colonies in small numbers of ET patients, it is likely that in most ET patients, the vast majority of V617F-positive cells are heterozygous for the mutation. Based on the overall median mutant level in ET of 22%, this implies that on average fewer than half of myeloid cells carry the mutation.

One possible explanation for the relatively low mutant level in ET is that the condition represents an early phase in the V617F-positive MPN spectrum and that PV and MF, in which the mutant levels are significantly higher, are later stages of the same disorder. It is possible that over time the mutant population outgrows the WT one leading to a change from ET to PV or MF. In support of this hypothesis, there is evidence from murine models that increasing the V617F-to-WT ratio changes the MPN phenotype. Tiedt *et al* (2008) generated a transgenic murine model in which expression of V617F in haemopoietic cells was controllable by Cre-lox

recombination and showed that when WT expression exceeded mutant expression, a thrombocytosis resulted whereas when mutant levels were greater than WT levels, polycythaemia was observed.

Mutant levels in ET, however, appear not to increase with time, according to a number of published studies. Gale *et al* (2007) sequentially analysed mutant levels in 19 V617F-positive ET patients over a median follow-up of 47 months. In 18 of these the mutant level remained stable during follow-up, within the limits of technical variation, including five patients who had not received cytoreductive therapy, suggesting that the stability of the mutant level was not due to treatment. Four subsequent studies, whilst primarily examining the effect of hydroxycarbamide therapy on mutant levels, have included untreated patients, with similar conclusions. Theocharides *et al* (2008) observed that in six untreated ET patients, mutant levels increased by a mean of 9% over a median follow-up of 26 months, which was considered to be within the limits of technical variation. Similarly, Girodon *et al* (2008) found no significant change in mutant levels in nine untreated ET patients during a median follow-up of 16 months, and Antonioli *et al* (2010) reported that in 24 untreated ET patients, followed for a median of 24 months, the median mutant level rose from 27% to 31%, which again was not significant. Finally, Besses *et al* (2010) observed a small but non-significant rise in mutant levels in 24 untreated ET patients over a median follow-up of 48 months, from a median of 26% to 29%. In summary, there is no evidence that mutant levels in ET do rise over time, even in the absence of cytoreductive therapy, or that the mutant-positive myeloid population outgrows the *JAK2* WT one. Instead a steady state appears to exist, with no further advantage for the *JAK2*-mutated cells, although the reason for this remains unclear.

4.1.2 *JAK2* V617F mutant levels in neutrophils and platelets

A second possible explanation for the relatively low mutant levels in ET is that this is a feature of analysing neutrophils rather than platelets or MKs. The majority of studies which have examined the issue have quantified mutant levels in neutrophils since these are abundant in the peripheral blood and are therefore the easiest myeloid cells to analyse. In ET, however, the most striking abnormality is in megakaryopoiesis and thrombopoiesis and therefore the mutant levels in MKs and platelets are perhaps more relevant than those in neutrophils. Because peripheral blood platelets contain no detectable DNA, quantification of their *JAK2* mutant level requires RNA analysis, and this has been done relatively infrequently.

Two studies did find that, in ET, mutant levels were higher in platelet RNA than in neutrophil DNA, suggesting that there may be differences in *JAK2* mutant levels in different myeloid lineages (Bellosillo *et al,* 2007; Moliterno *et al,* 2006). It is likely, however, that some patients in both studies had received treatment with cytoreductive agents, which have been reported to reduce mutant levels (Girodon *et al,* 2008; Kiladjian *et al,* 2008; Larsen *et al,* 2009). Consequently it remains uncertain whether V617F-positive platelets represent the majority of the platelet population in mutant-positive ET and the low mutant levels reported by the studies in Table 3.12 are an artefact of studying neutrophils, or whether mutant levels in platelets and neutrophils are similar.

Determining whether, in ET, V617F-positive and *JAK2* WT thrombopoiesis genuinely co-exist in a stable, long-term equilibrium has profound implications for our understanding of ET, since neoplastic conditions are usually characterised by an expansion of the abnormal population at the expense of the normal one.

4.1.3 Aims

The studies reported in this chapter set out to quantify *JAK2* V617F levels in neutrophils and platelets from a cohort of 10 patients with untreated ET in order to establish whether there was a difference in mutant levels between the two lineages, and to investigate the relative sizes of the V617F-positive and the *JAK2* WT platelet populations.

4.2 Patients, materials and methods

4.2.1 *JAK2* V617F mutant levels quantification in neutrophil and platelet RNA

Peripheral blood samples were collected from 10 ET patients prior to the initiation of cytoreductive therapy which might affect mutant levels. Each patient's platelet count at the time of collection was known. Neutrophils and platelets were purified as described in Section 2.1.3, RNA extracted as described in Section 2.1.4, and cDNA was generated using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit as described in Section 2.2.5. BIOTAQ™ PCR was then performed as described in Section 2.2.3.1 to amplify a 169bp fragment covering the mutation site in exon 14, using a 1 in 4 dilution of this cDNA as template. The primers used were *JAK2*ex14/(MM)F and an exon 15 fluorescently-labelled reverse primer (*JAK2*ex15/RF), both at a final concentration of 0.25µM. Only 30 cycles of PCR were performed in order to minimise the formation of heteroduplex products, since they would not be cut by *Afl*III and would therefore mimic V617F-positive product. Denaturation, annealing and extension times were 60s in each cycle, with an annealing temperature of 61°C.

Following *Afl*III digestion (as described in Section 3.2.3.1) the uncut mutant allele was identified by fragment analysis as a peak at 169bp, whilst the WT allele was cut to 28+141bp,

though only the 141bp peak was visible as a peak since the 28bp fragment had no fluorescent label. As described in Section 3.2.4, the mutant level was calculated by expressing the area beneath the mutant peak as a percentage of the total area beneath both peaks. In every case, each sample was analysed three times and the mean value for all three runs calculated.

4.2.2 Calculation of the numbers of V617F-positive and WT platelets

The mutant levels in platelet RNA (%Mut) and the total platelet count (PLT_T) at the time of sampling were used to calculate the number of mutant-positive platelets (PLT_M), based on the assumption that all the latter platelets were heterozygous for the mutation (Scott *et al,* 2006), using the following equation:

$$PLT_M = PLT_T \times 2(\%Mut \div 100).$$

The difference between the total and the mutant-positive platelet count represents the WT platelet count.

4.3 Results

4.3.1 Mutant allele levels in neutrophil DNA and RNA and platelet RNA

The median V617F mutant level in samples from 10 ET patients obtained prior to cytoreductive therapy was 15% (range 11%-27%) in neutrophil gDNA and 21% (range 12%-31%) in neutrophil RNA (Figure 4.1). There was no significant difference in mutant levels between the two groups (P=0.07, paired t-test). In eight patients, the mutant level was higher in neutrophil RNA than in gDNA (range 2% to 17% higher), whereas in two patients the reverse was observed (2% and

10% higher in neutrophil gDNA than in RNA). In the patient whose mutant level was 10% higher in neutrophil gDNA than in RNA (27% versus 17%) there was no evidence of sample transposition, and the quantification assay was repeated with similar results. If this patient's values were excluded, the median mutant level was significantly higher in neutrophil RNA than in gDNA (21% versus 14%, P=0.01, paired t-test).

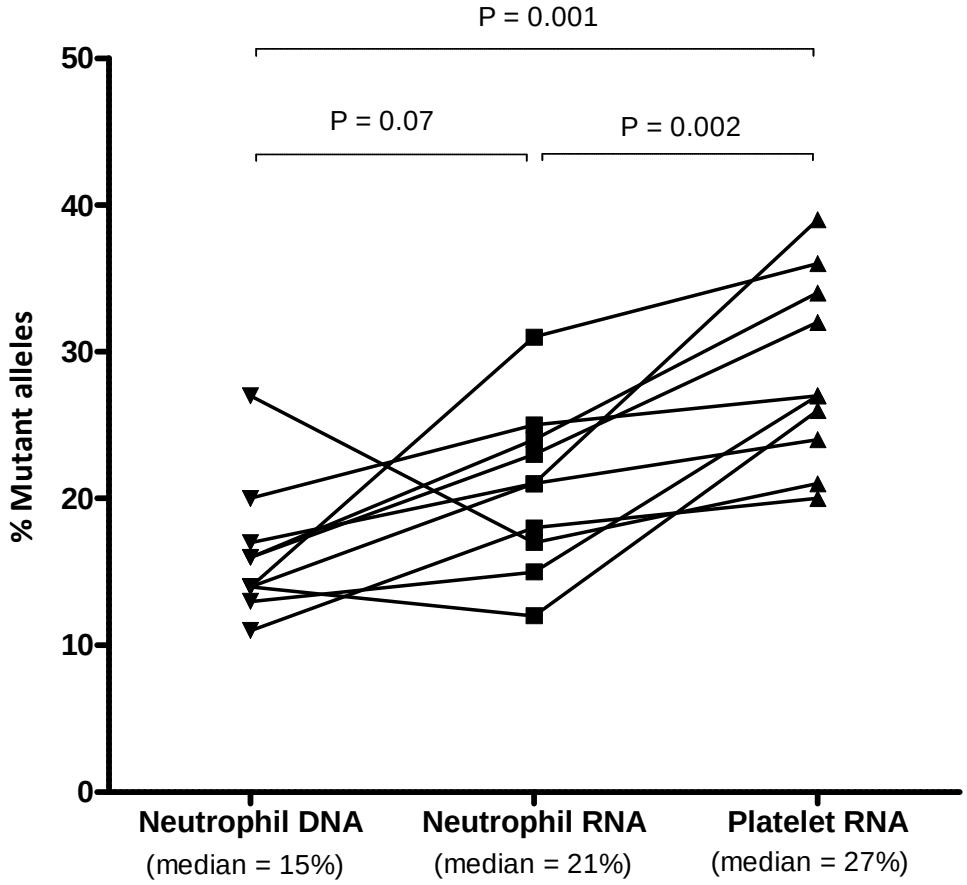


Figure 4.1 JAK2 V617F mutant levels in neutrophil DNA and RNA, and platelet RNA in 10 ET patients

The median mutant level in platelet RNA was 27% (range 20-39%), significantly higher than in neutrophil gDNA and neutrophil RNA (P=0.001 and P=0.002 respectively, paired t-test). The

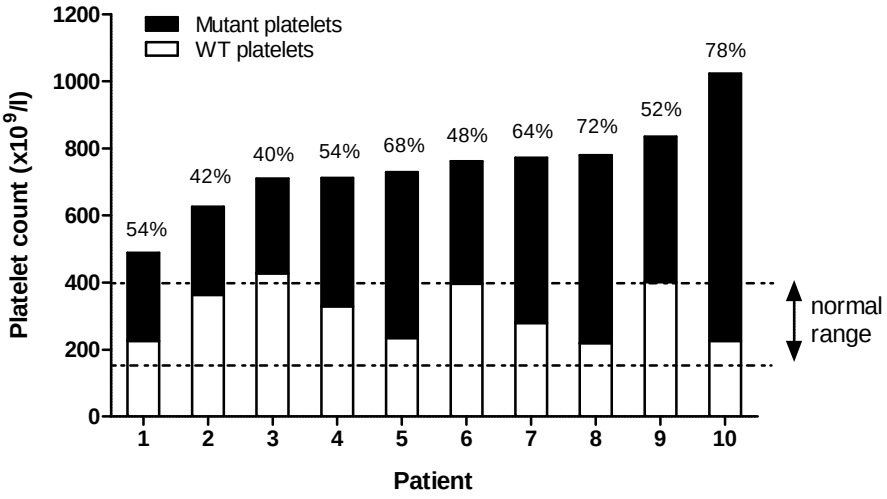
mutant level was higher in platelet RNA than in neutrophil RNA in every patient (range 2% to 18% higher), and higher than in neutrophil gDNA in 9 of the 10 patients (range 6% lower to 22% higher). The one exception was the patient described in the paragraph above (mutant level in neutrophil gDNA 27% versus 21% in platelet RNA).

4.3.2 Calculation of WT and mutant platelet counts

Using the equation described in Section 4.2.3, the WT and mutant-positive platelet counts were calculated for the 10 ET patients investigated (Figure 4.2A). The median total, WT, and mutant-positive platelet counts were 745×10^9/L (range 489-1023x10^9/L), 303×10^9/L (range 218-426×10^9/L), and 410×10^9/L (range 263-798×10^9/L) respectively. There was no difference between the median WT and mutant platelet counts (P=0.11, t-test; Figure 4.2B). In five patients the WT platelet count was higher than the mutant-positive platelet count, and in the other five it was lower.

In all 10 patients the WT platelet count was above the lower limit of normal for the laboratory (150×10^9/L) and in two patients it was above the upper limit of normal (400×10^9/L). There was no correlation between mutant level and total platelet count (r^2=0.325, P=0.085; Figure 4.3A), between total platelet count and WT platelet count (r^2=0.003, P=0.87, Figure 4.3B), or between mutant platelet count and WT platelet count (r^2=0.30, P=0.10, Figure 4.3C).

A



B

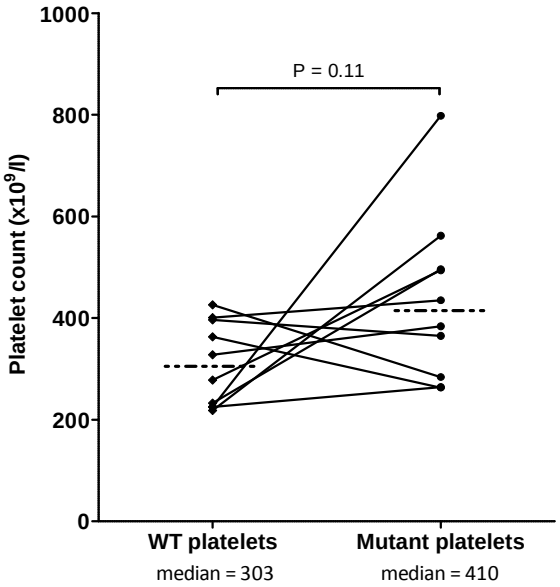


Figure 4.2 Calculation of *JAK2* wild-type and V617F-positive platelets in 10 ET patients

A. The whole bar represents the total platelet count at the time of testing. The open bar indicates the WT platelet count, and the filled bar the V617F-positive platelet count. The figure above the bar is the percentage of *JAK2* V617F-mutant positive platelets calculated from the mutant level in platelet RNA. The dashed horizontal lines indicate the normal range in our laboratory.

B. Comparison between WT and mutant platelets for each patient. The dashed horizontal lines indicates the median value for each group.

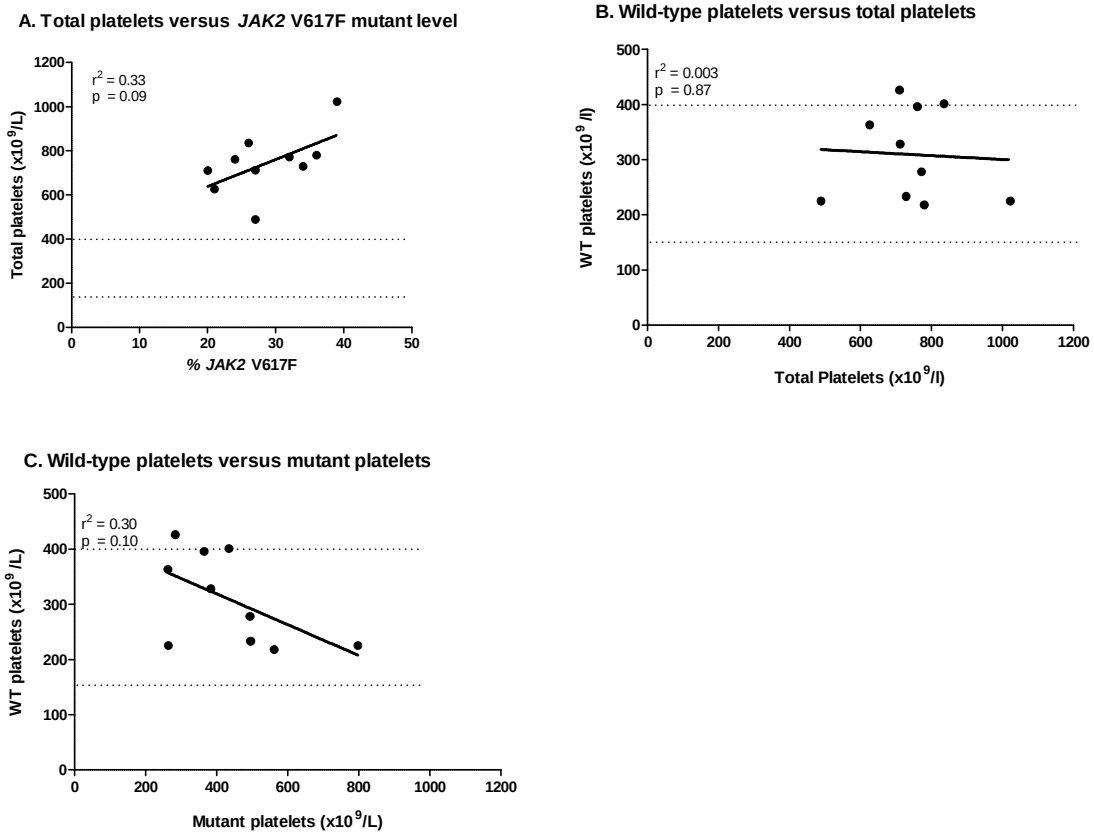


Figure 4.3 Correlation between platelet counts and *JAK2* V617F mutant level

4.4 Discussion

JAK2 mutant levels in V617F-positive ET patients are usually low, and on average fewer than half of their neutrophils carry the mutation. Moreover, mutant levels in these patients usually remain stable over many years. These observations are not keeping with the evidence that acquisition of *JAK2* V617F confers a proliferative advantage on affected cells but may simply be a consequence of analysing neutrophils, rather than the biologically more relevant platelets. The studies reported in this chapter, therefore, set out to investigate whether mutant levels in

neutrophils and platelets differ in ET, and to examine the effect of the presence of a mutant-positive platelet population on the size of the *JAK2* WT one.

Mutant levels were quantified in neutrophil gDNA and RNA, and platelet RNA from 10 untreated ET patients. The results indicate that, whilst median V617F levels were similar in neutrophil gDNA and RNA (15% and 21% respectively, P=0.07), the median level in platelet RNA (27%) was higher than in both neutrophil gDNA and RNA (P=0.001 and P=0.002 respectively). Nonetheless, the difference between the median level in neutrophil RNA and platelet RNA of 6% is, in absolute terms, small – especially in view of the technical variation for the assay of 10% (Gale *et al,* 2007). Considering the patients individually, all 10 had mutant levels which were higher (by between 2% and 18%) in platelet RNA than in neutrophil RNA and, with one exception, than in neutrophil gDNA (by between 7% and 22%). The sample from the patient whose mutant level was higher in neutrophil gDNA than in both other samples was checked and re-analysed; whilst there was no evidence of sample transposition, this could not be verified absolutely. If this patient's results are excluded, mutant levels were significantly higher in neutrophil RNA than in neutrophil gDNA (21% versus 15%, P=0.01), but the other comparisons were unaffected.

These observations are in keeping with those of Moliterno *et al* (2006) who found that in 13 ET patients the median mutant level was significantly higher in platelet RNA than in neutrophil gDNA than in (42% versus 22%, P=0.001). The difference in median mutant level between the platelet RNA and neutrophil gDNA, 20%, was more marked than that observed in the cohort studied in this chapter, 12%, but since the number of patients receiving cytoreduction was not reported, it is difficult to compare the two studies. A larger study of 43 mutant-positive ET patients by Bellosillo *et al* (2007) also found a higher median mutant level in platelet RNA than in neutrophil RNA (13% versus 10%, P = 0.02). In this study approximately one-third of the ET patients were receiving cytoreductive therapy at the time of sampling, which might account

for the relatively low overall mutant levels compared to the majority of other studies (Table 3.12).

Whilst the data presented in this chapter does suggest that in ET the mutant level in platelets is higher than in neutrophils, the actual figure of 27% is still considerably lower than the median reported by most studies for neutrophils in PV or MF (Table 3.12). If V617F-positive cells are usually heterozygous for the mutation in ET, as Scott *et al* (2006) suggest, this implies that on average approximately half of the platelets are mutant-positive and half are *JAK2* WT. These data therefore appear to confirm the notion that in ET the V617F-positive populations (both neutrophils and platelets) do not rapidly outgrow their WT counterparts, but seem to co-exist, typically representing 40-60% of the neutrophils and platelets in the peripheral blood.

To further explore the relationship between *JAK2* WT and mutant-positive platelets in ET, the number of WT and mutant-positive platelets at diagnosis were calculated for each of the 10 ET patients, based on platelet counts and simultaneous mutant levels quantified from samples taken prior to cytoreductive therapy. The overall median WT platelet count was similar to the median mutant platelet count (303×10^9/L versus 410×10^9/L, P=0.11; Figure 4.2B), and in no patient was the WT platelet count below the lower limit of normal. In two patients the WT platelet count (401×10^9/L and 426×10^9/L) was marginally above the upper limit of normal for this laboratory (400×10^9/L) (Figure 4.2A). Moreover, there was no correlation between mutant level and platelet count (Figure 4.3A), nor any evidence that the WT platelet count fell as the total platelet count or mutant-positive platelet count increased (Figures 4.3B and 4.3C).

The fact that production of *JAK2* WT platelets was not suppressed in the presence of a thrombocytosis caused by mutant-positive platelets suggests that the regulation of thrombopoiesis generally (i.e. of both *JAK2* WT and V617F-positive platelets) is defective in ET. One possible mechanism for this relates to the role of c-Mpl in the normal homeostatic feedback pathway for thrombopoiesis (see Chapter 1). Thrombopoiesis is principally regulated

by plasma levels of the c-Mpl ligand, TPO, which, in the absence of an inflammatory stimulus, vary inversely with platelet count in haematologically normal individuals. Clearance of TPO is mediated by its internalisation and degradation by platelets and megakaryocytes via c-Mpl. Impaired c-Mpl expression in ET has been reported by several groups (Harrison *et al,* 1999b; Kralovics *et al,* 2003; Li *et al,* 2000) and this may account for the elevated TPO levels often seen in the disorder (Table 1.2). Moreover, *JAK2* V617F may play a role in the impaired expression of c-Mpl. Moliterno *et al* (2006) observed an inverse correlation between mutant levels and platelet c-Mpl expression in ET, suggesting that the abnormal *JAK2* molecule may interfere with expression of the receptor and therefore lead to impaired TPO clearance. They also noted that c-Mpl platelet expression was similarly impaired in *JAK2* WT ET patients, and concluded that in these cases a different molecular mechanism was responsible for the abnormal receptor expression.

It is, therefore, possible that impaired c-Mpl expression results in disruption of the normal thrombopoietic feedback system and is responsible for the lack of suppression of *JAK2* WT platelets in the presence of a V617F-positive population and thrombocytosis. As a consequence, despite the presence of a thrombocytosis, there is sufficient circulating TPO to stimulate the remaining WT megakaryocytes to produce WT platelets in normal numbers. Over time, even if the absolute number of V617F-positive MKs and platelets were to increase, their defective c-Mpl expression would render them unable to clear TPO in the normal way, and WT thrombopoiesis would therefore be unaffected.

Mutant levels in PV are markedly higher than in ET, and this may in part relate to the regulation of erythropoiesis which is mediated by EPO. EPO levels are controlled by a quite different mechanism than that of TPO: elevated haemoglobin levels result in suppression of renal EPO production, and thereby downregulate bone marrow erythroid activity. In PV, EPO levels are usually suppressed (Johansson *et al,* 2002; Mossuz *et al,* 2004), and correspondingly little WT erythropoiesis would be expected. *JAK2* V617F-positive erythroid progenitors are,

however, hypersensitive to EPO (Dupont *et al,* 2007) and would therefore be able to proliferate despite the low EPO levels. Defective TPO clearance might account for the persistence of WT thrombopoiesis at normal levels in V617F-positive ET; it does not, however, explain why mutant levels in neutrophil DNA, which is what most studies have measured, from ET patients are relatively low and remain so over prolonged follow-up.

An alternative hypothesis to explain the persistence of relatively low mutant levels in ET in both platelets and neutrophils is that the degree of expansion of the V617F-positive population depends on the molecular background on which the mutation arises, since this could affect the relative proliferative advantage which acquisition of the mutation confers. If *JAK2* V617F occurred in an individual whose myeloid progenitors already possessed an unusually high proliferative rate – whether due to hereditary or acquired factors – the V617F-positive progenitor might possess a lesser growth advantage compared to *JAK2* WT progenitors than if it arose in a completely haematologically normal individual. The subsequent expansion of its progeny would be less marked and, since the *JAK2* WT myelopoiesis is more proliferative than normal it would not necessarily be suppressed by the V617F-positive myelopoiesis.

The possible nature of such a pre-existing 'hyper-proliferative' tendency remains largely speculative, but there is evidence for a pre-*JAK2* V617F event, at least a proportion of ET cases. Kralovics *et al* (2006) identified six ET patients whose neutrophil mutant level was less than 25% (i.e. fewer than 50% of granulocytes carried the mutation), who had evidence for monoclonal haemopoiesis by XCIP analysis or using a cytogenetic marker (del20q). This, they concluded, indicated that in a proportion of MPD patients *JAK2* V617F had arisen on a background of clonal haemopoiesis due to a prior somatic mutation. In keeping with this, one of the patients reported in Chapter 3 of this thesis was found to have a clonal population which was larger than the V617F-positive one.

Further evidence for a possible pre-*JAK2* event came from data from Campbell *et al* (2006a) and Theocharides *et al* (2007) who observed that in the majority of V617F-positive ET cases which transformed to AML, the leukaemic blasts were WT. They found no evidence for mitotic recombinations, deletions or point-mutations, which could account for the loss of V617F from a V617F-positive progenitor, and concluded that in some cases at least, it was more likely that the blasts had arisen from a pre-*JAK2* clonal population.

Alternatively, the speculative pre-*JAK2* abnormality might be inherited. Pardanani *et al* (2008) found that at three different *JAK2* SNPs, there was a strong association between one allele and PV, and the other allele and ET. The three SNPs were all in linkage disequilibrium with specific *JAK2* haplotypes, and it was suggested that the phenotype-determining allele may lie within these haplotypes. There is also evidence that the variation in haematological parameters in normal individuals may be determined by hereditary factors. In a study of nearly 14,000 individuals, Soranzo *et al* (2009) identified 22 SNPs which were associated with full blood count variability, including four which were associated with variation in platelet counts. Acquisition of *JAK2* V617F in an individual who harboured an inherited tendency to have a high-normal platelet count might be enough to result in a thrombocytosis, and simultaneously deprive the mutant-positive cells of the marked proliferative advantage required to dominate haemopoiesis.

In conclusion, the data presented here suggest that, whilst *JAK2* V617F levels are higher in platelets than neutrophils in ET, this difference is, in absolute terms, small. Further, it appears that in V617F-positive ET, WT thrombopoiesis is not suppressed by the presence of a V617F-positive population, and the mutant-positive progenitors do not rapidly outgrow their WT counterparts, contrary to what may be expected in a neoplastic disorder. This raises the question of whether ET is necessarily a clonal neoplasm, which is considered further in the following chapter.

5 Testing for more than one *JAK2* V617F-positive population in patients with mutant-positive ET

5.1 Introduction

In 2008, the WHO reclassified what had hitherto been called myeloproliferative disorders as myeloproliferative neoplasms (Swerdlow *et al,* 2008). This change in terminology served to emphasize the belief that these were neoplastic diseases which arose as a consequence of clonal expansion of haemopoietic progenitors (Tefferi and Vardiman 2008). Central to this view of the nature of MPNs was the perception that the acquisition of a number of somatic mutations, most commonly affecting *JAK2* exons 12 and 14 (V617F) and *MPL* exon 10, is critical to the pathogenesis of these neoplasms.

Parallels between the Philadelphia-negative MPNs and CML, and their respective constitutively-activated tyrosine kinases were soon drawn and the role of *JAK2* V617F in the Philadelphia-negative MPNs was equated to that of BCR-ABL in CML. ET and PV were likened to CML in chronic phase, MF to accelerated-phase CML, and the leukaemic transformation of all 3 Philadelphia-negative MPNs to the blast crisis phase of CML (Campbell and Green 2006). Conceptualising the Philadelphia-negative MPNs in this way was intellectually appealing since it allowed a rationalised classification of all 4 major MPNs. Moreover, it implied that the advances in the understanding of CML which followed the discovery of BCR-ABL might be rapidly emulated in the V617F-positive MPNs, facilitating the development of novel, targeted therapies. However, classifying ET as a clonal neoplasm akin to chronic-phase CML ignores marked differences in the natural history of the two disorders, and comparing *JAK2* V617F to

BCR-ABL as the pathogenic mutation overlooks several fundamental differences between the two molecular abnormalities.

5.1.1 Is V617F-positive ET a clonal neoplasm initiated by *JAK2* V617F?

Four observations suggest that V617F-positive ET is not analagous to chronic-phase CML, and that *JAK2* V617F does not play the same pathogenic role in ET that BCR-ABL does in CML. First, ET does not clinically behave as a neoplastic disease. The mortality associated with ET is low and is usually due to a thrombotic event. In the absence of leukaemogenic medications the rate of malignant transformation is low – approximately 1-3% over 10 years – and the median survival is between 15 and 25 years (Passamonti *et al,* 2004; Passamonti *et al,* 2008; Wolanskyj *et al,* 2006). By contrast in untreated CML there is a 25% per annum risk of transformation to acute leukaemia (Champlin and Golde 1985) and a median survival of less than 5 years (Cervantes and Rozman 1982).

Second, in the small proportion of ET patients who do develop acute leukaemia, the malignant clone is often V617F-negative even when the preceding ET was V617F-positive (Campbell *et al,* 2006a; Theocharides *et al,* 2007), whereas in CML loss of Philadelphia-positivity is not observed following leukaemic transformation – indeed BCR-ABL expression usually increases (Elmaagacli *et al,* 2000; Gaiger *et al,* 1995). Campbell *et al* (2006a) studied four V617F-positive MPN patients who developed AML (two ET and two PV), and observed that in three cases the blast cells were *JAK2* WT, although the mechanism for this was unclear. If the blasts were derived from a V617F-positive progenitor, mitotic recombination might explain the loss of the V617F mutation. This was, however, considered unlikely for two reasons. Firstly, in one of the three patients reported, the pre-AML V617F-positive granulocytes carried cytogenetic abnormalities – del20q and t(1;9) – which were not detectable in the blast cells, suggesting

that the blast cells were not derived from the V617F-positive ones. Secondly, in two patients, the blast cells were shown by microsatellite analysis to be heterozygous for two chromosome 9p SNPs, located either side of the *JAK2* gene, suggesting that the blasts cells had not undergone mitotic recombination. Consequently, the authors speculated on the existence of a pre-*JAK2* clonal population from which both the V617F-positive MPN cells and the *JAK2* WT blasts had arisen. Theocharides *et al* (2007) also reported that the majority of V617F-positive MPN patients who developed AML had *JAK2* WT blasts, found in nine out of 14 interpretable cases. Again, microsatellite analysis did not suggest the occurrence of mitotic recombination, but interestingly in one patient the V617F-positive MPN granulocytes and the *JAK2* WT blasts shared a common cytogenetic abnormality (del11q), which was interpreted by the authors as in keeping with a pre-*JAK2* transformed population. By implication, therefore, *JAK2* V617F was not the initiating event in MPN, at least in these individuals.

Third, in V617F-positive ET patients, the mutant-positive population is usually small and remains stable over many years. The median mutant level observed in neutrophil DNA in our patient cohort (17%) was similar to that reported by most published studies (overall median 22%; Table 3.12), and implies that on average approximately 30-40% of myeloid cells carried the mutation (see Chapter 4). In the majority of ET patients, the V617F level remains stable for many years (Gale *et al,* 2007; Theocharides *et al,* 2008) and, as demonstrated in the previous chapter, *JAK2* WT thrombopoiesis is not suppressed by the presence of a mutant-positive population. This is very different to the situation in CML where the cytogenetically normal progenitors are suppressed, and the Philadelphia-positive progenitors expand rapidly to constitute more than 99% of dividing myeloid precursors (Dubé *et al,* 1984a; Dubé *et al,* 1984b).

Finally, most studies examining XCIPs in ET have reported that a proportion, on average 40%, of patients have polyclonal, or at least oligoclonal, myelopoiesis (Table 1.4). Even in *JAK2* V617F-positive ET patients, haemopoiesis is frequently poly- or oligoclonal and there is no

correlation between V617F-positivity and clonality (Antonioli *et al,* 2005; Levine *et al,* 2006). In our cohort, 72% of V617F-positive ET patients with an interpretable XCIP had a polyclonal pattern, compared to 55% of *JAK2* WT patients. Taken together, these four observations suggest that V617F-positive ET is not necessarily a clonal neoplasm in the mould of CML but is instead a more complex, heterogeneous disorder.

5.1.2 *JAK2* V617F and clonality in ET

Of the observations presented above, the lack of association between the presence of *JAK2* V617F and myeloid clonality is perhaps the most surprising, since detection of a cell population carrying an acquired mutation is usually assumed to indicate the presence of a clonal disorder. One possible explanation for this observation, discussed in Chapter 3, is that in ET the mutant population is often too small to affect the overall myeloid clonality status. Nonetheless, an association between high mutant levels and myeloid clonality might still be expected, yet Levine *et al* (2006) demonstrated that in ET no such association exists, whereas in PV it does. It is also notable that in one patient discussed in Chapter 3 (Table 3.10, patient 2), T-cell and neutrophil XCIPs were nearly identical (72%:28% and 73%:27% respectively), despite a mutant level of 26%.

An alternative explanation is that, in some ET patients the V617F-positive population is not monoclonal and therefore does not necessarily result in myeloid clonality even in the presence of high mutant levels. This idea is not in keeping with conventional views of the role of somatic mutations in haematological disorders and implies that the V617F-positive population is derived from more than progenitor, each of which has acquired the mutation independently. Individual MPN patients have occasionally been reported to harbour more than one MPN-associated mutation. Pardanani *et al* (2006) studied 1182 MPN patients and identified six

individuals (0.5% of the cohort), including two with ET, who had both *JAK2* V617F and *MPL* W515 mutations. Of these six patients, two had *MPL* W515L and W515K mutations as well as *JAK2* V617F. Beer *et al* (2009; Jones *et al,* 2009; Olcaydu *et al,* 2009) described 13 MPN patients with two different genetic lesions, including five ET patients, four with *JAK2* V617F and *MPL* W515L, one with *JAK2* V617F and del20q. They used colony analysis to investigate the clonal relationship of the different mutant-positive populations and found that in seven of the 13 patients, including all five ET patients, some colonies carried one mutation, others carried the second mutation, but none was positive for both. They inferred from this that there were two or more distinct clones. They also noted that the frequency of patients with biclonal disease – seven out of approximately 1000 patients screened (0.7%) – was much greater than would be expected by chance and speculated about the existence of either an inherited or an environmental factor which predisposed to acquisition of these genetic abnormalities.

There is increasing evidence that some individuals have a hereditary predisposition to develop *JAK2* V617F-positive MPNs (discussed in detail in Chapter 6). Moreover, the data presented in Chapter 4 suggests that in most mutant-positive ET patients, *JAK2* WT myelo- and thrombopoiesis is not suppressed by the mutant-positive population, indicating the long-term persistence of WT myeloid progenitors. If these *JAK2* WT cells are particularly susceptible to acquisition of *JAK2* V617F, multiple mutant-positive clones might result. It is therefore feasible that the mechanism which predisposed these individuals to acquire *JAK2* V617F in the first place may impart a tendency to do so repeatedly.

The suggestion that in ET there may be several co-existing independent V617F-positive clones, rather than one dominant one, is contentious since it implies that the disorder is oligoclonal. The obstacle to examining this hypothesis is finding a means to identify multiple occurrences of the same mutation. To overcome this, two different approaches have been adopted. One technique uses an informative A/G SNP, rs2230724 located in exon 19 of the *JAK2* gene, to identify the *JAK2* allele (maternal or paternal) on which the mutation has arisen; the presence

of the mutation on both alleles in a single individual would be indicative of more than one mutational event. The other technique uses XCIP analysis to determine whether mutant-positive haemopoietic colonies from a single individual share a common clonal origin.

5.1.3 Aims

The experiments reported in this chapter aimed to investigate the hypothesis that in V617F-positive ET, multiple mutant-positive independent clones exist.

5.2 Patients, materials and methods

5.2.1 Patient selection

The possibility that more than one V617F-positive population exists in mutant-positive ET patients was initially investigated using a *JAK2* SNP, rs2230724, and a PCR-restriction enzyme digestion-PCR approach to identify the allele on which the mutation had arisen. This involved generating a PCR product spanning both the *JAK2* V617F mutation site (exon 14) and the SNP locus (exon 19). Since exons 14 and 19 are too far apart to allow a PCR product spanning both the mutation and SNP loci to be amplified from gDNA (8.0Kb), cDNA was used (798bp product). For analysis using this technique, therefore, patients had to be V617F-positive and heterozygous for the exon 19 SNP, and have cDNA available.

5.2.2 Determining *JAK2* status

5.2.2.1 Screening for JAK2 V617F using gDNA

PCR for *JAK2* exon 14 and restriction enzyme digestion with *Afl*III was used to screen DNA for the V617F mutation as described in Section 3.2.3.

5.2.2.2 Screening for JAK2 V617F using cDNA

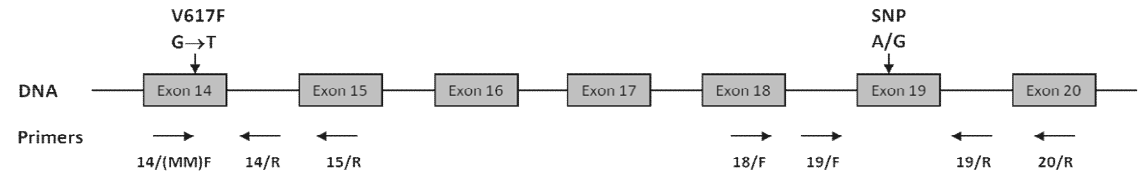
To screen cDNA for the V617F mutation (see Section 5.2.4), a similar PCR and restriction enzyme digest technique to that described in Section 3.2.3 was used, except that a reverse primer which annealed to exon 15 was used (*JAK2*ex15/R; Figure 5.1A). A 169bp fragment spanning exon 14 was amplified using BIOTAQ™ PCR with primers *JAK2*ex14/(MM)F and *JAK2*ex15/R. Thirty-five cycles of PCR were performed using an annealing temperature 61°C. PCR products were then digested with *Afl*III using 7µL PCR product in a reaction mixture containing 5 units *Afl*III enzyme, 1x manufacturer's buffer 3 and 100µg/mL BSA, made up to a 10µL total reaction volume with ddH_20 and incubated for 6 hours at 37°C. *Afl*III cut the WT allele to 28 and 141bp whilst the mutant allele remained uncut (169bp). Digestion products were separated by agarose electrophoresis (3% gel) and visualised by UV transillumination.

5.2.3 *JAK2* exon 19 SNP (rs2230724) genotyping

5.2.3.1 Exon 19 SNP genotyping using gDNA

Patient T-cell gDNA was genotyped for the *JAK2* exon 19 A/G SNP by PCR and restriction enzyme digestion. T-cell, rather than neutrophil, gDNA was used to avoid interference by possible acquired loss of homozygosity of chromosome 9 in myeloid cells. A 214bp fragment covering *JAK2* exon 19 and part of the flanking introns was amplified by PCR using BIOTAQ™

A



B

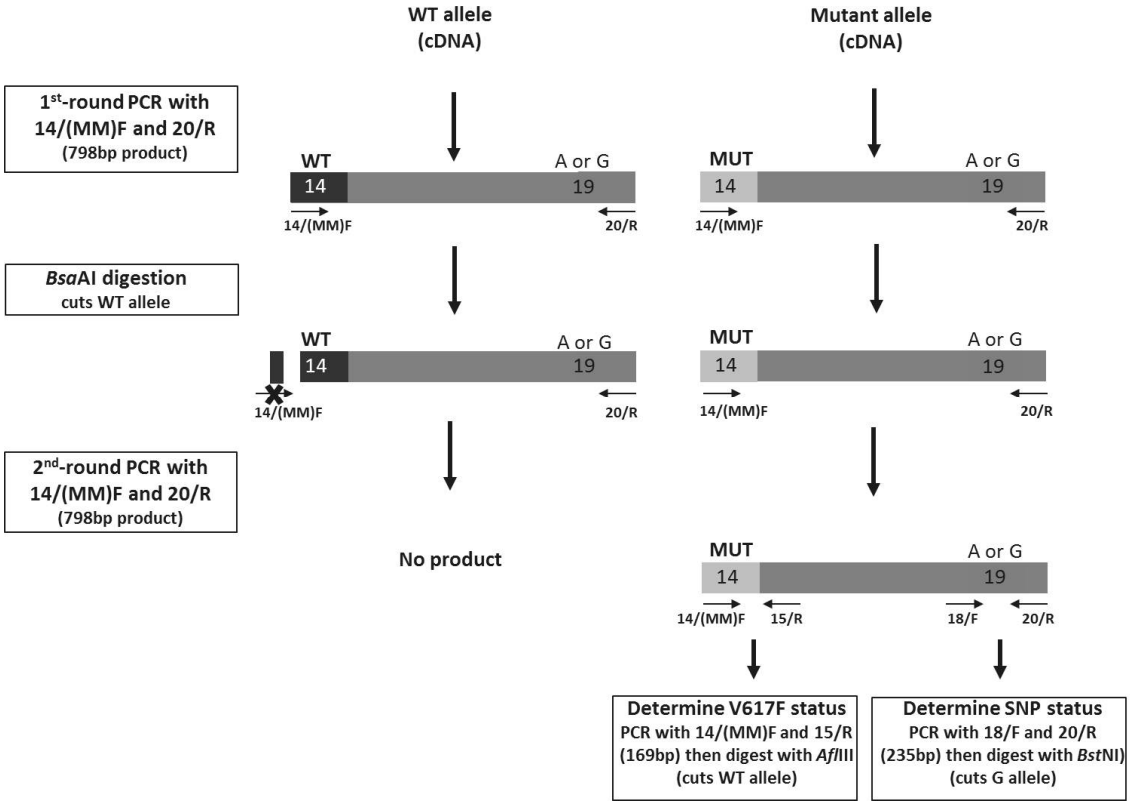


Figure 5.1 Schematic representation of the technique used to determine the *JAK2* exon 19 SNP (rs2230724) genotype of V617F positive-mutant alleles

A. Genomic structure of *JAK2* gene showing primers used.

B. PCR-restriction enzyme digestion-PCR technique used to determine the *JAK2* exon 19 SNP genotype of *JAK2* V617F mutant-positive alleles using cDNA

polymerase as described in Section 2.2.3.1, with primers *JAK2*ex19/F and *JAK2*ex19/R (Figure 5.1A), an annealing temperature of 60°C and 35 cycles of PCR, using 50ng of T-cell gDNA as template per reaction. Following this, a digestion mixture was prepared containing 5 units *Bst*NI enzyme, 1x manufacturer's buffer 2 and 100µg/mL BSA, made up to 5µL with ddH_20, and to this 5µL PCR product was added and incubated for 2 hours at 60°C. Digestion products were separated by agarose electrophoresis (3% gel) then visualised by UV transillumination. *Bst*NI cut the G-allele to 93 and 121bp but did not cut the A-allele (214bp).

5.2.3.2 Exon 19 SNP genotyping using cDNA

To genotype cDNA for the exon 19 SNP (see Section 5.2.4), a 235bp fragment was amplified using BIOTAQ™ PCR as described in Section 2.2.3.1, with exonic primers *JAK2*ex18/F and *JAK2*ex20/R. Thirty-five cycles of PCR were performed using an annealing temperature 62°C. PCR products were then digested with *Bst*NI using 7µL PCR product in a reaction mixture containing 5 units *Bst*NI enzyme, 1x manufacturer's buffer 2 and 100µg/mL BSA, made up to a 10µL total reaction volume with ddH_20 and incubated for 2 hours at 60°C. *Bst*NI cut the G-allele to 108 and 127bp whilst the A-allele remained uncut (235bp).

5.2.4 *JAK2* exon 19 SNP (rs2230724) genotyping of V617F-positive alleles by PCR-restriction enzyme digestion-PCR

JAK2 V617F-positive patients who were heterozygous for SNP rs2230724, and for whom cDNA was available, were investigated for the presence of more than one V617F-positive population using a PCR-restriction enzyme-PCR technique (Figure 5.1B).

cDNA was generated from neutrophil RNA by reverse transcription using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit as described in Section 2.2.5. A 798bp

fragment covering *JAK2* exons 14-20 was then amplified from neutrophil cDNA using BIOTAQ™ polymerase, as described in Section 2.2.3.1, with primers *JAK2*ex14/(MM)F and *JAK2*ex20/R, and 1µL cDNA diluted 1 in 4 in ddH_2O as template. Thirty-eight cycles of PCR, each of 60 seconds at 95°C, 60 seconds at 62°C, 90 seconds at 72°C were performed, followed by a final 5 minute extension at 72°C. Products were visualised on 2% agarose gel containing ethidium bromide.

Products were digested with *Bsa*AI to cut the WT but not the mutant allele. *Afl*III could not be used for this digestion due to the presence of an *Afl*III site common to both WT and mutant alleles which would destroy the ability to re-amplify the mutant products. For the digestion, a reaction mixture was made up containing 5 units *Bsa*AI enzyme and 1x manufacturer's buffer 3, made up to 5µL with ddH_20, and to this 5µL PCR product was added. The mixture was incubated for 6 hours at 37°C.

This digest was then used as template for a second round of PCR. Undiluted digests were too concentrated to yield interpretable second-round PCR products and a range of dilutions of the digests in ddH_20 was therefore made (1 in 50, 1 in 250, 1 in 1,000 and 1 in 10,000). For the second round of PCR, 1 µL of each dilution was used as template. As in the first round PCR, a 798bp fragment spanning exons 14-20 was amplified using BIOTAQ™ polymerase with primers *JAK2*ex14/(MM)F and *JAK2*ex20/R. Thirty-two cycles of PCR were performed, each of 60 seconds at 95°C, 60 seconds at 62°C, 90 seconds at 72°C, followed by a final 5 minute extension at 72°C.

The final stage of the process was to determine the *JAK2* V617F status and exon 19 SNP genotype of the resulting 798bp PCR product, by PCR and restriction enzyme digestion as described in Sections 5.2.2.2 and 5.2.3.2 respectively. For both PCRs, a 1 in 100 dilution of the second-round PCR product was used as template.

5.2.5 *JAK2* exon 19 SNP genotyping of V617F-positive alleles by TA cloning

In the process described above, incomplete *Bsa*AI digestion of the WT first-round PCR products, even if minor, would result in some WT allele amplification in the second-round PCR. Consequently it is possible that the *JAK2* exon 19 SNP genotyping performed in the final step of the process might be from a mix of WT and mutant-positive alleles, and would therefore be misleading.

To overcome this, the exon 19 SNP genotype of V617F-positive alleles was studied by TA cloning using the Invitrogen TOPO TA Cloning® Kit (Figure 5.2). For the five patients studied, cDNA was generated from neutrophil RNA as described in Section 5.2.4; cDNA was also generated from platelet RNA for two of the five patients, and from RNA obtained from CD34-positive cells for one patient. Using 1 in 4 dilutions of the cDNA as template, a 798bp fragment spanning *JAK2* exons 14-20 was amplified by BIOTAQ™ PCR, as described in Section 5.2.4. The entire 20µL PCR product obtained from each reaction was run on a 1.5% low-melting point agarose gel, visualised by UV transillumination and cut out from the gel. Gel-purification of the PCR products was necessary to remove the primers which interfered with subsequent reactions. The gel fragment containing the PCR product was melted by warming to 60°C. Depending on the strength of the original band as assessed visually, between 1 and 4µL of the molten gel was used in the ligation step of the TA cloning process which was performed according to manufacturer's instructions (see Section 2.2.10). Bacterial clones were grown on LB-agar-carbenicillin plates coated with X-gal and transformed clones containing the *JAK2* exon 14-20 PCR product were identified by their white or light-blue colour. These were picked and expanded by incubation in 50µL LB medium containing carbenicillin at 37°C overnight.

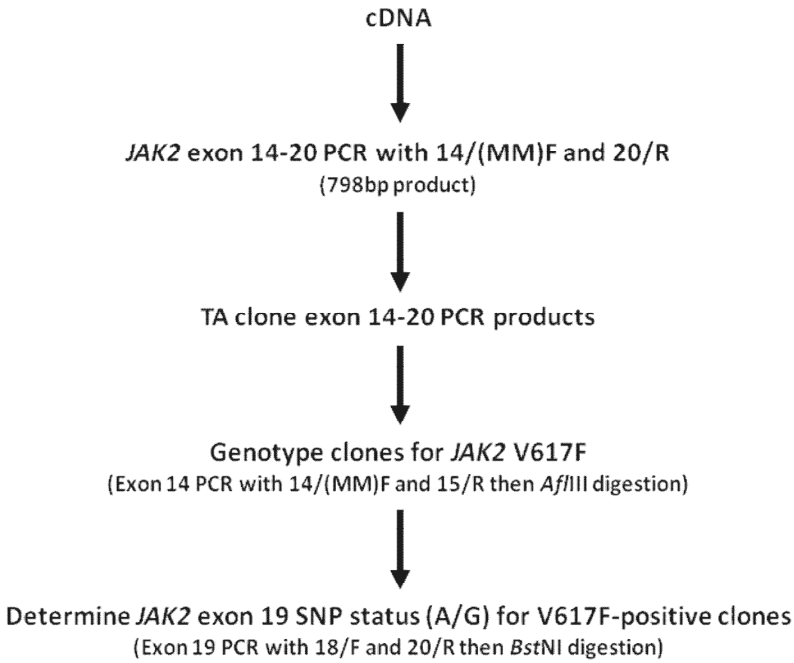


Figure 5.2 Schematic representation of the TA cloning technique used to determine the *JAK2* exon 19 SNP (rs2230724) genotype of *JAK2* V617F mutant-positive alleles using cDNA

The *JAK2* V617F status of individual clones was determined by PCR and restriction enzyme digestion (Section 5.2.2.2) using 1µL bacterial suspension in the 20µL PCR mix as template. Prior to the first cycle of PCR, the reaction mix was incubated for 2 minutes at 95°C to lyse the bacteria. V617F-positive clones were then genotyped for the *JAK2* exon 19 SNP by PCR and restriction enzyme digestion (Section 5.2.3.2), again using 1µL bacterial suspension in the 20µL PCR mix as template and a 2 minute 95°C bacterial lysis step prior to the first PCR cycle.

5.2.6 Using a *JAK2* intron 14 SNP (rs12343687) to genotype V617F-positive alleles

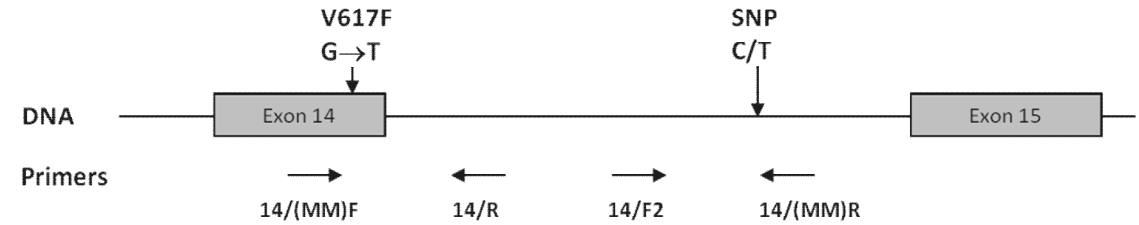
Whilst this work was underway, data on several previously unrecognised *JAK2* SNPs was published (Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009), including one in intron

14 which was sufficiently close to the V617F locus to allow a 482bp PCR product spanning both sites to be amplified from gDNA. The patients who had been studied using the exon 19 SNP were therefore investigated using the intron 14 SNP to establish whether RNA and gDNA-based approaches gave concordant results. The process described in Sections 5.2.4 and 5.2.5 was therefore repeated but here the *JAK2* intron 14 SNP, rs12343687 (C/T), was studied instead of the *JAK2* exon 19 SNP.

5.2.6.1 Genotyping patients for intron 14 rs12343687 SNP status

In order to identify which of the patients studied in Sections 5.2.4 and 5.2.5 were heterozygous for the intron 14 SNP, a 164bp fragment spanning this SNP was amplified using primers *JAK2*ex14/F2 and *JAK2*ex14/(MM)R (Figure 5.3A). The reverse primer introduced a mismatch immediately downstream of the SNP to create a recognition site so that *Bsa*AI would cut products with the C allele whilst leaving the T-allele products uncut. T-cell gDNA was used as template and 35 cycles of BIOTAQ™ PCR were performed as described in Section 2.2.3.1, with an annealing temperature of 59°C. Products were run on a 2% agarose gel and visualised by UV transillumination. Digestion with *Bsa*AI was then performed: a reaction mix was made up containing 4 units *Bsa*AI enzyme and 1x manufacturer's buffer 3, made up to a 4µL ddH_20, and to this 4µL PCR product was added and the mixture incubated for 4 hours at 37°C. Products were run on a 3% agarose gel and visualised by UV transillumination. *Bsa*AI cut the C allele to 31 and 133bp whilst the T allele remained uncut (164bp).

A.



B.

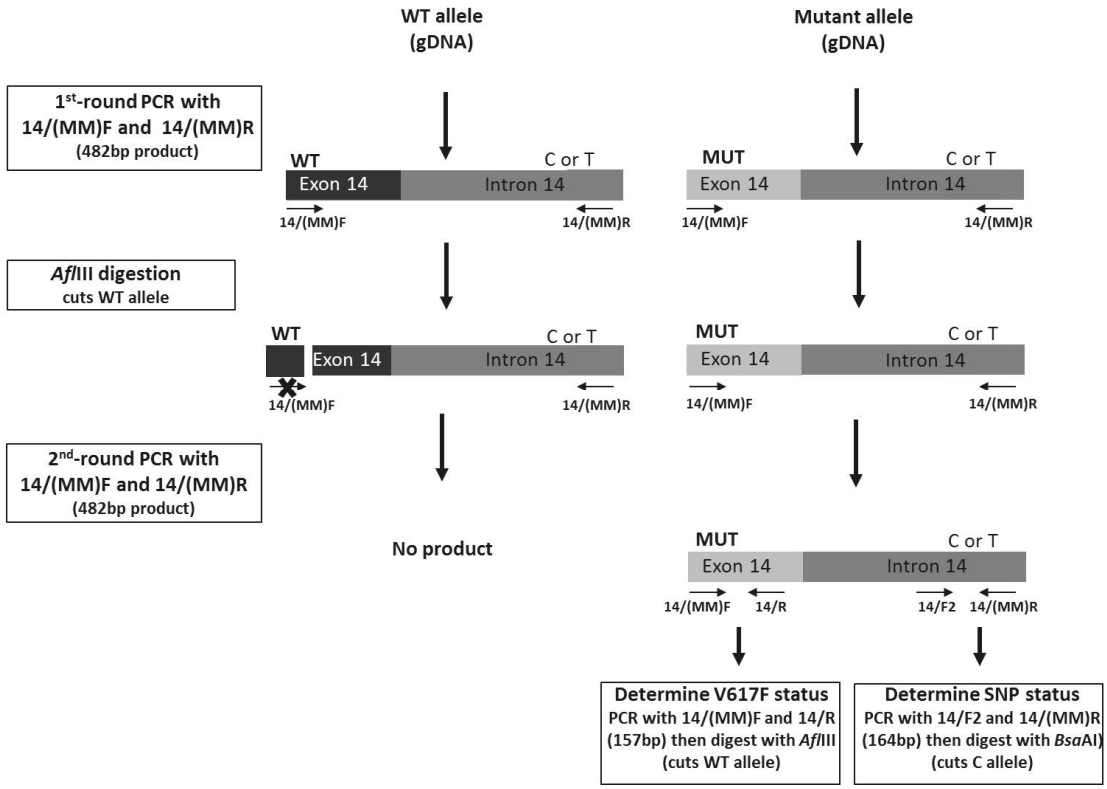


Figure 5.3 Schematic representation of *JAK2* exons 14 and 15 showing primers used to genotype V617F-positive alleles using intron 14 SNP (rs12343687)

A. Genomic structure of *JAK2* exons 14 to 15 showing primers used.

B. PCR-restriction enzyme digestion-PCR technique used to determine the *JAK2* intron 14 SNP genotype of *JAK2* V617F mutant-positive alleles using gDNA

5.2.6.2 JAK2 intron 14 SNP genotyping of V617F-positive alleles by PCR-restriction enzyme digest-PCR

Neutrophil gDNA from patients who were heterozygous for rs12343687 was then studied using a similar PCR-restriction enzyme digest-PCR technique to that described in Section 5.2.4 except that a 482bp fragment was amplified covering the *JAK2* exon 14 mutation site and the rs12343687 SNP in intron 14 (Figure 5.3B). A first round PCR was performed using GoTaq® DNA polymerase, primers *JAK2*ex14/(MM)F and *JAK2*ex14/(MM)R, 35 cycles of PCR at an annealing temperature of 59°C. PCR products were digested using *Afl*III as described in Section 3.2.3 which cut the WT allele so that only mutant-positive PCR products were re-amplified in a second round of PCR. Conditions for the second round PCR were identical to those used in the first-round PCR but with 1µL of the digest diluted 1 in 20 as template. The *JAK2* V617F status of the second-round PCR products was then determined using the PCR-restriction digestion technique described in Section 5.2.2.1, followed by determination of their rs12343687 status as described in Section 5.2.6.1.

5.2.6.3 JAK2 intron 14 genotyping of V617F-positive alleles by TA cloning

TA cloning was performed in three patients, using the same approach to that described in Section 5.2.5 except that competent bacteria were transformed with the 482bp PCR product which spanned the *JAK2* exon 14 mutation site and rs12343687 in intron 14. This was amplified from neutrophil gDNA using primers *JAK2*ex14/(MM)F and *JAK2*ex14/(MM)R as described in Section 5.2.6.2. V617F-positive clones were identified by PCR and restriction enzyme digestion as described in Section 5.2.2.1 and the rs12343687 allele present in these clones was determined as described in Section 5.2.6.1. For both PCRs, 1µL bacterial suspension was added to the 20µL PCR mix as template and prior to the first cycle of PCR, the reaction mix was incubated for 2 minutes at 95°C to lyse the bacteria.

5.2.7 X-chromosome inactivation analysis of erythroid burst-forming units (BFU-Es)

To establish whether V617F-positive haemopoietic progenitors shared a common clonal origin, BFU-Es were cultured from mononuclear cells (MNCs) from patients with mutant-positive ET. *JAK2* V617F-positive BFU-Es were then identified and analysed to determine their inactivated X-chromosome.

5.2.7.1 Culturing BFU-Es from peripheral blood

To minimise contamination all processing except centrifugation was performed in a laminar air flow cabinet. Approximately 30mL peripheral blood was collected in EDTA and centrifuged for 15 minutes at 200g at room temperature and the platelet-rich plasma was removed. The buffy coat of the remaining sample was then harvested and MNCs purified by Ficoll-Hypaque density centrifugation as described in Section 2.1.3. They were resuspended in 15mL RPMI containing 10% FCS (RF10%) and spun for 5 minutes at 800g at room temperature. The supernatant was removed, the pellet resuspended in 5mL RF10% and the resulting MNC suspension was counted using a Sysmex KX21N whole blood cell analyser.

MACS HSC-CFU media complete with Epo (Miltenyi Biotec, Bisley, UK) was thawed at 37°C and divided into 2.5mL aliquots. To each aliquot, an appropriate volume of the MNC suspension was added to generate the following dilutions: $0.5x10^5$ cells/mL, $1.0x10^5$ cells/mL, $2.0x10^5$ cells/mL and $4.0x10^5$ cells/mL. At least four aliquots at each concentration were made up. Each aliquot was vortexed and gently layered into a single well of a 6 well tissue-culture plate using a 5mL syringe and kwill, ensuring no air bubbles were introduced into the media. Approximately 3mL sterile PBS was added to the spaces between the wells, to prevent dehydration of the media, and each plate was incubated at 37°C in a humidified atmosphere containing 4% CO_2.

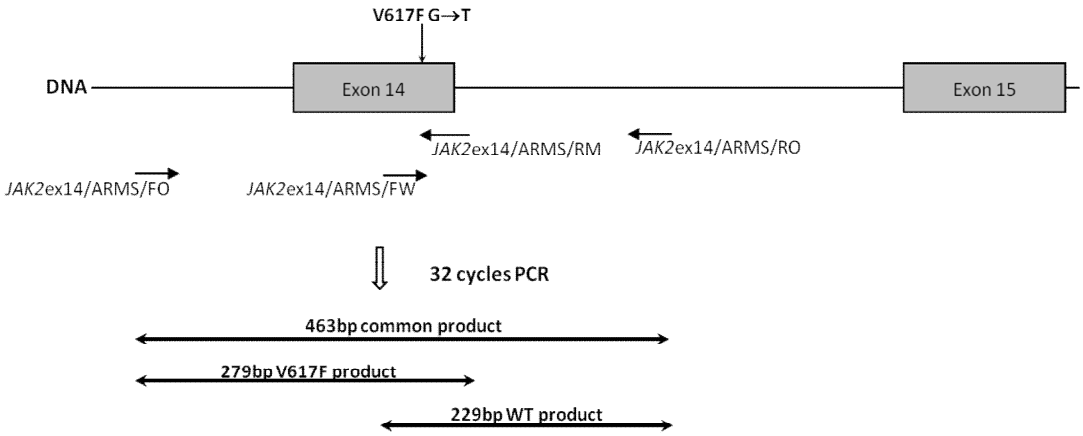
After 14 days, each well was inspected under a dissecting microscope and BFU-Es identified. Single, discrete BFU-Es were harvested into 200µL PBS using a sterile fine-tip pastette and centrifuged at 2000g for 5 minutes at room temperature. The supernatant was removed and to each pellet 43µL TNE lysis buffer (10mM Tris-HCl, 400mM NaCl, 2mM EDTA) was added. Samples were incubated on ice for 2 minutes, and 5µL 10% SDS and 1µL 20mg/mL proteinase K added. Samples were then vortexed, incubated at 56°C for one hour and placed at -20°C for at least 12 hours. After thawing, DNA was extracted by the addition of 1µL glycogen, 5µL 3M sodium acetate and 150µL absolute ethanol, followed by centrifugation at 14,000g for 15 minutes at room temperature. The supernatant was removed, 200µL 75% ethanol added, and the sample spun at 14,000g for 5 minutes at room temperature. The supernatant was again removed, the DNA pellet allowed to air dry and resuspended in 15µL ddH_20.

5.2.7.2 Identification of V617F-positive colonies

JAK2 V617F-positive colonies were identified by amplification refractory mutation system (ARMS) PCR technique using the methodology of Jones *et al* (2005; Figure 5.4). This single-step technique allowed more rapid analysis of large numbers of colonies than the PCR and restriction enzyme digestion described in Section 5.2.2. Since mutant-positive colonies were expected to be heterozygous for V617F, with a mutant level of 50%, a high-sensitivity technique was not required. For this PCR, four primers were used in order to generate three possible products – a 463bp common (control) product, a 229bp WT product, and a 279bp mutant product. Two common intronic primers were used, both at 1µM: a forward primer (*JAK2*ex14/ARMS/FO) located in intron 13, upstream of the mutation site, and a reverse primer (*JAK2*ex14/ARMS/RO) located in intron 14, downstream of the mutation site. These two primers generated the 463bp common product regardless of the presence of V617F. In addition, a WT-specific forward primer (*JAK2*ex14/ARMS/FW) was used at 0.5µM, which only annealed to the WT *JAK2* sequence and together with *JAK2*ex14/ARMS/RO generated the

229bp WT product. Finally, a V617F-specific reverse primer (*JAK2*ex14/ARMS/RM) was used at 0.5µM, which, in the presence of V617F and *JAK2*ex14/ARMS/FO, generated the 279bp mutant product. GoTaq® PCR was performed as described in Section 2.2.3.2 except for the use of the 4 primers at the specified concentrations, with 1µL colony DNA as template. Thirty-two cycles of PCR were performed, with an annealing temperature of 60°C, and products electrophoresed and visualised as before.

A.



B.

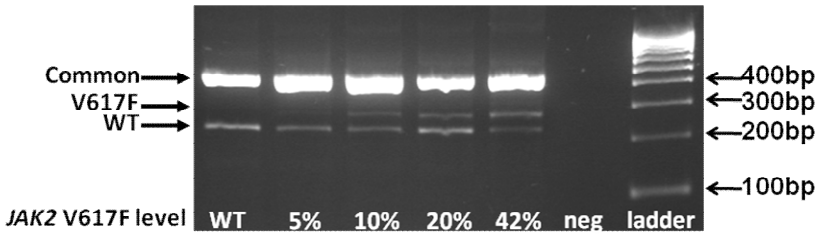


Figure 5.4 *JAK2* exon 14 ARMS PCR to detect V617F (based on Jones *et al*, 2005)

A. Schema for PCR showing *JAK2* exons 14 and 15, the four primers used, and the three possible products
B. Agarose gel (2%) showing the three possible products in four V617F-positive ET patients with known mutant levels (indicated at the bottom of the gel), and one *JAK2* WT control ('WT'). 'Neg' indicates negative control and 'ladder' indicates Bioline Hyperladder 4.

5.2.7.3 Inactivated X-chromosome analysis of V617F-positive colonies

The inactivated X-chromosome of V617F-positive colonies was determined using a modified HUMARA technique (Section 3.2.5). A digestion mixture containing 12.5 units *Hpa*II, 2.5 units *Rsa*I and 1x manufacturer's buffer 1 was prepared and made up to 2.5µL with ddH_2O. To this mix 2.5µL colony DNA was added and the mixture incubated overnight at 37°C. A PCR product spanning the VNTR sequence in the first exon of the *HUMAR* gene was then amplified. A reaction mix containing 1x manufacturer's flexibuffer, 0.2mM each dNTP, 1ng/µL each HUMARA primer and 1 unit GoTaq® Hot Start polymerase was prepared, made up to 15µL with ddH_20, and added to the 5µL digestion mix. Twenty-five cycles of PCR were performed, using an annealing temperature of 68°C, and a final extension of 72°C for 15 minutes. Cycling conditions were as described in Section 2.2.3.2.

PCR products were then analysed by fragment analysis, as described in Section 2.2.7, in order to determine the inactivated *HUMAR* allele. Since all cells in each colony were descended from a single cell, all would be expected to inactivate the same *HUMAR* allele. Consequently, only a single primary peak was expected on fragment analysis. The presence of more than one primary peak implied the presence of either two separate colonies or incomplete enzyme digestion, and such results were repeated and excluded if persistent.

5.3 Results

5.3.1 *JAK2* exon 19 SNP (rs2230724) genotyping in haematologically normal controls and ET patients

A cohort of 114 haematologically normal controls, 111 ET patients and 26 PV patients was screened to determine the rs2230724 genotype (AA, AG or GG). There was no difference in the

distribution of SNP genotypes in ET patients, regardless of mutational status, or in PV patients compared to normal controls (Table 5.1). However, when compared to the expected genotype frequency based on HapMap European population data, there was a significant excess of A-alleles in ET patients overall, V617F-positive ET patients, and PV patients (P<0.001, P= 0.03 and P=0.04 respectively, Chi-square).

5.3.2 *JAK2* exon 19 SNP genotyping of V617F-positive alleles using SNP rs2230724

Twenty-three V617F-positive ET patients were heterozygous for rs2230724, and of these 11 had cDNA available. Samples from these 11 patients were therefore screened for more than one V617F-positive population, initially using the PCR-restriction enzyme digestion-PCR technique. PCR products were visualised by agarose gel electrophoresis. Inspection of the gel revealed that adequate first-round and second-round PCR products were amplified in 10 of the 11 patient samples studied; in the remaining patient (Table 5.2, patient E10) both PCR products were too weak to allow accurate genotyping for V617F and the exon 19 SNP.

In the 10 samples where good PCR products were obtained, re-amplification of only mutant-positive and not WT alleles in the second-round PCR was confirmed by exon 14 PCR and *Afl*III digestion (Figure 5.5A). SNP genotyping of the second round PCR products from nine of the 10 patients showed a mixture of A and G alleles (Figure 5.5B) with only one patient (patient E1, lane 3) showing only A alleles. This patient had a relative mutant level of 100%, consistent with the presence of chromosome 9pLOH. The relative A:G allele proportions, as determined by visual estimation of the two bands (Figure 5.5B), varied in the other nine patients (Table 5.2). In six patients, the mutation arose principally on the A-allele (Table 5.2, patients E2,E3,E6, E8, E9 and E10), in three it arose principally on the G-allele (patients E4, E7 and E11), and in one patient the distribution between the two alleles was approximately equal (patient E5). This

suggested that *JAK2* mutations were present on both chromosomes in most of the patients studied.

To ensure that these results were not due to incomplete *Bsa*AI digestion of the WT PCR product, which would mimic the mutant product, full-length PCR products covering the V617F mutation and the SNP (exons 14-20) were TA cloned using neutrophil cDNA from five of these patients. V617F-positive clones were identified and the exon 19 SNP present in these clones determined. Between 29 and 33 V617F mutant-positive clones were genotyped from each sample. In each case, both allele A and allele G clones were detected (Table 5.2, patients E7-E11). In three cases, the majority of clones had allele A: 82%, 76% and 77% of clones respectively in patients E8-E10. In one case allele A and G clones were approximately equal (patient E7, 41% A clones), and in the remaining case there were more allele G clones (patient E11, 23% A clones). In all these individuals, the results from the TA cloning approach mirrored those from the PCR-restriction enzyme digest-PCR approach (Table 5.2).

Platelet samples from two patients gave comparable results to the neutrophil samples: 82% and 78% allele A clones respectively in neutrophils and platelets from patient E8; 77% and 72% from patient E9. Neutrophil samples taken from the same patient 23 months apart (patient E9) were analysed and gave comparable results with 77% and 87% allele A clones respectively. RNA from a purified bone marrow CD34+ sample was also available from this patient and yielded similar results to the neutrophil sample, 78% allele A clones.

Of the 26 V617F-positive PV patients studied, ten were heterozygous at the exon 19 SNP and cDNA was available for five of these. Neutrophil cDNA samples from these five patients were screened using the PCR-restriction enzyme digestion-PCR technique for the presence of more than one mutational event. In all five cases, the V617F-positive alleles were associated with a single exon 19 SNP allele (allele A in every case; Figure 5.6).

Table 5.1 Incidence of *JAK2* exon 19 SNP rs2230724 genotypes in normal controls, ET and PV patients

Diagnosis	*n*	*JAK2* exon 19 SNP genotype						Observed versus normal control (P-value) †	Observed versus expected* (P-value)†
		Observed n (%)			Expected n*				
		AA	AG	GG	AA	AG	GG		
Normal controls	114	33 (29%)	57 (50%)	24 (21%)	23	56	34	-	0.17
ET All patients:	111	39 (35%)	55 (50%)	17 (15%)	22	55	34	0.43	<0.001
V617F positive:	43	16 (37%)	23 (53%)	4 (9%)	9	21	13	0.20	0.03
JAK2 WT:	68	23 (34%)	32 (47%)	13 (19%)	14	34	21	0.79	0.13
PV‡	26	13 (50%)	10 (38%)	3 (12%)	5	13	8	0.11	0.04

* expected frequencies of AA = 20%, AG = 49%, GG = 30%, based on HapMap allele frequency of SNP rs2230724 (A:G = 0.448:0.552) in a European population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2230724)

† Chi-square P-values shown

‡ all 26 PV patients were *JAK2* V617F-positive

Table 5.2 *JAK2* exon 19 and intron 14 SNP analysis of PCR products from 11 patients with *JAK2* V617F mutant-positive ET

Patient	% Mutant *JAK2* (Neutrophil DNA)	*JAK2* exon 19 SNP (rs2230724)		*JAK2* intron 14 SNP (rs12343687)	
		SNP allele A:G ratio in V617F PCR products* (Neutrophil RNA)	% of SNP allele A V617F+ clones† (No. clones examined)	SNP allele C:T ratio in V617F PCR products* (Neutrophil DNA)	% of SNP allele C V617F+ clones in neutrophil DNA (No. clones examined)
E1	100	A	-	Not informative	-
E2	13	A>G	-	Not informative	-
E3	40	A>G	-	C>T	87% (16)
E4	25	A<G	-	Not informative	-
E5	24	A=G	-	C=T	-
E6	42	A>G	-	C=T	-
E7	26	A<G	41% (31)	C<T	14% (22)
E8a (Oct 2000)	20	A>G	82% (33)	Not informative	-
E8b (Dec 2004)	33	A>G	Platelets 78% (27)		
E9a (Jul 2000)	27	A>>G	77% (33)		
			Platelets 72% (29)		
E9b (Jun 2002)	20	A>>G	87% (31)	C=T	100% (14)
			CD34+ 78% (32)		
E10	14	PP	76% (29)	Not informative	-
E11	11	A<G	23% (31)	C<T	-

* visual estimates from gel

† neutrophil RNA unless otherwise indicated

PP indicates poor product unsuitable for analysis; dash (-) indicates not done; not informative indicates patient's gDNA was homozygous at this SNP

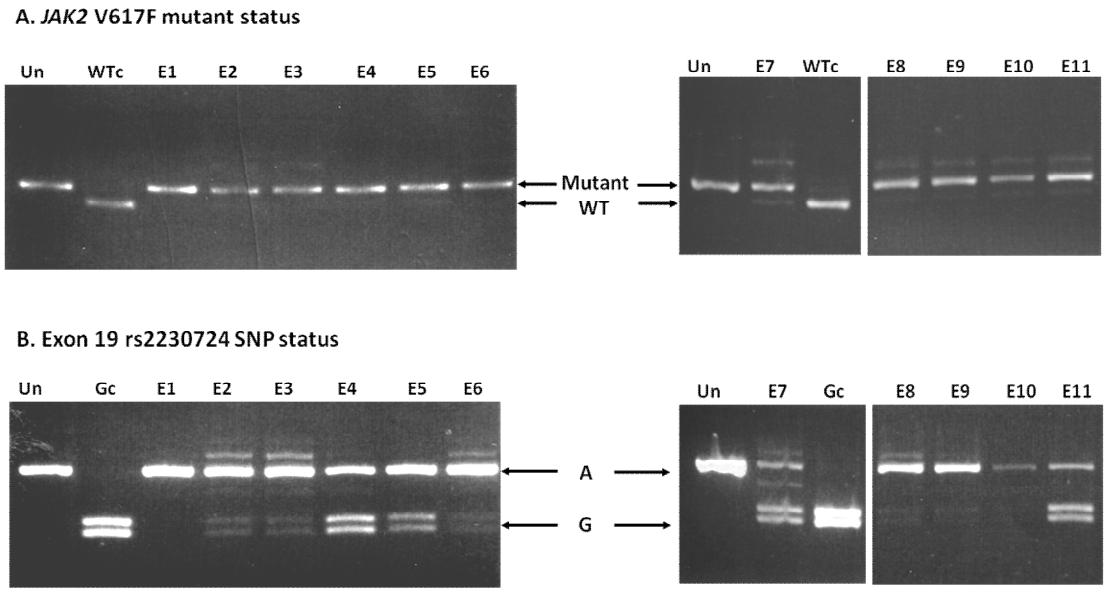


Figure 5.5 ***JAK2*** **exon 19 SNP genotype analysis of V617F mutant alleles in 11 ET patients**

A. Gel showing *Afl*III restriction enzyme digest of 14/(MM)F + 15/R PCR products amplified from the 798bp second-round PCR product. WT products were cut (28+141bp), V617F mutant-positive products remained undigested (169bp).

B. Gel showing *Bst*NI restriction enzyme digest of 18/F + 20/R PCR products amplified from the 798bp second-round PCR product. Exon 19 SNP allele G products were cut (108+127bp), but allele A products remained undigested (235bp).

Un=no restriction enzyme added. WTc = wild-type control. Gc=allele G control.

Breaks within a single panel indicate spliced lanes from a single run

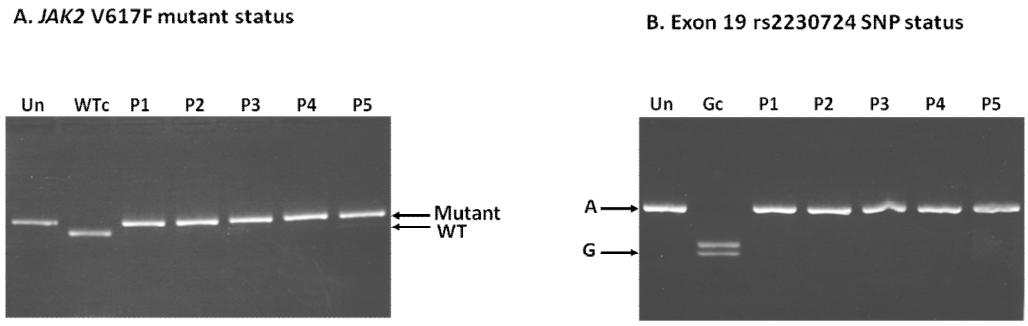


Figure 5.6 *JAK2* exon 19 SNP genotype analysis of V617F mutant alleles in 5 PV patients

A. Gel showing *Afl*III restriction enzyme digest of 14/(MM)F + 15/R PCR products amplified from the 798bp second-round PCR product. WT products were cut (28+141bp), V617F mutant-positive products remained undigested (169bp).
B. Gel showing *Bst*NI restriction enzyme digest of 18/F + 20/R PCR products amplified from the 798bp second-round PCR product. Exon 19 SNP allele G products were cut (108+127bp), but allele A products remained undigested (235bp).

Un=no restriction enzyme added. WTc = wild-type control. Gc=allele G control.

5.3.3 *JAK2* intron 14 SNP genotyping of V617F-positive alleles using SNP rs12343687

DNA samples from the 11 ET patients investigated in Section 5.3.2 were genotyped for the intron 14 SNP (rs12343687). Six were heterozygous for the intron 14 SNP and could therefore be assessed for the presence of more than one V617F-positive population by PCR-restriction enzyme digestion-PCR or TA cloning. Using the former technique, in all six cases the second-round PCR amplified only V617F-positive PCR products (Figure 5.7A) and these mutant-positive products contained a mix of C and T alleles at SNP rs12343687 (Figure 5.7B). In one patient (E3), the mutation arose principally on the C allele, in two patients (E7 and E11) it arose principally on the T allele, and in three patients (E5, E6 and E9) the distribution between the two alleles was approximately equal.

For three patients, the 482bp PCR product spanning the mutation site and the intron 14 SNP was TA cloned. In two of these (Table 5.2, patients E3 and E7), the results from these confirmed the results from the PCR-digest-PCR technique – i.e. mutant-positive clones were a mix of C and T alleles at the intron 14 SNP. For patient E3, both the PCR-restriction enzyme digest-PCR and the TA cloning approach indicated that *JAK2* V617F had arisen principally on the C allele, and in patient E7 both techniques indicated that the mutation arose principally on the T allele. However, in one (patient E9) only one intron 14 SNP allele (C) was associated with V617F-positive clones even though the PCR-digest-PCR technique suggested that the mutation arose on both the C and T alleles (Figure 5.7B).

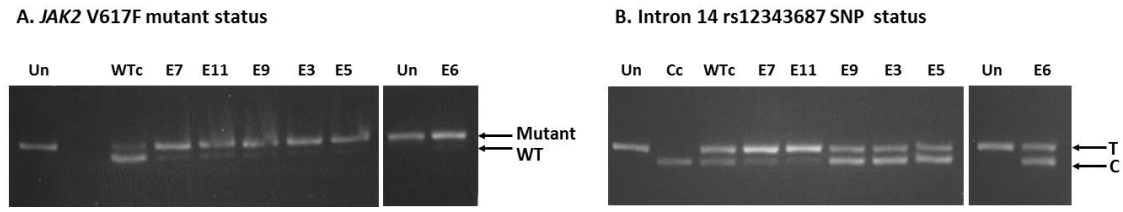


Figure 5.7 *JAK2* intron 14 SNP (rs12343687) genotype analysis of V617F mutant alleles in 6 ET patients

A. Gel showing *Afl*III restriction enzyme digest of 14/(MM)F + 14/R PCR products amplified from the 482bp second-round PCR product. WT products were cut (28+129bp), V617F mutant-positive products remained undigested (157bp).
B. Gel showing *Bsa*AI restriction enzyme digest of 14/F2 + 14/(MM)R products amplified from the 782bp second-round PCR product. Intron 14 SNP allele C products were cut (31+133bp), but allele T products remained undigested (164bp).

Un=no restriction enzyme added. WTc = wild-type control. Cc=allele C control.
Breaks within a single panel indicate spliced lanes from a single run

These results were then compared with those obtained using the exon 19 SNP (rs2230724) for the six patients for whom data for both SNPs was available. In two patients (E7 and E11) in whom the mutation arose principally on the T allele at the intron 14 SNP, it arose principally on the G allele at the exon 19 SNP. Conversely, in the single patient (E3) in whom the mutation

arose principally on the C allele of the intron 14 SNP, it arose principally on the A allele at the exon 19 SNP, and in one patient (E5) the mutation was equally distributed between the two alleles at both SNPs. In two further patients (E6 and E9), however, the mutation was distributed equally between both intron 14 SNP alleles but arose preferentially on the A allele at the exon 19 SNP.

5.3.4 Genotyping of peripheral blood erythroid burst-forming units (BFU-Es)

Freshly-collected peripheral blood from female V617F-positive ET patients was required for this assay. Of the eighteen appropriate patients under active follow-up, six were available to provide samples and erythroid colonies from these six individuals were cultured and analysed (Table 5.3). The mutant level in these patients varied between 5% and 35%, implying that approximately 10% to 70% of the myeloid cells were mutant-positive, assuming that all mutant-positive cells were heterozygous for the mutation (Scott *et al,* 2006). Two of the six patients (E8 and E12) had polyclonal neutrophil XCIPs, two had clonal ones (E13 and E14), and two had uninterpretable ones – one due to age (E6) and one due to constitutional skewing (E7).

For each of the six patients studied, between 53 and 192 colonies were harvested and underwent *JAK2* V617F mutation screening. There was a significant correlation between the V617F mutant level in neutrophil DNA and proportion of mutant-positive colonies for the six patient samples ($P=0.04$, $r^2=0.71$). Patients E6 and E7 had the highest mutant level, in keeping with 70% and 46% of their cells being mutant-positive respectively, and the greatest proportion of their colonies were V617F-positive, 98% and 35% respectively (Table 5.3). Patient E13 had the lowest mutant level, equivalent to 10% mutant-positive cells, and the lowest proportion of mutant-positive colonies, 4%. The other three patients had intermediate

values for both the mutant levels and the proportion of erythroid colonies which were mutant-positive.

Table 5.3 Summary of XCIP analysis of V617F-positive BFU-Es from 6 ET patients

Patient	% *JAK2* V617F (neuts)	% V617F+ cells*	XCIP CD3+	XCIP neuts	Total colonies harvested *n*	V617F+ colonies *n* (% of total)	Colonies with HUMARA result *n*	A-allele inactivated n (% of total)	B-allele inactivated n (% of total)
E6	35%	70%	46% : 54%	20% : 80%	53	52 (98%)	17	0 (0%)	17 (100%)
E7	23%	46%	83% : 17%	82% : 18%	184	64 (35%)	37	37 (100%)	0 (0%)
E8	13%	26%	38% : 62%	45% : 55%	126	16 (13%)	15	0 (0%)	15 (100%)
E12	16%	32%	31% : 69%	31% : 69%	58	14 (24%)	13	5 (38%)	8 (62%)
E13	5%	10%	64% : 36%	82% : 18%	96	4 (4%)	3	3 (100%)	0 (0%)
E14	10%	20%	40% : 60%	18% : 82%	192	48 (25%)	23	0 (0%)	23 (100%)

* Assumes V617F-positive cells are heterozygous for the mutation

The inactivated *HUMAR* allele for every V617F-positive colony was analysed. In a proportion of colonies it could not be established due to the presence of more than one peak on fragment analysis, indicating contamination by another colony. For this reason the number or colonies with a HUMARA result was generally smaller than the number of V617F-positive colonies identified (Table 5.3). In five of the six patients, all the mutant-positive colonies from a single patient inactivated the same *HUMAR* allele – the A allele in two patients (E7 and E13), and the B allele in three patients (E6, E8 and E14). In one patient (E12), however, five (38%) of the 13 V617F-positive colonies inactivated the A allele, and eight (62%) inactivated the B allele. The neutrophil and T-cell XCIPs in this patient were both 31%:69%.

Three of these six patients (E6-E8) had been investigated using TA cloning and found to have more than one V617F-positive population (Section 5.3.2). All three of these patients appeared to have the same inactivated X-chromosome in V617F-positive erythroid colonies.

5.3.5 Calculation of neutrophil XCIP based on T-cell and BFU-E XCIPs and mutant levels

The calculation described in Section 3.3.4 uses the T-cell XCIP and V617F mutant levels to generate two different neutrophil XCIPs, depending on which X-chromosome is inactivated in the V617F-positive population. The data presented in Table 5.3 allows the inactivated X-chromosome of the mutant-positive populations to be deduced from the XCIP of the BFU-Es, and the calculation can therefore be refined as follows:

Predicted neutrophil XCIP (A%:B%) = $[(y/100 \times A_{C)} + (z/100 \times \%A_T)] : [(z/100 \times \%B_T) + (y/100 \times B_C)]$

where y is the percentage mutant-positive cells, z is the percentage WT cells, the T-cell XCIP is $A_T\%:B_T\%$, and the ratio of inactivated A to B alleles in the V617F-positive colonies is $A_C:B_C$.

This calculation generates only a single predicted neutrophil XCIP, which was compared to the observed value (Table 5.4). For the six cases studied the median difference between the predicted and the observed neutrophil XCIP values was 12% (range 2-17%).

5.4 Discussion

The experiments reported in this chapter have investigated the possibility that there is more than one *JAK2* V617F-positive population in mutant-positive ET patients. Two different *JAK2* SNPs were exploited in order to identify the allele on which the V617F mutation had arisen, initially using a PCR-restriction enzyme digestion-PCR technique and then confirmed by TA cloning. Erythroid colonies were subsequently cultured from PB MNCs from V617F-positive female ET patients and mutant-positive colonies analysed to determine the inactivated X chromosome in order to investigate whether they shared a common clonal origin.

The results indicate that, in this small cohort of mutant-positive ET patients, the majority had evidence for more than one V617F-positive myeloid population. In 10 of the 11 patients studied the V617F mutation had arisen on both *JAK2* alleles. This implies that, in these individuals, the mutation was acquired on at least two different occasions. Because there are only two possible alleles on which the mutation could arise, it is impossible to determine the exact number of times the mutation has been acquired, except that it must have been more than once. Poisson statistics suggest that there is likely to be more than two independent mutational events in most cases: if there were just two events in a single individual, the probability of that person acquiring each event on different alleles is 0.5, and therefore on average only about half of the 11 individuals would be expected to harbour V617F on different alleles. In fact 10 of the 11 cases studied had biallelic acquisition of V617F, and the probability of this occurring by chance if each individual acquired the mutation just twice is small – equal to $11/2^{11}$ or 0.005. This suggests that the majority of the patients studied had more than two mutational events. By contrast, in all five PV patients there was no evidence for more than one mutational event.

In six ET patients, the mutation arose principally on the A-allele (Table 5.2, patients E2,E3,E6, E8, E9 & E10), in three it arose principally on the G-allele (patients E4, E7 & E11), and in one patient the distribution between the two alleles was approximately equal (patient E5). In the two patients in whom platelet or CD34+ cell cDNA was analysed (patients E8 and E9), the A:G ratio was comparable in these cells to that obtained in neutrophils. Likewise, analysis of samples from the one patient (E9) at separate time points yielded equivalent results. This suggests that the A:G ratio is similar regardless of cell lineage studied, which is compatible with the idea of the mutational events occurring in a multipotent progenitor, and that it remains stable over time.

The findings from the experiments using the *JAK2* intron 14 SNP (rs12343687) supported these conclusions. In the six patients who were heterozygous for this SNP, the PCR-restriction

enzyme digest-PCR technique showed that mutant-positive alleles were associated with a mix of C and T alleles. Data from the TA cloning approach was in keeping with the acquisition of *JAK2* V617F on both alleles in two of the three patients studied (E3 & E7; Table 5.2). In one patient, however, (E9; Table 5.2) the mutant-positive clones carried only A-alleles at the intron 14 SNP, even though the PCR-restriction enzyme digest-PCR technique suggested a mix of mutant-positive alleles. The reason for this discrepancy is unclear, but may be due to chance since only 14 mutant-positive clones were analysed.

Data from haplotype analysis (see Chapter 6) suggests that the C allele at rs12343687 and the A allele at rs2230724 are both part of the 46/1 haplotype. In keeping with this, the patient (E3) who predominantly acquired V617F on the C allele at rs12343687 also predominantly acquired the mutation on the A allele at rs2230724. Conversely, the two patients (E7 and E11) who predominantly acquired V617F on the T allele at rs12343687 also predominantly acquired the mutation on the G allele at rs2230724. On patient (E5) appeared to acquire the mutation equally on both alleles for both SNPs investigated. In two patients (E6 and E9) the two SNPs gave discordant results – in both cases the SNP rs12343687 data suggested that the mutation was acquired equally on both alleles whereas the SNP 2230724 data indicated preferential acquisition of the mutation on the A allele.

Overall, these results strongly suggest that ET patients may acquire *JAK2* V617F more than once. This is not the first evidence for the existence of multiple mutant-positive myeloid populations in MPN patients. Pardanani *et al* (2008) and Beer *et al* (2009) both reported that approximately 0.5-1% of MPN patients harboured more than one MPN-associated mutation (see Section 5.1.2). However, the data reported in this chapter goes further and suggests that the majority (10 of 11 patients studied, 91%) of ET patients have more than one V617F-positive population.

Data from Olcaydu *et al* (2009) supports the assertion that MPN patients may have more than one V617F-positive population, although they observed a markedly lower prevalence of this phenomenon. In their seminal study reporting the existence of a haplotype associated with acquisition of *JAK2* V617F, they analysed a PCR product spanning both the mutation site and the intron 14 SNP by allele-specific PCR and observed that V617F preferentially arose on the 46/1 haplotype allele (93 of 109 patients, 85%). In three cases (3%), however, the mutation appeared to have arisen on both alleles. The authors concluded that there was evidence for more than one V617F events in a minority of MPN patients. They speculated that the true prevalence was likely to be greater than 3% since patients in whom all the mutations had arisen on the same allele would appear to have just a single mutational event using this technique.

This does not, however, explain the marked difference between the proportion of patients with biallelic *JAK2* V617F mutations reported by Olcaydu *et a*l (2009) and that observed in our cohort – 3% and 91% respectively. It may be in part due to differences in the patient cohort. Of the five PV patients reported in this thesis, none had evidence for more than one V617F-positive event, and it may be that the frequency of this phenomenon is lower in PV than in ET. Consequently, the presence of PV patients in the Olcaydu *et al* (2009) series might have reduced the apparent frequency of biallelic mutations. Since the composition of their MPN cohort is not reported, this cannot be verified.

Another suggested explanation for this difference is that the apparent proportion of patients with biallelic mutations in our cohort was exaggerated by PCR artefact due to use of relatively high PCR cycle numbers, resulting in template switching during PCR extension and the generation of chimeric DNA molecules (Beer *et al,* 2010b). They argued that the true prevalence of more than one V617F event in ET is 5-10%. Although this is a possibility, if the findings presented in this chapter were due to random PCR error, it is improbable that two different samples, either from two different lineages or two separate time points, from the

same patient would consistently yield similar A:G ratios whilst samples from different patients produced different ones. Moreover, in all five PV patients studied *JAK2* V617F was present only on one allele (the A-allele), despite using identical methodology.

Regardless of the precise figure, it is apparent that a proportion of ET patients have more than one V617F-positive myeloid population. This does not, however, prove that the disorder is truly polyclonal, since all the mutant-positive populations may be derived from a pool of clonally-related progenitors which were themselves derived from a single transformed precursor, implying the existence of a pre-V617F event. In this case, in female patients all the V617F-positive cells would share a common inactivated X-chromosome. Alternatively, the mutant-positive cells may be derived from 'normal' polyclonal progenitors, implying that acquisition of *JAK2* V617F was the initial pathogenic event, and the disorder is truly oligo- or polyclonal. In this case the V617F-positive cells would not necessarily share a common inactivated X-chromosome. For this reason, establishing the clonal relationship of the V617F-positive cells was particularly important in providing an insight into the pathogenesis of the disease.

XCIP analysis of V617F-positive BFU-Es was performed in six patients. In one patient 38% of colonies inactivated one X-chromosome whilst 62% of colonies inactivated the other – a ratio which was similar to the patient's constitutional XCIP (31%:69%). It therefore appears that in this patient the mutation arose independently in more than one clonally unrelated progenitor. Results from the other five patients suggested that in these cases all the V617F colonies inactivated a single X-chromosome, in keeping with the progenitors which acquired *JAK2* V617F being phylogenetically related and therefore the progeny of a single transformed precursor. It is also possible, however, they were not clonally related and simply shared a common inactivated X-chromosome by chance. The chance of two unrelated progenitors sharing the same inactivated X-chromosome will depend on the patient's constitutional (i.e. T-cell) XCIP – for instance in patient E7 (Table 5.3), whose T-cell XCIP was 82%:18%, the chance

of the mutation arising in two unrelated progenitors which both inactivate the A-allele is approximately $(82\%)^2$ i.e. 67%. Given the relatively small number of colonies with XCIP results (between 3 and 37), it is possible that in some cases the colonies shared the same inactivated X-chromosome by chance.

If the inactivated X-chromosome observed in the V617F-positive colonies is taken to reflect the overall XCIP of the mutant-positive population, it can be used to predict the neutrophil XCIP of these six patients, based on the T-cell XCIP and the size of the mutant-positive population, as described in Section 5.3.5 (Table 5.4). In every case, the difference between the expected and the observed neutrophil XCIP was within the technical limits of the HUMARA (20%). This is in keeping with one of the conclusions drawn in Chapter 3 – that the neutrophil XCIP is a function of the T-cell XCIP, the mutant level, and the XCIP of the mutant-positive population – but refines it by accommodating the possibility that the V617F-positive cells may not necessarily all inactivate the same X-chromosome, as in patient E12.

Whilst it is not possible to draw firm conclusions from such a small cohort, the data suggests that at least some patients may harbour more than one V617F-positive population which are not clonally related and have arisen from polyclonal progenitors, although in the majority the mutant-positive populations are phylogenetically related and derived from a single transformed precursor. Studies in larger numbers of patients are required to confirm these observations.

These finding are compatible with those of Beer *et al* (2009) who determined the XCIP of the mutant-positive colonies in three female patients that carried two distinct mutant populations, either V617F and *MPL* W515, or V617F and a *JAK2* exon 12 mutation. They observed that in two, including one ET patient, the colonies carrying different mutations inactivated different X-alleles, proving that in these two patients the two clones had arisen independently and were not from a shared founder clone. Mutant-positive colonies from the third patient all expressed

Table 5.4 Prediction of neutrophil XCIP in 6 patients, based on their CD3-positive XCIP, mutant levels in neutrophil DNA and the XCIP of V617F-positive colonies

Patient	% *JAK2* V617F (neuts)	% V617F+ cells*	% WT cells	CD3+ XCIP		Observed neutrophil XCIP		XCIP in V617F-positive BFU-Es†		Predicted neutrophil XCIP‡		%difference (observed-predicted)
				A%	B%	A%	B%	**A%**	**B%**	A%	B%	
		'z'	'y'	'A_T'	'B_T'			'A_C'	'B_C'			
E6	35%	70%	30%	46	54	20	80	0	100	14	86	6
E7	23%	46%	54%	83	17	82	18	100	0	91	9	9
E8	13%	26%	74%	38	62	45	55	0	100	28	72	17
E12	16%	32%	68%	31	69	31	69	38	62	33	67	2
E13	5%	10%	90%	64	36	82	18	100	0	68	32	14
E14	10%	20%	80%	40	60	18	82	0	100	32	68	14

* Assumes V617F-positive cells are heterozygous for the mutation

† Based on inactivated X-chromosome ratios of V617F-positive colonies (Table 5.3)

‡ Using the calculation: predicted neutrophil XCIP (A%:B%) = $[(y/100 \times A_C) + (z/100 \times \%A_T)] : [(z/100 \times \%B_T) + (y/100 \times B_C)]$,
where y is the percentage mutant-positive cells, z is the percentage WT cells, the T-cell XCIP is $A_T\%:B_T\%$, and the ratio of inactivated A to B alleles in the V617F-positive colonies is $A_C:B_C$

the same X-chromosome. This would suggest that the sequence of mutational events varies between patients.

In keeping with this, studies which have attempted to determine the timing of acquisition of V617F in relation to other mutations have come to contradictory conclusions. Some published reports have suggested that *JAK2* V617F is primarily a secondary molecular event in MPN. Levine *et al* (06 2006) reported that in 23% of V617F-positive ET patients, the clonal myeloid population based on XCIP analysis was larger than the V617F-positive one. They argued that this implied the presence of a pre-*JAK2* clone, from which the *JAK2* V617F-population was derived. Kralovics *et al* (2006) came to the same conclusion, also by comparing XCIP and V617F data, as did Delhommeau *et al* (2009) but using a different method. Delhommeau *et al* (2009) employed haematopoietic colony analysis to determine the sequence of mutational events in five patients with *JAK2* V617F-positive *TET2*-mutated MPN, including one case of ET. In all five cases, they detected *TET2*-mutated haemopoietic colonies which were *JAK2* WT, but they did not identify any V617F-positive, *TET2* WT colonies. They interpreted this as evidence that in these patients acquisition of *TET2* mutations preceded acquisition of *JAK2* V617F. Similarly Beer *et al* (2010a) identified an individual with post-ET AML who had both *MPL*-mutated and V617F-positive populations, each of which was shown to be derived from a common *TET2*-mutated clone.

There is, however, evidence that *JAK2* V617F may precede other molecular abnormalities. Colony analysis by Schaub *et al* (2009) suggested that in three of nine MPN patients with both *JAK2* V617F and del20q, including 2 ET patients, V617F preceded del20q, whereas in two patients the reverse was true. In the remaining four cases there was evidence for multiple del20q and 9pLOH events, and the sequence was uncertain. The same group subsequently investigated eight patients with V617F-positive, *TET2*-mutant MPN and found that in four patients the *TET2* mutation appeared to arise first, in two patients V617F arose first, and in the remaining two patients there were two distinct populations each carrying a single mutation

(Schaub *et al,* 2010). Saint Martin *et al* (2009) also identified a single PV patient who appeared to have acquired *JAK2* V617F prior to *TET2* mutations.

Taken together, these reports emphasise the heterogeneity of the molecular pathogenesis of V617F-positive ET, and that the role of the mutation in the disorder is not straightforward. This concept is in keeping with the main finding from the experiments presented in this chapter – that a large proportion of ET patients have more than one V617F-positive population, and that in some patients these populations are clonally distinct whereas in others they are likely to be phylogenetically related. It thus appears that whilst some patients have a monoclonal mutant-positive population, others have an oligoclonal population with more than one independent V617F-positive clone. This latter situation is reminiscent of paroxysmal nocturnal haemoglobinuria (PNH). Multiple clones, each with a different mutation in the phosphatidylinositol glycan anchor (*PIGA*) gene, are seen in some PNH patients, and the extent to which the clones expand varies widely among patients (Parker 2007).

PNH is generally considered to be a benign disorder and shares a number of key characteristics with V617F-positive ET. In both conditions, the expansion of mutant-positive clones is limited and the peripheral blood is a relatively stable mosaic of normal and abnormal cells, the most common serious complications are thrombotic, and transformation to AML occurs more frequently than in haematologically normal individuals but is nonetheless uncommon (Parker 2007). Consequently some patients with *JAK2* V617F may have a benign disorder, and the presence of the mutation should not invariably be taken to indicate the presence of a malignant neoplasm.

These findings raise two further questions. If the mutation does arise repeatedly in V617F-positive ET patients, why does the mutant level remain stable, often for many years; and what causes a specific mutation which is not observed in normal controls to occur repeatedly in some individuals?

Addressing the first question, it is not known why repeated acquisition of *JAK2* V617F mutations in WT progenitors does not result in a progressive increase in the mutant level. One possibility is that the V617F-positive progenitors have a limited lifespan, resulting in an equilibrium in which the size of the V617F-positive population is determined by the rate at which old mutant-positive progenitors die and new ones arise. Alternatively, there may be some limit on the total size of the mutant-positive population, governed perhaps by the proliferative advantage conferred by *JAK2* V617F compared to its *JAK2* wild-type counterparts. If *JAK2* V617F is a second event and arises in pre-transformed progenitors, those *JAK2* WT progenitors which are also part of the pre-transformed clone may have a proliferative or survival advantage compared to truly normal progenitors. The relative advantage of acquiring *JAK2* V617F might then be less marked than if it were acquired on a normal background, and expansion of V617F-positive cells would be correspondingly slower. In this way it is possible that the nature of the pre-*JAK2* mutation determines the size of the stable V617F-positive population, and therefore the disease phenotype (ET or PV). The second question, why a specific mutation should occur repeatedly in some individuals, is even more intriguing but there is increasing evidence for an inherited predisposition to developing V617F-positive MPNs. The observation that PV patients and those with V617F-positive ET had a significant excess of A-alleles at the *JAK2* exon 19 SNP (Table 5.1) suggested an association between these disorders and this SNP. In fact, this SNP is part of a wider MPN-associated haplotype, a subject which is explored in detail in the next chapter.

In conclusion, the findings presented in this chapter suggest that some, and possibly most, patients with *JAK2* V617F-positive ET have more than one mutant-positive population. In many cases these appear to be clonally related and may therefore have arisen from a larger clonal population, but in at least some cases they are unrelated and may indicate that *JAK2* V617F has been acquired by a number of polyclonal haematopoietic progenitors. This is compatible with other studies which have indicated that ET is a heterogeneous disease, and suggests that

the role of *JAK2* V617F in the pathogenesis of ET is complex and not directly analogous to that of BCR-ABL in CML. Finally, these results show that at least some V617F-positive ET patients have an oligoclonal disorder, in keeping with its benign clinical course, and more generally they indicate that the presence of an acquired mutation in a haematological disorder does not necessarily imply the presence of a malignant disease.

6 Investigations into the 46/1 haplotype

6.1 Introduction

Inherited causes of thrombocytosis which show classical Mendelian inheritance are well described but uncommon (Table 1.3). Moreover, they do not behave like sporadic ET, since individuals with these conditions typically develop a polyclonal thrombocytosis at an early age, do not manifest the increased risk of thrombotic and leukaemic complications seen in the sporadic variant, and very rarely harbour the mutations associated with sporadic ET (Skoda 2009; Teofili *et al,* 2007). There is, however, increasing evidence that at least a proportion of patients with sporadic ET have an inherited predisposition to develop the disorder. This chapter will examine this phenomenon and investigate possible underlying mechanisms.

6.1.1 Familial clustering of ET

Familial clustering of individuals with classical, adult-onset ET and other MPNs has been repeatedly described (Brubaker *et al,* 1984; Fickers and Speck 1974; Janssen *et al,* 1990) but was for many years regarded as a rare phenomenon of questionable significance. It was thought that, whilst this might have suggested the existence of a genetic or environmental factor which predisposed to the development of MPNs in a small number of cases, it may equally well have been due to chance (Perez-Encinas *et al,* 1994). A recent Swedish Registry study (Landgren *et al,* 2008), however, has convincingly demonstrated that this clustering is unlikely to be due to chance. This study determined the relative risk (RR) of developing an MPN for 24,577 first-degree relatives of 11,039 MPN patients, compared to 99,542 first-degree relatives of 43,550 haematologically normal matched controls. The overall RR was 5.6 times

greater in relatives of MPN patients compared to relatives of controls, although it varied depending on the MPN subtype of the proband. First degree relatives of ET patients had a RR of 6.8 compared to control relatives of developing an MPN, not necessarily ET, but this figure was 4.9 and 2.7 for relatives of PV and MF patients respectively. Relatives of ET patients were at greatest risk of developing ET (RR = 8.0) but also had an increased risk of developing PV (RR = 5.4), whilst relatives of PV patients had a similar risk of developing both PV and ET (RR = 6.0 and 5.4 respectively).

Familial clustering of MPN cases, therefore, appears to occur more frequently than would be expected by chance, but the mechanism for this is unknown. Moreover, the majority of MPN cases do not arise in familial clusters and it is possible that the clustered cases represent a different disease than the much commoner sporadic ones, with a different pathogenesis. A number of recent reports, however, suggest that this is not the case and that MPN patients within family clusters frequently harbour the same genetic mutations as those with the sporadic disorder.

Bellanné-Chantelot *et al* (2006) screened 46 'MPN families' – defined as having at least two members, mostly first-degree relatives, diagnosed with MPN – for the presence of *JAK2* V617F. They found that in 6 families the mutation was not detectable in any individual, in 22 families all affected individuals carried the mutation, and most significantly, in 18 families some affected individuals carried the mutation whilst others did not. This last finding suggested that *JAK2* V617F was not a prerequisite for development of MPN, even in patients with V617F-positive relatives. Furthermore, in those family members with V617F-positive MPN, the mutation was detectable in myeloid cells but, with one exception, not in their lymphoid cells. None of their haematologically normal relatives harboured the mutation. This would suggest that the mutation was acquired rather than constitutional. Combined with the observation that in some families only a proportion of members with an MPN carried *JAK2* V617F, this might imply the existence of a heritable factor which predisposed to acquisition of several

mutations, including *JAK2* V617F, which in turn contributed to development of the MPN. Another group subsequently reported similar findings in their study of 10 Italian MPN families investigated with regards to *JAK2* V617F status (Rumi *et al,* 2006). They observed that in four families some, but not all, of the affected individuals carried *JAK2* V617F, and in the V617F-positive individuals the mutation was confined to myeloid cells. Of the 11 informative female MPN patients studied using XCIP analysis, eight had clonal haemopoiesis, of whom seven were V617F-positive.

The similarity between cases of sporadic MPN and those occurring within family clusters was further highlighted by a later study which reported on 458 patients with apparently sporadic MPN (Rumi *et al,* 2007). Detailed investigation of family members revealed that 35 (8%) of these 458 patients in fact had a relative with an MPN. The natural history of the disorder was similar regardless of whether or not the patient had an identifiable relative with MPN. Of note, second-generation patients appeared to develop the disorder significantly earlier than first-generation ones and exhibited telomere shortening, indicative of disease anticipation. As noted in the previous studies, in some families only a proportion of MPN patients were *JAK2* V617F-positive.

In summary, there is increasing evidence for the existence of a mechanism which confers an inherited susceptibility to acquiring MPN-associated mutations such as *JAK2* V617F in the familial MPN clusters. Because sporadic MPN shares a number of clinical and molecular characteristics with MPN occurring in familial clusters, such a mechanism may be common to both variants. One possibility is that in the familial variant there is a germline mutation in an unknown gene which results in a tendency to acquire further genetic events, and that this mutation is acquired in the truly sporadic condition. Since mutations in *TET2* occur in up to 17% of sporadic MPN patients (Delhommeau *et al,* 2009; Tefferi *et al,* 2009), and often precede *JAK2* V617F in patients who harbour both mutations, and because *TET2* is believed to be a tumour suppressor gene, inherited mutations in this gene were proposed as a mechanism

for familial MPNs (Saint-Martin *et al,* 2009). However, two studies have failed to support the suggestion that germline mutations in *TET2* underlie the familial occurrence of MPN (Olcaydu *et al,* 2010; Saint-Martin *et al,* 2009).

6.1.2 The 46/1 haplotype

Currently, therefore, no single mutation has been found to convey an increased risk of acquiring MPN-associated mutations. Instead, there is evidence that a specific haplotype within the *JAK2* gene may do so, at least in sporadic MPN. Three initial studies reported an association between this haplotype and V617F-positive MPNs, though it was unclear whether the haplotype also predisposed to V617F-negative MPNs (Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009). All three studies, published simultaneously, arrived at similar conclusions using different approaches.

Jones *et al* (2009) set out to investigate whether *JAK2* V617F arose in association with a particular *JAK2* haplotype by studying six *JAK2*-spanning SNPs (Figure 6.1): rs7864782 (A/G), rs10758669 (A/C), rs7046736 (A/C), rs1234241 (G/C), rs10974947 (A/G) and rs 2031904 (A/G). They selected 142 MPN patients who were heterozygous for these SNPs and had a mutant level greater than 50%, which was taken to indicate the presence of 9pLOH. They predicted that 9pLOH should result in preferential amplification of the SNP allele which was in *cis* with the mutant allele. By determining the allelic ratios at each SNP using pyrosequencing, they established the SNP allele sequence on which the mutation had arisen. In 109 (77%) of the 142 patients studied, the mutation had been acquired in *cis* with one particular haplotype, characterised by the SNP allele sequence ACAGGG at the six loci studied.

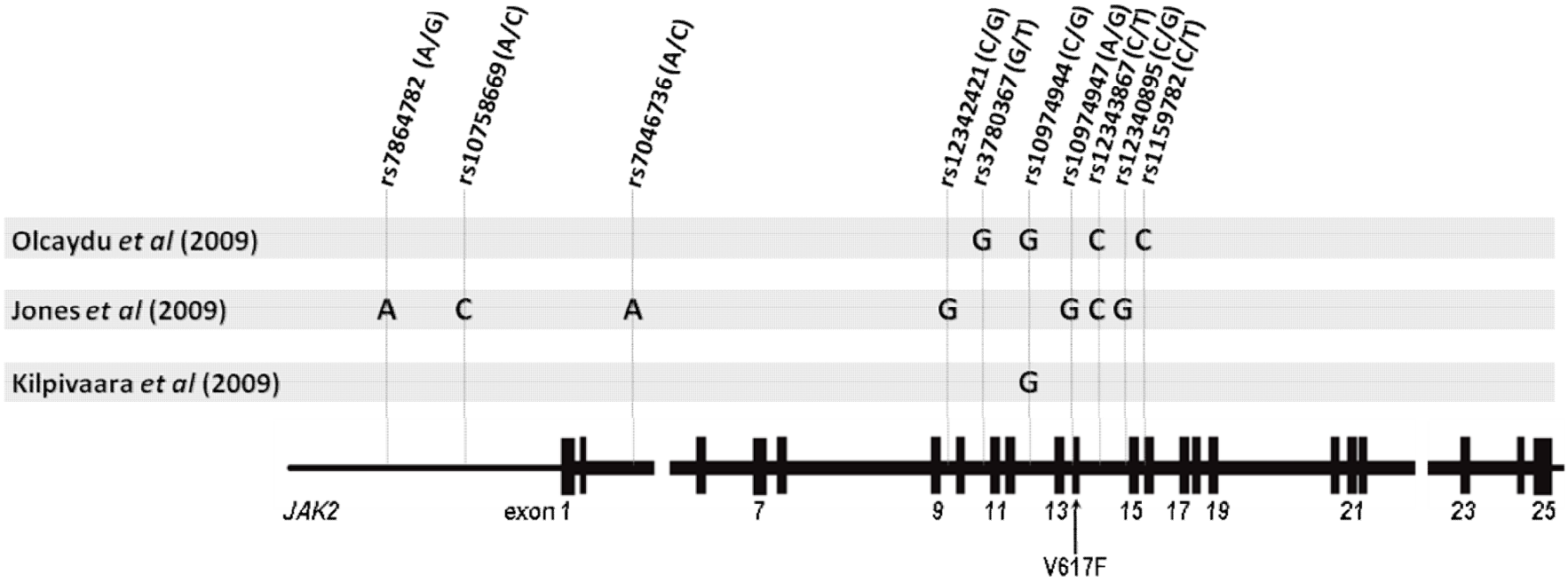


Figure 6.1 *JAK2* SNPs investigated in the initial haplotype studies

The 46/1-associated SNP alleles are shown beside the reference

They then analysed the Wellcome Trust Case Control Consortium (WTCCC) SNP data from 1,500 UK blood donors and concluded that, in these haematologically normal controls, a 280kb linkage disequilibrium (LD) block existed spanning *JAK2* and two neighbouring genes, *INSL4* and *INSL6*. Within this block, 92 *JAK2* haplotypes were apparent and one of these, designated number 46, had the allele sequence ACAGGG at the six SNPs initially studied. Another haplotype, number 1, had five of these six alleles at the same loci. Jones *et al* (2009) focused on a total of 20 *JAK2* SNPs, and at 19 of these, haplotypes 1 and 46 were mutually identical and were therefore referred to jointly as the 46/1 haplotype. This combined haplotype represented 24% of the WTCCC cohort. Using C/G SNP rs12340895, which was not one of the six SNPs originally investigated but was in complete LD with the 46/1 haplotype, they determined the haplotype frequency amongst a larger cohort of MPN patients and haematologically normal controls. The G allele, which tagged the 46/1 haplotype at rs12340895, was over-represented in PV and V617F-positive ET patients, and in one of the two groups of V617F-negative ET patients studied. Using allele-specific PCR, they then amplified a fragment spanning V617F and rs12343867, a C/T SNP located in *JAK2* intron 14, which was also in LD with 46/1. Sixty-six patients who were heterozygous for both the mutation and the SNP were investigated and in 49 (74%) cases the mutation arose in *cis* with the C allele, which tagged the 46/1 haplotype. They concluded that the 46/1 haplotype predisposed to the development of V617F-associated MPNs, either by increasing the somatic mutation rate at this locus (the 'hypermutability' hypothesis) or by enhancing the selective advantage of the mutation when acquired in the presence of it (the 'fertile ground' hypothesis).

Olcaydu *et al* (2009) arrived at similar conclusions in their study which initially set out to examine the relationship between *JAK2* V617F, del13q and del20q in a patient with MF using haemopoietic colony analysis. They noted that two V617F-positive populations were apparent, each of which carried a different cytogenetic abnormality and appeared to have acquired *JAK2* V617F independently. Like Jones *et al* (2009), they used allele-specific PCR to amplify a product

spanning V617F and SNP rs12343867 in *JAK2* intron 14, but here the purpose was to screen MPN patients for multiple V617F events. Analysis of 109 V617F-positive MPN patients who were heterozygous for this SNP revealed that in three cases (2.8% of the cohort) the mutation had arisen in *cis* with both SNP alleles in a single individual, in keeping with more than one V617F event. Moreover, in the 106 individuals in whom there was no evidence of multiple V617F events, the mutation had arisen in *cis* with the C allele in 93 of them (88%). Examination of a larger cohort of 333 MPN patients, of whom 213 were V617F-positive, revealed an association between the C allele at SNP rs12343867 and V617F-positivity. Similar analysis of a further seven *JAK2* SNPs revealed that at three of them, rs3780367, rs10974944 and rs1159782, a specific allele (G,G and C respectively) was associated with V617F-positivity. The authors concluded that this defined a haplotype, termed 'GGCC', which predisposed to acquisition of *JAK2* V617F. Their work suggested that this haplotype was confined to a 10kb region of *JAK2*, located within the larger LD block identified by Jones *et al* (2009). Since this 10kb haplotype did not contain the promoter regions of the gene, which would affect its expression, they suggested that a difference in mutability between the two haplotypes might explain their findings.

In the third study, Kilpivaara *et al* (2009) performed genome-wide SNP analysis on neutrophil DNA from 181 ET and PV patients to investigate the hypothesis that germline variation contributed to both predisposition to MPNs and phenotypic differences between the disorders. They observed that, compared to 1500 haematologically normal controls, V617F-positive MPN patients – but not, *JAK2* WT ones – were significantly more likely to have a G rather than a C allele at SNP rs10974944, located in *JAK2* intron 12. This association was present regardless of whether neutrophil or buccal DNA from the MPN patients was studied, suggesting it was an inherited phenomenon and not due to somatic abnormalities in neutrophils, such as acquired UPD of chromosome 9. Using allele-specific PCR to amplify a DNA fragment spanning both the SNP and *JAK2* exon 14, they also noted that 38 of 45 (84%) V617F-

positive patients who were heterozygous for the SNP had acquired the mutation in *cis* with the G allele. They concluded that their data indicated the presence of a *JAK2* SNP allele which favoured the in-*cis* acquisition of *JAK2* V617F.

Taken together, the three seminal studies described above suggested that V617F-positive MPN patients were more likely to have a specific *JAK2* haplotype than haematologically normal controls, and that the mutation usually, but not invariably, arose in *cis* with this haplotype. Subsequent studies using a single tagged SNP have confirmed the association between the 46/1 haplotype and V617F-positive, but not *JAK2* WT, MPNs (Andrikovics *et al,* 2010; Pardanani *et al,* 2010; Trifa *et al,* 2010).

Establishing whether or not the association is confined to V617F-positive disorders is important since it will strongly indicate which of the two postulated mechanisms is more likely. If the association extends to *JAK2* WT MPN, the argument for the 'fertile ground' hypothesis is greatly strengthened, since it is difficult to explain how a *JAK2* haplotype could influence the mutability of other genes. Consequently, the recent finding that the 46/1 haplotype is associated with *MPL* W515 mutations is potentially of great significance and supports the 'fertile ground' hypothesis, since the *MPL* gene is located on chromosome 1p (Jones *et al,* 2010). This study pooled data from UK, German, Italian and Greek cohorts and reported that the frequency of 46/1 alleles in 176 *MPL* W515-mutated ET patients was significantly higher than in controls (0.37 versus 0.28, P=0.0003). However much of this difference was due to a relatively high 46/1 allele frequency in the small Greek and Italian cohorts (*n* = 24 and 27 respectively) of 0.46 and 0.43 respectively. By contrast, in the larger UK and German cohorts (*n* = 56 and 69 respectively), the 46/1 allele frequency was 0.34 and 0.36 respectively, and not significantly higher than in controls (P=0.164 and 0.267 respectively). Furthermore, a similar study by Patnaik *et al* (2010) found that the 46/1 allele frequency amongst 22 *MPL* W515-positive MPN patients (0.25) was not significantly higher than in local or WTCCC controls. The association between the 46/1 haplotype and *MPL* W515 is, therefore, not strong. It is possible

that both the 'hypermutability' and 'fertile ground' mechanisms are involved, and this would explain the stronger association between the haplotype and V617F-positive MPN than with *JAK2* WT MPN.

6.1.3 Possible mechanisms underlying the association between the 46/1 haplotype and MPNs

In order to explain the 'fertile ground' mechanism, Jones *et al* (2009) speculated on the existence of a functional variant in LD with 46/1 which interacted with *JAK2* V617F so that acquisition of the mutation on the background of this haplotype was more likely to result in clinical disease than if it arose on the non-46/1 haplotype. They postulated that, if the 46/1 haplotype was preferentially expressed, then in-*cis* acquisition of *JAK2* V617F might result in increased expression of the mutant allele, potentially resulting in a proliferative advantage for the mutated cells. They, therefore, investigated expression of two *JAK2* SNPs which were part of the 46/1 haplotype – rs2230724 in exon 19 in 29 normal controls, and rs10429491 in exon 6 in 40 normal controls – and found that at both loci expression of the 46/1 allele was no different to that of the non-46/1 allele. Since only normal controls were examined, however, the possibility of aberrant expression of the 46/1 allele in individuals with MPN, possibly due to a functional interaction with *JAK2* V617F, could not be excluded.

Another possible mechanism for the 'fertile ground' hypothesis postulated by Jones *et al* (2009) is that the 46/1 haplotype is in LD with a functional variant which results in different baseline myeloid proliferative activity in 46/1-positive individuals compared to non-46/1 individuals. Acquisition of *JAK2* V617F might have different effects in these different contexts. They therefore, assessed myeloid colony formation in 56 normal controls with and without the 46/1 haplotype. Peripheral blood derived from individuals who were either homo- or

heterozygous for 46/1 grew significantly fewer granulocyte-macrophage colony-forming units than blood from individuals who were homozygous for the non-46/1 haplotype, although there was no difference in the number of BFU-Es. The authors interpreted this as evidence that the 46/1 haplotype affected myeloid proliferation and that this was more suggestive of the 'fertile ground' rather than 'hypermutability' hypothesis. MPN patients were not studied, and the authors did not speculate further on potential mechanisms for this functional interaction. Two studies have evaluated presentation blood counts in 46/1-positive and 46/1-negative MPN patients and found no difference in any parameter, suggesting that if any difference exists it is very subtle (Jones *et al,* 2010; Pardanani *et al,* 2010).

Although no association between the 46/1 haplotype and blood counts has yet been described, a genome-wide analysis of 13,943 patients did identify an association between another chromosome 9 SNP and variability in platelet counts (Soranzo *et al,* 2009). This SNP, rs505534, is located in an intergenic region of chromosome 9p23 approximately 8 megabases upstream from *JAK2*. If one of the rs505534 alleles is associated with a platelet count towards the upper limit of normal then acquisition of a mutation such as *JAK2* V617F or *MPL* W515 by a patient with a particular rs505534 genotype might be sufficient to generate the ET phenotype. In this case, an association might be expected between a particular rs505534 SNP allele and ET.

A further hypothesis to explain the association between V617F-positive MPNs and the 46/1 haplotype which has not been explored to date is that the latter affects *JAK2* methylation. In the presence of the 46/1 haplotype, at least two SNPs, rs12343867 and rs12340895, both located in *JAK2* intron 14, generate CpG dinucleotides which are lost in the absence of the 46/1 haplotype. Since cytosine bases within CpG dinucleotides may be methylated to form 5-methylcytosine, the presence of the 46/1 haplotype might thus alter the number of CpG dinucleotides available for methylation. Aberrant gene methylation has been implicated in the pathogenesis of many human malignancies, including numerous haematological cancers

(Esteller 2003; Feinberg and Vogelstein 1983). The best described mechanism involves the hypermethylation of promoter regions of tumour suppressor genes which normally prevent cells with DNA damage from proliferating. The consequent reduction in their expression allows genetically-mutated cells to accumulate (Esteller 2008). Even when promoter regions are not affected, aberrant methylation can predispose to hypermutability by altering the DNA conformation and inhibiting DNA mismatch repair (Muheim *et al,* 2003). Furthermore, the tendency of 5-methylcytosine to undergo spontaneous deamination to thymine (Coulondre *et al,* 1978) can also lead to hypermutability.

Mutations in *TET2*, a member of the *TET* family of putative tumour suppressor genes which are believed to be involved in epigenetic regulation, have been described in a small number of ET patients and it has been suggested that these may result in abnormal methylation (Mullighan 2009; Tahiliani *et al,* 2009). It is possible that mutations in *TET2*, or other genes regulating methylation, could interact with the 46/1 haplotype and lead to aberrant *JAK2* methylation thereby predisposing to MPN by increasing the *JAK2* mutation rate.

6.1.4 Aims

The work in this chapter aimed to study the incidence of the 46/1 haplotype in the cohort of patients described earlier in this thesis, and to investigate possible mechanisms for the 'fertile ground' and 'hypermutability' hypotheses, namely preferential expression of the 46/1 haplotype, an association between an rs505534 allele and MPN, and aberrant *JAK2* methylation in the presence of the 46/1 haplotype.

6.2 Patients, materials and methods

6.2.1 Screening *JAK2* V617F-positive patients for the 46/1 haplotype using 5 *JAK2* SNPs

Initially, DNA from the 11 well-characterised ET patients described in sections 5.2.2 and 5.2.3 was screened for the presence of the 46/1 haplotype by PCR and restriction enzyme digestion. Five SNPs which were reported to be associated with the 46/1 haplotype were selected (Figure 6.2). Primers were designed to amplify a fragment spanning the SNP and then allow discrimination between the two possible alleles by restriction enzyme digestion. For two SNPs (rs3780367 and rs10974944), restriction enzymes were available which could distinguish between the two SNP alleles directly but for the other three SNPs mismatched primers were required to generate a cutting site. Using the reagents and conditions described in Table 6.1, GoTaq® PCR (see Section 2.2.3.2) was performed using T-cell gDNA as template for each of the five SNPs to amplify a PCR product which was then digested using the specified restriction enzyme. A digestion mix was prepared containing 5 units of the appropriate enzyme, 1x appropriate buffer and where specified 100µg/mL BSA. The mix was made up to 3µL with ddH_2O and to this 7µL PCR product was added. The resulting mix was incubated using the conditions shown in Table 6.1. Following 3% agarose gel electrophoresis, digestion products were visualised by UV transillumination and the alleles identified by their size (Table 6.1). All primer sequences are listed in Appendix 1.

6.2.2 Screening for the presence of the 46/1 haplotype using SNP rs12343867

To screen the whole ET patient cohort and a group of haematologically normal controls for the presence of the 46/1 haplotype, each individual was genotyped for a single tagged SNP,

Table 6.1 PCR and restriction digestion conditions used to determine the genotype of five SNPs within the *JAK2* gene

SNP name (locus on *JAK2* gene)	Possible alleles (relative frequency in control population)*	Allele in 46/1 haplotype	Primers (T_a, expected size)	Restriction enzyme (incubation conditions, buffer)	Expected post-digestion allele sizes (bp)
rs3780367 (intron 10)	G/T (0.26/0.74)	G	*JAK2*ex10/F *JAK2*ex10/R (59°C, 172bp)	*Mse*I (37°C for 4 hours, buffer 2, BSA)	G: 24 + 148 T: 21 + 24 + 127
rs10974944 (intron 12)	C/G (0.75/0.25)	G	*JAK2*ex12/F2 *JAK2*ex12/R2 (59°C, 205bp)	*Bcl*I (50°C for 4 hours, buffer 3)	G: 31 + 174 C: 31 + 37 + 137
rs12343867 (intron 14)	C/T (0.29/0.71)	C	*JAK2*ex14/F2 *JAK2*ex14/(MM)R (59°C, 164bp)	*Bsa*AI (37°C for 4 hours; buffer 3)	C: 31 + 133 T: 164 (uncut)
rs12340895 (intron 14)	C/G (0.74/0.26)	G	*JAK2*ex14/(MM)F3 *JAK2*ex14/R2 (58°C, 178bp)	*Bst*UI (60°C for 2 hours; buffer 4)	G: 22 + 156 C: 178 (uncut)
rs1159782 (intron 15)	T/C (0.74/0.26)	C	*JAK2*ex15/(MM)F *JAK2*ex15/R2 (56°C, 169bp)	*Sca*I (37°C or 4 hours; buffer 4)	C: 24 + 145 T: 169 (uncut)

* From HAPMAP European Cohort

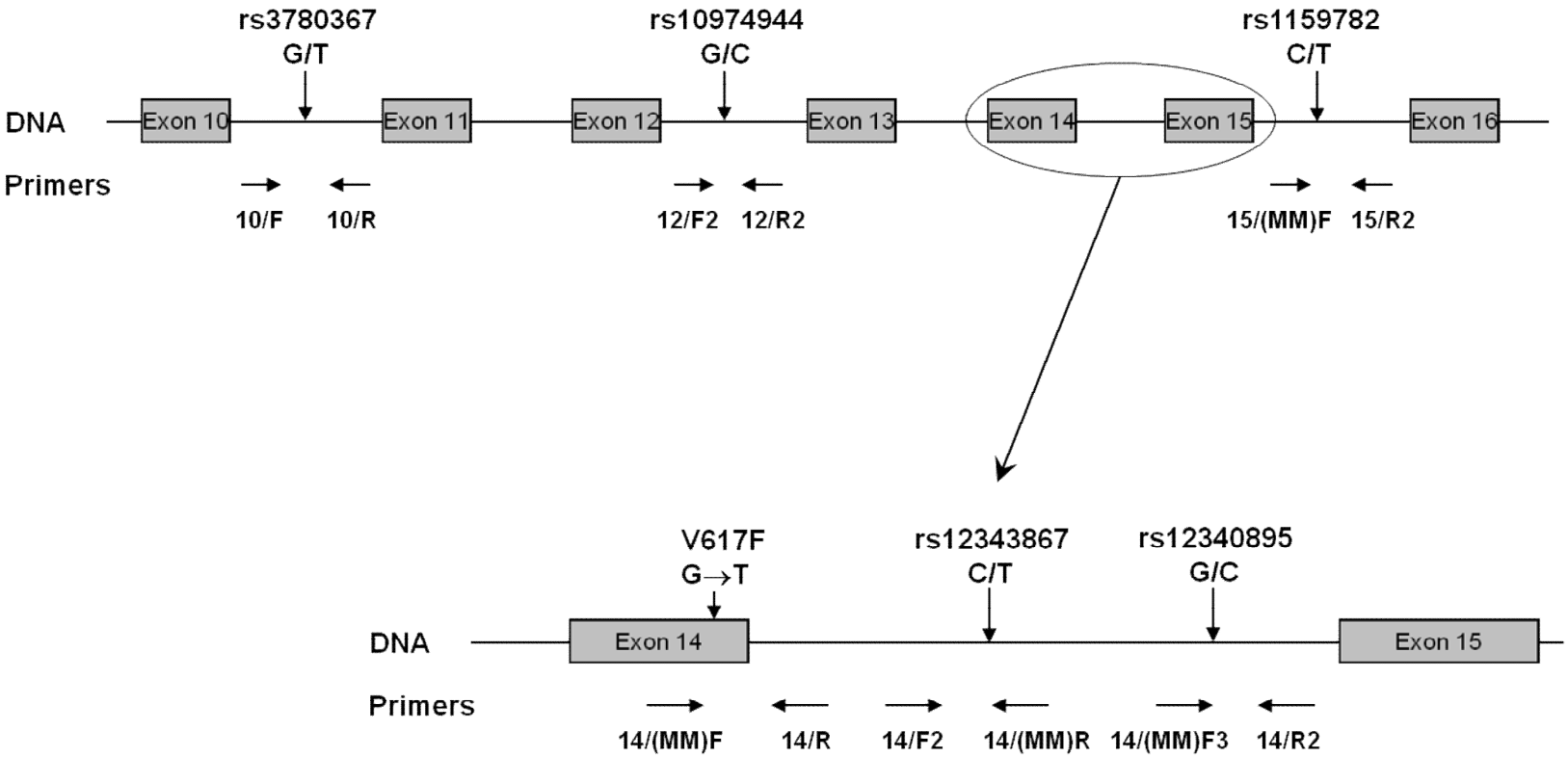


Figure 6.2 Schematic representation of *JAK2* exons 10 to 16 showing the five SNPs studied and their corresponding PCR primers

For each SNP, the 46/1-associated allele is given first

rs12343867, which is in complete LD with the 46/1 haplotype. Using primers *JAK2*ex14/F2 and *JAK2*ex14/(MM)R which spanned this SNP (Figure 6.2), a 164bp PCR product was amplified from T-cell gDNA by GoTaq® PCR with 35 cycles of PCR and an annealing temperature of 59°C. A digestion mix was then prepared containing 5 units *Bsa*AI enzyme and 1x manufacturer's buffer 3, made up to 3µL with ddH_2O, and to this 7µL PCR product was added. The resulting mix was incubated for 4 hours at 37°C. Following 3% agarose gel electrophoresis, digestion products were visualised by UV transillumination. *Bsa*AI cut the C-allele to 133 and 31bp but did not cut the T-allele (164bp).

6.2.3 Expression analysis of the 46/1 and non-46/1 alleles

To investigate the hypothesis that preferential expression of one of the 46/1 alleles might account for the association between *JAK2* V617F and the 46/1 haplotype, the relative expression of the two alleles (A/G) of rs2230724, a 46/1-associated SNP located in exon 19 of *JAK2* was analysed using cDNA. A PCR-restriction enzyme digestion approach was adopted to distinguish between the two alleles, and the relative amounts of each one quantified by fragment analysis.

6.2.3.1 Validation of the technique using neutrophil gDNA

To ensure the technique was quantitative, analysis was initially performed using neutrophil gDNA from 22 normal controls who were heterozygous for rs2230724 where a 1:1 allelic ratio was expected. A 214bp fragment covering *JAK2* exon 19 and part of the flanking introns was amplified by PCR using BIOTAQ™ polymerase as described in Section 2.2.3.1, with primers *JAK2*ex19/F and fluorescently-labelled *JAK2*ex19/RF, an annealing temperature of 60°C, 25 cycles of PCR, and approximately 50ng of neutrophil gDNA as template per reaction (Figure 6.3A). A digestion mix was prepared containing 5 units *Bst*NI, 1x manufacturer's buffer 2,

100µg/mL BSA, made up to 3µL with ddH_2O and 5µL PCR product added. This was incubated for 2 hours at 60°C then separated by fragment analysis using the CEQ 8000 Genetic Analysis system. *Bst*NI cut the G allele to 121 and 93bp but the A allele remained uncut (214bp). The relative amount of each allele was calculated from the area under the appropriate peak and expressed as a proportion of the total area of both peaks.

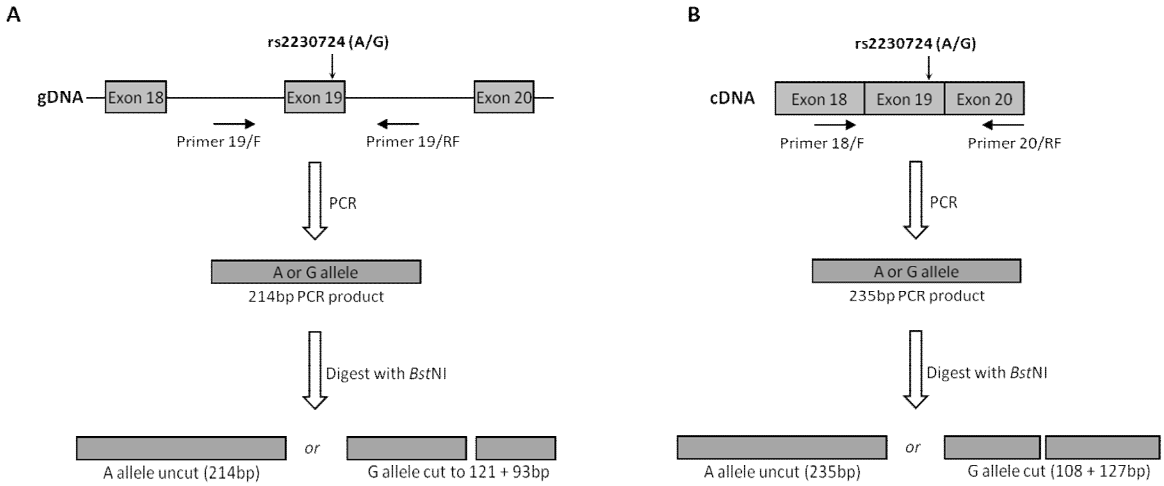


Figure 6.3 Schematic representation of PCR of *JAK2* exon 19 followed by *Bst*NI digestion to determine expression of A and G alleles at SNP rs2230724

A. Using gDNA as template

B. Using cDNA as template

6.2.3.2 Expression analysis in neutrophil cDNA

To be suitable for analysis, subjects had to be heterozygous for the SNP and have neutrophil RNA available. Neutrophil cDNA was generated using RNA from normal controls, *JAK2* V617F-positive ET patients, and *JAK2* WT ET patients using the Invitrogen Superscript III kit as described in Section 2.2.5. A 235bp fragment spanning *JAK2* exons 18-20 was amplified by BIOTAQ™ PCR with primers *JAK2*ex18/F and fluorescently-labelled *JAK2*ex20/RF (Figure 6.3B).

Twenty-five cycles of PCR were performed, with an annealing temperature of 62°C and using 2µL of the cDNA diluted 1 in 4 in ddH_20 as template per reaction. The PCR product was incubated with *Bst*NI as above which cut the G allele to 108 and 127bp but left the A allele uncut (235bp), analysed on the CEQ 8000 system, and the relative amounts of the A and G alleles determined by calculating the area under the appropriate peaks.

6.2.4 Analysis of SNP rs505534

The cohort of ET patients was genotyped for rs505534, a C/T SNP located on chromosome 9p, 8Mb upstream of the *JAK2* gene, to investigate the possibility that it might be a 46/1-associated variant. A 222bp fragment spanning the SNP was amplified using GoTaq® PCR (see Section 2.2.3.2) with T-cell gDNA as template, a mismatched forward primer rs505534/(MM)F, reverse primer rs505534/R, an annealing temperature of 57°C and 35 cycles of PCR. Products were digested with *Bsm*AI. A reaction mix was prepared containing 5 units *Bsm*AI and 1x manufacturer's buffer 4, made up to 2µL with ddH_2O, and to this 8µL PCR product was added and incubated for 2 hours at 55°C, then run on a 3% agarose gel. *Bsm*AI cut the C allele to 116, 75 and 29bp, and the T allele to 145 and 75bp.

6.2.5 Methylation analysis

To investigate the possibility that the 46/1 haplotype may affect *JAK2* methylation, bisulphite sequencing followed by pyrosequencing was used to investigate the methylation status of two *JAK2* intron 14 loci: the rs12343867 C/T SNP, and the cytosine nucleotide immediately upstream of the rs12340895 C/G SNP, both of which potentially form a CpG dinucleotide in the presence of the 46/1 haplotype (Figures 6.4A,B). This approach allowed methylated and non-

methylated cytosine bases to be distinguished since treatment of DNA with bisulphite converts unmethylated cytosine to uracil, which is converted to thymine by subsequent PCR, but does not affect 5-methylcytosine. The ratio of cytosine to thymine at the locus being investigated, which can be determined by pyrosequencing, reflects the degree of methylation in the original DNA.

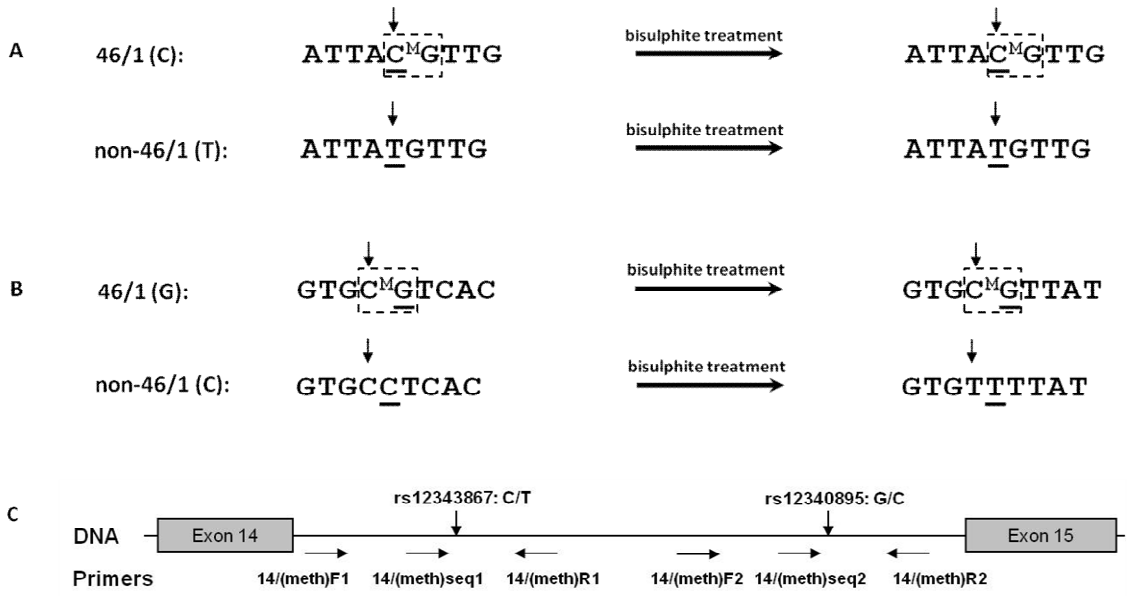


Figure 6.4 Effect of bisulphite treatment on possible CpG dinucleotides that involve SNPs rs12343867 and rs12340895 (*JAK2* intron 14)

Assumes that C nucleotides immediately prior to G nucleotides are methylated (C^M), and 100% conversion of unmethylated C nucleotides to T nucleotides. Vertical arrows indicate the nucleotide for which the methylation status was determined by pyrosequencing, and the possible CpG dinucleotide is indicated by a dashed box

A. DNA sequence around C/T SNP rs12343867 (underlined)
B. DNA sequence around C/G SNP rs12340895 (underlined)
C Schematic representation showing primers used for methylation assays

6.2.5.1 Bisulphite treatment

Bisulphite treatment was performed using the Zymo EZ DNA methylation-gold kit (Cambridge Bioscience Ltd, Cambridge UK) according to manufacturers' instructions. Neutrophil gDNA, 500ng in 20µL ddH_2O, was added to 130µL CT conversion agent, mixed and incubated at 98°C for 10 minutes, 64°C for 2.5 hours, then stored at 4°C for up to 20 hours. The reaction mixture was added to 600µL M-binding buffer in a Zymo-Spin™ IC column, mixed and spun at 14,000g for 30 seconds at room temperature. The run-through liquid was discarded and 100µL M-wash buffer was added to the column, which was spun again for 30 seconds at 14,000g. The run-through was discarded and 200µL M-desulphonation buffer was added to the IC column, incubated at room temperature for 20 minutes, then centrifuged for 30 seconds at 14,000g at room temperature. Two washes were performed each consisting of the addition of 200µL M-wash buffer to the IC column and a 30 second room-temperature spin at 14,000g. The IC column was then transferred to a 1.5mL microcentrifuge tube and 16µL M-elution buffer added. The bisulphite-treated DNA was eluted by centrifugation at 14,000g for 30 seconds and stored at -20°C.

6.2.5.2 Qualitative analysis of SNP rs12343867 methylation status

The methylation status of C/T SNP rs12343867 within *JAK2* intron 14 was determined non-quantitatively by direct sequencing of T-cell DNA from 2 haematologically normal controls, one known to be CC, the other TT at this locus. A 198bp product spanning rs12343867 was amplified by GoTaq® PCR using 1.5µL bisulphite-treated DNA as template, with forward primer *JAK2*ex14/(meth)F1 and reverse primer *JAK2*ex14/(meth)R1 (Figure 6.4C). Other reagents and conditions were as described in Section 2.2.3.2. Thirty-three cycles of PCR were performed, each 30 seconds at 95°C, 30 seconds at 56°C, 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. PCR products were visualised by UV transillumination following 2% agarose gel electrophoresis, then purified using the peqGold micro-spin cycle-pure kit and sequenced

by the UCL Wolfson Institute for Biomedical Research DNA sequencing service using *JAK2*ex/14(meth)F1 as the sequencing primer.

6.2.5.3 Quantitative analysis of SNP rs12343867 methylation status

Pyrosequencing was used to quantify the relative amounts of the two alleles (C or T) present at the SNP following bisulphite treatment, from which the relative degree of methylation could be calculated. If the sample analysed was known to be homozygous CC at the SNP, and these cytosine bases were completely methylated, then the post-bisulphite treatment C:T ratio is predicted to be 100%:0%. If the actual percentage C (%C) determined by pyrosequencing was less than 100%, this would imply incomplete methylation of the cytosine at this SNP. Similarly, if the sample was heterozygous CT, the predicted %C is 50%, and an observed %C of less than this would indicate incomplete methylation.

Generation of a calibration curve

In order to establish whether the assay was quantitative throughout its range, a calibration curve was first established using standards generated by mixing T-cell DNA from two normal controls, one known to have rs12343867 genotype CC, the other TT. Results from the previous stage indicated the C allele was completely methylated in control samples (see Section 6.3.5.1.). The control DNA samples were therefore mixed to give an expected %C of 0%, 2%, 5%, 10%, 25%, 50%, 75%, 90%, 95%, 98% and 100% and then bisulphite treated using the Zymo EZ DNA methylation-gold kit as described above. A 198bp PCR product spanning the SNP was then amplified in a 50µL GoTaq® PCR using 2.5µL bisulphite-treated DNA with forward primer *JAK2*ex14/(meth)F1 and a biotinylated reverse primer *JAK2*ex14/(meth)R1, both at 0.08mM. Fifty cycles of PCR were performed, each of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 45 seconds followed by a 5 minute 72°C extension stage. Samples were analysed using the PyroMark™Q96 MD pyrosequencing system (Qiagen, Crawley, UK) according to manufacturers' recommendations by the UCL Wolfson Institute for Biomedical Research DNA sequencing

service, using *JAK2*ex14/(meth)seq1 as the sequencing primer. For each of the standard mixes, the %C determined by pyrosequencing was then plotted against the expected %C.

Analysis of neutrophil gDNA

To be suitable for analysis, subjects were either heterozygous or homozygous for the C allele at SNP rs12343867. Neutrophil gDNA from a cohort of normal controls and ET patients was bisulphite treated, and the 198bp PCR product spanning SNP rs12343867 amplified by PCR and analysed by pyrosequencing as described in the preceding paragraph. Samples were analysed in duplicate.

6.2.5.4 Quantitative analysis of methylation status near to SNP rs12340895

The degree of methylation of the cytosine immediately upstream of a second C/G SNP, rs12340895, was analysed in the same group of normal controls and ET patients (Figure 6.4B). A 192bp PCR product spanning SNP rs12340895 was amplified from bisulphite-treated neutrophil gDNA with conditions as described in Section 6.2.5.3 except that here forward primer *JAK2*ex14/(meth)F2 and biotinylated reverse primer *JAK2*ex14/(meth)R2 were used (Figure 6.4C). Samples were then analysed by pyrosequencing using primer *JAK2*ex14/(meth)seq2 as the sequencing primer.

6.3 Results

6.3.1 Screening *JAK2* V617F-positive patients for the 46/1 haplotype

The 11 ET patients studied in detail in Chapter 5 were initially selected for analysis. For each patient, the genotype at each of five *JAK2* SNPs was determined (Table 6.2). Of the 11 patients, five were homozygous at all five SNPs: TT, CC, TT, CC and TT at rs3780367, rs10974944,

rs12343867, rs12340895 and rs1159782 respectively. From these patients a haplotype could be deduced, with the allele sequence TCTCT, i.e. these five patients were homozygous for the non-46/1 haplotype. The remaining six patients were heterozygous at all 5 SNPs: GT, GC, CT, GC and CT. It therefore appeared that these six patients were heterozygous for the TCTCT haplotype determined above, and for a second haplotype, GGCGC, i.e. the 46/1 haplotype. No patient was homozygous for the 46/1 haplotype and none was homozygous at some SNPs and heterozygous at others.

Table 6.2 *JAK2* SNP genotypes of 11 *JAK2* V617F-positive ET patients

SNP	rs3780367	rs10974944	rs12343867	rs12340895	rs1159782	rs2230724
***JAK2* locus**	Intron 10	Intron 12	Intron 14	Intron 14	Intron 15	Exon 19*
46/1 allele	G	G	C	G	C	
Patient E1	TT	CC	TT	CC	TT	AG
Patient E2	TT	CC	TT	CC	TT	AG
Patient E3	GT	GC	CT	GC	CT	AG
Patient E4	TT	CC	TT	CC	TT	AG
Patient E5	GT	GC	CT	GC	CT	AG
Patient E6	GT	GC	CT	GC	CT	AG
Patient E7	GT	GC	CT	GC	CT	AG
Patient E8	TT	CC	TT	CC	TT	AG
Patient E9	GT	GC	CT	GC	CT	AG
Patient E10	TT	CC	TT	CC	TT	AG
Patient E11	GT	GC	CT	GC	CT	AG

* Determined in section 5.2.1

6.3.2 Genotyping normal controls and ET patients for the presence of the 46/1 haplotype using SNP rs12343867

SNP rs12343867, which tagged the 46/1 haplotype, was genotyped in 101 haematologically normal controls, 122 ET patients and 24 PV patients (Table 6.3). Within each patient category, the total number of 46/1 (i.e. C) alleles and of non-46/1 (T) alleles was calculated, assuming

that patients who were CC homozygous at rs12343867 contributed two C alleles, those who were heterozygous contributed one C allele, and TT homozygotes contributed none, and vice-versa to calculate the total number of T alleles. The frequency of C alleles (as a proportion of total alleles) in each patient group was then compared to those observed in the control cohort, and to equivalent data published by Jones *et al* (2009) from the WTCCC analysis of 1,500 UK blood donors.

Table 6.3 SNP rs12343867 genotypes of the normal controls, ET and PV patients studied

			rs12343867 genotype* *n* (percent of patients)		
		n	**CC**	**CT**	**TT**
Normal controls		101	8 (8%)	42 (42%)	51 (50%)
ET patients					
Overall		122†	15 (12%)	50 (40%)	57 (46%)
By *JAK2* status	V617F+	46†	6 (13%)	23 (50%)	17 (37%)
	V617F-	76	9 (12%)	27 (36%)	40 (53%)
By exon 19 SNP status‡	AA	39	11 (28%)	19 (49%)	9 (23%)
	AG	55	4 (7%)	26 (47%)	25 (45%)
	GG	17	0 (0%)	0 (0%)	17 (100%)
PV patients		24	2 (8%)	16 (67%)	6 (25%)

* The C allele is part of the 46/1 haplotype

† The rs12343867 status was not known for 3 *JAK2* V617F-positive ET patients

‡ The exon 19 SNP status was not known for 14 ET patients.

Complete 9pLOH in myeloid cells would convert a patient who was heterozygous for the *JAK2* SNPs in their germline DNA to homozygosity in myeloid cells and confound the calculation above. To prevent this, T-cell gDNA was used for normal controls and ET patients. Since T-cell gDNA was not available for PV patients, only those with V617F levels of less than 80% were selected – i.e. without evidence of complete 9pLOH.

The frequency of the C allele at SNP rs12343867 was 0.33 in ET patients overall, 0.38 in V617F-positive ET patients, 0.29 in *JAK2* WT ET patients, 0.42 in PV patients and 0.29 in local controls. In no patient group was the frequency of the C allele significantly different from local controls (P=0.41, P=0.14, P=0.91 and P=0.09 respectively, Chi square test; Table 6.4). If PV patients were combined with V617F-positive ET patients, the C allele frequency in this group was just significantly higher than in local controls (0.39 versus 0.29, P=0.05, Chi-square test; OR 1.61, 95%CI 1.02-2.54). Compared to the WTCCC cohort of normal controls, the rs12343867 C allele frequency was significantly higher in ET patients overall, in V617F-positive ET patients, and in PV patients (P=0.003, P=0.003 and P=0.008 respectively, Chi square test) but not in *JAK2* WT ET patients (P=0.14, Chi square test).

The exon 19 SNP studied in Section 5, rs2230724, has been reported to be part of the 46/1 haplotype (Jones *et al,* 2009). In the cohort studied here, an association was noted between the presence of an A allele at rs2230724 and the C allele at rs12343867 (P=0.02, Chi-square test; Table 6.4), indicating that the A allele is in LD with the 46/1 haplotype.

Of the three *MPL* W515-positive patients, two were heterozygous for the 46/1 haplotype and one was homozygous for the non-46/1 haplotype.

Table 6.4 Frequency of rs12343867 alleles in each group studied and comparison with local and WTCCC controls

Group	*n*	C alleles	T alleles	C alleles/ total alleles	Comparison versus local controls			Comparison versus WTCCC controls		
					P-value	OR	95% CI	P-value	OR	95% CI
Local controls	101	58	144	0.29				0.15	1.28	0.93 - 1.75
WTCCC controls*	1500	720	2280	0.24	0.15	1.28	0.93 - 1.76			
ET patients										
All patients	122	80	164	0.33	0.41	1.21	0.81 - 1.81	0.003	1.55	1.17 - 2.04
V617F-positive patients	46	35	57	0.38	0.14	1.53	0.91 - 2.56	0.003	1.94	1.27 - 3.00
JAK2 WT patients	76	45	107	0.29	0.91	1.04	0.66 - 1.66	0.140	1.33	0.93 - 1.91
AA genotype at exon 19 SNP	39	41	37	0.53	0.0003	2.75	1.6 - 4.72	<0.0001	3.50	2.23 - 5.52
AA or AG genotype at exon 19 SNP	94	75	113	0.40	0.02	1.65	1.08 - 2.51	<0.0001	2.10	1.55 - 2.85
PV patients	24	20	28	0.42	0.09	1.77	0.93 - 3.40	0.008	2.26	1.27 - 4.04
V617F-positive ET and PV patients combined	70	55	85	0.39	0.05	1.61	1.02 - 2.54	<0.0001	2.05	1.45 - 2.91

All P-values were derived using the chi-square test

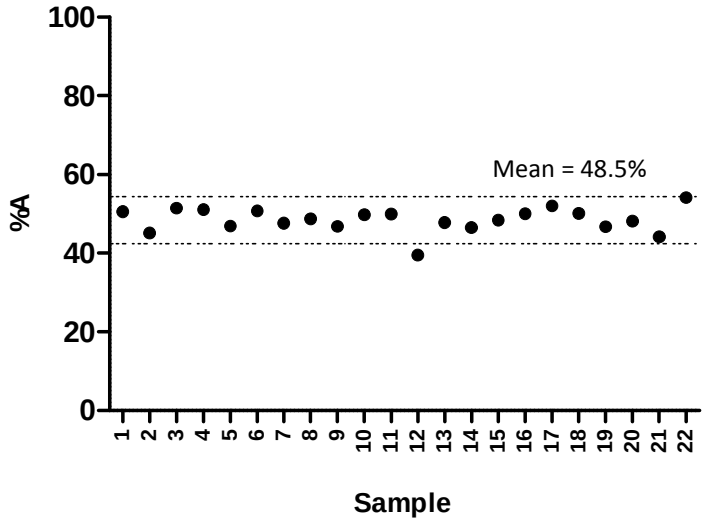
* Taken from Jones *et al* (2009)

6.3.3 Expression analysis of the 46/1 and non-46/1 alleles

In order to investigate the hypothesis that differential expression of the 46/1 haplotype might account for its association with V617F-positive MPN, the relative expression of the 46/1-associated and non-46/1 alleles was analysed at the *JAK2* exon 19 SNP, rs2230724. The A allele at this locus is in LD with the 46/1 haplotype (see previous section). In patients who were heterozygous for the *JAK2* exon 19 SNP, rs2230724, the relative expression of the two possible alleles (A/G) was determined by PCR and restriction enzyme digestion with *Bst*NI, using a fluorescently-labelled reverse primer to allow quantification by fragment analysis. Validation that the technique was quantitative was performed using neutrophil gDNA from 22 controls, where a 50%:50% ratio of A to G alleles was expected. The mean level of the A allele, which is part of the 46/1 haplotype, in these subjects was 48.5% of the total, with an SD of 3.2%, giving a technical range of 42.1 – 54.9% (mean ±2SD).

This technique was then used to determine relative expression of the two SNP alleles in individuals who were heterozygous at the SNP and had neutrophil cDNA available. Eight normal controls, 17 *JAK2* V617F-positive and 8 *JAK2* WT ET patients were analysed. There was no difference in the relative expression of the A allele expression between the groups, with a mean level of 54%, 52% and 52% in normal controls, V617F-positive ET and *JAK2* WT ET respectively (Figure 6.5B).

A.



B.

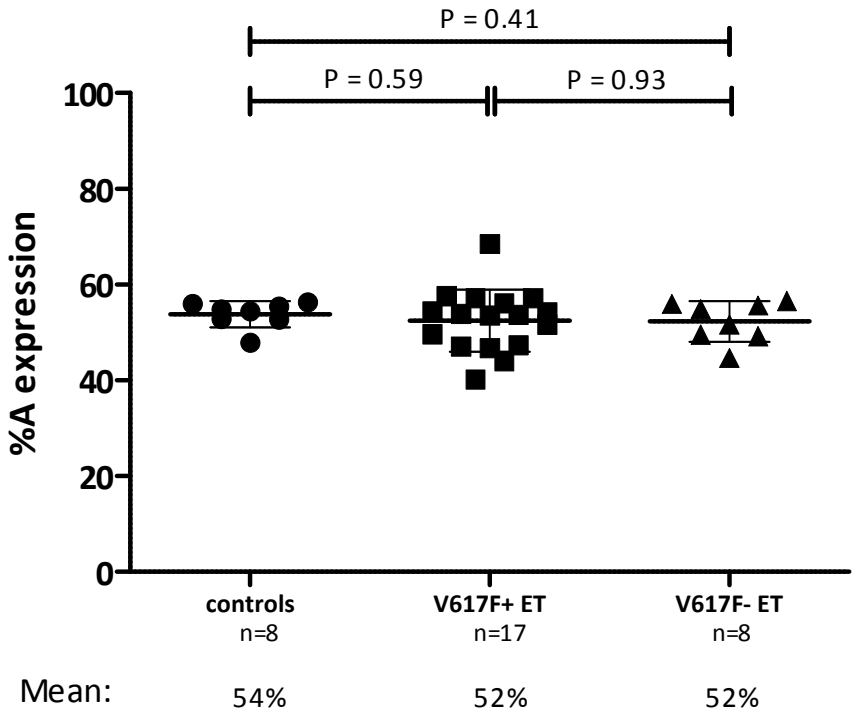


Figure 6.5 Quantification of the relative levels of SNP rs2230724 alleles A/G

A. Levels of allele A in neutrophil genomic DNA from 22 normal controls
   Dashed lines indicate mean ± 2 standard deviations (42.1 – 54.9%)

B. Levels of allele A expressed in neutrophil cDNA from normal controls, *JAK2* V617F-positive ET patients, and *JAK2* wild-type patients.
   Figures immediately above the graph give two-tailed t-test P-values, for the indicated comparisons

6.3.4 Analysis of SNP rs505534

The C/T SNP, rs505534, located on chromosome 9p, has been reported to be associated with variability in platelet count (Soranzo *et al,* 2009). The genotype frequency of this SNP was therefore examined to determine whether it was in LD with the 46/1 haplotype and thereby provide a mechanism for the association between 46/1 and MPNs. According to HapMap data, the relative frequency of the C and T alleles in Northern European controls is 0.975 and 0.025 respectively, giving an expected CC, CT and TT genotype frequency of 95%, 5% and 0% respectively. Of the 122 ET patients analysed, the rs505534 genotype was available for 114. The genotype was CC in 109 (96%) patients, CT in 5 (4%) and no patient was genotype TT. There was, therefore, no association between rs505534 genotype and ET, regardless of *JAK2* V617F, rs2230724 or rs12343867 genotype (Table 6.5).

Table 6.5 SNP rs505534 genotypes of 114 ET patients

Group	*n*	Total alleles	C-alleles	T-alleles	Frequency of C allele (% of total)	P-value (Chi-square)
Expected	114	228	222*	6*	98	
All ET patients	114	228	223	6	98	1.00
ET patients by *JAK2* status						
V617F-positive patients	45	90	88	2	98	1.00
JAK2 WT patients	67	134	131	3	98	1.00
ET patients by SNP rs2230724 genotype						
AA genotype	36	72	70	2	97	1.00
ET patients by SNP rs12343867 genotype						
CC genotype	13	26	24	2	92	0.19
CT genotype	45	90	87	3	97	0.71
TT genotype	53	106	106	0	100	0.18

*Based on HapMap Northern European control cohort (frequency of C allele = 0.975, T allele = 0.025)

6.3.5 Methylation analysis of normal controls and ET patients

6.3.5.1 Qualitative analysis of SNP rs12343867 methylation status

At two *JAK2* SNPs, rs12343867 and rs 12340895, the 46/1 haplotype introduces two CpG dinucleotides which are not present the non-46/1 haplotype. In order to investigate the hypothesis that the 46/1 haplotype may affect *JAK2* methylation, the degree of methylation at these two 46/1-associated *JAK2* SNPs was determined. Neutrophil gDNA was bisulphite treated to convert unmethylated cytosine bases to thymine but leave methylated cytosine bases unchanged. Direct sequencing of the bisulphite-treated DNA from 2 normal controls showed that the individual with SNP rs12343867 genotype CC had a single 'C' peak at the appropriate nucleotide, in keeping with complete methylation at that locus (Figure 6.6B). All the other cytosine bases within the amplicon had been converted to thymine. As expected, the individual with rs12343867 genotype TT had a single 'T' peak at the appropriate nucleotide (Figure 6.6C).

6.3.5.2 Quantitative analysis of SNP rs12343867 methylation status

Quantitative analysis of the degree of methylation at this SNP was then performed by pyrosequencing. To establish linearity of the assay throughout the range, 11 DNA mixes of CC and TT gDNA were analysed, with an expected %C which varied between 0% and 100%, assuming that all C alleles were completely methylated. A good correlation was observed between the expected %C in the calibration samples and the observed %C at the rs12343867 locus ($P<0.0001$, $r^2=0.999$; Figure 6.7). The Y-intercept was 2.5%, indicating that at the lower end of the calibration range, the assay overestimated the %C by 2.5%. At the upper end of the calibration range, the assay underestimated the %C by 4%, i.e. when the sample contained 100% C, the observed %C was 96%, due either to technical error or to incomplete methylation of C alleles.

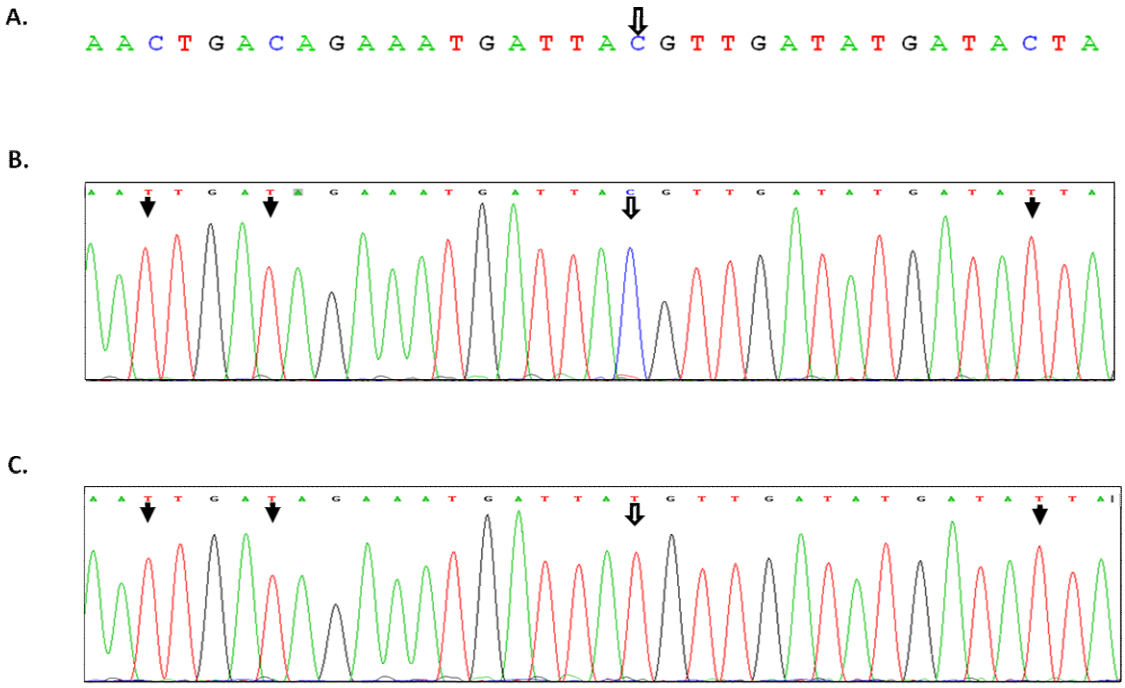


Figure 6.6 Sequences of normal control DNA showing the region around SNP rs12343867 (C/T)

A. DNA sequence prior to bisulphite treatment. SNP rs12343867 is indicated by a hollow arrow

B. Sequence chromatogram following bisulphite treatment in a normal control with rs12343867 genotype CC. Solid arrows indicate unmethylated cytosine bases which have been converted to thymine bases

C Sequence chromatogram following bisulphite treatment in a normal control with rs12343867 genotype TT

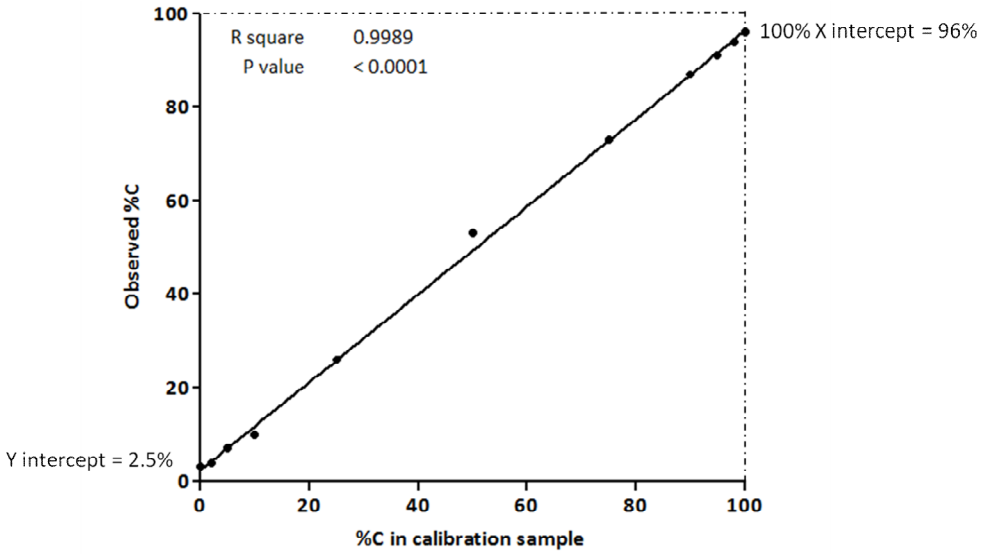


Figure 6.7 Calibration curve of pyrosequencing analysis of cytosine methylation at SNP rs12343867 using artificially created mixes

For SNP rs12343867 results were obtained for 12 normal controls (all CT heterozygotes) and 64 ET patients (examples shown in Figure 6.8). The assay was performed in duplicate, with good concordance for the two results for each sample ($P<0.0001$, $r^2=0.996$; Figure 6.9). The mean difference between values in the two runs was 0.1% (range 0-8%, SD=1.3%), indicating a technical variation of 5.3% (mean±2SD).

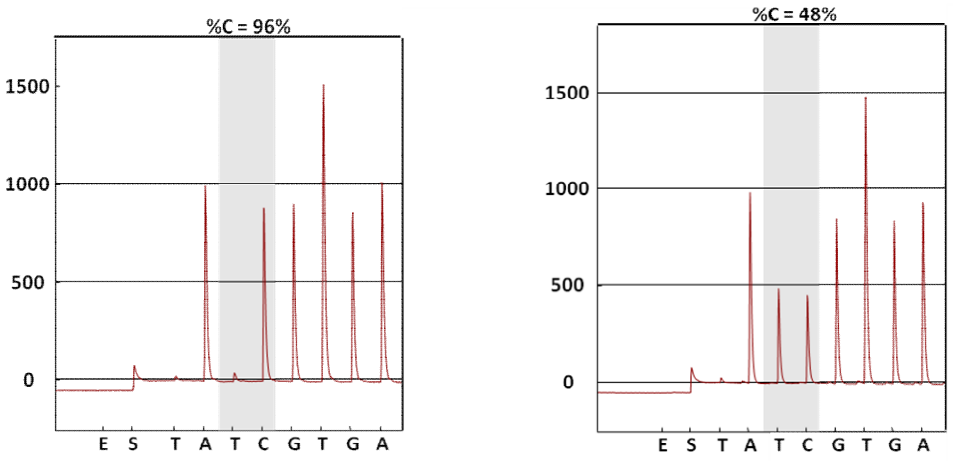


Figure 6.8 Pyrosequencing of C/T SNP rs12343867 following bisulphite treatment of neutrophil DNA

SNP rs12343867 is indicated by the shaded grey box. The relative percentage of each allele is calculated using the peak heights

A Pyrosequencing trace from an ET patient with SNP genotype CC

B Pyrosequencing trace from an ET patient with SNP genotype CT

The maximum %C at the SNP locus was dependent on the individual's SNP genotype – i.e. for CC individuals, it was 100% and for CT individuals it was 50%. The results are therefore analysed according to SNP genotype (Figure 6.10). For SNP heterozygous (CT) individuals the median %C was 48% in controls (range 46-52%), 47% in *JAK2* WT ET patients (range 44-64%) and 48% in V617F-positive ET patients (range 44-75%). No patient group was different from the controls. Of note, seventeen SNP heterozygous individuals had %C levels greater than the theoretical maximum of 50%, of whom nine had %C more than 2 standard deviations above

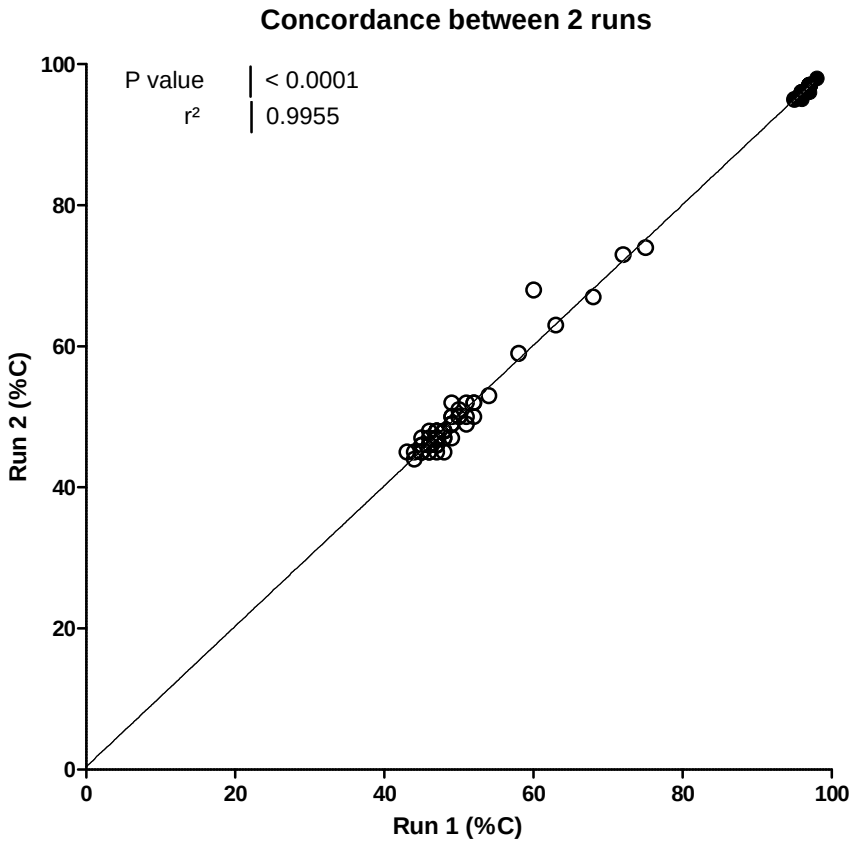


Figure 6.9 Concordance between duplicate pyrosequencing analyses of the percentage methylation at SNP rs1234386 in normal controls and ET patients

Open circles indicate SNP heterozygous individuals, closed circles indicate SNP homozygous individuals

the group median, comprising two *JAK2* WT patients and seven V617F-positive patients. Five of the seven V617F-positive patients had mutant levels greater than the median mutant level for the group (19%).

For SNP homozygous (CC) individuals, the median %C was 96% in *JAK2* WT ET patients (range 95-97%) and 97% in V617F-positive ET patients (range 96-97%). Reference to the calibration curve suggests that within technical limits these values were equivalent to 100% methylation

(Figure 6.7). The difference in median %C between *JAK2* WT and V617F-positive groups was not significant (P=0.19, t-test).

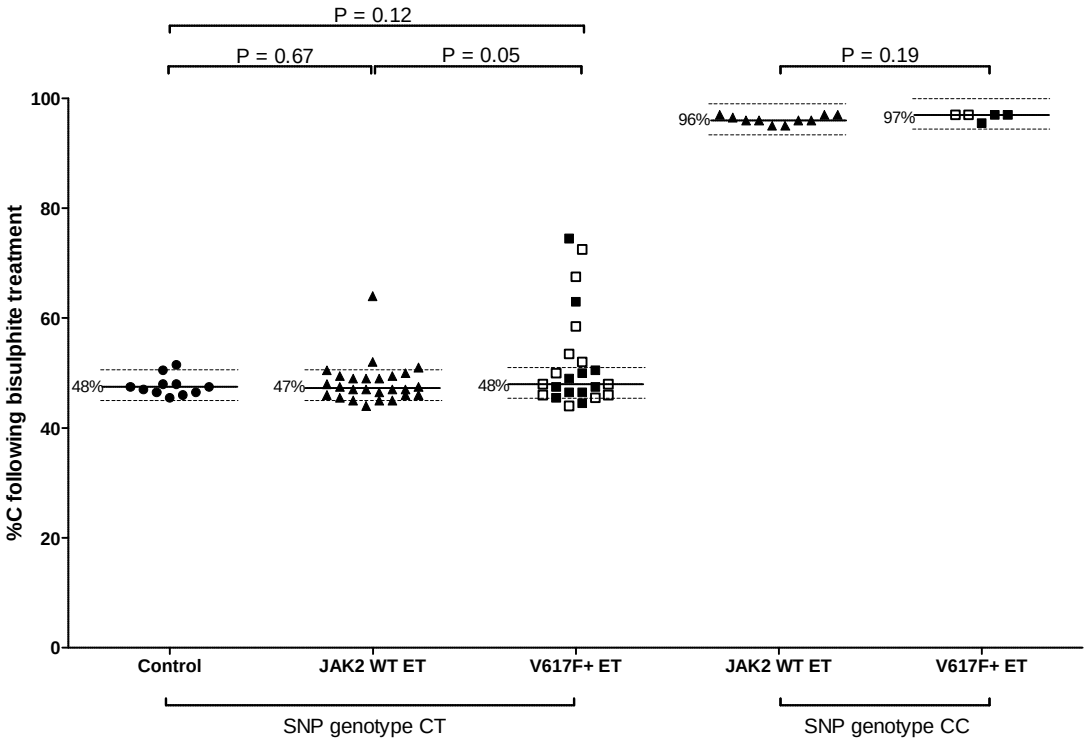


Figure 6.10 Pyrosequencing analysis of methylation at SNP rs12343867 in normal controls and ET patients

Solid horizontal lines indicates median (value shown to the left); dashed lines indicate median ±2 standard deviations

For the V617F-positive patients, patients with mutant levels below group median (19%) are represented by filled squares, those with mutant levels above group median are represented by open squares

6.3.5.3 Quantitative analysis of methylation status near to SNP rs12340895

The methylation of the cytosine base which was immediately upstream of C/G SNP rs12340895 was then studied (Figure 6.4B). At this locus, a CpG dinucleotide was only possible when a G allele was present at SNP rs12340895. For individuals who were GG homozygous at the SNP, CpG dinucleotides were generated on both alleles and their maximum expected %C was, therefore, 100%. For CG SNP heterozygotes, the maximum expected %C was 50%. For SNP heterozygous individuals the median observed %C was 23% in controls (range 20-27%), 21% in *JAK2* WT ET patients (range 18-27%) and 24% in V617F-positive ET patients (range 18-52%; Figure 6.11). The difference between the latter two groups was statistically significant (P=0.009, t-test), due to five patients in the V617F-positive group with %C greater than two standard deviations above the group median. No difference between any of the other groups was observed. For SNP homozygous individuals, the median %C was 96% in both *JAK2* WT ET patients (range 23-97%) and V617F-positive ET patients (range 96-97%) in keeping with complete methylation, within technical limits. One JAK2 WT ET patient who was classified as GG homozygous at this SNP had an observed %C of 23%, suggesting a sampling error and that this patient was SNP heterozygous.

6.3.5.4 Comparison of methylation data for SNP rs12343867 and SNP rs12340895

There was a strong correlation between the observed %C at the two SNPs studied (r^2=0.887, P<0.001, Figure 6.12). The four SNP-heterozygous individuals with the highest %C at SNP rs12343867 also had the highest %C at SNP rs12340895.

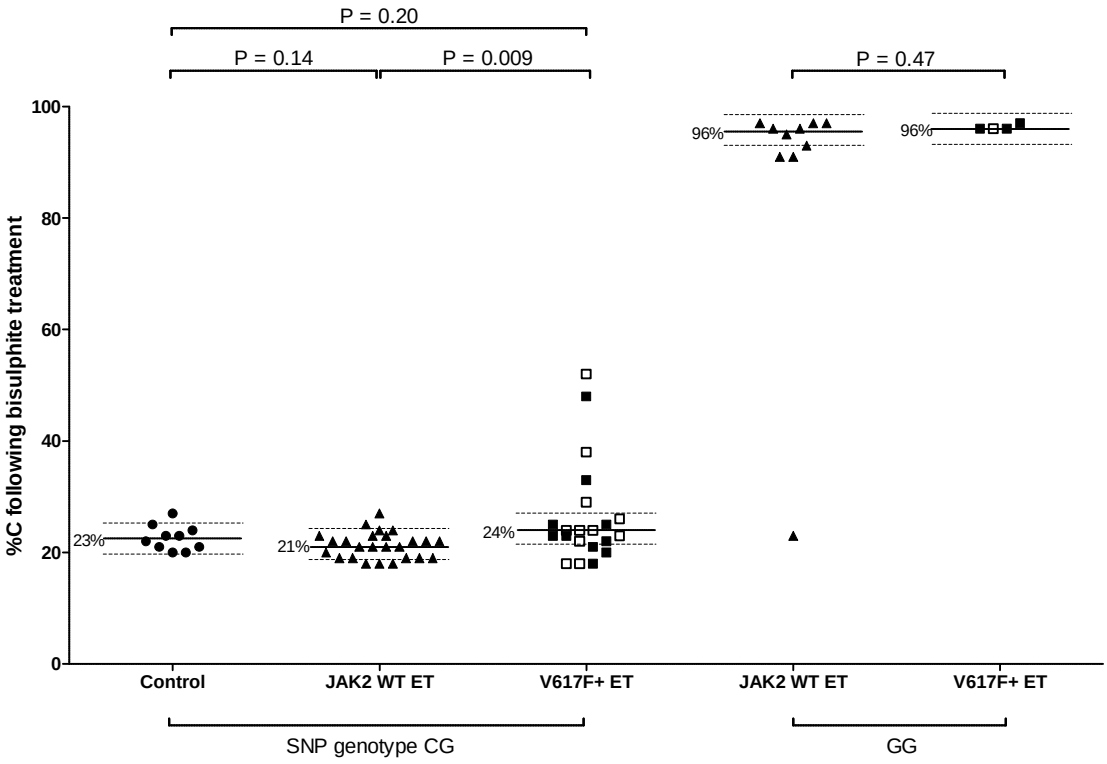


Figure 6.11 Pyrosequencing analysis of methylation at SNP rs12340895 in normal controls and ET patients

Solid horizontal lines indicates median (value shown to the left); dashed lines indicate median ±2 standard deviations

For the V617F-positive patients, patients with mutant levels below group median (19%) are represented by filled squares, those with mutant levels above group median are represented by open squares

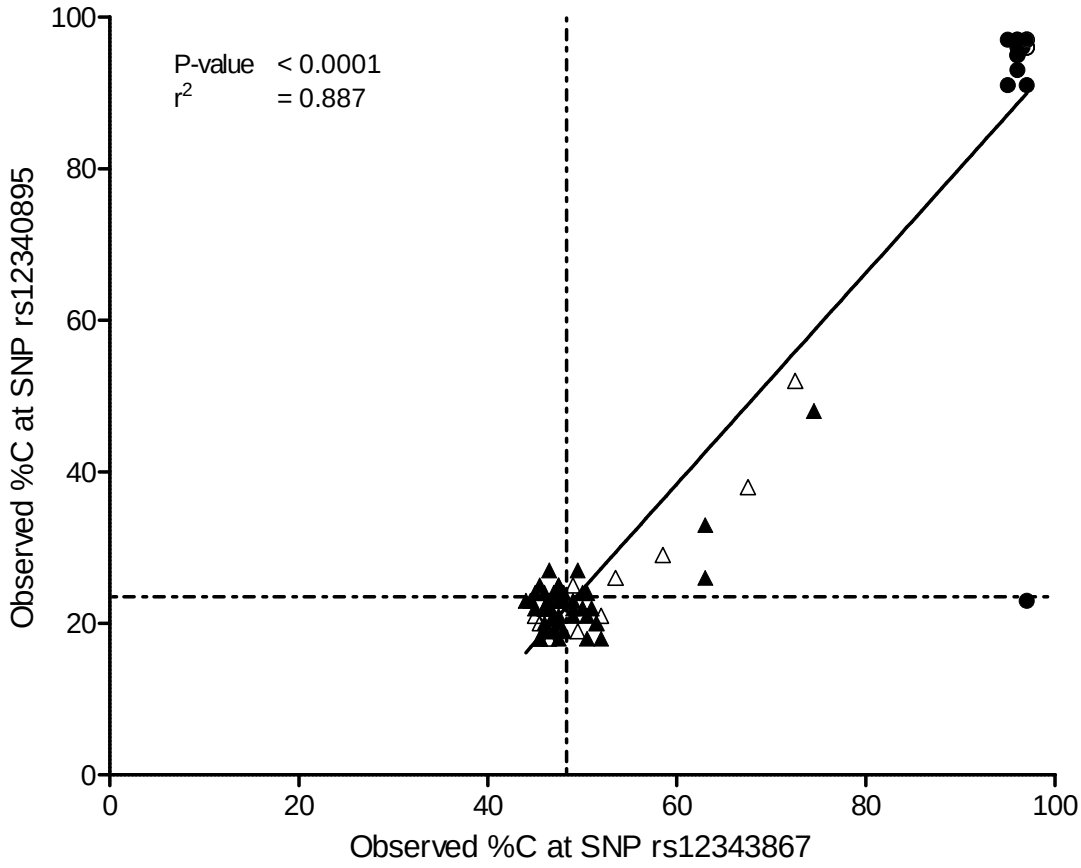


Figure 6.12 Comparison of methylation analysis results at the two SNPs studied

Dashed lines indicate median %C values at the two SNPs

Triangles indicate SNP heterozygotes, circles indicate SNP homozygotes

For the V617F-positive patients, those with mutant levels below the group median (19%) are represented by filled symbols and those with mutant levels above the group median are represented by open symbols

6.4 Discussion

The surprising finding that a specific *JAK2* haplotype predisposes to the development of an MPN has now been corroborated by numerous independent studies (Andrikovics *et al,* 2010; Guglielmelli *et al,* 2010; Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009; Trifa *et al,* 2010; Patnaik *et al,* 2010). The mechanism, however, remains obscure. The experiments

reported in this chapter were designed to investigate whether there was an association between the 46/1 haplotype in the cohort reported in this thesis and to examine potential mechanisms.

Eleven ET patients had been studied in detail in Chapter 5 and these individuals were studied initially by genotyping five *JAK2* SNPs reported to be involved in the 46/1 haplotype (Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009). Five patients were homozygous at all five SNPs, although it is notable that they were all homozygous for the non-46/1 alleles (TCTCT), and the other six patients were heterozygous for all five SNPs – i.e. they had one copy of the 46/1 haplotype. The fact that all 11 patients were either homozygous or heterozygous for all five SNPs – and none was homozygous at some loci, but heterozygous at others – is consistent with the presence of a haplotype spanning the region studied. A sixth SNP, rs2230724, located in *JAK2* exon 19 and which was studied in Chapter 5, appeared not to be in complete LD with the haplotype, since five patients were heterozygous for rs2230724 but homozygous for the other five SNPs. However, the presence of an A allele at rs2230724 was strongly associated with a C allele at rs12343867 (P=0.0003; Table 6.4), indicating the A allele at this locus is part of the 46/1 haplotype, in keeping with data from Jones *et al* (2009).

A cohort of normal controls, ET and PV patients was then screened for the presence of the 46/1 haplotype by genotyping a tagged intron 14 SNP, rs12343867. The frequency of the C allele, which tagged the 46/1 haplotype, was significantly greater in PV and V617F-positive ET patients combined than in local controls (0.39 versus 0.29, P=0.05; Table 6.4). In all other patient groups, the C allele frequency was similar to local controls. Compared with WTCCC controls, however, it was significantly higher in all patient groups except *JAK2* WT patients (P=0.008 for PV patients, P=0.003 for ET patients overall, P=0.003 for V617F-positive ET patients, P<0.0001 for PV and V617F-positive ET patients combined, P=0.14 for *JAK2* WT ET patients). These results suggest that in this cohort there was evidence for an association between the 46/1 haplotype and the development of PV and V617F-positive ET, although it

was subtle and was only apparent if the two groups were combined or individual groups were compared with a large WTCCC control group rather than a smaller cohort of local controls. No association was observed in *JAK2* WT ET patients.

These findings are broadly in keeping with the six published studies which have examined the relationship between 46/1 and MPNs (Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009; Andrikovics *et al,* 2010; Pardanani *et al,* 2010; Trifa *et al,* 2010). Olcaydu *et al* (2009) and Kilpivaara *et al* (2009) analysed the frequency of particular genotypes (e.g. CC versus TT) whereas the other studies assessed the allele frequency (i.e. C and T) in affected individuals compared to controls. Table 6.6, therefore, shows the data from all six studies re-analysed by allele frequency, with the results from the cohort reported in this chapter included for comparison. All the studies which considered PV, V617F-positive ET, or both PV and V617F-positive ET together reported that the 46/1 allele frequency was significantly higher in the patient groups compared to local controls (Jones *et al,* 2009; Kilpivaara *et al,* 2009; Olcaydu *et al,* 2009; Andrikovics *et al,* 2010; Pardanani *et al,* 2010; Trifa *et al,* 2010). Combining these studies, the overall 46/1 allele frequency was 0.51 in PV patients, 0.46 in V617F-positive ET patients, and 0.50 in both groups combined, compared to 0.26 in controls ($P<0.0001$ for all three comparisons). The data from these studies provides strong evidence for an association between the 46/1 haplotype and both PV and V617F-positive ET. This association was weaker in the cohort reported in this thesis than in published studies, particularly comparing patient groups to local controls, most likely due to smaller patient numbers in the cohort reported here.

It is not clear, however, whether the 46/1 haplotype is associated with the development of *JAK2* WT ET, particularly if comparison is made to local rather than WTCCC control groups. Although Olcaydu *et al* (2009) did report a difference compared to local controls, the P-value of 0.05 was borderline, and Jones *et al* (2009) observed a difference in only one of the two cohorts they studied ($P=0.009$ for the UK cohort, $P=0.09$ for the Greek cohort). Three further

Table 6.6 Published studies examining the association between the 46/1 haplotype and ET and PV

Reference	SNP alleles (46/1: non-46/1)	Controls		PV			V617F-positive ET			PV and V617F-positive ET combined			*JAK2* WT ET		
		n	46/1 allele frequency	*n*	46/1 allele frequency	P*	*n*	46/1 allele frequency	P*	*n*	46/1 allele frequency	P*	*n*	46/1 allele frequency	P*
(Jones *et al,* 2009) UK cohort	rs12343867 (C:T)	188	0.24	184	0.54	<0.0001	78	0.51	<0.0001	262	0.53	<0.0001	47	0.38	0.009
(Jones *et al,* 2009) Greek cohort	rs12343867 (C:T)	108	0.25	-	-	-	143	0.43	<0.0001	143	0.43	<0.0001	136	0.33	0.09 0.002§
(Kilpivaara *et al,* 2009)	rs10974944 (G:C)	1500‡	0.25	-	-	-	-	-	-	245	0.50	<0.0001§	74	0.34	0.005§
(Olcaydu *et al,* 2009)	rs12343867 (C:T)	99	0.28	-	-	-	-	-	-	213	0.57	<0.0001	120	0.38	0.05
(Pardanani *et al,* 2010)	rs12343867 (C:T)	57	0.28	-	-	-	118	0.44	0.005	118	0.44	0.005	108	0.38	0.09 <0.0001§
(Andrikovics *et al,* 2010)	rs12343867 (C:T)	331	0.29	153	0.48	<0.0001	78	0.47	<0.0001	231	0.49	<0.0001	53	0.33	0.43 0.04§
(Trifa *et al,* 2010)†	rs10974944 (G:C)	150	0.26	-	-	-	-	-	-	107	0.50	<0.0001	42	0.32	0.27 0.11§
Overall (weighted mean)		**2433**	**0.26**	**337**	**0.51**	**<0.0001**	**417**	**0.46**	**<0.0001**	**1319**	**0.50**	**<0.0001**	**580**	**0.36**	**<0.0001**
Reported in this thesis	rs12343867 (C:T)	101	0.29	24	0.42	0.09 0.008§	46	0.38	0.14 0.003§	70	0.39	0.05 <0.0001§	76	0.29	0.91 0.14§

* Chi-square P-value, compared to local controls unless stated otherwise

† Includes 15 PMF patients (9 V617F-positive and 6 *JAK2* WT); ‡ WTCC cohort; § Compared to WTCCC cohort

studies did not observe a difference compared to local controls (Andrikovics *et al,* 2010; Pardanani *et al,* 2010; Trifa *et al,* 2010). All five of these studies did report that the 46/1 allele frequency was significantly higher in *JAK2* WT ET patients than in the WTCCC group, but whether this was a valid control group for the non-UK studies is questionable. The overall 46/1 allele frequency amongst *JAK2* WT ET patients, 0.36, was significantly higher than that amongst all controls combined, 0.26 (P<0.0001). For the cohort reported in this thesis, no association between the 46/1 haplotype and *JAK2* WT ET was observed due to a lower 46/1 frequency in the *JAK2* WT ET patients compared to the published studies overall (0.29 versus 0.36) and a higher 46/1 frequency in the normal controls (0.29 versus 0.26). These differences may reflect variations in the ethnic composition of the groups or be due to chance.

Although the 46/1 haplotype is clearly an important risk factor for the development of some MPNs, its role should not be overstated. Amongst the patients reported in this thesis, 46% of ET patients and a 25% of PV patients were homozygous for the non-46/1 haplotype, suggesting that whilst the 46/1 haplotype may predispose to the development of MPNs, it is not a pre-requisite. In keeping with this, three of the patients reported in Chapter 5 in whom there was evidence that *JAK2* V617F had occurred more than once, the mutation arose predominantly in *cis* with the non-46/1 allele (G), suggesting that the haplotype predisposes to acquisition of the mutation on either allele. Similarly, in two of the three MPN patients reported by Olcaydu *et al* (2009) to have acquired *JAK2* V617F more than once, the mutation had arisen more frequently on the non-46/1 allele.

In order to explain the association between the 46/1 haplotype and MPNs, two potential hypotheses have been advanced. The hypermutability hypothesis suggests that this haplotype predisposes to the acquisition of *JAK2* V617F, and possibly other MPN-associated mutations. The fertile ground hypothesis proposes that in 46/1-positive individuals, mutations arise at the normal rate but a functional variant in LD with the haplotype means that 46/1-positive

individuals who acquire MPN-associated mutations are more likely to develop clinical disease than those who do not have this haplotype. In this chapter, both hypotheses have been considered. To investigate the fertile ground hypothesis, the possibility of preferential expression of the 46/1 allele was first investigated, since this might result in enhanced expression of V617F if it arose in *cis* with the 46/1 haplotype. The relative expression of the two possible alleles at an exonic *JAK2* A/G SNP, rs2230724, was therefore studied. The A allele at this SNP is in LD with the 46/1 haplotype (see Section 6.3.2). Expression of the two rs2230724 alleles in neutrophil cDNA was similar in all three groups studied, 54% A-expression in normal controls, 52% A-expression in both V617F-positive and *JAK2* WT ET patients (P>0.05 for all three comparisons, Figure 6.5B). All three values suggest the expression of the A and G alleles are balanced (i.e. 50%:50%) within the limits of technical variability for the technique. These results do not, therefore, support the notion that preferential expression of the 46/1 allele accounts for its association with V617F-positive MPNs, although the small size of the cohort studied means that subtle differences in expression cannot be excluded. These findings are in keeping with those of Jones *et al* (2009) who investigated the relative expression of rs2230724 alleles in 29 normal controls and of rs10429491 alleles, another 46/1-associated SNP located in exon 6 of *JAK2*, in 40 normal controls. They found no difference in expression of the 46/1 and the non-46/1 alleles.

Another possible mechanism to explain the fertile ground hypothesis is that 46/1 is in LD with rs505534, a chromosome 9 SNP located 8Mb upstream from *JAK2* that was associated with variability in platelet counts in a study of nearly 14,000 healthy German and British controls (Soranzo *et al,* 2009). If the *JAK2* V617F mutation arises in the presence of an rs505534 allele associated with higher platelet counts than average, then a clinical MPN might be more likely to result than if it arose on the other SNP allele. There was, however, no difference in allele frequency (C versus T) in any of the patient groups studied compared to the HapMap Northern

European control cohort and therefore no suggestion af an association between this SNP and ET or the 46/1 haplotype.

To investigate a possible mechanism to account for the hypermutability hypothesis, the possibility that the 46/1 haplotype might affect DNA methylation was considered, since changes in methylation may affect DNA conformation and influence susceptibility to mutations. In humans, DNA methylation affects cytosine bases that precede guanine bases (dinucleotide CpGs), and two of the reported 46/1-tagging SNPs (rs12343867 and rs12340895, both in intron 14) result in CpG sites when the 46/1-associated allele is present but not when the non-46/1 allele is present. To quantify the degree of methylation at these SNPs, pyrosequencing of bisulphite-treated neutrophil DNA from normal controls and ET patients, both *JAK2* WT and V617F-positive, was performed. At SNP rs12343867, the median post-bisulphite treatment %C was 47-48% in SNP heterozygous individuals, and 96% in SNP homozygous individuals (Figure 6.10), in keeping with complete methylation at this locus within the technical limits of the assay.

The degree of methylation was similar in normal controls and SNP heterozygous ET patients, regardless of *JAK2* status. It was notable, however, that a seven SNP heterozygous, mutant-positive patients had post-bisulphite %C values of greater than 50%, a figure which is not compatible with being CT heterozygous at this locus. Five of these patients had relatively high V617F mutant levels and the simplest explanation for the %C being greater than 50% in these patients was the presence of chromosome 9p LOH in some of their myeloid cells, resulting in amplification of the C allele to more than 50%. This phenomenon would not have been apparent when the patients' SNP genotypes was originally determined (Section 6.2.2) since this was done using T-cells, which are unlikely to be affected by 9pLOH. To further investigate this, it would be necessary to repeat the pyrosequencing analysis in tandem, using bisulphite-treated and non-bisulphite treated DNA from each individual, and then to compare the two

results from each individual. Overall, the C allele at SNP rs12343867 appeared to be completely methylated in both ET patients and normal controls.

At the second SNP studied, rs12340895, the median %C was 96% in SNP homozygous ET patients, but less than 25% in all three SNP heterozygous groups: 23% in controls, 21% in *JAK2* WT ET patients and 24% in V617F-positive ET patients (Figure 6.11). The %C was significantly different in these latter two groups (P=0.009), due to five mutant-positive SNP heterozygous patients with %C which was more than two standard deviations greater than the group median. These patients, three of whom had relatively high mutant levels, all had %C greater than 50% at SNP rs12343867 (Figure 6.12). The most plausible explanation is, again, the presence of 9pLOH. No other statistically significant difference was observed between any of the comparable groups at this SNP.

Amongst SNP heterozygous individuals the degree of methylation at SNP rs12340895 appeared to be markedly lower than that at rs12343867 in both controls and ET patients (overall median %C 22% versus 48%, P<0.0001). This was due, at least in part, to a software limitation which precluded accurate calculation of %C in patients who were heterozygous at SNP rs12340895 (all the controls and 49 of the 64 ET patients). The pyrosequencing analysis software could only interpret sequence data which varied a one locus, but the presence of a C allele at SNP rs12340895 affected the DNA sequence at two loci: at the SNP itself and at the preceding cytosine base, which in the presence of a C allele at the SNP was unmethylated and, therefore, converted to thymine during bisulphite treatment (Figure 6.4B). If analysis is, therefore, confined to ET patients who were homozygous for the 46/1 allele at the two SNPs, within technical limits both loci were completely methylated (median %C was 96% at SNP rs12343867 versus 97% at SNP 12340895, P=0.22). Overall, the data from these experiments suggests that the presence of the 46/1 haplotype may influence the degree of methylation of the *JAK2* gene, potentially affecting its conformation and susceptibility to acquiring mutations. It is notable that at least two of 46/1-tagging SNPs which might affect *JAK2* methylation, rs12343867 and

rs12340895, are located in intron 14, close to the V617F mutation site in exon 14. Clearly further study of other *JAK2* SNPs in larger cohorts of controls and patients is required.

In conclusion, the experiments reported in this chapter provide further evidence for a specific *JAK2* haplotype which is associated with the development of V617F-positive MPNs, though the association was weaker than that reported by other studies and did not extend to *JAK2* WT ET. Furthermore, nearly a half of ET patients and a quarter of PV patients were homozygous for the non-46/1 haplotype, indicating that the presence of the 46/1 haplotype is not required for the development of an MPN. There was no evidence that the predisposition to an MPN observed in 46/1-positive individuals is due to preferential expression of the 46/1 allele or an association with a nearby 'platelet variability' SNP. However, the two CpG dinucleotides introduced by the 46/1 haplotype within the *JAK2* gene were found to be completely methylated, and the possibility that this may affect the susceptibility to mutations merits further study.

7 Conclusions and future directions

7.1 Summary of findings

Although ET has been recognised as a clinical entity for nearly 80 years, the identification in 2005 of a recurrent acquired point mutation, *JAK2* V617F, in approximately 50% of cases resulted in a fundamental change in the way the disorder is perceived, leading to its reclassification by the WHO as a myeloproliferative neoplasm in 2008. This reclassification reflected a belief that ET is a clonal neoplastic disease which arises as a consequence of one or more acquired mutational events. However, it overlooked several characteristics of this disorder, in particular that ET does not typically behave as a malignant neoplasm and that a substantial proportion of cases are polyclonal according to XCIP data. The studies reported in this thesis, therefore, set out to investigate the molecular origins of ET, and in particular to consider the role of *JAK2* V617F in the pathogenesis of the disease and its relationship to the concept of clonality in ET. The aim was to gain a better understanding of this heterogeneous disease so that its clinical phenotype might be explicable in molecular terms.

The clinical characteristics of a cohort of 133 ET patients were evaluated and their association with haematological and molecular features examined (Chapter 3). In terms of demographic and clinical features, as well as haematological and molecular characteristics, these patients were similar to those described in most published studies. In keeping with most other series, the clinical course in the majority of patients studied was relatively benign, and the most common complications were thrombotic. Over a median time since diagnosis of 12.6 years, the thrombotic rate was 16% and the mortality was less than 5%. Although patients who had had a thrombosis were older at diagnosis than those who did not (median age 53 versus 44 years, P=0.03), presenting blood counts and a history of prior thrombosis did not predict thrombotic

risk. Of the 62 evaluable female patients, 24 (39%) had a clonal XCIP. These patients were younger at diagnosis and had higher presenting platelet counts than those with a polyclonal XCIP, and appeared to have a higher rate of thrombotic complications, though this did not reach statistical significance (53% versus 28%, P=0.11), probably due to the relatively small cohort studied. When these results were combined with data from other groups, however, a significant association between XCIP status and thrombotic rate was observed (38% in patients with a clonal XCIP versus 18% in patients with a polyclonal XCIP, P=0.008).

The *JAK2* V617F mutation was detectable in 55 (41%) of patients and, in keeping with the majority of published studies, these individuals were older at presentation than those with *JAK2* WT ET, had lower platelet counts and higher haemoglobin concentrations. The overall thrombotic risk in the two groups was not significantly different (45% versus 26%, P=0.10), but the median mutant level was higher in those who had had a thrombosis than in those who did not (11% versus 5%, P=0.02). This is consistent with data from other groups and suggests that the mutant level, rather than the presence of the mutation per se, may determine the thrombotic risk. It is also in keeping with the notion that the phenotype of V617F-positive ET lies somewhere between *JAK2* WT ET and PV in terms of presenting platelet counts and thrombotic risk. The presence of *JAK2* V617F is often taken to indicate the presence of a clonal myeloid population, yet the rate of myeloid clonality was similar amongst mutant-positive and *JAK2* WT patients (27% versus 45%, P=0.19). This may have been due to the inability of the XCIP technique to detect small clones, and conversely to the presence of *JAK2* WT patients with clonal myelopoiesis who are likely to carry other mutations.

The relative size of *JAK2* WT and V617F-positive platelet populations in ET patients was then investigated (Chapter 4). A number of prior studies had shown that, in ET, on average less than half the myeloid population carried the V617F mutation and that the size of the mutant-positive population appeared to remain stable over many years, both characteristics which might be considered unexpected for a neoplastic disease. To address the possibility that this

phenomenon was an artefact of studying neutrophils rather than the biologically more relevant platelets, mutant levels were quantified in platelet and neutrophil RNA from 10 V617F-positive ET patients, obtained prior to the initiation of cytoreduction. Whilst mutant levels were significantly higher in platelet RNA than in neutrophil RNA (27% versus 21%, P=0.002), the results indicated that on average only about half of the circulating platelets in ET were mutant-positive. None of the 10 patients studied had evidence that *JAK2* WT thrombopoiesis was suppressed in the presence of a thrombocytosis. Whilst this might have been due to defective regulation of thrombopoiesis in ET, possibly caused by impaired c-Mpl-mediated TPO clearance, this observation might also suggest that the *JAK2* WT platelets were abnormal in some way. Regardless of the mechanism, the implied persistence of *JAK2* WT progenitors, combined with recent evidence that some individuals harbour an inherited predisposition to acquire the mutation, raised the possibility that the *JAK2* V617F event might occur more than once in a single individual.

The possibility that more than one mutant-positive myeloid population might exist due to the acquisition of *JAK2* V617F on more than one occasion was, therefore, investigated (Chapter 5). The chromosome on which the mutation had arisen was determined in 11 V617F-positive ET patients who were heterozygous for a *JAK2* exon 19 SNP by examining which SNP allele was in *cis* with the mutation. In order to study only V617F-positive alleles, a PCR fragment which spanned both the mutation site and the SNP was amplified from neutrophil cDNA. Restriction enzyme digestion was then performed to cut *JAK2* WT products so that in a second-round PCR, only V617F-positive products were re-amplified. Analysis of the *JAK2* exon 19 SNP present in mutant-positive products revealed that in 10 of the 11 individuals studied, a mixture of SNP alleles was present. In each of these 10 patients, therefore, the mutation appeared to have arisen on both chromosomes, suggesting that the majority of ET patients studied had evidence for the independent acquisition of V617F on more than one occasion in different haematopoietic progenitors. Poisson statistics would suggest that the mutation had arisen

more than twice. These results were confirmed by subcloning the PCR product from five patients and determining the exon 19 SNP allele present in the mutant-positive clones. In all five patients the mutation appeared to have arisen more than once. The SNP allele with which the mutation was predominantly associated varied between individuals. Where samples obtained from a single patient at different time points or from different cells (platelets rather than neutrophils) were analysed, the SNP allele on which the mutation had predominantly arisen was the same in the two different samples. The concordance between different samples in a single individual suggests that the results genuinely indicated the presence of multiple V617F events and were not due to random PCR artefact, although this cannot be fully excluded. By contrast, in five PV patients studied there was no evidence for multiple mutant-positive populations.

The inactivated X-chromosome was then determined in V617F-positive erythroid colonies derived from six mutant-positive ET patients, including three previously shown to have more than one mutant-positive population using the approaches described above. In one patient, different mutant-positive colonies inactivated different X-chromosomes, indicating that in this case the V617F- positive populations were clonally distinct and that in this individual *JAK2* V617F had arisen more than once in phylogenetically unrelated progenitors. In the other five cases the mutant-positive colonies inactivated the same X-chromosome. This might suggest that in these patients – i.e. the majority – the mutant-positive populations were clonally related and that *JAK2* V617F arose more than once in different myeloid progenitors which were all derived from a single transformed progenitor. It is also possible, however, that the V617F-positive erythroid colonies from these five patients inactivated the same X-chromosome by chance. These results highlight the biological heterogeneity of ET, and suggest that in some cases at least, ET resembles a polyclonal non-malignant condition rather than a clonal neoplastic disorder.

The role of the 46/1 haplotype in the development of ET and the acquisition of *JAK2* V617F was also investigated (Chapter 6). A number of recently-published studies suggest that individuals who harbour this haplotype may be at higher risk of developing MPNs than those who do not. In keeping with this, an association was observed between the 46/1 haplotype and V617F-positive MPN amongst the patients reported in this thesis, although it was weaker than that reported by other groups. Moreover, nearly 50% of ET and 25% of PV patients were homozygous for the non-46/1 haplotype, indicating that this haplotype is not required for the development of an MPN. No association between the 46/1 haplotype and *JAK2* WT MPN was observed.

The mechanism underlying the association between the 46/1 haplotype and MPNs is unknown, but two hypotheses have been proposed. One suggests that the haplotype increases the mutational rate within the *JAK2* gene. The other argues that the mutation does not occur any more frequently than in non-46/1 individuals, but that when it does arise it is more likely to result in clinical disease, possibly due to the presence of a functional variant associated with the haplotype. A series of experiments was then performed to explore these two potential mechanisms. The possibility that the haplotype affected the *JAK2* expression or was in LD with a SNP which was associated with platelet variability was investigated, but the results did not support either hypothesis. The 46/1 haplotype was noted to introduce at least two CpG dinucleotides into the *JAK2* gene, which may be methylated and therefore alter the DNA conformation and render it more susceptible to mutations. The methylation at two 46/1-associated SNPs was, therefore, investigated using bisulphite treatment followed by pyrosequencing. Both loci were observed to be completely methylated in the presence of the 46/1 haplotype, in both controls and ET patients, which might affect the mutability of the gene.

7.2 Future directions

Over the past decade, a number of important advances have been made in our understanding of the pathogenesis of ET, in particular the identification of the *JAK2* V617F and *MPL* W515 mutations and the recognition that the 46/1 haplotype may be a risk factor in the development of ET. Nonetheless, many challenges remain, both in comprehending the biology of the disease and in managing patients with it. Nearly fifty percent of patients with ET have no detectable molecular abnormality, and in these cases making the diagnosis can be problematic. Future work is, therefore, required to identify the causative mutations in these cases. It is notable that in 17 (71%) of the 24 ET patients with a clonal XCIP reported in this thesis, neither *JAK2* V617F nor *MPL* W515 mutations could be detected. Such patients would be suitable subjects for investigation by genome-wide approaches to mutation detection using high-throughput technology. Screening ET patients using high-resolution SNP microarray-profiling has not revealed any common regions of LOH or copy number alteration suitable for candidate gene mutational analysis (Kawamata *et al,* 2008; Stegelmann *et al,* 2010). High-throughput sequencing, for instance using sequencing by synthesis microarray technology, might, therefore, be the most appropriate method to address this issue.

The agents currently used to treat ET can cause a range of side-effects and consequently their use should be confined to patients at highest risk of complications. Prognostication in ET, however, remains imprecise and is still predominantly based on clinical factors, despite attempts to identify molecular markers of increased thrombotic risk. Myeloid XCIP status has previously been proposed as one such marker, but the association between XCIP status and thrombotic risk is relatively weak and only becomes clearly evident when multiple studies are combined. Moreover, this association relates predominantly to events prior to rather than after diagnosis and the assay is only performed in a research setting and cannot be used to analyse men and some women. Similarly, *JAK2* status does not appear to have a useful role in

risk stratification in ET, though the mutant level might do, at least in patients with very high mutant levels. It would worth investigating whether, in addition to the size of the V617F-positive population, the phylogenetic relationship of its constituent cells affects prognosis. Data presented in Chapter 5 of this thesis suggests that most ET patients have more than one V617F-positive population, and in at least a minority of cases these populations are not clonally related. Larger patient cohorts might usefully be studied, therefore, to determine whether patients with more than one phylogenetically unrelated V617F-positive population have a different natural history than those whose mutant-positive populations appear to be derived from a common founder clone.

ET is incurable with current conventional therapies. Whilst the disease may not affect the life expectancy of older adults, this is probably not the case in younger patients, especially those with aggressive disease and in these cases at least, novel agents are required. Furthermore, the majority of existing treatments target the end result of the pathogenic process, the proliferating MK, and require lifelong, daily administration. If the initiating event could be identified and targeted, durable medication-free remissions or even cure may become possible. A number of new drugs which inhibit *JAK2* are in development and whilst some are showing some promise, none is currently able to reproduce in MPNs the remarkable activity of BCR-ABL inhibitors seen in CML (Druker *et al,* 2006; Pardanani *et al,* 2011; Verstovsek *et al,* 2010). There is evidence for a so-far uncharacterised 'pre-*JAK2*' event in at least some V617F-positive individuals (Campbell *et al,* 2006a; Theocharides *et al,* 2007), suggesting that V617F may not necessarily be the pathogenic abnormality in MPN, and this might explain the relative lack of efficacy of *JAK2* inhibitors. In order to develop more effective targeted therapies, determining the pathogenic event is critical. The observation that *JAK2* WT myelopoiesis and thrombopoiesis were not suppressed by the presence of a V617F-positive population (Chapter 4) raised the possibility that these *JAK2* WT cells were themselves not normal, possibly due to a non-*JAK2* abnormality. For this reason, the *JAK2* WT cells present in mutant-positive

individuals warrant further investigation – for instance by growing up haemopoietic colonies and selecting the *JAK2* WT ones for analysis by high-throughput sequencing.

The evidence that most ET patients have more than one V617F-positive population (Chapter 5) also has implications for therapy. Any future attempts to eliminate the mutant-positive haemopoietic progenitor altogether and hence cure patients of the disease may prove futile if they have a pre-existing tendency to acquire *JAK2* V617F repeatedly. The role of the 46/1 haplotype in the development of MPNs warrants further research since a clearer understanding of how this inherited genotype results in predisposition to an MPN in individuals who harbour it may reveal potential therapeutic targets. For instance, if the 46/1 haplotype does result in increased *JAK2* methylation and thereby increases its susceptibility to acquiring mutations, then consolidating anti-*JAK2* therapy with hypomethylating agents might be appropriate. Methylation analysis of the entire *JAK2* gene in larger groups of normal controls and ET patients would, therefore, be useful to determine whether the presence of the 46/1 haplotype has a significant effect on overall gene methylation. Analysis of mutational rates in individuals according to 46/1 haploptype status might also be informative.

Finally, the question of whether ET is always a clonal, neoplastic disorder, as stated in the 2008 WHO classification, requires careful consideration, in view of the data presented in Chapters 4 and 5 of this thesis. Labelling ET as a neoplasm can generate unnecessary anxiety in patients if this is taken to indicate the presence of a malignant disease. Recognition that at least some ET patients have a disorder characterised by a mutant-positive population which is not monoclonal and remains stable in size over many years, should prompt further discussion as to whether ET is invariably a neoplastic condition.

7.3 Overall conclusions

In conclusion, the results presented in this thesis provide evidence for the biological heterogeneity of ET, and that accurate prognostic markers remain to be defined. Using XCIP analysis, a substantial proportion of ET patients appeared to have polyclonal myelopoiesis, including the majority of V617F-positive ones. Furthermore, in the majority of mutant-positive ET patients there was evidence which suggested that *JAK2* V617F had arisen more than once, and in at least one patient this had occurred in phylogenetically unrelated progenitors. In other patients, the multiple mutant-positive populations may have arisen from a clonal progenitor pool, in keeping with the existence of a pre-*JAK2* mutational event. Finally, these results provide further evidence for the presence of a specific *JAK2* haplotype which predisposes to the development of V617F-positive MPNs, but the mechanism is unclear. Together, these observations suggest that the role of *JAK2* V617F in the pathogenesis of ET is more complex than it being a single initiating event, that this role may vary between patients, and that the presence of the mutation does not necessarily indicate the presence of a monoclonal disorder.

Appendix 1: Primer sequences

Primer name	Primer sequence	Primer melting temperature (°C)
HUMAR/U	5'-TCCAGAATCGTTCCAGAGCGTGC-3'	67
HUMAR/D	5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'	66
*JAK2*ex10/F	5'-TGGAACGTAGGTTGTATGTTGGC-3'	64
*JAK2*ex10/R	5'-CATGTAAGAAGGCAATGATAATTAATAGC-3'	64
*JAK2*ex12/F2	5'-GCTGTTCAAGGGTCAACTGTAG-3'	63
*JAK2*ex12/R2	5'-ACTGAGGATGTATTTACTTAAGTGTC-3'	62
*JAK2*ex14/ARMS/FO	5'-TCCTCAGAACGTTGATGGCAG-3'	62
*JAK2*ex14/ARMS/FWT	5'-GCATTTGGTTTTAAATTATGGAGTATATG-3'	63
*JAK2*ex14/ARMS/RMU	5'-GTTTTACTTACTCTCGTCTCCACAAAA-3'	64
*JAK2*ex14/ARMS/RO	5'-ATTGCTTTCCTTTTTCACAAGAT-3'	56
*JAK2*ex14/F2	5'-CAAAAGAGATTATGGCAGGTTCAAC-3'	63
*JAK2*ex14/(meth)F1	5'-TGTTTATGATAGTAAAAGAGATTATGG-3'	60
*JAK2*ex14/(meth)R1	5'-ATTTCTATAAACACCTAAATTTAACCAAAA-3'	60
*JAK2*ex14/(meth)seq1	5'-AATGGAATTGATAGAAATGATT-3'	52
*JAK2*ex14/(meth)F2	5'-AAATTTATGATTTGTTTTATTATGGTAGT-3'	58
*JAK2*ex14/(meth)R2	5'-CAATTTTCAAAAATATAAAAAAATATCATACA-3'	59
*JAK2*ex14/(meth)seq2	5'-TTTAGAGGTATGTTTTTATTTTAGT-3'	55
*JAK2*ex14/(MM)F	5'-CAAGCATTTGGTTTTAAATTATGGAGTACGT-3'	66
*JAK2*ex14/(MM)F3	GAGGTATGCCTTTATTTTAGCGC-3'	62
*JAK2*ex14/(MM)R	5'-AGCCAAAAAATATATCTAGTATCATATCTAC-3'	64
*JAK2*ex14/R	5'-TAAATTATAGTTTACACTGACACCTAG-3'	61
*JAK2*ex14/R2	5'-CATATAACAGGTTCCAAACCACTG-3'	62

*JAK2*ex15/F	5’-CTTAGAACTCATGTGAAATGGC-3’	59
*JAK2*ex15/(MM)F	5’-ATTCATTTCATCACAAGCATAGAGTA-3’	61
*JAK2*ex15/R2	5’-CTGGAGCATTTAGTATGTTCAAG-3’	60
*JAK2*ex18/F	5’-TCAGGCCTTCTTTCAGAGCCA-3’	62
*JAK2*ex19/F	5’-GTATATCAGTTTAGTCCAGAGAATG-3’	61
*JAK2*ex19/R	5’-GACAATTTACCTTGCCAAGTTGC-3’	62
*JAK2*ex20/R	5’-TGTAGAGGGTCATACCGGCAC-3’	64
*MPL*ex10/F	5’-TGGATGAGGGCGGGGCTCCG-3’	69
*MPL*ex10/F3	5’-GTGGGCCGAAGTCTGACCCT-3’	65
*MPL*ex10/R2	5’-CGCTCTGTGACCCCAGATCTC-3’	66
*MPL*W515L/F	5’-TGCTGCTGAGGTTGCAGTTTCCTGC-3’	70
*MPL*W515L/R	5’-GTGCAGGAAACTGCAACCTCAGCA-3’	68
rs505534/(MM)F	5’-GTTCATATATTTCTATCCAAAATTGTCT-3’	61
rs505534/R	5’- GCTAAGATTATACAACAATATTGTTAAG-3’	61

Appendix 2: Publications arising from experiments reported in this thesis

Lambert, J.R., Everington, T., Linch, D.C. & Gale, R.E. (2009) In essential thrombocythemia, multiple JAK2-V617F clones are present in most mutant-positive patients: a new disease paradigm. *Blood,* **114,** 3018-3023.

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